A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*

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Vertically transmitted symbionts of arthropods have been implicated in several reproductive manipulations of their hosts. These include cytoplasmic incompatibility (CI), parthenogenesis induction in haplodiploid species (PI), feminization and male killing. One symbiont lineage in the α-Proteobacteria, *Wolbachia*, is the only bacterium known to cause all of these effects, and has been thought to be unique in causing CI, in which the fecundity of uninfected females is reduced after mating with infected males. Here, we provide evidence that an undescribed symbiont in the *Bacteroidetes* group causes CI in a sexual population of the parasitic wasp *Encarsia pergandiella*. Wasps were crossed in all four possible combinations of infected and uninfected individuals. In the cross predicted to be incompatible, infected (I) males × uninfected (U) females, progeny production was severely reduced, with these females producing only 12.6% of the number of progeny in other crosses. The incompatibility observed in this haplodiploid species was the female mortality type; dissections showed that most progeny from the incompatible cross died as eggs. The 16S rDNA sequence of this symbiont is 99% identical to a parthenogenesis-inducing symbiont in other *Encarsia*, and 96% identical to a feminizing symbiont in haplodiploid *Brevipalpus* mites. Thus, this recently discovered symbiont lineage is capable of inducing three of the four principal manipulations of host reproduction known to be caused by *Wolbachia*.

Keywords: *Wolbachia*; parthenogenesis; male killing; Aphelinidae; Hymenoptera; CFB group

1. INTRODUCTION

Vertically transmitted bacterial symbionts of arthropods have diverse effects on their hosts. Whereas some of these symbionts increase their frequency by directly enhancing host fitness (Moran & Telang 1998; Oliver *et al.* 2003), others alter the reproductive behaviour of their hosts in ways that enhance their own transmission (Werren & O'Neill 1997). The latter have been called 'reproductive parasites', and may cause: (i) cytoplasmic incompatibility (CI), in which uninfected female hosts are reproductively incompatible with infected males; (ii) parthenogenesis induction (PI) in haplodiploid systems, where haploid host eggs double their chromosome complement and develop as diploid females; (iii) feminization, in which genetic males develop as females; and (iv) male killing, in which males are killed during development. Because of their dependence on their hosts for transmission, vertically transmitted symbionts become intimate partners in the evolutionary trajectory of their hosts, and have been implicated in adaptive radiation (Moran & Telang 1998), reproductive isolation (Bordenstein *et al.* 2001), and in the evolution of sex determination systems (Rigaud 1997) of host lineages.

Among reproductive parasites, *Wolbachia*, in the α-Proteobacteria, appears to be the master manipulator of host reproduction. Although several widely taxonomically distributed bacteria have been shown to be responsible for some of these four different phenotypes, only *Wolbachia* induces all four of them (Stouthamer *et al.* 1999). In addition, the production of non-viable offspring due to CI

has appeared to be limited to *Wolbachia* (Stouthamer *et al.* 1999; Weeks *et al.* 2002). In CI, uninfected females produce non-viable offspring when mated with infected males, and are thus at a reproductive disadvantage to infected females that are able to produce viable offspring when mated to both infected and uninfected males. In addition, males and females infected with different strains of *Wolbachia* are reproductively incompatible. Cytoplasmic incompatibility is by far the most predominant phenotype caused by *Wolbachia* infection, and has been found in most major insect groups as well as in mites (Hoffmann & Turelli 1997). The mechanism of CI fits a 'modification–rescue' model in which sperm from infected males are modified in such a way that karyogamy, the union of sperm and egg nucleus, can only occur when both parents are infected by the same or very closely related *Wolbachia* lineages (Werren 1997; Poinsot *et al.* 2003). Recent evidence suggests a simple timing delay in decondensation of the sperm nucleus might prevent karyogamy in incompatible crosses (Tram & Sullivan 2002). However, the means by which *Wolbachia* in males ensures that embryo development occurs only when females are infected with the same *Wolbachia* strain is still a mystery. This specificity of the interaction adds to the perception that CI is an especially deft manipulation of the host by the bacterium, and thus presumably not easily evolved by bacterial lineages outside of *Wolbachia* (Stouthamer *et al.* 1999).

