Single and Double Infections with Wolbachia in the Parasitic Wasp *Nasonia vitripennis*: Effects on Compatibility

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Manuscript received September 10, 1995
Accepted for publication February 19, 1996

ABSTRACT

Wolbachia are cytoplasmically inherited bacteria responsible for reproductive incompatibility in a wide range of insects. There has been little exploration, however, of within species Wolbachia polymorphisms and their effects on compatibility. Here we show that some strains of the parasitic wasp *Nasonia vitripennis* are infected with two distinct bacterial strains (A and B) whereas others are singly infected (A or B). Double and single infections are confirmed by both PCR amplification and Southern analysis of genomic DNA. Furthermore, it is shown that prolonged larval diapause (the overwintering stage of the wasp) of a double-infected strain can lead to stochastic loss of one or both bacterial strains. After diapause of a double-infected line, sublines were produced with AB, A only, B only or no Wolbachia. A and B sublines are bidirectionally incompatible, whereas males from AB lines are unidirectionally incompatible with females of A and B sublines. Results therefore show rapid development of bidirectional incompatibility within a species due to segregation of associated symbiotic bacteria.

BACTERIA of the genus *Wolbachia* are intracellular rickettsia harbored by a wide number of diverse arthropod species, mainly insects (BARR 1980; WADE and STEVENS 1985; ROUSSET *et al.* 1992; STOUTHAMER *et al.* 1993; WERREN *et al.* 1995a,b). These cytoplasmically inherited microorganisms are known to alter host reproduction in three ways: reproductive incompatibility (YEN and BARR 1971; HOFFMANN *et al.* 1986; BREEUWER and WERREN 1990; O'NEILL and KARR 1990), parthenogenesis (STOUTHAMER *et al.* 1993), and feminization of genetic males (RIGAUD *et al.* 1991, ROUSSET *et al.* 1992) and fertilization potential of males (WADE and CHANG 1994). These reproductive alterations enhance vertical transmission of the bacteria and therefore their maintenance in host populations.

Wolbachia-induced cytoplasmic incompatibility (CI) results in embryonic lethality in diploid insects (HOFFMANN *et al.* 1986) and in production of male offspring in haplodiploid insects (BREEUWER and WERREN 1990). Both phenomena result from an aborted karyogamy in the first mitotic division of the egg (RYAN and SAUL 1968; O'NEILL and KARR 1990; REED and WERREN 1995). In the parasitic wasp *Nasonia vitripennis,* it has been established that the paternal chromosomes fail to condense properly and are eventually fragmented and lost (RYAN and SAUL 1968; BREEUWER and WERREN 1990; REED and WERREN 1995). CI occurs when sperm from an infected male fertilizes an uninfected egg or an egg infected with a different bacterial type. In the former case, incompatibility is unidirectional since the reciprocal cross is compatible. The latter case can either result in unidirectional or bidirectional incompatibility. A number of studies have found that bidirectional incompatibility is due to differences between bacteria strains, rather than genetic differences between the hosts. These could consist in either genetic differences in bacterial strains (BREEUWER and WERREN 1993a; BRAIG *et al.* 1994; ROUSSET and DE STORDEUR 1994), differences in bacterial density (BREEUWER and WERREN 1993b; BRESSAC and ROUSSET 1993), or both. The interspecific and intraspecific variability of the compatibility pattern, particularly well documented in Drosophila and Nasonia species, raises the question of how new compatibility types arise.

Phylogenetic relationships of Wolbachia present in diverse insect taxa have been investigated, based on sequencing of the 16S rDNA gene (BREEUWER *et al.* 1992; O'NEILL *et al.* 1992; ROUSSET *et al.* 1992) and *ftsZ* cell-cycle gene (WERREN *et al.* 1995a). Phylogenetic studies based on the bacterial *ftsZ* gene reveal two major subdivisions of Wolbachia, designated as A and B (WERREN *et al.* 1995a). These two major divisions of Wolbachia diverged from each other 58–67 mya, based upon synonymous substitution rates.

