Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont Spiroplasma

Marie Pollmann, Logan D. Moore, Elena Krimmer, ..., Matthew J. Ballinger, Johannes L.M. Steidle, Yuval Gottlieb
jsteidle@uni-hohenheim.de

Highlights

- A Spiroplasma strain induces cytoplasmic incompatibility (CI) in a parasitoid wasp
- Spiroplasma and CI are efficiently transferred to naive hosts by hemolymph injection
- The CI-Spiroplasma genome contains potential CI-causing genes, but no Wolbachia cif
- Absence of cif genes in the Spiroplasma genome suggests an independent origin of CI

Pollmann et al., iScience 25, 104335
May 20, 2022 © 2022 The Author(s).
https://doi.org/10.1016/j.isci.2022.104335
Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont Spiroplasma

Marie Pollmann, Logan D. Moore, Elena Krimmer, Paul D’Alvise, Martin Hasselmann, Steve J. Perlman, Matthew J. Ballinger, Johannes L.M. Steidle, and Yuval Gottlieb

SUMMARY

Cytoplasmic incompatibility (CI) is a form of reproductive manipulation caused by maternally inherited endosymbionts infecting arthropods, like Wolbachia, whereby matings between infected males and uninfected females produce few or no offspring. We report the discovery of a new CI symbiont, a strain of Spiroplasma causing CI in the parasitoid wasp Lariophagus distinguendus. Its extracellular occurrence enabled us to establish CI in uninfected adult insects by transferring Spiroplasma-infected hemolymph. We sequenced the CI-Spiroplasma genome and did not find any homologues of any of the cif genes discovered to cause CI in Wolbachia, suggesting independent evolution of CI. Instead, the genome contains other potential CI-causing candidate genes, such as homologues of high-mobility group (HMG) box proteins that are crucial in eukaryotic development but rare in bacterial genomes. Spiroplasma’s extracellular nature and broad host range encompassing medically and agriculturally important arthropods make it a promising tool to study CI and its applications.

INTRODUCTION

A number of maternally transmitted microorganisms in arthropods have evolved the remarkable ability to manipulate their hosts’ reproduction, in order to increase the frequency of infected hosts (Engelstädter and Hurst, 2009; Moran et al., 2008). The most common of these manipulations is cytoplasmic incompatibility (CI), whereby uninfected females produce few or no offspring upon mating with infected males (Shropshire et al., 2020; Werren, 1997). As a result, infected females have a significant advantage over their uninfected counterparts and can rapidly spread and replace them in the population (Shropshire et al., 2020; Werren, 1997).

In an important advance, the genetic basis of CI was recently discovered in the best studied CI microbe, the Alphaproteobacterium Wolbachia pipiensis, and involves two linked genes, termed cifA and cifB (for cytoplasmic incompatibility factor). They operate in a manner similar to toxin-antitoxin systems, with one or both cif genes modifying or poisoning infected male sperm, and cifA alone rescuing incompatibility in the eggs of infected females (Adams et al., 2021; Beckmann et al., 2017; LePage et al., 2017). Without cifA rescue, paternally derived chromosomes are destroyed, resulting in embryonic lethality (Beckmann et al., 2017) or haploid male development in some species with haplodiploid sex determination (Vavre et al., 2001).

There is great interest in using CI microbes to control arthropod pests and disease vectors. This can be done in two ways, both of which involve establishing a new, stable CI infection in the target host species (Brelsfoard and Dobson, 2009). First, analogous to sterile insect techniques, large quantities of infected incompatible males can be released in the field, which will result in population crashes due to their mating with wild uninfected females (Brelsfoard and Dobson, 2009). Alternatively, CI microbes can be used to spread desired traits when females and males infected with a novel CI microbe are released in the wild (Brelsfoard and Dobson, 2009). This approach has been proven highly successful in reducing the prevalence of dengue virus in humans by releasing Aedes aegypti mosquitoes infected with a strain of the bacterial symbiont Wolbachia that causes both CI and suppresses viruses (Hoffmann et al., 2011; Utarini et al., 2021). Wolbachia-infected mosquitoes reach high frequencies due to CI, and this results in reduced dengue virus titer and transmission (Walker et al., 2011).
Wolbachia is by far the best known and most common CI-inducing microbe. It was first shown to cause CI in Culex pipiens mosquitoes in the 1970s and has since been found to cause CI in at least 10 arthropod orders. In 2003, a second unrelated bacterial symbiont, Candidatus Cardinium hertigii (hereafter referred to as Cardinium), in the Bacteroidetes, was found to cause CI in a parasitic wasp (Hunter et al., 2003), with later studies extending this phenomenon to mites (Gotoh et al., 2007), plantthoppers (Nakamura et al., 2012), and thrips (Nguyen et al., 2017). Although Cardinium-induced CI also involves modification of male sperm and rescue in females, sequencing its genome revealed that it does not contain homologues of the Wolbachia cif genes (Penz et al., 2012), indicating independent evolution of CI. More recently, a new bacterium in the same family as Wolbachia, called Candidatus Mesenet longicola (hereafter referred to as Mesenet), and a strain of the Gammaproteobacterium Rickettsiella were shown to cause CI in a beetle and a spider, respectively, but so far, little is known about them and the CI they induce (Rosenwald et al., 2020; Shropshire et al., 2020; Takano et al., 2017, 2021).

Here, we report the discovery of a surprising new CI microbe that infects Lariophagus distinguendus (Forster 1841) (Hymenoptera: Pteromalidae), a cosmopolitan parasitic wasp that is commonly used in biological control of beetle pests of stored products. Recently, we reported CI in L. distinguendus, marked by the absence of diploid female offspring in incompatible crosses (König et al., 2019). Interestingly, we detected neither Wolbachia nor Cardinium in wasps from the incompatible line (König et al., 2019).

Unexpectedly, we show that CI in L. distinguendus is caused by a maternally transmitted strain of Spiroplasma, a diverse lineage of microbes in the Gram-positive Mollicutes that includes pathogens and endosymbionts, as well as several vertically transmitted endosymbionts, and infects a wide range of invertebrates (Gasparich, 2002; Regassa and Gasparich, 2006). Some vertically transmitted Spiroplasma produce female-biased sex ratios as male killers, whereas others protect their hosts against parasitic nematodes, wasps, and pathogenic fungi (Gasparich, 2002; Regassa and Gasparich, 2006).

Spiroplasma’s localization in the hemolymph throughout host life facilitates rapid and efficient transfer of heritable infections to new hosts in the laboratory (Anbutsu and Fukatsu, 2011; Ballinger and Perlman, 2019). Indeed, we were able to transfer Spiroplasma and its CI phenotype to uninfected wasps using microinjection. This easy transferability and broad host range promise new possibilities for research into CI and its applications. Finally, we sequenced the genome of CI-inducing Spiroplasma. It contains no homologues of the Wolbachia cif genes, demonstrating a different genetic basis underlying Spiroplasma-induced CI. Instead, the genome harbors a number of interesting candidate genes, including novel toxins, genes containing ankyrin repeat domains, and eukaryotic high-mobility group (HMG) box proteins.

RESULTS
Bacteria induce CI in L. distinguendus
Tetracycline-treated and untreated females and males of the STU strain of L. distinguendus were crossed in all possible combinations, and offspring production was compared. Crosses between tetracycline-treated females and untreated males produced significantly fewer daughters (p < 1 × 10^{-3} *** in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons) and more sons (p < 0.001 *** in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons) with no difference in total offspring numbers (p > 0.05 n.s. in single comparisons with all other combinations, GLM, family = negative binomial, followed by Tukey test for multiple comparisons), which is indicative of the presence of CI-inducing bacteria (Figure 1, for full test statistics see Table S1). Increased male production is common in CI in hosts with haplodiploid sex determination, with incompatible fertilized embryos developing into males (Vavre et al., 2001).

Spiroplasma is the only reproductive manipulator
Individual wasps were tested for endosymbionts known to act as reproductive manipulators using PCR. Previously, we showed that the STU strain is not infected with either Wolbachia or Cardinium (König et al., 2019). Here, we also screened for Rickettsia, Arsenophonus, Mesenet, and Rickettsiella, as well as Spiroplasma. All tested STU females (n = 10) and 90% of the tested STU males (n = 10) were found to carry Spiroplasma, hereafter referred to as sDistinguendus (sDis); none of the other symbionts were present. In order to reveal a potential involvement of other bacteria, we used 16S rRNA amplicon sequencing of the whole bacterial community in five separate pooled samples of STU females. Overall, we found 12 amplicon sequence variants (ASVs), of which only five were present in at least four of the five samples
sequenced. One ASV was identified as *Spiroplasma* and was present in all samples with an average of 8,258.4 total reads. Three ASVs were members of the Proteobacteria: a strain of *Yersinia* (4/5 samples, average of 22.6 total reads), a strain of *Pseudomonas* (4/5 samples, an average of 20.6 total reads), and an unidentified member of the Enterobacteriaceae (5/5 samples, average of 8411.6 total reads). The role of the unknown Enterobacteriaceae in CI was dismissed, as the rate of infection did not differ between tetracycline-treated (n = 5, 100% infection) and untreated (n = 4, 75% infection) STU females as well as CI-inducing (n = 4, 100% infection) and non-CI-inducing (n = 3, 100% infection) STU males (2x4 Fisher’s Exact Test for Count Data, p = 0.6875). A standard nucleotide BLASTn (Altschul et al., 1990) of the consensus sequence resulted in many close hits of the same rank (Table S3) and a comparison with only the NCBI rRNA/ITS database found *Enterobacter cancerogenus*, for which a resistance to tetracycline has been shown before (Zwenger et al., 2008), to be the best match (99% coverage, 99.53% identity, E value = 0.0, GenBank: NR_044977.1).