Studies of *Wolbachia* dominate current research efforts on reproductive parasites, in part because of the wide distribution and importance of this symbiont, but perhaps also because of the ease with which it is detected (Stouthamer *et al.* 1999). The accessibility of *Wolbachia*-specific polymerase chain reaction (PCR) primers has led to rapidly

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Proc. R. Soc. Lond. B (2003) **270**, 2185–2190
DOI 10.1098/rspb.2003.2475

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increasing numbers of reports of *Wolbachia* infection. Further, molecular detection of *Wolbachia* is becoming increasingly sensitive and recent authors have discovered previously undetected infections, or have shown multiple strains of *Wolbachia* in the same host (Jiggins *et al.* 2001; Jamnongluk *et al.* 2002). However, there are also many reports of infection with intracellular symbionts other than *Wolbachia* or obligate bacteriocyte symbionts, including observations of multiple infections with both *Wolbachia* and non-*Wolbachia* symbionts (Moran *et al.* 2003). Because of the well-documented association of *Wolbachia* with certain host phenotypes, it is easy to assume that *Wolbachia*, when present, is the causal agent of particular effects. However, the findings of multiple infections suggest that *Wolbachia* may sometimes be implicated in host phenotypes that should be properly credited to other symbionts (Weeks *et al.* 2002). Further, the failure to find *Wolbachia* in arthropods with one of the common phenotypes attributed to this symbiont does not rule out the possibility that another reproductive parasite is responsible.

We show that a recently discovered bacterial symbiont from the *Bacteroidetes* (= Cytophaga–Flexibacter–Bacteroides or CFB) group induces CI in the parasitic wasp *Encarsia pergandiella*. We believe that this is the first example in which a symbiont other than *Wolbachia* has been shown to induce mating incompatibility leading to non-viable offspring. This case extends the portfolio of reproductive alterations caused by this particular undescribed monophyletic lineage, here provisionally called '*Cytophaga*-like organism', or CLO, to include CI as well as PI (Zchori-Fein *et al.* 2001), and feminization (Weeks *et al.* 2001), thus demonstrating that *Wolbachia* is not unique in its versatility. Further, the CI-causing CLO symbiont appears very closely related to the strain associated with parthenogenesis in a different population of the same host species, suggesting that like *Wolbachia*, closely related strains of this symbiont may have very different effects on their hosts.

2. MATERIAL AND METHODS

(a) *Study system*

Encarsia (Hymenoptera: Chalcidoidea: Aphelinidae) is a speciose genus of minute parasitic wasps with an unusual biology. Almost all sexual *Encarsia* species are autoparasitoids; while females develop internally on a whitefly or armoured scale insect (the primary host), male *Encarsia* are obligate hyperparasitoids, and develop only on conspecific females or other parasitoids developing within the primary host (Hunter & Woolley 2001). Parthenogenesis is also common in *Encarsia*. All parthenogenetic *Encarsia* that have been sampled have been shown to be infected with bacterial endosymbionts, including six lineages with closely related CLO (Zchori-Fein *et al.* 2001), and one, *E. formosa*, with *Wolbachia* (van Meer *et al.* 1999). One of these parthenogenetic, CLO-infected species, *E. hispida,* readily produces males upon treatment with antibiotics (Giorgini 2001). In addition, one sexual population of *E. pergandiella* from Texas, USA, was also shown to harbour a CLO endosymbiont. This symbiont showed *ca.* 99% sequence similarity at 16S rDNA with the endosymbiont implicated in parthenogenesis induction of the population of *E. pergandiella* from Brazil, but unmated females of this population were unable to produce female offspring, ruling out a PI symbiont (Zchori-Fein *et al.* 2001). The Texas *E. pergandiella* was the subject of the current study.