There is increasing evidence that individual insects and strains can harbor double infections with different types of Wolbachia. BREEUWER *et al.* (1992) first detected the presence of two different Wolbachia ribosomal types within individual strains of *Nasonia vitripennis* (Hymenoptera: Pteromalidae) and hypothesized that this was either due to infection with two different

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Wolbachia strains or a single Wolbachia strain with two divergent 16S rDNA genes. ROUSSET and SOLIGNAC (1995) found two different 16S rDNA types within individual strains of *Drosophila simulans* and *D. sechellia* by polymerase chain reaction (PCR) amplification and interpretated this result as evidence for double infection. MERCOT *et al.* (1995) identified a double-infected strain in *D. simulans* and succeeded in separating two bacterial types during backcrossing experiments. SINKINS *et al.* (1995a) also detected double infections (by PCR) in the mosquito *Aedes albopictus.*

WERREN *et al.* (1995a) detected double Wolbachia infections in the seven species by PCR amplification using both 16S rDNA and *ftsZ* cell cycle genes. They found a concordance between presence of 16S rDNA and *ftsZ,* a protein coding gene. An additional survey of 154 neotropical insects by WERREN *et al.* (1995b) detected double Wolbachia infections in nine of 26 infected species, or 35%. Standard wild-type strains of all three Nasonia species were found to harbor double infections with A group and B group Wolbachia (WERREN *et al.* 1995a). All the methods above have employed PCR to detect double infections by Wolbachia.

The existence of double infection has not been confirmed by methods other than PCR and cloning (*e.g.,* by Southern hybridization of DNA from infected individuals) in any of the above studies. The effect of double infection on reproductive compatibility in Nasonia species had not been previously determined. Our preliminary studies in Nasonia had also suggested that larval diapause (the overwintering stage of this insect) could result in reduction in bacterial infections and production of single-infected individuals from double-infected strains.

The purpose of this study is to demonstrate the occurence of single and double infection in the parasitic wasp *N. vitripennis* by Southern hybridization as well as PCR amplification, investigate the effects of diapause on bacterial infection level and the production of single infections from a double-infected strain, and determine the effect of double and single infections on reproductive compatibility in Nasonia.

MATERIALS AND METHODS

Lab strains: All wasps in these experiments were reared on *Sarcophaga bullata* (fleshfly) pupae reared in the laboratory. These flies do not harbor Wolbachia. The following standard strains are maintained by mass rearing at 25° under constant light: LbII, R511, AsymC [a LbII strain cured of the CI bacteria by tetracycline treatment in 1986 (BREEUWER and WERREN 1990)] and Ti277. The compatibility relationships among these strains are well studied (SAUL 1961; BREEUWER and WERREN 1993b); LbII and R511 males are incompatible with Ti277 and AsymC females, Ti277 males are (partially) incompatible with AsymC females, and all other crosses are compatible. These strains are used as controls for testing compatibility and for studies of double and single infection.

Field strains: Field strains were established from single females collected in summer 1989 and 1991. These strains were maintained into diapause shortly after collection. At the time of collection, a sample of F1 progeny was frozen at −80° for future analysis by molecular methods. Strains were brought out of diapause every 1 or 2 yr and then immediately rediapaused. In some cases, rediapause took up to four generations to reinitiate. These strains had thereby undergone the diapause cycle one to four times. A second set of strains were established from single females collected in summer 1994. These were maintained at 21° without diapause. PCR amplification using A group and B group specific primers (WERREN *et al.* 1995a) were used to screen the prediapause (frozen) females, post diapause females and nondiapause females for Wolbachia, to investigate the effects of diapause on infection status.

R511 postdiapause lines: Preliminary observations indicated that prolonged diapause can cause loss of bacteria and alter compatibility. To further investigate the effect of diapause on infection pattern, 15 single (isofemale) lines were initiated from R511 females that had been in larval diapause for 2 yr at 4°; however, only 12 were successfully established. At the second generation, six of these isofemale lines were selected based on preliminary PCR evidence, suggesting some of these lines harbored single A and B infections, because this is what we were attempting to recover. Ten sublines were established for each line by mating F_2 females to AsymC males. AsymC males were used to avoid possible loss of sublines due to variation of infected status within sublines, because AsymC males are compatible with both infected and uninfected females. Among the sublines, 13 were chosen at the F_3 generation (based on preliminary crossing results indicating single infections) and set as five single females per line, mated to brothers. Of the 75 sublines, referred to as R511D sublines, 16 were maintained following the F_3 generation as single sibmated females until the F_8 generation, and since have been maintained as mass cultures.