The final ASV was identified as *Enterococcus* (phylum Firmicutes) with an average of 138.6 total reads (see Table 1 for full ASV count table). As sDis was the dominant bacterium and only potential reproductive manipulator, we set about characterizing it and establishing its role in CI.

**sDis is present in the ovaries of *L. distinguendus***

To determine the presence of sDis in wasp ovaries, we performed fluorescent in situ hybridization (FISH) using the *Spiroplasma*-specific probe SPR and anti-sense and no-probe controls. Specific, localized signals were obtained with SPR in the ovaries of STU females (Figure 2).

**sDis can be transferred to a noninfected host and induce CI***

To demonstrate that the CI phenotype is ultimately induced by sDis, we performed a transinfection experiment. As a prerequisite, hemolymph from STU females was tested for infection with sDis. For the
transinfection, hemolymph from STU females was injected into endosymbiont-free STU females. Of these, 27 (69.2%) were positive for sDis at the end of the experiment. These females were consecutively offered different batches of koi pellets infested with host beetle larvae to parasitize for two to three days each. The first three host batches parasitized by positive females produced sDis-negative offspring. Positive males started to emerge from the fourth batch and increased in proportion up to 100% in the last two batches (Figure 3 A). Eventually, 15 of the positive injected females (56%) were found to have sDis-positive F1 male offspring.

Newly hatched F1 male offspring of injected females were mated to endosymbiont-free STU females, and the presence of CI was inferred from the number of F2 females and F2 males. sDis-positive F1 males (n = 82) sired significantly fewer F2 females than uninfected F1 males (n = 36) (Wilcoxon rank-sum test with continuity correction, W = 2,718; p = 3.58 × 10^{-13}***; Figure 3 B), whereas the number of F2 male offspring was significantly higher in crosses with positive F1 males (Wilcoxon rank-sum test with continuity correction, W = 839, p = 0.0001971***), and total F2 offspring number did not differ between the crosses (Welch two sample t test, t = 1.341, p = 0.1853 n.s.) (Figure 3 B), recreating the CI phenotype shown by Figure 1. This demonstrated that CI was induced by sDis and that sDis could be transferred into previously uninfected wasps by injection of hemolymph from infected wasps, maintaining its CI-inducing effect.

In order to examine whether the strength of CI was influenced by Spiroplasma titer, we conducted qPCR on the DNA samples of mated sDis-positive F1 male offspring of receiver females using dnaA as a target gene. There was no correlation between Spiroplasma titer in F1 male wasps and proportion of female offspring (number of female F2 offspring divided by total number of F2 offspring) used as proxy for CI level (Spearman’s rank correlation, n = 36, rho = 0.171, p = 0.319).

**Genome of sDis**

To explore the genetic basis of sDis-induced CI, we sequenced total genomic DNA extracted from adult *L. distinguendus* strain STU to 30x coverage (Figure S1). Phylogenomic analysis supported the position of sDis within an understudied Spiroplasma group, the Ixodetis clade, which is the most basal of the Spiroplasma clades (Gasparich et al., 2004) and includes strains that induce male killing in diverse insect hosts, including butterflies (Jiggins et al., 2000; Tabata et al., 2011) and beetles (Hurst et al., 1999; Majerus et al., 1999; Tinsley and Majerus, 2006) (Figure 4 A).

Genome completeness was estimated at 96% based on the presence of all but five conserved single-copy Mollicutes orthologs in the BUSCO database. We searched manually for the missing orthologs and identified near-complete ORFs encoding four: recA, rlmB, rplL, and metS. We did not find a putative homologue of the remaining gene, rsml, an rRNA ribose-2’-O-methyltransferase, in sDis or in any other Ixodetis clade Spiroplasma genome. Although this gene has a conserved role in bacterial translation, it appears to have been lost in this clade. Thus, the 96% BUSCO estimate is an underestimate. The draft assembly is

| Total # of reads | Average # of reads | Class | Order | Family | Genus | Species |
|------------------|-------------------|-------|-------|--------|-------|---------|
| 42,058           | 8411.6            | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | N/A | N/A |
| 41,292           | 8258.4            | Mollicutes | Entomoplasmatales | Spiroplasmataceae | Spiroplasma | secondary |
| 693              | 138.6             | Bacilli | Lactobacillales | Enterococccaeae | Enterococcus | N/A |
| 113              | 22.6              | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Yersinia | N/A |
| 103              | 20.6              | Gammaproteobacteria | Pseudomonadales | Pseudomonadaeae | Pseudomonas | N/A |
| 12               | 2.4               | Bacilli | Bacillales | Bacillaceae | N/A | N/A |
| 11               | 2.2               | Gammaproteobacteria | Pseudomonadales | Pseudomonadaeae | Pseudomonas | N/A |
| 10               | 2                 | Alphaproteobacteria | Rhodobacteriales | Rhodobacteriaceae | Rubellimicrobiun | N/A |
| 6                | 1.2               | Bacilli | Lactobacillales | Lactobacillaceae | Lactobacillus | N/A |
| 4                | 0.8               | Actinobacteria | Microcccales | Microccocaceae | N/A | N/A |
| 2                | 0.4               | Bacteroidia | Flavobacteriales | Flavobacteriaceae | Flavobacterium | resistens |

Table 1. ASV count table from the NGS analysis of the microbiome of the *L. distinguendus* strain STU. See Table S3 for BLASTn matches to the 16S rRNA sequence of the Enterobacteriaceae.
1.16 Mb in length and includes two closed plasmids (Table S6). Intact and pseudogenized insertion sequence transposes are abundant, comprising about 7.3% of the draft assembly. Of 1,175 predicted protein-coding genes, 462 could be assigned functional predictions in the form of KEGG numbers. Additional assembly statistics are presented in comparison to recently sequenced Ixodetis clade Spiroplasma in Table 2.

The sDis symbiont encodes complete ATP synthase and glycolysis pathways, as well as transport components for glucose, fructose, GlcNAc, glycerol, and possibly mannose (Figure 5). Amino acid and lipid biosynthesis pathways are incomplete or absent, as reported previously for hemolymph-dwelling Spiroplasma species, including those in the Ixodetis clade (Martin et al., 2020; Vera-Ponce León et al., 2021; Yeoman et al., 2019). Incomplete pathway components were identified for lysine (dapE), valine, leucine, and isoleucine (ilvA), and arginine (OTC) synthesis. In addition to the PTS genes for carbon import, ABC membrane proteins for transport of phosphate (ptsABCS), nucleosides (bmpA, nupABC), and micronutrients (ecfa1A2T) could be identified. A comparative summary of membrane transport, biosynthesis, and metabolism genes in sDis and two other Ixodetis clade genomes is present in Table S4.

Known CI-related genes are absent from sDis

We searched the sDis genome for potential homologues of the Wolbachia CI genes (i.e. cifA and cifB), as well as the Spiroplasma male killing toxin gene spaid and the candidate Wolbachia male killing gene wmk. We did not find any cif, spaid, or wmk gene homologues. We found two OTUs and two PDDEXK genes that contained domains associated with type V cifB-like proteins, but closer investigation revealed only distant homology to the domains found in CI-inducers. Specifically, the OTU cysteine proteases of sDis are related to OTU domains of other Spiroplasma strains, while Wolbachia cifB OTU domains are in a different clade, and cluster with some Rickettsia, Occidentia, and Mesenet OTU domains (Figure S3, see Table S5 for a summary of search results for bacterial toxins and manipulation factors).

Candidate manipulation genes and virulence factors

Genome annotation and targeted tBLASTn searches revealed numerous symbiont genes with potential to impact key host structures and processes including development regulation, protein translation, protein degradation, and cell membrane integrity. The genome encodes four eukaryotic high-mobility group (HMG) box proteins. HMG1–3 bear domain similarity to HMG box proteins in the SOX-TCF family of DNA binding transcription factors that play crucial roles in embryonic development (Figure 4B). HMG2, 3, and 4 are found on small contigs (< 2000 bp), whereas HMG1 is present on a 25 kbp unclosed plasmid-like contig (MAEMCOA_7; Table S6). HMG1 and the genes flanking it share greater sequence similarity with other Ixodetis clade Spiroplasma and with spirochetes than with Apis and Ctri clade.
 Spiroplasma. Flanking genes include plasmid partitioning and replication genes, conjugation proteins, insertion sequence elements, and uncharacterized proteins (Table S6).