Encarsia pergandiella was collected in the Rio Grande Valley of Texas, USA, on *Bemisia tabaci,* the sweet potato whitefly in the late 1990s. In the laboratory, *E. pergandiella* appears to be fixed for the symbiont infection. *Encarsia pergandiella* was maintained in the laboratory on *B. tabaci* on cowpea (*Vigna unguiculata*) at 25 °C, 16 L : 8 D photoperiod at ambient humidity. To supplement production of the hyperparasitic male wasps, virgin female *E. pergandiella* wasps were provided with pupae of another parasitoid of *B. tabaci, Eretmocerus eremicus* (Hymenoptera: Aphelinidae).

(b) *Testing for cytoplasmic incompatibility*

Wasps were cured of their bacterial infection by feeding adults antibiotics for three generations. Young adults were held together in vials containing 50 mg ml^{-1} of rifampicin in honey solution for 48 h. After this period, females were allowed to oviposit for 6–8 h on a leaf disc with whitefly nymphs to deplete the number of mature and possibly infected oocytes in their ovaries. Both uninfected (U) and infected (I) wasps were then maintained separately in small cages containing cowpea seedlings bearing either early fourth instar whitefly nymphs (for female production), or prepupal or early pupal *E. eremicus* (for male production).

Two blocks of experimental crosses were set up in the fourth and fifth generation, during which time no antibiotics were administered to any wasps. Experimental U and I virgin females were paired individually with either U or I males of similar age. After 24 h, pairs were separated, and females were placed in individual experimental arenas for 4 h. The arenas consisted of a 35 mm diameter cowpea leaf disc bearing 30 third–early fourth instar whitefly nymphs, set in a 2–4 mm layer of 5% cooling water agar in a 65 mm Petri dish. The number and stages of whiteflies were standardized by removing the excess nymphs. After female wasps were introduced, a piece of filter paper was inserted into the top of the Petri dish to absorb condensation, and the dishes were inverted. After the females were removed, the dishes were incubated at 27 °C, 14 L : 10 D, and 65% r.h. until pupation of the wasps. At this time, we categorized the whiteflies as 'emerged', 'dead' and 'developmentally arrested'. The latter did not successfully eclose, but were still alive at the time the wasps had pupated. We later confirmed that almost all of these arrested whiteflies were parasitized nymphs in which the wasp did not develop beyond the egg stage (see below). Wasps were differentiated as dead larvae, early pupae or healthy pupae. The pupal wasps we observed in arenas of all of the crosses appeared to be females, based on their pigmentation. In the first block, all of the pupae from the predicted incompatible cross, I male × U female, as well as sample pupae from other treatments were isolated and reared until emergence. This confirmed our assessment that all pupae were female.

After the first experiment of this type was completed, two more independent lines of uninfected wasps were established from the culture by antibiotic treatment for three generations, as described above, and these lines were used in experiments identical to the one described above, but with fewer (*n* = 10) replicates per treatment.

Comparisons of the frequencies of different outcomes among treatments were made using generalized linear modelling techniques available in the statistical software package GLIM. Arenas in which 27 or more of the 30 whiteflies in the arenas eclosed were excluded from the analyses. The frequency data were compared in two factor models with Poisson errors, with treatment and block as factors. This type of analysis yields estimates of

significance that are approximately χ^2 distributed. When the residual deviance of the analysis was larger than the residual degrees of freedom, indicating a greater than Poisson variance, a heterogeneity factor was fitted to correct the χ^2 estimates (Crawley 1993).

A second type of experiment was conducted to determine when non-viable offspring from the incompatible cross stopped developing. Two crosses were performed with individuals in the sixth generation since the end of antibiotic curing; the I male × U female (the incompatible cross) and U male × U female (a control cross). The experiment was performed as described for the earlier experiment, but half of the arenas were incubated for only 5 days. All of the whitefly nymphs in these arenas were then dissected, and the stage of the parasitoid, when present, was recorded. Pupae from the other half of the arenas were isolated in 1.2 ml vials and incubated until emergence.