Cytology: To determine presence and density of bacteria, eggs were collected from females, fixed, stained in lacmoid and then viewed under a light microscope (BREEUWER and WERREN 1990).

To collect eggs, females were first set on hosts (fleshfly pupae) for 2 days after emergence and then deprived of hosts overnight. Single females were allowed to oviposit on a host for three periods of 3, 2 and 2 hr. The parasitized hosts were cracked open immediately at the end of the oviposition period, and the eggs collected, fixed in Carnoy's (6:3:1 proportions of 99% EtOH:chloroform:acetic acid by volume) and stored at 4°. For mounting, eggs were placed on a microscope slide in a drop of 70% EtOH and a drop of 2.5% lacmoid [2.5% lacmoid (Pfaltz and Bauer Inc., Stamford, CT) by weight in 1:1:1 H_2O:acetic acid:lactic acid by volume]. The chorion was mechanically removed from the egg by gently pressing down and moving the coverslip. The slide was sealed by nail polish and stained at 4° for 12–48 hr. Before examination, the preparation was slightly destained by allowing a drop of 50% acetic acid to pass under the coverlip. Slides were examined with a light microscope (15 × 100, phase, oil).

Bacterial density was estimated by placing a grid over the posterior end of the egg, opposite the micropyle, an area where the bacteria are localized (BREEUWER and WERREN 1990). The 5 × 5 squares grid was successively placed in three nonoverlapping areas within the posterior end of the egg, and the bacteria were counted in five squares of 10 × 10 μm (four corners and the central square) each time. In each of these 15 squares, the focus was adjusted during counting, to score the bacteria in the column of cytoplasm under the square. The

data obtained by this method are given as the total number of bacteria scored in 15 squares for an egg and are not, therefore, an estimate of total bacterial density in the egg.

PCR assay: A PCR assay was used to detect A and B group Wolbachia, using specific *ftsZ* gene primers for these two bacterial groups (WERREN *et al.* 1995a). Female wasps were frozen at −80° before DNA extraction. Each female was washed in a drop of 5% filter sterilized sodium hydrochloride (Chlorox) and rinsed three times in drops of sterile distilled water. The wasp was then transferred and homogenized using a micropipette tip in a microtube containing 50 μl of 5% Chelex 100 Resin (Bio-Rad) and 0.01% proteinase K (WALSCH *et al.* 1991). Tubes were vortexed at high speed for 10 sec, incubated at 56° for 35 min, vortexed again for 10 sec and placed at 95° for 15 min. The sample was then vortexed for 10 sec, centrifuged at 14,000 rpm for 3 min and stored at −20° (short term) or −80° (long term, after PCR) until needed.

Two microliters of DNA solution were used per PCR reaction. Amplification was performed in a 25 μl volume containing 2.5 μl 10× buffer (GIBCO BRL), 0.75 μl $MgCl_2$ (50 mM), 0.5 μl nucleotide mix (10 mM each), 0.35 μl 20 mM primer 1, 0.35 μl 20 mM primer 2, 0.25 μl Taq DNA polymerase (GIBCO BRL) and ddH2O q.s.p. The mixture was covered with mineral oil before amplification in a Ericomp thermal cycler programmed as follows: 1 min at 94°, 1 min at 55° and 3 min at 72° for an initial cycle; 15 sec at 94°, 1 min at 55°, 3 min at 72° for 35 cycles and 15 sec at 94°, 1 min at 55°, 10 min at 72° for a terminal cycle.

After PCR, 5 μl of amplification product were separated electrophoretically on a 1% agarose gels at 80 V and visualized by ethidium bromide staining and UV fluorescence. For each individual analyzed, the two PCR reactions using A specific primers and B specific primers were performed. Primers sequences specific for the A type and B type of *ftsZ* gene are in WERREN *et al.* (1995a).

Southern hybridization: To confirm the presence of single and double infections in different insect species, Southern hybridizations of genomic DNA were performed. The DNA was digested with a restriction enzyme that gives different sized *ftsZ* fragments for the two bacterial groups and hybridized using *ftsZ* radiolabeled probe.