We identified two translation-targeting toxins: a ribosome-inactivating protein (RIP) and a putative tRNA-inactivating nucleotidyltransferase distantly related to type IV abortive infection systems and absent from almost all other Spiroplasma (Cai et al., 2020) (Figure S3). RIPs have been implicated in Spiroplasma-mediated protection against natural enemies (Hamilton et al., 2016). We also identified one gene related to a pore-forming ETX/MTX2 toxin (Figure S3). Ankyrin repeat domains were identified in twelve genes (Figure S4). Several sDis ankyrin genes share similarities with Wolbachia and Cardinium genes (Table S6).

Two sDis proteins contain OTU-like cysteine protease domains. This protease is one of three functional domains in the male killing Spiroplasma toxin, spaid (Harumoto and Lemaitre, 2018), although neither of the sDis domains bears strong amino acid sequence similarity to the spaid domain (Figure S3). One of the two OTU domains is closely related to homologues in the Ixodetis clade Spiroplasma symbionts of Danaus chrysippus and Dactylopius coccus, whereas the second is phylogenetically separate. The latter is present within a large hypothetical protein (957 aa) and is predicted to have a C-terminal domain with structural homology to staphopain and staphostatin, a cysteine protease and cysteine protease inhibitor from Staphylococcus sp., respectively.

DISCUSSION

In this study, we demonstrate that the widespread symbiotic bacterium Spiroplasma causes cytoplasmic incompatibility in an insect host and show that the symbiont and phenotype can be efficiently transmitted by adult recipients following hemolymph microinjection. The benefits of understanding and applying CI have motivated decades of studies developing and optimizing protocols for symbiont transfer.

Prior to the present study, all CI-inducing bacteria have been primarily intracellular symbionts. There have been successful transfers of Wolbachia using hemolymph injection (Frydman, 2007), but most transfections of CI-inducing symbionts require challenging techniques and specialized equipment for transfer into very young recipient embryos, often with limited success (Duplouy et al., 2013; Hughes and Rasgon, 2014; Walker et al., 2011; Xi et al., 2005; Zabalou et al., 2004).
Spiroplasma's ecology as a hemolymph-dwelling bacterium bypasses all of these challenges.

In our study system, we were able to establish CI-Spiroplasma by adult-to-adult microinjection, mirroring previous results showing high success of Spiroplasma transfer between fruit fly hosts using this method (Ballinger and Perlman, 2019; Haselkorn and Jaenike, 2015). Our horizontal transfer experiments also suggest that the strength of CI in Spiroplasma is not related to overall bacterial titer, which also has positive implications for the success of transferring CI to new hosts.

As a new phenotype for Spiroplasma, this discovery presents exciting new avenues for basic and applied research in arthropod reproductive manipulation. Like other facultative symbionts, Spiroplasma has a broad host range that does not reflect host or symbiont phylogeny (Binetruy et al., 2019; Gasparich et al., 2004). Closely related Spiroplasma ixodetis clade strains infect fruit flies, aphids, ticks, sawflies, scale insects, and spiders. Reproductive manipulations performed by members of this group can also affect many different arthropod hosts, as evident by the male killing strains in butterflies and beetles (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 1999; Tabata et al., 2011; Tinsley and Majerus, 2006)—and now a CI strain in wasps. This broad host manipulation range suggests a potential for the transfer and maintenance of sDis to diverse arthropod hosts of agricultural, medical, and basic research relevance that should be a focus of future studies. Beyond the Ixodetis clade, the host range of Spiroplasma is even broader, with strains infecting terrestrial and aquatic arthropods (Regassa and Gasparich, 2006; Wang et al., 2011), jellyfish (Cortés-Lara et al., 2015), and sea cucumbers (He et al., 2018). If the ease of Spiroplasma transfer can be replicated for interspecific transfers of sDis as well, it will facilitate similar investigations of sDis's reproductive effects in other insect hosts. Finally, a number of Spiroplasma strains, including vertically transmitted male killers, can be grown in cell-free media and genetically transformed (Masson et al., 2018, 2020), which has the potential to greatly facilitate the study and manipulation of CI.

The discovery that Spiroplasma can cause CI adds to a growing list of microbial symbionts, such as Rickettsiella and Mesenet, that join Wolbachia and Cardinium in the small club of CI microbes. As infection with multiple symbionts is common, this suggests that if we observe CI, we should be careful not to assume that it is being caused by Wolbachia. However, it is possible to use differing susceptibility to antibiotics to link CI to a specific symbiont; for example, Wolbachia is highly susceptible to rifampicin, whereas Spiroplasma is resistant (Jaenike et al., 2010).
The sDis genome provides a first look at gene candidates potentially involved in Spiroplasma CI. The notable absence of cif homologues mirrors observations from the genome of CI-Cardinium, suggesting independent evolution of CI. It is striking that the cytological phenotype of CI is similar in both Wolbachia and Cardinium despite being caused by the expression of different genes, suggesting a convergent development to affect the same targets in the hosts (Gebiola et al., 2017; Mann et al., 2017; Penz et al., 2012). Although it is possible that some sDis genes are missing from our draft assembly, we note that our genome is more complete than the 96% estimate obtained by BUSCO analysis, as four of the five missing genes could be identified by manual BLAST and the last is absent from all Ixodetis clade Spiroplasma genomes sequenced to date. Identification of eukaryotic HMG box DNA-binding proteins in sDis may be of interest given the shared chromatin-bridging phenotype in these other systems. For example, in Drosophila melanogaster, HMG box-like proteins play a crucial role in chromatin condensing during spermatogenesis (Doyen et al., 2015; Rathke et al., 2010). Interestingly, depletion of maternally provided chromatin decondensing proteins during fertilization results in complete sterility in Drosophila. With the exception of spirochetes, homologous HMG genes are rare in bacteria, yet we identified gene families in sDis as well as two closely related Spiroplasma symbionts, including the male killing symbiont of Danaus chrysippus (DCF). Indeed, it is interesting that closely related Spiroplasma Ixodetis clade strains cause male killing and CI, as previous studies have demonstrated a close link between CI and male killing in Wolbachia. For example, multiple Wolbachia CI-inducing strains have been shown to act as male killers following introgression into different host genetic backgrounds (Jaenike, 2007; Sasaki et al., 2002, 2005).

The sDis genome also encodes genes with a collection of protein domains, such as OTU-like cysteine proteases and ankyrin repeats, that are present in spaid, the toxin that causes male killing in Spiroplasma poulsonii (Harumoto and Lemaitre, 2018). Both the cif and spaid are evolutionarily dynamic genes—with cif being widespread and rapidly evolving components of WO prophage regions in Wolbachia genomes (Lepage et al., 2017; Lindsey et al., 2018) and spaid being plasmid-encoded (Masson et al., 2018). In this context, plasmid-encoded genes may be the strongest candidates for CI in sDis. The two closed plasmids and two additional candidate plasmids of sDis encode an HMG box protein and ankyrin-domain-containing proteins, as well as numerous hypothetical proteins absent functional predictions. A common feature of facultative symbiont genomes and those of Spiroplasma especially is an abundance of uncharacterized protein coding genes (Duplouy et al., 2013; Paredes et al., 2015; Siozios et al., 2019). Nearly half of the predicted genes of sDis lack functional annotations based on their amino acid sequences. Future studies will be crucial to identify further gene candidates and characterize their role in this new CI system.

Limitations of the study

We successfully transferred Spiroplasma and the CI it induces to uninfected Lariophagus wasp hosts via hemolymph injection. A critical next step will be to determine the potential host range of this strain of Spiroplasma, along with its ability to induce CI, by attempting to establish infections in different and more distantly related host species. Also, although our nearly complete sDis draft genome suggests several CI candidate genes and an independently evolved route to CI, the function of these candidates and their potential role in CI have not yet been tested. In addition, further sequencing is required to close the sDis assembly and confirm its completeness.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
METHOD DETAILS
- Crossing experiments
- Specific testing for bacteria
- Next generation sequencing of the microbiome
- Testing for Enterobacteriaceae
- Fluorescent in-situ hybridization
- Transinfection experiments
- Testing for Spiroplasma in the hemolymph
- Transfer of hemolymph
- Testing for CI with male offspring
- Effect of Spiroplasma titer on CI level
- qPCR standards
- qPCR
- Genome sequencing, assembly, and annotation

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104335.

ACKNOWLEDGMENTS
MP and EK were financially supported by the Ministry for Science, Research and the Arts of Baden-Württemberg as part of the partnership fund of the Robert H. Smith Faculty of Agriculture, Food and Environment of the Hebrew University of Jerusalem (HUJI) in Rehovot and the Faculties of Agricultural and Natural Sciences of the University of Hohenheim (UHOH) to JLMS and YG. The authors would like to thank Einat Kapri and Maya Lalzar for conducting some of the experiments. SJP acknowledges funding support from a Sinergia grant from the Swiss National Science Foundation (grant no. CRSII3_154396) and from the Discovery Grant program of the Natural Sciences and Engineering Research Council of Canada. MJB is supported in part by a Center of Biomedical Research Excellence grant from the National Institute of General Medical Sciences (Grant P20 GM103646) of the National Institutes of Health.