(c) *Confirmation of bacterial infection via polymerase chain reaction and cloning*

We monitored the infection status of infected and uninfected laboratory stocks via PCR, using the CLO-specific primers, EPS-f and EPS-r, and PCR protocol described in Zchori-Fein *et al.* (2001). DNA was extracted by grinding wasps in *ca.* 40 µl of lysis buffer (consisting of 1 mg of proteinase K, 5 µl of 1.0 M Tris, 1 µl of 0.5 M EDTA, 5 µl of detergent, and 989 µl of sterile water). Samples were incubated at 65 °C for 15 min, and then at 95 °C for 10 min.

To determine whether any symbionts other than the CLO are present in the sexual population of *E. pergandiella*, we used the universal bacterial 16S rDNA primers, fD1 and rP2 (Weisburg *et al.* 1991) to amplify the bacterial sequence from a single wasp and then cloned the resulting PCR product using the TOPO TA Cloning kit from Invitrogen (chemical transformation with kanamycin selection). These primers are commonly used to amplify bacterial symbiont 16S rDNA, including *Wolbachia* (e.g. Arakaki *et al.* 2001). Thirty-one positive clones were purified and sequenced on an ABI 3700 sequencer at the Genomic Analysis and Technology Core (GATC) at the University of Arizona. The sequence of each clone was compared with other sequences in GenBank using BLAST (www.ncbi.nlm.nih.gov/ blast) (Altschul *et al.* 1997). We were unable to obtain PCR product from single wasps using two other general bacterial 16S rDNA primer pairs, 63F and 1387R, and 27F and 1392R, that have been used in environmental sampling of diverse bacteria (see Marchesi *et al.* 1998; O'Sullivan *et al.* 2002). Finally, we used two *Wolbachia*-specific primer pairs, 16S rDNA and *ftsZ* (O'Neill *et al.* 1992; Werren *et al.* 1995) to test for the presence of *Wolbachia* in *E. pergandiella*.

3. RESULTS

(a) *Testing for cytoplasmic incompatibility*

The number of pupal offspring produced was significantly different among the four crosses (figure 1*a*; table 1). Females in the predicted incompatible cross produced 12.6% of the number of offspring produced in the other crosses. The number of developmentally arrested whiteflies was also highly significantly different among crosses (figure 1*b*; table 1); here many more arrested whiteflies were produced in the cross predicted to be incompatible than in any of the other crosses. The separate dissection experiment described below suggested that these developmentally arrested whiteflies were parasitized but the wasp
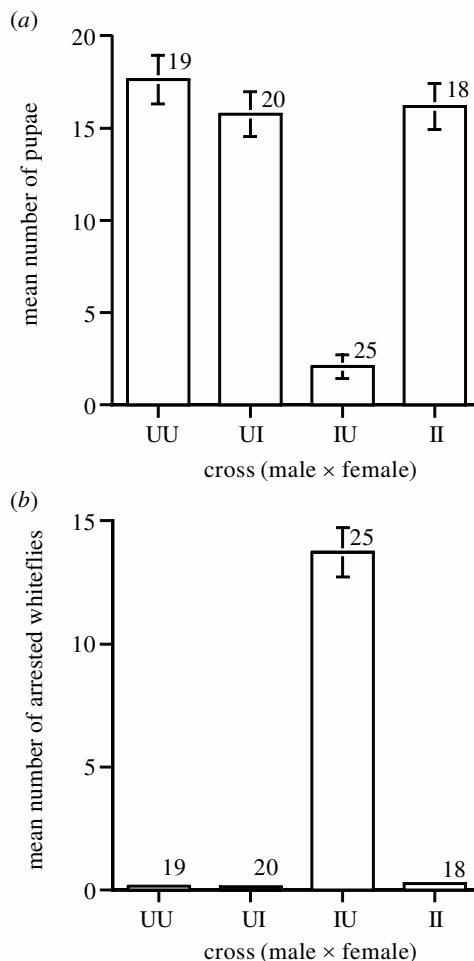