Total genomic DNA was extracted from ~200 females that were fed on hosts for 2 days, to stimulate egg maturation and ovarian development. After phenol-chloroform extraction, pellets were resuspended in TrisHCl-EDTA buffer and RNAase-treated. Around 1 μg of DNA extract was *Dde*I digested. A clone containing the A type of *ftsZ* gene (LbIIA) and a clone containing the B type of *ftsZ* gene (LbIIB) were used as controls. An amplification of a 1:10,000 dilution of each clone by PCR was performed with *ftsZ* general primers and the product was digested with *Dde*I. *Dde*I gives a characteristic 838-kb fragment in A-group Wolbachia and a 666-kb fragment in B-group Wolbachia.

Digested DNA was electrophoresed through 0.8% agarose gel using 1× TAE as buffer. The gel was pretreated following Stratagene's instructions for southern transfer with a PosiBlot pressure station. The DNA was transferred to a nylon filter (Duralon UV, Stratagene), dried and UV cross-linked.

Probe was prepared using a random labeling reaction with ^{32}P dATP following the manufacturer's recomandations (Amersham). A mix of approximately equal amounts (based on ethidium bromide stains of DNA before dilution) of LbIIA clone and LbIIB clone DNA was used as the template for the synthesis of the probe. The probe was denature 10 min at 99° and added to the hybridization solution (2× SSC, 5× Denhardt's, 0.1% sodium pyrophosphate, 25 mM sodium phosphate, 0.1% SDS, and 125 μg/ml calf thymus DNA). Hybridization was carried out overnight at 61°. After two washes of 20 min at 65° (4× SSC, 0.1% SDS, and 2× SSC, 0.1% SDS), the filter was dried and exposed to an autoradiographic film for 4 days in −80°.

Crossing experiments: Normally, *N. vitripennis* females produce strongly female-baised sex ratios when ovipositing alone on hosts (WHITING 1967). In contrast, incompatibility in Nasonia is expressed as production of all or nearly all-male families. This occurs because paternal chromosome loss results in (haploid) male production in this haplodiploid insect. Thus, a sex ratio shift is the standard phenotypic assay for incompatibility. Strains from the different experiments were tested for compatibility by crossing to strains of known compatibility type. We examined the compatibility type of five field strains maintained without diapause by mating females to LbII males and males to AsymC females. Both crosses were mated in sets of five females and two males per test tube. The five females were given five hosts for egg laying, and the overall offspring number and sex ratio were scored.

Crossing experiments to characterize the compatibility type of R511D sublines (AB, A, B and uninfected) were performed at the ninth generation after diapause. Females were mated in groups of 25 with five males for 24 hr. Males were removed and females were set with two hosts for egg laying.

Statistics: The analysis of cytogenetic observations of cytogenetic observation of percent infected eggs was performed using a logistic regression on bimodal data (GLIM, logit link function, AITKIN *et al.* 1988). The probability distribution of percent infected eggs within a lineage was assumed to be binomial. A line effect and a female effect within lines were introduced in a basic model with a fixed probability of infection for all eggs. Model selection was based on the use of likelihood ratio tests (LRT) and of AKAIKE's information criterion (AIC) (AKAIKE 1973). The significance of the change in deviance [DEV = $-2\mathrm{Ln}(L)$ where L is the maximum likelihood] after introducing the effect to be tested (line effect or female effect within line) was estimated by a chi-square test.

For the analysis of bacterial density, uninfected females were removed and density was normalized after a Box-Cox transformation, following the formula: $Y' = (Y^{\lambda} - 1)/\lambda$ (SOKAL and ROLF 1981) with $\lambda = 1/3$. The fitting to a normal distribution was tested with a one sample Kolmogorov-Smirnov test (SIEGEL and CASTELLAN 1988). Between line and within line-between female differences were tested by a two-way nested analysis of variance.

In the crossing experiments, differences in sex ratio were tested by a two-tailed Mann-Whitney U test. Mean comparisons of family size were performed by a t-test, with $P < 0.05$.

RESULTS

Double and single infection in standard laboratory strains: Four standard laboratory strains (LbII, R511, Ti277 and AsymC) were compared for A+B group Wolbachia and compatibility. LbII and R511 are wild-type strains, whereas Ti277 is an eye-color mutant strain that was originally discovered to be unidirectionally incompatible with standard laboratory strain (SAUL 1961). AsymC is a Wolbachia-free strain produced by antibiotic treatment and maintained free of the bacteria since 1986 (BREEUWER and WERREN 1990).