AUTHOR CONTRIBUTIONS
MP and LDM performed research, analyzed data, and wrote the paper; EK and PDA performed research and analyzed data; MH, MJB, JLMS, and YG designed research; SJP designed research and wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: January 24, 2022
Revised: April 6, 2022
Accepted: April 26, 2022
Published: May 20, 2022

SUPPORTING CITATIONS
The following reference appears in the Supplemental Information: Benson et al., 2012; Massey and Newton, 2021

REFERENCES
Adams, K.L., Abernathy, D.G., Willett, B.C., Selland, E.K., Itoe, M.A., and Catteruccia, F. (2021). Wolbachia cifB induces cytoplasmic incompatibility in the malaria mosquito vector. Nat. Microbiol. 6, 1575–1582. https://doi.org/10.1038/s41564-021-00998-6.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. 215, 403-410. https://doi.org/10.1016/0022-2836(90)90360-2.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925. https://doi.org/10.1128/aem.56.6.1919-1925.1990.

Anbutus, H., and Fukatsu, T. (2011). Spiroplasma as a model insect endosymbiont. Environ. Microbiol. Rep. 3, 144–153. https://doi.org/10.1111/j.1758-2229.2010.00240.x.

Ballinger, M.J., and Perlman, S.J. (2019). The defensive Spiroplasma. Curr. Opin. Insect
The image contains a page with scientific text. Due to the density and quality of the text, converting it into a readable format requires transcription. Here is a simplified representation of the text:

**iScience Article**

Sci. 32, 36–41. https://doi.org/10.1016/j.cais.2018.10.004.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Pyshelski, A.D., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–473. https://doi.org/10.1089/cmb.2012.0021.

Beckmann, J.F., Ronau, J.A., and Hochstrasser, M. (2017). A Wolbachia debiquitylating enzyme induces cytoplasmic incompatibility. Nat. Microbiol. 2, 17007. https://doi.org/10.1038/nmicrobiol.2017.7.

Benson, D.A., Karash-Mizrachi, I., Clark, K., Lipman, D.J., Ostell, J., and Sayers, E.W. (2012). Genbank. Nucleic Acids Res. 40, D48–D53. https://doi.org/10.1093/nar/gkr1202.

Binetruy, F., Bailly, X., Chevillon, C., Martin, O.Y., Bernasconi, M.V., and Duron, O. (2019). Phylogenetics of the Spiroplasma oxadexid endosymbiont reveals past transfers between ticks and other arthropods. Ticks Tick Borne Dis. 10, 575–584. https://doi.org/10.1016/j.ttbdis.2019.02.001.

Breffield, C.L., and Dobson, S.L. (2009). Wolbachia-based strategies to control insect pests and disease vectors. Asia Pac. J. Mol. Biol. Biotechnol. 17, 55–63.

Bushnell, B. (2014). BBMap: a fast, accurate, splice-aware aligner. https://www.osti.gov/servlets/purl/1241166.

Cai, Y., Usher, B., Gutierrez, C., Tolcan, A., Moshkin, Y.M., and Engelstad, J., and Hurst, G.D.D. (2009). The ecology and evolution of microbes that manipulate host reproduction. Annu. Rev. Ecol. Evol. Syst. 40, 127–149. https://doi.org/10.1146/annurev.ecolsys.110308.102004.

Finn, R.D., Clements, J., and Eddy, S.R. (2011). HMMER web server: interactive sequence similarity searching. Nucleic Acids Res. 39, W29–W37. https://doi.org/10.1093/nar/gkr367.

Fox, J., and Weisberg, S. (2019). An R companion to applied regression, third edition (Sage).

Frank, J.A., Reich, C.I., Sharma, S., Weisbbaum, J.S., Wilson, B.A., and Olsen, G.J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl. Environ. Microbiol. 74, 2461–2470. https://doi.org/10.1128/AEM.02272-07.

Frydman, H. (2007). Wolbachia bacterial infection in Drosophila. J. Vis. Exp. 15. https://doi.org/10.3791/1586.

Gasparich, G.E. (2002). Spiroplasmas: evolution, adaptation and diversity. Front. Biosci. 7, d619–40. https://doi.org/10.2741/A799.

Gasparich, G.E., Whitcomb, R.F., Dodge, D., French, F.E., Glass, J., and Williamson, D.L. (2004). The genus Spiroplasma and its non-heclal descendants: phylogenetic classification, correlation with phenotype and roots of the Mycoplasma mycoides clade. Int. J. Syst. Evol. Microbiol. 54, 893–918. https://doi.org/10.1099/ijsem.0.02688-0.

Gebiola, M., Giorgini, M., Kelly, S.E., Doremus, M.R., Ferree, P.M., and Hunter, M.S. (2017). Cytological analysis of cytoplasmic incompatibility induced by Cardinium suggests convergent evolution with its distant cousin Wolbachia. Proc. R. Soc. B 284, 20171433. https://doi.org/10.1098/rspb.2017.1433.

Gotóh, T., Noda, H., and Itou, S. (2007). Cardinium symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98, 13–20. https://doi.org/10.1038/sj.hdy.6800881.

Gund, X.D., Jiggins, F.M., Schulenburg, J.H.G.v.d., Bertrand, D., West, S.A., Goriacheva, I.I., Zakharov, I.A., Venner, J.H., Stouthamer, R., and Magerus, M.E.N. (1999). Male-killing Wolbachia in two species of insect. Proc. R. Soc. B 266, 735–740. https://doi.org/10.1098/rspb.1999.0698.

Haeuser, K., and Lemaitre, B. (2018). Male-killing toxin in a bacterial symbiont of Drosophila. Nature 557, 252–255. https://doi.org/10.1038/s41586-018-0086-2.

Haselkorn, T., and Jaenike, J. (2015). Macroevolutionary persistence of heritable endosymbionts: acquisition, retention and expression of adaptive phenotypes in Spiroplasmas. Mol. Ecol. 24, 3752–3765. https://doi.org/10.1111/mec.13261.

He, L.-S., Zhang, P.-W., Huang, J.-M., Zhu, F.-C., Danchin, A., and Wang, Y. (2018). The enigmatic genome of an obligate ancient Spiroplasma symbiont in a halophilic bacterial strain. Environ. Microbiol. 84, e01965–17. https://doi.org/10.1128/AEM.01965-17.

Henneberg, K.A., Hammond, R.L., and Bourke, A.F.G. (2003). Non-lethal sampling of DNA from bumble bees for conservation genetics. Insect Soc. 50, 277–285. https://doi.org/10.1077/s00040-003-0672-6.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. Biom. J. 50, 346–363. https://doi.org/10.1002/bimj.200810425.

Hughes, G.L., and Rasgon, J.L. (2014). Transfection: a method to investigate Wolbachia-host interactions and control arthropod-borne disease. Insect Mol. Biol. 23, 141–151. https://doi.org/10.1111/imb.12066.

Hunter, M.S., Perlman, S.J., and Kelly, S.E. (2003). A bacterial symbiont in the Bacteroides induces cytoplasmic incompatibility in the parasitoid wasp Encarsia pergandiella. Proc. R. Soc. B 270, 2165–2190. https://doi.org/10.1098/rspb.2003.2475.

Grubb, F.E. (1950). Sample criteria for testing outlying observations. Ann. Math. Statist. 21, 27–58. https://doi.org/10.1214/aoms/1177729885.