Figure 1. (*a*) The mean number of viable pupae of *Encarsia pergandiella* (± s.e.m.) produced in crosses of infected (I) and uninfected (U) males and females. In the cross designations the male type is listed first. The numbers above the bars indicate the number of replicates. All pupae produced were female. The mean number of pupae produced was highly significantly different among crosses (analysis of deviance, Poisson errors, $\chi^2_{3\,\text{d.f.}} = 137.10$, $p < 0.001$), with the IU treatment producing only 12.6% the number of progeny produced in the other three crosses. (*b*) The mean number of developmentally arrested whitefly nymphs (± s.e.) in arenas of the four cross types. The number of developmentally arrested whiteflies was highly significantly different among crosses (analysis of deviance, Poisson errors, $\chi^2_{3\,\text{d.f.}} = 721.00$, $p < 0.001$), with the most being produced in the IU cross. Dissections of developmentally arrested whiteflies in a later experiment indicated that most were parasitized, but that the eggs did not hatch.

progeny did not complete development. The other variables, number of emerged whiteflies, number of dead whiteflies and number of dead late larval–early pupal wasps, were not significantly different with respect to cross, nor were block or the block × treatment interaction (table 1). Finally, we obtained very similar results when we repeated the experiment with two more independent, uninfected lines obtained by antibiotic curing (data not shown).

Dissections of whiteflies indicated that the non-viable offspring of the incompatible cross do not progress beyond the egg stage (figure 2). Five days after removal of females, unparasitized whiteflies had eclosed, and only apparently parasitized, developmentally arrested whiteflies were left

Table 1. Whitefly hosts and wasp offspring production in crosses of uninfected (U) and infected (I) individuals, with males listed first. In each arena, 30 whitefly nymphs were presented to individual wasps.

cross type	n	*Encarsia pergandiella* pupae[a]	developmentally arrested whiteflies[b]	emerged whiteflies	dead whitefly nymphs	late larval–early pupal dead *E. pergandiella*
I × U	25	2.08 ± 0.63[c]	13.72 ± 0.99	11.32 ± 1.03	2.32 ± 0.21	0.52 ± 0.19
U × U	19	17.63 ± 1.31	0.16 ± 0.09	10.00 ± 1.42	1.84 ± 0.21	0.58 ± 0.18
U × I	20	15.75 ± 1.20	0.15 ± 0.08	11.45 ± 1.27	1.60 ± 0.17	1.05 ± 0.25
I × I	18	16.17 ± 1.24	0.28 ± 0.11	10.50 ± 1.51	2.11 ± 0.40	1.06 ± 0.31
deviance						
cross		137.10***	721.00***	0.71	3.66	4.57
block		0.58	0.10	1.98	4.34	0.13
cross × block		3.85	1.93	2.768	0.01	0.69
d.f.		3	3	3	3	3

[a] All pupae were female.
[b] This category includes all *E. pergandiella* that died early in development, before the whitefly was consumed by the wasp larva.
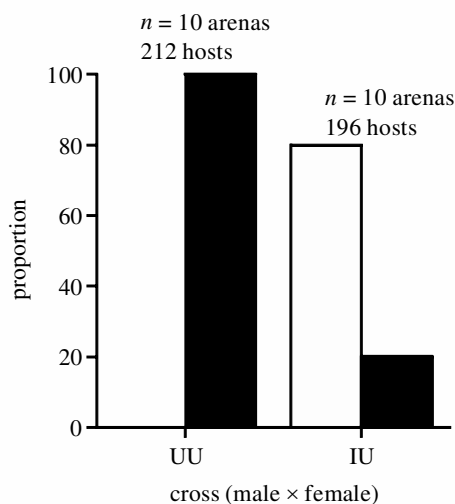[c] Mean ± s.e.m.
*** $p < 0.001$.