PCR amplification using group A and group B specific primers indicated that the wild-type strains are infected with both bacterial types, whereas Ti277 strain

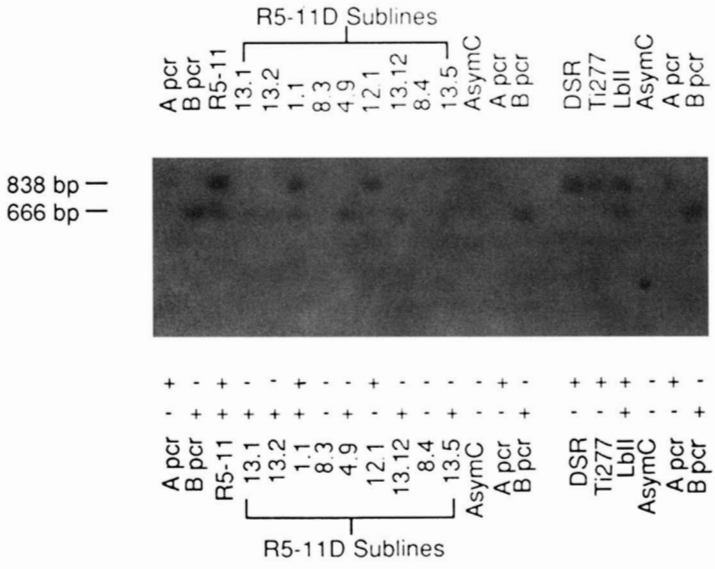

FIGURE 1.—Autoradiogram of Southern hybridization performed on total genomic DNA of ~200 females of four standard laboratory strains and of nine R511D sublines at the F_3 generation after diapause. DNA was digested with *Dde*I and hybridized to a probe made from a mix of A-type clone and B-type clone of the *ftsZ* Wolbachia gene. AsymC, *N. vitripennis* asymbiotic strain (tetracycline treated); R5–11, Ti277 and LbII, *N. vitripennis* standard strains; DSR, *D. simulans* Riverside; A pcr and B pcr, products of the PCR amplification with A group and B group specific *ftsZ* primers, using A-type clone and B-type clone as template, respectively.

is infected with only A group bacteria. As expected, AsymC failed to amplify product for either group (data not shown).

To verify results from the PCR, we performed Southern hybridizations using genomic DNA from each strain probed with radiolabeled Wolbachia *ftsZ* DNA (Figure 1). Genomic DNA was digested with a restriction enzyme (*Dde*I) that gives a characteristic restriction fragment for A and B group Wolbachia. Southern hybridizations confirmed that LbII and R511 carry both A and B group bacteria whereas Ti277 carries only A group bacteria. Only the 838-kb fragment typical of A bacteria was detected in Ti277 females (corresponding to the restriction sites of *Dde*I in the A type of *ftsZ* gene), whereas a fragment of 666 kb was detected in LbII and R511 DNA samples (corresponding to the restriction sites of *Dde*I in the B type of *ftsZ* gene). No hybridization occurred to the digested DNA of AsymC females (Figure 1). The results provide confirmation of the occurrence of single- and double-infected laboratory strains of *N. vitripennis* and thereby confirm the existence of two distinct types of Wolbachia.

Double and single infections are associated with compatibility type (Table 1). LbII and R511 show complete undirectional incompatibility with Ti277. Crosses between Ti277 females and males of the two standard strains yielded all male progeny whereas the reciprocal crosses yielded regular sex ratios (Table 2). Recall that incompatibility causes paternal genome loss, resulting in production of male (haploid) progeny in this haplodiploid wasp. Crosses between Ti277 males and AsymC females produced a low proportion of females (17%), significantly different from the sex ratio of the offspring from the reciprocal crosses (Mann-Whitney U test = 0, $P < 0.001$). The production of 17% females in the former cross shows that Ti277 expresses only a partial incompatibility with the AsymC. AsymC males were compatible with females from Ti277, LbII and R511 strains, as expected because of the uninfected status of the AsymC strain.