Hamilton, P.T., Peng, F., Boulanger, M.J., and Perlman, S.J. (2016). A ribosome-inactivating protein in a Drosophila defensive symbiont. Proc. Natl. Acad. Sci. U S A 113, 350–355. https://doi.org/10.1073/pnas.1518648113.
transcriptome sequencing reveals novel M., Hunter, M.S., and Schmitz-Esser, S. (2017). 1999.00151.x. Microbiologia 7, 113. https://doi.org/10.1186/ s40168-019-0244-4. Jiggins, F.M., Hurst, G.D.D., Jiggins, C.D., Schulenburg, J.H.G.v.d., and Majerus, M.E.N. (2000). The butterfly Danaus chrysippus is infected by a male-killing Spiroplasma bacterium. Parasitology 120, 439–446. https://doi.org/10.1017/s0031182099005867. Kanehisa, M., Sato, Y., and Morishima, K. (2018). MAFFT genome and metagenome sequences. tools for functional characterization of Kanesawa, M., Sato, Y., and Morishima, K. 1017/S0031182099005867. https://doi.org/10.1093/nar/gkz268. Katoh, K., and Standley, D.M. (2013). MAFFT incompatibility in parasitoid wasps. Ecol. Evol. 9, 120. https://doi.org/10.1093/nar/gks1219. R Core Team 2019, R a language and environment for statistical computing (R Foundation for Statistical Computing). https://www.R-project.org. Rathke, C., Barckmann, B., Burkhard, S., Jayaramaiah-Raja, S., Roote, J., and Renkawitz-Pohl, R. (2010). Distinct functions of Mst77F and protamines in nuclear shaping and chromatin condensation during Drosophila spermiogenesis. Eur. J. Cell Biol. 89, 326–338. https://doi.org/10.2741/2027. Regassa, L.B., and Gasparich, G.E. (2006). Spiroplasmas: evolutionary relationships and biodiversity. Front. Biosci. 11, 2983–3002. https://doi.org/10.2741/2027. Rosenwald, L.C., Sitvarin, M.I., and White, J.A. (2020). Endosymbiotic Rickettsiella causes cytoplasmic incompatibility in a spider host. Proc. R. Soc. B 287, 20201107. https://doi.org/10.1098/rspb.2020.1107. RTstudio Development Team (2020). RStudio: integrated development for R. (Boston, MA: RStudio, Inc.). https://www.rstudio.com. Sasaki, T., Kubo, T., and Ihekawa, H. (2002). Interspecific transfer of Wolbachia between two lepidopteran insects expressing cytoplasmic incompatibility: A Wolbachia variant naturally infecting Cadra cautella causes male killing in Euphorbia kuehnii. Genetics 162, 1313–1319. https://doi.org/10.1093/genetics/162.3.1313. Sasaki, T., Massaki, N., and Kubo, T. (2005). Wolbachia variant that induces two distinct reproductive phenotypes in different hosts. Heredity 95, 389–393. https://doi.org/10.1038/sj. hdy.6800373. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089. Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2688–2690. https://doi.org/10.1093/bioinformatics/btu153. Shropshire, J.D., Leigh, B., and Bordenstein, S.R. (2009). Symbiont-mediated cytoplasmic incompatibility: what have we learned in 50 years? eLife 9, e01989. https://doi.org/10.7554/eLife.61989. Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., and Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy
Symbiont. PeerJ 7 (2019). The draft genome of strain Spiroplasma novemdecimpunctata confirms insect Cardinium are not a monophyletic group and reveals a novel gene family expansion in a symbiont. PeerJ 7, e6448. https://doi.org/10.7717/peerj.6448.

Tabata, J., Hattori, Y., Sakamoto, H., Yukihiro, F., Fujii, T., Kugimiya, A., Mochizuki, A., Ishikawa, Y., and Kageyama, D. (2011). Male killing and incomplete inheritance of a novel Spiroplasma in the moth Ostrinia zaguljai. Microb. Ecol. 61, 254–263. https://doi.org/10.1007/s00248-010-9799-y.

Takano, S.-I., Gotoh, Y., and Hayashi, T. (2021). “Candidatus Mesenet longicola”: novel endosymbionts of Brontispa longissima that induce cytoplasmic incompatibility. Microb. Ecol. 82, 512–522. https://doi.org/10.1007/s00248-021-01686-y.

Takano, S.-I., Tuda, M., Takasu, K., Furuya, N., Imamura, Y., Kim, S., Tashiro, K., Iyama, K., Tavares, M., and Amaral, A.C. (2017). Unique clade of alphaproteobacterial endosymbionts induces complete cytoplasmic incompatibility in the coconut beetle. Proc. Natl. Acad. Sci. U S A 114, 6110–6115. https://doi.org/10.1073/pnas.1618094114.

Thao, M.L., and Baumann, P. (2004). Evidence for multiple acquisition of Arsenophonus by whitfly species (Stercornylithica: Aleyrodidae). Curr. Microbiol. 48, 140–144. https://doi.org/10.1007/s00284-003-1417-7.

Tinsley, M.C., and Majerus, M.E.N. (2006). A new male-killing parasite: Spiroplasma bacteria infect the ladybird beetle Anisosticta novemdecimpunctata (Coleoptera: Coccinellidae). Parasitology 132, 757–765. https://doi.org/10.1017/S0031182006009789.

Utarini, A., Indriani, C., Ahmad, R.A., Tantowiyo, W., Arguni, E., Ansari, M.R., Supriyati, E., Wardana, D.S., Meitika, Y., Enessia, I., et al. (2021). Efficacy of Wolbachia-infected mosquito deployments for the control of dengue. N. Engl. J. Med. 384, 2177–2186. https://doi.org/10.1056/NEJMoa2030243.

Vavre, F., Dedeine, F., Quillon, M., Fouillet, P., Fleury, F., and Bouléreau, M. (2001). Within-species diversity of Wolbachia-induced cytoplasmic incompatibility in haplodiploid insects. Evolution 55, 1710–1714. https://doi.org/10.1111/j.0014-3820.2001.tb00691.x.

Vera-Ponce Leon, A., Dominguez-Mirazo, M., Bustamante-Brito, R., Higareda-Alverez, V., Rosenblum, M., and Martinez-Romero, E. (2021). Functional genomics of a Spiroplasma associated with the carmine cochineals Dactylopus coccus and Dactylopus puntae. BMC Genom. 22, 240. https://doi.org/10.1186/s12864-021-07540-2.

Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Trentu, F.D., McMeniman, C.J., Leong, Y.S., Dong, Y., Axford, J., Kriesner, P., et al. (2011). The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature 476, 450–453. https://doi.org/10.1038/nature10335.

Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., et al. (2016). Improved bacterial 16S RNA gene (V4 and V4-S) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 1, e00009-15. https://doi.org/10.1128/mSystems.00009-15.

Wang, W., Gu, W., Gasparich, G.E., Bi, K., Ou, J., Meng, Q., Liang, T., Feng, G., Zhang, J., and Zhang, Y. (2011). Spiroplasma erocheiris sp. nov., associated with mortality in the Chinese mitten crab, Eriocheir sinensis. Int. J. Syst. Evol. Microbiol. 61, 703–708. https://doi.org/10.1099/ijsem.0.05259-0.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.

Wrenn, J.H. (1997). Biology of Wolbachia. Annu. Rev. Entomol. 42, 587–609. https://doi.org/10.1146/annurev.ento.42.1.587.

Wick, R.R., Judd, L.M., Gorrie, C.L., and Holt, K.E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13, e1005595. https://doi.org/10.1371/journal.pcbi.1005595.

Xi, Z., Dean, J.L., Kho, C., and Dobson, S.L. (2005). Generation of a novel Wolbachia infection in Aedes albopictus (Asian tiger mosquito) via embryonic microinjection. Insect Biochem. Mol. Biol. 35, 903–910. https://doi.org/10.1016/j.ibmb.2005.03.015.

Yeoman, C.J., Brutscher, L.M., Esen, O.C., Ibaoglu, F., Fowler, C., Eren, A.M., Wanner, K., and Weaver, D.K. (2019). Genome-resolved insights into a novel Spiroplasma symbiont of the wheat stem sawfly (Cephus cinctus). PeerJ 7, e7548. https://doi.org/10.7717/peerj.7548.

Zabolou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., and Bourtzis, K. (2004). Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control. Proc. Natl. Acad. Sci. U S A 101, 15042–15045. https://doi.org/10.1073/pnas.0403853101.

Zimmermann, L., Stephens, A., Nam, S.Z., Rau, D., Kubler, J., Lozajic, M., Gabler, F., Söding, J., Lupas, A.N., and Alva, V. (2018). A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. J. Mol. Biol. 430, 2237–2243. https://doi.org/10.1016/j.jmb.2017.12.007.