Figure 2. The proportion of parasitized, developmentally arrested whiteflies containing egg (open bar) and larval (filled bars) *Encarsia pergandiella* in the control (UU) and predicted incompatible (IU) cross.

to dissect. In the control cross, almost all (99%) dissected hosts were parasitized. In 100% of these parasitized hosts, dissections revealed healthy late instar larvae (figure 2). In the incompatible cross, only 65% of the dissected hosts contained detectable wasp eggs or larvae (figure 2), but of these, 80% were eggs and 20% healthy late instar larvae. The eggs found were sometimes fragile relative to healthy eggs. We suspect that the difference in apparent rates of parasitism of the developmentally arrested whiteflies between the treatments may be due to disintegration of fragile, dead eggs before or during dissection in the incompatible cross arenas. Consistent with this interpretation, if one assumes that the apparently unparasitized, arrested whiteflies in the incompatible cross contained eggs that were not detected, the proportion of wasp progeny that were healthy larvae in this cross was 12.7%. This estimate is remarkably close to that of the progeny production of the incompatible cross relative to the other crosses found in the experiment described above (12.6%).

(b) *16S rDNA cloning*

Twenty-six out of the 31 16S rDNA clones we sequenced were identical, yielding a 1487 bp sequence that was 99% similar to the 16S rDNA sequence of the PI symbiont of Brazilian *E. pergandiella* (GenBank accession number AY026335). The sequences of the five remaining clones were all singletons, and were most probably wasp gut or general contaminant bacteria. The apparent identity of these clones was: (i) *Pseudomonas aeruginosa* (e.g. 99% similarity to AJ249451); (ii) a clone that was 97–99% similar to *Sphingomonas* sp. (e.g. AF494538, X94100 and U37341); (iii) an '*Acidobacterium*'-like clone that was 97–99% similar to accessions such as AY154482 and AF312219; (iv) an unidentified α-Proteobacteria with 95% similarity to the uncultured sample corresponding to AF293006; and (v) an unidentified *Enterobacter* with 98–99% similarity to accessions such as *Enterobacter aerogenes* (AF395913), *Enterobacter asburiae* (AB004744) and *Cedecea neteri* (AB086230). None of the clone sequences was *Wolbachia*, and, as was found previously (Zchori-Fein *et al.* 2001), we were unable to amplify DNA from either sexual or asexual *E. pergandiella* lineages using *Wolbachia*-specific primers.

4. DISCUSSION

(a) *Cytoplasmic incompatibility in* E. pergandiella

The results presented here strongly suggest that the CLO symbiont induces cytoplasmic incompatibility in the sexual *E. pergandiella* from Texas. To our knowledge, this is the first study demonstrating that bacteria other than *Wolbachia* are able to induce the type of CI that results in host non-viability. There is one described case of CI in *Drosophila paulistorum* (Diptera: Drosophilidae) that results in the production of sterile male offspring but fertile females. This unusual type of CI is caused by streptococcal L-form bacteria (Somerson *et al.* 1984). By contrast, the CI phenotype induced by the CLO symbiont appears indistinguishable from that caused by CI inducing *Wolbachia*. We observed an incompatibility that was severe but not complete. Females in the incompatible cross produced, on average, 12.6% of the number of daughters produced by females in

the compatible crosses. Incomplete compatibility is common in CI *Wolbachia* infections (Hoffmann & Turelli 1997). After 5 days, parasitized whitefly hosts in the incompatible cross did not generally show the advanced stages of parasitism seen in the other crosses but were developmentally arrested, and when dissected, most were found to contain dead or dying wasp eggs. The proportion of healthy larvae found upon dissection of hosts in this treatment was similar to the proportion of viable offspring produced in the experiment in which offspring were reared to pupation. *Wolbachia* infections associated with CI similarly lead to egg mortality in the incompatible cross in most instances (Hoffmann & Turelli 1997). In haplodiploid systems, incompatible eggs may sometimes develop as normal males after the paternal set of chromosomes is destroyed (Breeuwer & Werren 1990), or female embryos may die (Breeuwer 1997; Vavre *et al.* 2000) or both patterns may be exhibited, even within crosses (Perrot-Minnot *et al.* 2002). In this study, the type of CI appears to be the embryo mortality type rather than the male production type, but the interpretation of this is complicated by the biology of the parasitoid hosts. In autoparasitoid species such as *E. pergandiella*, males do not ordinarily develop in whitefly hosts, even if male eggs are laid there (Hunter & Woolley 2001). Thus, the results obtained could also be consistent with the male production type of haplodiploid CI, if eggs died simply because male embryos were unable to develop in unsuitable whitefly hosts.