The offspring numbers are similar in compatible and incompatible crosses, as expected because in haplodiploid species CI causes production of (haploid) males rather than zygotic lethality. However, Ti277 females overall had higher fecundity than both AsymC females ($t = 5.34$, $P < 0.001$), R511 females ($t = 2.39$, $P < 0.05$) and LbII females ($t = 2.64$, $P < 0.01$). It is not known whether this is due to genetic differences in the strains or to differences in their infection status.

These data confirm previously reported compatibility relationships of LbII, Ti277 and AsymC (BREEUWER and WERREN 1993b). Unidirectional incompatibility occurs between males of two strains doubly infected with A and B Wolbachia and females of a strain singly infected with A Wolbachia.

Bacterial density in the eggs of Ti277 females (median 71.8, range 40.3–404.4, $N = 19$) is significantly

TABLE 1

Bacterial density and infection type of standard laboratory strains

	Sample size	Bacterial density		Infection type
		Median	Range	
LabII	14 (106)	206.9	78–647	AB
R511	15 (96)	163.3	72–334	AB
Ti277	19 (136)	71.8	8–491	A
AsymC	7 (30)	0	—	∅

Bacterial density of a strain is given as the median and the range over eggs. Sample size is the number of females (and overall number of eggs). Infection type: A, A group of Wolbachia; B, B group of Wolbachia; AB, A and B group of Wolbachia; ∅, no Wolbachia. Southern hybridization was used to determine infection type.

TABLE 2

Compatability relationships of Ti277 with standard laboratory strains

Crosses male × female	Sample size	Sex ratio	Family Size
Ti277 × LbII	20	0.86 ± 0.03	99.2 ± 22.2
LbII × Ti277	19	0 ± 0	124.9 ± 29.6
LbII × LbII	19	0.88 ± 0.03	92.9 ± 27.7
Ti277 × Ti277	17	0.89 ± 0.04	100.0 ± 26.0
Ti277 × R511	18	0.89 ± 0.03	90.1 ± 27.4
R511 × Ti277	17	0 ± 0	120.6 ± 30.9
R511 × R511	21	0.86 ± 0.15	95.9 ± 38.3
Ti277 × Ti277	17	0.84 ± 0.22	101.9 ± 26.5
Ti277 × AsymC	28	0.17 ± 0.21	88.2 ± 28.8
AsymC × Ti277	28	0.84 ± 0.23	116.5 ± 31.6
AsymC × AsymC	29	0.90 ± 0.03	91.2 ± 27.9
Ti277 × Ti277	26	0.82 ± 0.14	118.8 ± 19.4

Sex ratio is given as the proportion of females in the progeny. Family size corresponds to the adult offspring number. Both are given as means ± SD.

lower than in the eggs of LbII females (206.9, 126.6–388.1, $N = 14$) (Mann-Whitney U test = 40.5, $P < 0.01$) and of R511 females (163.3, 120.5–241.4, $N = 15$) (Mann-Whitney U test = 58, $P < 0.01$). An analysis of variance on normalized data indicates that 32.9% of the variance in bacterial density is due to strain effects, whereas the female effect (variation among females within a strain) accounts for 41.5% of the variance. The residual variance, which includes variation between eggs within a female, accounts for 25.6%. There is a significant variation between females within a strain, suggesting that selection could act upon the trait. Consistent with this, we have found that bacterial density can be increased in the Ti277 strain by selecting on incompatibility level (M.-J. PERROT-MINNOT and J. H. WERREN, unpublished data).

Effect of diapause on Wolbachia infection: Nasonia wasps overwinter as diapausing larvae. Exposure of the ovipositing female to short photoperiod and cool temperatures results in her larvae arresting development and entering diapause before pupation (WHITING 1967). Larval diapause is used as a method for storing stocks in the laboratory, because they can be kept alive in the refrigerator for up to 2 yr.

Preliminary results indicated that prolonged refrigeration during larval diapause can result in reduction and loss of Wolbachia. As a part of a study of incompatibility, we removed eight lines from refrigeration that had been rotated in diapause for 2–6 yr. Each line had been derived from a single inseminated female collected from field populations. We found that each of the lines was unidirectionally incompatible with the standard double-infected (AB) laboratory strain LbII and was compatible with the uninfected strain AsymC (data not shown), a pattern suggesting that these isofemale lines were bacterial free. Subsequent cytogenetic examination of the eggs revealed the Wolbachia were indeed absent from these lines.