Zwenger, S.R., Welsch, T., and Gillock, E.T. (2008). Bacteria resistant to ciprofloxacin, chloramphenicol and tetracycline isolated from western Kansas feedlots. Trans. Kansas Acad. Sci. 111, 125–135. https://doi.org/10.1660/0022-8443(2008)111[125:BRCTCA]2.0.CO;2.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Tetracycline hydrochloride | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany | Cat#87130 |
| Molecular biology grade proteinase K | Sigma-Aldrich Products Ltd., Rehovot, Israel | Cat#3115887001 |
| IGEPAK®-CA-630 | Sigma-Aldrich Products Ltd., Rehovot, Israel | Cat#8896 |
| **Critical commercial assays** | | |
| nexttect™ 1 Step DNA Isolation Kit for Tissue & Cells | nexttect Biotechnologie GmbH, Hilgertshausen, Germany | Cat#10N.050 |
| DNase® Blood & Tissue Kit | QIAGEN LLC, Germantown MD, USA | Cat#69504 |
| GoTaq®Green Master Mix 2X | Promega, Madison WI, USA | Cat#M7122 |
| Green GoTaq® Reaction Buffer | Promega, Madison WI, USA | Cat#M7911 |
| dNTPs | Promega, Madison WI, USA | Cat#U1330 |
| GoTaq® G2 DNA Polymerase | Promega, Madison WI, USA | Cat#M7841 |
| ROTI®Pal TaqS Red-Mix (2x) | Carl Roth GmbH + Co. KG, Karlsruhe, Germany | Cat#9241.1 |
| pegGREEN | WWR International GmbH, Darmstadt, Germany | Cat#732-3196 |
| RedSafe™ Nucleic Acid Staining Solution | iNTRON Biotechnology, Inc. | Cat#21141 |
| LowRanger 100bp DNA | Norgen Biotek Corp., Canada | Cat#11500 |
| equimolar 100bp ladder | Carl Roth GmbH + Co. KG, Karlsruhe, Germany | Cat#T834.1 |
| GeneJet Gel extraction and DNA Cleanup Micro Kit | Thermo Fisher Scientific, Waltham MA, USA | Cat#K0831 |
| pGEM®-T Easy Vector kit | Promega, Madison WI, USA | Cat#1380 |
| peqGold Plasmid Miniprep Kit I | WWR International GmbH, Darmstadt, Germany | Cat#12-6942-1 |
| Platinum® SYBR® Green qPCR SuperMix-UDG | Invitrogen, Carlsbad CA, USA | Cat#11733038 |
| DreamTaq™ Green DNA Polymerase with DreamTaq™ Green Buffer | Thermo Fisher Scientific, Waltham MA, USA | Cat#EP0711 |
| Oxford Nanopore MinION | Oxford Nanopore Technologies, Oxford Science Park, UK | Cat#Min1018 |
| **Deposited data** | | |
| NGS sequences | This paper | GenBank: OL684700-OL684711 |
| Spiroplasma sp. sequence | This paper | GenBank: OM334830 |
| Enterobacter sp. sequence | This paper | GenBank: OM273870 |
| WGS sequences | This paper | NCBI BioProject: PRJNA826431 |
| **Experimental models: Organisms/strains** | | |
| Lariophagus distinguendus strain dbSTU-D1 | Dept. of Chemical Ecology, University of Hohenheim | N/A |
| **Oligonucleotides** | | |
| See Table S2 for list of primers | N/A | N/A |
| Fluorescent probe Eub338-Cy3 5’-Cy3-ACT CCT ACG GGA GGC AGC-3’ | (Amann et al., 1990) | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Johannes L.M. Steidle (jsteidle@uni-hohenheim.de).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
All data generated in this study will be publicly available as of the date of publication. Accession numbers for sequences submitted to the NCBI database are listed in the key resources table.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Fluorescent probe SPR-Cy3 5'-Cy3-CCC ACC TTC CTC TAG CTT AC-3' | This study | N/A |

**Software and algorithms**

| Software and algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| GENtLe v. 1.9.4. | (Manske, 2006) | http://gentle.magnusmanske.de/ |
| Axiovision 4.6 | Carl Zeiss AG, Oberkochen, Germany | https://www.micro-shop.zeiss.com/en/us/system/software+axiovision+axiovision+program+axiovision+software/10221/ |
| ImageJ 1.x | (Schneider et al., 2012) | https://imagej.nih.gov/ij/ |
| GIMP v. 2.10.24 | Kimball, Mattis and the GIMP Development Team | https://www.gimp.org/ |
| R v. 4.0.3 | (R Core Team, 2020) | https://www.R-project.org/ |
| RStudio v. 1.3.1093 | (RStudio Team, 2020) | https://www.rstudio.com/ |
| multcomp v.14.-15, R package | (Hothorn et al., 2008) | http://multcomp.R-forge.R-project.org |
| car v. 3.0-10, R package | (Fox and Weisberg, 2019) | https://socialsciences.mcmaster.ca/jfox/Books/Companion/ |
| dada2 v. 1.14.0, R package | (Callahan et al., 2016) | https://github.com/benjjneb/dada2/releases/tag/v1.14 |
| BBMap | (Bushnell, 2014) | https://sourceforge.net/projects/bbmap/ |
| SPAdes 3.10.1 | (Bankevich et al., 2012) | https://github.com/alab/spades |
| KAT | (Mapleson et al., 2017) | https://kat.readthedocs.io/en/latest/# |
| BUSCO | (Simão et al., 2015) | https://busco.ezlab.org/ |
| gVolante | (Nishimura et al., 2017) | https://gvolante.riken.jp/ |
| Prokka v 1.14.5 | (Seemann, 2014) | https://github.com/tseemann/prokka |
| BLASTKoala | (Kanehisa et al., 2016) | https://www.kegg.jp/blastkoala/ |
| BLAST | (Altschul et al., 1990) | https://blast.ncbi.nlm.nih.gov/Blast.cgi |
| MAFFT 7.388 | (Katoh and Standley, 2013) | https://mafft.cbrc.jp/alignment/software/ |
| Geneious Prime | Geneious 2022.0.1 | https://www.geneious.com/ |
| Unicycler | (Wick et al., 2017) | https://github.com/rwick/Unicycler |
| FastTree 2.1.11 | (Price et al., 2010) | http://microbesonline.org/fasttree/ |
| HHMMER 3.3 | (Finn et al., 2011) | http://hmmner.org/ |
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

*Lariophagus distinguendus*

In our experiments, we used the *L. distinguendus* strain dbSTU-D1 (STU), which was collected in households in 2007 in Stuttgart-Bad Cannstatt, Germany. The strain was reared on six-week-old larvae of *Stegobium paniceum* in koi pellets (Hikari Friend, Kamihata Fish Industry Group, Kyorin Corporation, Japan) in glass jars with a ventilated lid (diameter 12 cm, height 16 cm). Beetles were reared by placing about 1 g of newly emerged unsexed adult beetles (about 700 beetles) on 80 g koi pellets. After six weeks, freshly emerged *L. distinguendus* were placed on the infested pellets. The cultures were kept at 26 °C and 45% RH with a natural light:dark cycle. To obtain virgin wasps for the experiments, they were isolated in the pupal stage by dissecting the koi pellets and kept individually in 1.5 ml tubes until eclosion. An endosymbiont-free line, termed STU(-), was obtained by tetracycline treatment for at least three generations. Newly emerged wasps were placed in a Petri dish containing filter paper and a piece of cotton wool soaked in a solution of tetracycline (1 mg/ml) and sucrose (100 mg/ml). After 24 h, wasps were placed on host-infested substrate as described above. The loss of endosymbionts was confirmed by PCR (as described below) for randomly chosen individuals prior to using these lines in experiments.

**METHOD DETAILS**

**Crossing experiments**

1-day-old single virgin wasp males and females of the antibiotic and untreated lines were placed together to enable mating in all possible combinations. All pairs were subsequently transferred to host-infested substrate for oviposition, i.e. 5 g of koi pellets containing *S. paniceum* larvae. After 4 to 5 weeks, the number and sex of emerging offspring were recorded. Crossings without any offspring were excluded from the analysis.

**Specific testing for bacteria**

The wasps were tested for the presence of *Spiroplasma*, *Rickettsia*, *Arsenophonus*, *Mesenet*, and *Xenella*. For the DNA extraction, two different methods were used. One method consisted of crushing single wasps in 10 μl of a lysis buffer (9.95 μl TE-buffer with 10 mM Tris, 0.5 mM EDTA, 3 mg molecular biology grade proteinase K (Sigma-Aldrich Products Ltd., Revohov, Israel), and 0.5 μl IGEPA@-CA-630 (Sigma-Aldrich Products Ltd., Revohov, Israel)) and the subsequent incubation of the mixture with another 30 μl or 90 μl of the same buffer at 65°C for 15 min and 95°C for 10 min using a heating block. Alternatively, wasp DNA was extracted using the nexttetc™ 1 Step DNA Isolation Kit for Tissue & Cells (nexttetc Biotechnologie GmbH, Hilgertshausen, Germany) following the corresponding protocol. The PCR conditions generally consisted of an initial denaturation step at 94°C to 95°C for 35 cycles of 92°C or 95°C for 30 s, 30 s at the respective annealing temperatures of 55 to 58°C and 30 to 60 s at 72°C followed by a final extension at 72°C for 5 min (see Table S2 for details). The reaction mix consisted either of 20 μl Promega GoTaq®Green Master Mix 2X (Promega, Madison WI, USA), with 4 μl of each primer and 10 μl of double distilled water per 2 μl sample, 5 μl Promega 5X Green GoTaq® Reaction Buffer with 0.5 μl 10mM dNTPs (Promega, Madison WI, USA), 1 μl of each primer, 16.375 μl double distilled water and 0.125 μl Promega GoTaq® G2 DNA Polymerase (Promega, Madison WI, USA) per 1 μl sample or 12.5 μl of ROTI®Pol TaqS Red-Mix (2X) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with 1 μl of each primer and 9.5 μl of double distilled water per 1 μl sample. The PCRs were performed using a Biometra professional Basic Thermocycler (Analytik Jena AG, Jena, Germany), a Biometra TGradient 96 Thermocycler (Biometra, Jena, Germany) or a Techn@ Prime Thermocycler (Cole-Parmer, Stone, UK). Gel electrophoresis was conducted on a 1 to 2% agarose gel using 5 μl peqgreen (VWR International GmbH, Darmstadt, Germany) per 100 ml TAE buffer or 5 to 10 μl RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology, Inc.) for DNA staining and Norgen LowRange 100 bp DNA (Norgen Biotek Corp., Canada) or an equimolar 100 bp ladder (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as ladder. Of each sample, 5 to 10 μl were transferred to the gel. All PCR experiments in this study followed these procedures unless stated otherwise. We used the primers ApDnaAF1 (5’-ATT CCT CAG TAA AAA TGC TTG GA-3’ (Fukatsu et al., 2001)) and ApDnaAR1 (5’-ACA CAT TTA CCT CAT GCT ATT GA-3’ (Fukatsu et al., 2001)) to test for *Spiroplasma*, and RB-F (5’-CGT CAG AAC GAA CGC TAT C-3’ (Gottlieb et al., 2006)) and RB-R (5’-GAA GGA AAG CAT CTC TGC TGC -3’ (Gottlieb et al., 2006)), respectively, for *Rickettsia*. Ars23S-1 (5’-CGT TTT ATG AAT TCA TAG TCA AA-3’ (Takano et al., 2004)) and Ars23S-2 (5’-GGT CCT CCA GTT AGT GTT ACC CAA C-3’ (Thao and Baumann, 2004)) were used to test for *Arsenophonus*, L35SF (5’-GCT ATG CGG CGT GAG TGA TT-3’ (Takano et al., 2004)) were used to test for *Mesenet*, and LARI (5’-AAT CTT TAA GGT GGC AAC TAA ATT TGA AAT TCA TAT GGA GGC TAT C-3’ (Gottlieb et al., 2006)) were used to test for *Xenella*. The wasps were tested for the presence of *Spiroplasma*, *Rickettsia*, *Arsenophonus*, and *Xenella*.
Next generation sequencing of the microbiome