(b) *A new lineage of symbiont causes multiple host reproductive effects*

The cytoplasmic incompatibility documented in this study is the third reproductive manipulation phenotype associated with infection of arthropod hosts with the CLO symbiont. Earlier studies showed an association of this symbiont with parthenogenesis in *Encarsia* parasitoids (Zchori-Fein *et al.* 2001) and feminization in the mite *Brevipalpus phoenicis* (Weeks *et al.* 2001). In the latter case, haploid, incipient male eggs develop as females when infected with the symbiont. These findings make the CLO second only to *Wolbachia* in the diversity of host phenotypes induced. *Wolbachia* has also been associated with male killing (Stouthamer *et al.* 1999) and mutualistic relationships with its arthropod and nematode hosts (Bandi *et al.* 2001; Dedeine *et al.* 2001). However, given the recent discovery of the CLO, it seems likely that other host phenotypes will also be found for this lineage.

This study further implicates the large and understudied *Bacteroidetes* group of bacteria in diverse symbioses. In addition to the reproductive manipulations of the CLO described here, *Bacteroidetes* group representatives also serve as the primary symbionts of cockroaches where they are housed in specialized cells in the fat body and appear to act as mutualists (Bandi *et al.* 1994). *Bacteroidetes* symbionts have also been implicated in male killing in coccinellid beetles (Hurst *et al.* 1999), and have been found as symbionts of sharpshooter insects (Moran *et al.* 2003) and acanthamoebae (Horn *et al.* 2001).

How do closely related symbionts cause diverse effects on their hosts? The symbionts of the sexual and parthenogenetic lineages of *E. pergandiella* share *ca.* 99% sequence similarity in 16S rDNA and yet induce completely different phenotypes, PI and CI, in different populations of the same host species. This may suggest that the mechanisms for these two phenotypes are similar, and that, like *Wolbachia*, transitions between phenotypes occur readily. Very closely related *Wolbachia* lineages have been found to cause different phenotypes in their host (van Meer *et al.* 1999). Further, *Wolbachia* can induce completely different phenotypes when experimentally transferred into novel hosts (Sasaki *et al.* 2002). No current models adequately explain the relative role of host and symbiont factors in determining the host phenotype in *Wolbachia*. Similarly, the mechanism by which host phenotypes are induced is still largely unknown, although both CI and PI in *Wolbachia*-infected hosts involves targeting and manipulation of host chromosomes (Gottlieb *et al.* 2002; Tram & Sullivan 2002). Clearly, the relationship between CI and PI in CLO-infected wasps requires further exploration. Experimental transfers of the symbionts will help elucidate whether host or symbiont factors are more influential in host phenotype.

The similarity in both the range of reproductive alterations produced by *Wolbachia* and the CLO, as well as the apparent facility of transitions among host phenotypes, begs the question of whether convergence or homology explains the remarkable similarity between the effects of these two unrelated symbiont lineages. Within *Wolbachia* lineages, the lack of concordance among phylogenies for particular gene sequences suggests recombination among strains (Werren & Bartos 2001), and the abundance of phage and transposon sequences within the *Wolbachia* genome may suggest a mechanism of exchange (Masui *et al.* 2000). Lateral gene transfer across unrelated bacterial lineages is one of the most important forces shaping bacterial evolution (Ochman *et al.* 2000). Interestingly, a recent experimental study documented high rates of lateral gene transfer between bacteria coinfecting flea guts, suggesting a potential for transfer between insect associates (Hinnebusch *et al.* 2002). With the imminent publication of several *Wolbachia* genomes, it may soon be possible to determine the genetic bases of reproductive parasitism, as well as whether other reproductive parasites share homologous genes with *Wolbachia*.

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