The occurrence of bacteria-free lines could be explained by either a loss of Wolbachia during diapause or absence of bacteria before the strains being placed into diapause. To investigate the latter possibility, we tested five strains established from single females, collected from the vicinity of Rochester, NY, and maintained without diapause for 2–4 mon. All five strains were bidirectionally compatible with LbII and their bacterial density was not significantly lower than density in the standard strains (Table 3). PCR amplification using A and B *ftsZ* primers confirmed the presence of double infections in these strains.

To further investigate the effects of diapause on presence of Wolbachia, we examined 10 strains collected in 1991 and four collected in 1989. These strains had been maintained for one generation (F_1) before being placed under diapause conditions. Some F_1 progeny had been frozen at −70° for future molecular analysis. Although precise records of the diapause history of these strains were not kept, the 1989 strains had been subjected to at least two diapause cycles and the 1991 strains to at least one diapause cycle. To test for presence of A and B Wolbachia, the PCR assay was employed on single F_1 prediapause females and single postdiapause females from each strain. F_1 prediapause females from all 10 1991 strains and all four 1989 strains showed double infections (AB). However, after one diapause cycle of 2 yr (1991 strains), three out of 10 females analyzed were still doubly infected, three females harbored only the A Wolbachia and four females were uninfected. After at least two diapause cycles (1989 strains), three females from the four strains analyzed were no longer infected, and one female still harbored an AB infection. The proportion of uninfected females after at least two diapause cycles is significantly higher than after just one (Fisher exact test, $P = 0.027$).

Single infections from R511 postdiapause lines: The results above suggest that single-infected lines can be produced from a double-infected line after diapause. To investigate this possibility, 16 sublines were created from the double-infected line R511 after diapause (see MATERIALS AND METHODS). Nine R511D sublines have been extensively characterized by PCR assay, Southern hybridization, cytogenetics and crossing at different generations after diapause.

At the first generation (F_1) after diapause, a PCR assay on 30 females from 10 different sublines revealed polymorphisms for the presence of A and B group Wolbachia. Polymorphisms were detected both among and between lines (Table 4). For example, in line 13, two AB-infected, two A-infected and one uninfected females

TABLE 3

PCR analysis, bacterial density and compatibility relationships of five field strains collected in 1994

Field strains	Infection type				Compatibility relationships	
	PCR	Bacterial median	Density range	*t*-test	Female strain × male LbII	Male strain × female AsymC
51B	AB	234	77–379	abc	0.77 (0.05)[a]	0
13A	AB	159	78–249	a	0.82 (0.04)	0
14A	AB	237	157–410	c	0.86 (0.05)	0
18A	AB	277	171–479	c	0.80 (0.03)	0
19A	AB	155	97–262	a	0.80 (0.05)	0
R511	AB	163.3	72–334	a		
LbII	AB	206.9	78–647	b		

Bacterial density is based on three females per strain, five eggs per female. The median and range of density over eggs within a strain is shown. Differences in bacterial density among standard laboratory strains and field strains are tested by a *t*-test on normalized data. Mean densities in strains with the same letter are not significantly different ($P > 0.05$). Compatibility relationships are given as the mean proportion of females produced in crosses of each strain to LbII males and AsymC females.

[a] Parenthetical data are *P* values.

were detected. Line 15 contained two A, one B and two uninfected individuals.

Cytological examination of eggs was performed at the F_1 generation on six R511D lines. Significant differences were found between lines and between females within lines in both the proportion of infected eggs and the bacterial density. A model with a line effect on the proportion of infected eggs (AIC = 752.2, DEV = 202.3) provided a better fit to the data than a simple model with a fixed probability of infection for all eggs (AIC = 885.3, DEV = 359.3; $\chi^2 = 157$, d.f. = 5, $P < 0.001$). However, considering a female effect nested in the line effect (AIC = 644.3, DEV = 66.3) significantly improved the fit of the model ($\chi^2 = 136$, d.f. = 21, $P < 0.01$). Both factors therefore contribute to explaining differences in the proportion of infected eggs among F_1 females.