The whole microbiome of the STU strain was subjected to Next Generation Sequencing (16S rRNA amplicon sequencing) with five samples consisting of 50 females each, following the procedure described by (Dally et al., 2020). DNA extraction was conducted using the DNeasy® Blood and Tissue kit (QIAGEN, Germantown, MD, USA) according to the manufacturer’s instructions. PCR was conducted with the primer pair 51SF (5'-GTG YCA GCC GCC GCG GTA A-3')/926R (5'-CCG YCA ATT YMT TTR AGT TT-3'), targeting the V4-V5 variable regions of microbial 16S rDNA (Walters et al., 2016) to screen for microbes. Sequencing of the resulting amplicons was performed on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA) as described by (Jiang et al., 2019) using a MiSeq v3 flow cell (Illumina Inc., San Diego, CA). PCR, library preparation, and sequencing were performed at the University of Illinois at Chicago Sequencing Core (UICSCQC).

The DADA2 pipeline, provided by the R package “dada2” (v. 1.14.0) (Callahan et al., 2016) was implemented to process the obtained sequences by trimming them and filtering those with poor quality (“filter-AndTrim”, maxEE = 2, maxN = 0 and trimleft = 15). Error rate was estimated (“learnErrors”, randomize set to “TRUE”) and corrected sequences were inferred with the dada2 algorithm (“dada”). Complete sequences were assembled from the forward and reverse sequences via “mergePairs” and chimeras were identified and removed (“removeBimeraDenovo”). A count table containing the amplicon sequence variants (ASVs) and their respective counts was created (“makeSequenceTable”). The taxonomic matches of the ASVs were determined via the “assignTaxonomy” command (minimum bootstrap confidence value at 80%) using the SILVA non-redundant small subunit ribosomal RNA database (v.132) (Quast et al., 2013) as reference, and added to the table for further analysis.

Testing for Enterobacteriaceae

Tetracycline-treated and untreated STU females, as well as STU males of both CI and non-CI crossings were tested for the Enterobacteriaceae detected by NGS using the primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') (Weisburg et al., 1991), 1492r (5'-TAC CTT GTT ACG ACT T-3') (Frank et al., 2008), EnterobacterF (5'-AGG GGT GCA AGC GTT AAT CGG-3'), and EnterobacterR (5'-AGC GTC AGT CTT TGT CCA GGG-3') (also see Table S2). A sample of a tetracycline-treated STU female subjected to PCR with the primer combination 27f/1492r was chosen for Sanger Sequencing and sent to Microsynth Seqlab (Gottingen, Germany) for this purpose. The sequence was processed with GENtle v. 1.9.4. (© by Magnus Manske, University of Cologne, released under GPL, 2003) (Manske, 2006).

Fluorescent in-situ hybridization

In order to test the presence of Spiroplasma in the ovaries of L. distinguendus, the ovipositor with attached ovaries of live females was removed in 1 x PBS using fine needles. Ovaries were kept in FAA (5% acetic acid, 5% formaldehyde, 90% absolute ethanol) for fixation for 1 to 3 days. The fixed ovaries were washed in fresh 50% ethanol and subsequently incubated in fresh 50% ethanol, 80% ethanol and 100% ethanol for 15 min each. Afterwards, the ovaries were air-dried for 10 min and subsequently moved to hybridization buffer (20 mM pH 8.0 Tris-HCl, 0.9 M NaCl, 35% formamide, 0.01% SDS) pre-warmed at 46°C for an incubation period of 15 min. The hybridization buffer was then replaced by pre-warmed hybridization buffer containing the fluorescent probes. The Spiroplasma-specific probe SPR-Cy3 (5’-CAG GGT GCA AGC GTT AAT CGG-3’) and controls the probe anti-sense Eub338-Cy3 (5’-CAG GGT GCA AGC GTT AAT CGG-3’) (Amann et al., 1990), as well as a no-probe approach were used. The samples were incubated overnight at 46°C for hybridization. After incubation in 500 µl of pre-warmed washing buffer (20 mM Tris-HCl pH 8.0, 80 mM NaCl, 50 mM EDTA, 0.01% SDS) for 15 min, the samples were left in 1 ml of pre-warmed washing buffer for 30 min and subsequently washed twice with 1 x PBS. For mounting on a slide with 1 x PBS, the cuticles were removed from the ovaries, and a drop of mountant (glycerol, 1 x PBS, Hoechst staining) was added to the slide before it was covered with a coverslip that was fixed with nail polish. The slides were incubated in the dark for several minutes before being analyzed. Visual analysis was performed using a Zeiss LSM 700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with an AxioCam HSM video camera (Carl Zeiss AG, Oberkochen, Germany) and Axiovision 4.6 software (Carl Zeiss AG, Oberkochen, Germany).
ImageJ 1.x (Schneider et al., 2012) and the GNU Image Manipulation Program (GIMP, v. 2.10.24, © Spencer Kimball, Peter Mattis and the GIMP Development Team, www.gimp.org) were applied for processing.

**Transinfection experiments**

Transinfection experiments were performed to study if CI can be induced in an endosymbiont-free strain by infection with *Spiroplasma*. Hemolymph was transferred from infected female wasps (donor females) to uninfected STU(-) females (receiver females). Male offspring of receiver females were mated to endosymbiont-free females to check for CI between *Spiroplasma*-positive males and negative females. The status of *Spiroplasma* infection was initially tested for in the hemolymph of infected females and subsequently in the receiver females of the endosymbiont-free strain STU(-) and their male offspring by PCR with the primers ApDnaAF1 and ApDnaAR1 as described above (also see Table S2) following DNA extraction with the nexttec™ 1-Step DNA Isolation Kit for Tissue & Cells (nexttec Biotechnologie GmbH, Hilgertshausen, Germany) for all samples except the hemolymph. Positive results were confirmed by Sanger Sequencing (Microsynth Seqlab, Göttingen, Germany).

**Testing for *Spiroplasma* in the hemolymph**

To confirm the presence of *Spiroplasma*, hemolymph was extracted from 20 STU females. The wasps were fixed on a double-sided adhesive tape to a slide and as much hemolymph as possible was removed from the abdomens with a drawn-out glass capillary mounted on a micromanipulator and pooled in 30 μl of TE buffer and 3 μl of proteinase K (nexttec™ 1-Step DNA Isolation Kit for Tissue & Cells; nexttec Biotechnologie GmbH, Hilgertshausen, Germany). This mixture was incubated at 55°C for 30 min and 300 rpm and at 100°C for 10 min for DNA extraction as described by (Holehouse et al., 2003). The presence of *Spiroplasma* was tested for with PCR conditions slightly modified from those specified before by using 40 cycles instead of 35 and by increasing the template volume to 2 μl while decreasing the volume of double distilled water in the PCR mixture by 1 μl accordingly.

**Transfer of hemolymph**

Donor and receiver females were newly hatched and virgin. Prior to the experiment, receiver females were cooled in a refrigerator at 5°C to reduce their mobility. Hemolymph was extracted from a donor female as described above and subsequently injected with an Eppendorf FemtoJet (Eppendorf AG, Hamburg, Germany) into the abdomens of a receiver female that was carefully held using a spring steel forceps. The receiver females were transferred to batches of 2 g koi pellets infested with larvae of *S. paniceum* as hosts for oviposition. Host batches were replaced every two to three days until the death of the wasps. After their death, the receiver females were either transferred to 100% ethanol and kept at -20°C until DNA extraction, or immediately extracted. Because they remained unmated, they only produced male offspring. For the subsequent experiments, only male offspring of *Spiroplasma*-positive receiver females were used.

**Testing for CI with male offspring**

Newly hatched male offspring of positive receiver females were mated to virgin, uninfected STU(-) females. After copulation, the males were killed in 2 ml tubes (Sarstedt AG & Co. KG, Numbrecht, Germany) containing 100% ethanol and kept at -20°C until DNA extraction unless they were extracted immediately. All samples were subsequently checked for *Spiroplasma* infection. The uninfected STU(-) females were placed on 5 g koi pellets infested with larvae of *S. paniceum* as hosts for oviposition. After four weeks, the number and sex of the F2 offspring were recorded to study CI. Crosses without any offspring were excluded from the analysis.

**Effect of *Spiroplasma* titer on CI level**

In order to examine whether the strength of CI detected in matings between positive males and tetracycline-treated females was correlated to the *Spiroplasma* load in these males, we conducted quantitative PCR (qPCR) with all samples of mated *Spiroplasma*-positive F1 male offspring of receiver females. *DnaA*, amplified with the primers ApDnaAF1 and ApDnaAR1, was chosen as target gene.

**qPCR standards**

A PCR product of one of the studied samples was purified using the Thermo Scientific GeneJet Gel extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, Waltham MA, USA). The DNA content was checked with an Eppendorf BioPhotometer® D30 (Eppendorf AG, Hamburg, Germany), and the purified
PCR product was cloned into pGEM®-T Easy Vector (Promega, Madison WI, USA), whereby the ligation protocol was modified by using 0.5 μl pGEM®-T Easy Vector (50 ng), 1 μl DNA ligase (3 U/μl), 2.5 μl 2X Rapid Ligation Buffer, and 2 μl PCR product. The ligation was incubated overnight at 16°C. The ligation mix was then transformed into 45 μl of chemically competent E. coli JM109. The transformed bacteria were grown overnight at 37°C and 250 rpm in 20 ml test tubes filled with 5 ml LB-medium containing 100 μg/ml ampicillin. Plasmid extraction was performed using the peqGold Plasmid Miniprep Kit I (VWR International GmbH, Darmstadt, Germany). The plasmid extract was amplified with 2.5 μl DreamTaq™ Green Buffer (Thermo Fisher Scientific, Waltham MA, USA), 2.5 μl dNTPs, 0.125 DreamTaq™ Green DNA Polymerase (Thermo Fisher Scientific, Waltham MA, USA), 18.87 μl double distilled water, and 0.5 μl of each of the sequencing primers SP6/T7 (see Table S2 for details) and sent for Sanger Sequencing (Microsynth Seqlab, Gottingen, Germany) to confirm the Spiroplasma dnaA insert. The closest match in the NCBI database was Spiroplasma sp. Ozg dnaA gene (GenBank: AB586705.1) with 99% identity and 100% query coverage. The concentration of the plasmid extract was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham MA, USA), and the original extract was serially diluted (1:10) to serve as standards for qPCR. The standard curve had a slope of -3.501 and a y intercept of 39.256 with a regression coefficient R2 of 0.991. PCR efficiency was determined to be approximately 90%. The range of reliable quantification was determined to be 103 to 1010 target molecules/μl DNA extract, with a Cq variation of 0.25 at the lower boundary.

qPCR

qPCR was conducted on a CFX96 Real Time System (Bio-Rad Laboratories, Inc.) with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad CA, USA), in reaction volumes of 10 μl, consisting of 0.8 μl template (DNA extract or standard), 5 μl 2x qPCR supermix, 0.2 μl 10mM forward primer, 0.2 μl 10 mM reverse primer, 0.2 μl ROX Reference Dye and 3.6 μl nuclease-free water. The annealing temperature of 52°C was determined by gradient PCR prior to qPCR. A lower elongation temperature of 60°C had to be used to allow amplification of the AT-rich template. The resulting cycling conditions were therefore: 50°C for 2 min; 95°C for 2 min; 35 cycles of 95°C for 20 s, 52°C for 20 s and 60°C for 1 min; followed by recording of the melting curve (50 to 95°C in increments of 0.5°C). Two analytic runs were conducted, each with technical duplicates of all samples and standards as well as four non-template controls. The measurements of each sample were averaged for further analyses after excluding outliers according to Grubbs’s test (Grubbs, 1950). Spiroplasma dnaA copy numbers were calculated by linear regression to the standard curve.

Genome sequencing, assembly, and annotation

Nucleic acids were extracted from a pool of ten adult L. distinguendus strain STU using the phenol-chloroform method. Paired-end, 125 bp sequencing libraries were prepared from genomic DNA by Genome Quebec and sequenced on one third of an Illumina HiSeq 2500 lane. The sequencing run yielded 95.5 million reads from which adapters and low-quality sequences were trimmed using bbduk (Bushnell, 2014). A metagenome assembly was assembled using SPAdes 3.10.1 (Bankevich et al., 2012). The Spiroplasma metagenome assembly was covered to 30x Illumina read depth (Figure S1), but fragmented. High molecular weight DNA was extracted from a second pool of 25 adult L. distinguendus STU adults used to generate 2.1 Gbp of Oxford Nanopore MinION reads on an R9 flow cell. Counting only passed reads over 1000 bases, total sequencing yield was 1.8 Gbp. An initial hybrid metagenome was assembled using Unicycler (Wick et al., 2017). Spiroplasma-derived contigs were retained through a combination of metagenomic binning (Laczny et al., 2017) and manual curation. Manual curation involved targeted searches for sequences likely to contaminate the bin based on similar base composition, e.g. host mitochondrial genome, and was guided by kmer abundance analyses performed with KAT (Mapleson et al., 2017). Contigs associated with the lowest and highest 21-mer abundances (Figure S5) were investigated and retained only if BLASTn, BLASTp, or HMMER searches yielded significant similarity with other Spiroplasma. In general, the low abundance kmers were unidentifiable or attributable to L. distinguendus genome contamination and were removed, while the highest were insertion sequence elements common in ixodetid clade genomes. Genome completeness was estimated using BUSCO (Simão et al., 2015) implemented on the gVolante webserver (Nishimura et al., 2017). The draft genome was annotated using Prokka 1.14.5 (Seemann, 2014). KEGG numbers were assigned using BLASTKoala (Kanehisa et al., 2016). Spiroplasma homologues of interest, including potential toxins (Figure S3, Figure S4, Table S5), plasmid-associated genes (Table S6), and those used for phylogenetics, were identified by tBLASTn, BLASTn, BLASTp and alignments (Altschul et al., 1990). Nucleotide sequences were aligned with MAFFT 7.388
(Katoh and Standley, 2013) and phylograms were built using FastTree 2.1.11 (Price et al., 2010). The GTR substitution model was used to build phylograms from nucleotide alignments and the JTT model was used for amino acid alignments. Protein domains were identified with HMMER 3.3 (Finn et al., 2011) and PfamScan with an expect value threshold of .01 (Madeira et al., 2019). Proteins of interest, including those containing cif-like domain homologues were investigated further using HHpred to identify potentially hidden structural homologies (Zimmermann et al., 2018). BLASTp searches with relaxed significance thresholds (expect values ≤ 0.01) were also performed to ensure detection of more distantly related protein domains, if present.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted using RStudio (v. 1.3.1093 (R Core Team, 2020, RStudio Team 2020)) with the packages “multcomp” (Hothorn et al., 2008) and “car” (Fox and Weisberg, 2019) in addition to the pre-installed packages. Statistical significance was assumed at \( p < 0.05 \), with \( p < 0.01 \) and \( p < 0.001 \) indicating high and very high significance, respectively.

To test for CI in crossing experiments between tetracycline-treated and untreated STU individuals, the numbers of male and female F1 offspring as well as total F1 offspring were analyzed with generalized linear models, with negative binomial chosen for family as the best fit, since the data did not adhere to normal distribution. Significant differences and numbers of replicates are indicated in Figure 1; the corresponding \( p \) values are given in Table S1.

A 2x4 Fisher’s Exact Test for Count Data was used to compare the rate of infection with Enterobacteriaceae between tetracycline-treated and untreated STU females as well as CI-inducing and non-CI inducing STU males due to low replicate numbers. Numbers of replicates and \( p \) values are given in the corresponding section of the results text.

For the comparisons of F2 female offspring numbers as well as F2 male offspring numbers between crosses of STU(-) females with sDis-negative and positive F1 male offspring of hemolymph-injected STU(-), Wilcoxon rank sum tests with continuity correction were used due to the data being non-normally distributed. As normal distribution applied to the total F2 offspring numbers of these crosses, they were compared using a Welch two sample t-test. Information on \( p \), \( W \) and \( t \) values, levels of significance, and replicates are given in Figure 3B and its legend as well as the corresponding results text.

The correlation between dnaA copy number determined by qPCR and the level of CI represented by proportion of female offspring (number of female F2 offspring divided by total number of F2 offspring) was assessed using Spearman’s rank correlation, as further detailed in the respective part of the results section.