TABLE 4

Infection pattern of R511D lines (F_1) and sublines (F_3 and F_{11})

Lines	F_1 infection type	Sublines	F_3 infection type	F_{11} infection type
1	3 AB	1.1	AB	AB
		1.2	—	B
4	1 A, 4 ∅	4.9	B	B
8	2 A	8.3	∅	∅
		8.4	∅	∅
13	2 AB, 2 A, 1 ∅	13.1	B	B
		13.2	B	∅
		13.5	B	B
		13.12	B	B
12	5 ∅	12.1	A	A
15	2 A, 1 B, 2 ∅	15.12	—	A

Molecular characterization of Wolbachia strain in postdiapausing females of the R511D lines at the F_1 and F_{11} generation (PCR assay on single females) and at the F_3 generation (Southern hybridization on 200 females). Infection type is defined as in Table 1.

Following the analysis of proportion of infected eggs, the nine F_1 uninfected females among the 28 females analyzed were removed from the sample to test for differences in bacterial density. Bacterial density in the eggs of F_1 infected females was normally distributed after a Box and Cox transformation (Kolmogorov-Smirnov one sample test, $n = 141$, D = 0.04, NS). Differences between lines accounted for 53.1% of the variance and differences between infected females within line accounted for 16.8% of the variance in bacterial density. Moreover, mean bacterial density of all postdiapause F_1 females (median 27.3, range 0–257.2, $N = 28$) was significantly lower than mean bacterial density of R511 females from the nondiapause stock (163.3, range 118.1–241.4, $N = 15$) (Mann-Whitney *U* test = 39, $P < 0.001$). Three lines (1, 8, 13) had bacterial densities more similar to, but still lower than, the control R511 strain from which they were derived. Each of these lines showed a greater range in bacterial densities than did R511, specifically at the low end. Two lines (4 and 12) showed very low infection rate, with, respectively, one and two weakly infected females among the five females analyzed per line. These results clearly indicate that bacterial density was reduced immediately after diapause and that there was a greater stochastic variation in bacterial density, both between females and between eggs within females.

Southern hybridizations were performed at the F_3 generation on nine sublines, to confirm preliminary PCR results that these lines were segregating for A and B group Wolbachia. Methods were the same as for the standard laboratory strains and results are presented in Figure 1. One line (1.1) was doubly infected, four lines (13.1, 13.2, 13.12, 4.9) were singly infected

TABLE 5

Bacterial density and percent infection of eggs from the R511D F_1 lines, F_7 sublines and the standard R511 laboratory strain

F_1				F_7			
Lines	Inf. eggs (%)	Bacterial median	Density range	Sublines	Inf. eggs (%)	Bacterial median	Density range
1	100	98	27–339	1.1	100	344	215–604
				1.2.3	100	72	50–90
				1.2	100	251	87–388
4	20	0	0–101	4.9	100	37	11–188
				4.9.3	100	105	33–233
8	100	80	41–279	8.3	0	0	
				8.4	0	0	
13	70	51	0–273	13.1	100	64	41–125
				13.1.4	100	95	53–135
				13.2	0	0	
				13.5	100	79	24–115
				13.12	100	131	68–226
12	20	0	0–3	12.1	100	271	196–439
15	70	6	0–100	15.12	100	255	136–381
				15.12.3	100	167	73–398
R511	100	163.3	72–334	R511	100	163.3	72–334

The median and range of bacterial density over all eggs within a line are given at the F_1 generation (five females per line, 8–10 eggs per female) and F_7 generation (three females per subline, five eggs per female). R511D sublines are designed by the original female number followed by the subline number (*e.g.*, 13.1). Further sublines established from these are designed with an additional identifier (*e.g.*, 13.1.4).

with B, two lines (12.1 and 15.12) were singly infected with A and two lines (8.3 and 8.4) were uninfected. It is possible that a strain weakly infected with A or B could appear uninfected in a Southern hybridization. However, PCR analysis was carried out on the same DNA extracts and yielded the same infection patterns for those sublines (data not shown). Thus, both PCR and Southern hybridization confirm the separation of A and B group Wolbachia from a double-infected strain after diapause.

Bacterial density in some sublines was examined in the F_7 generation (Table 5). By contrast to the postdiapause F_1 females, mean bacterial density in the eggs laid by infected F_7 females (median 91.5, range 94.9–248.4, $N = 42$) was not significantly different than mean density of R511 females from the nondiapause stock (163.3, range 118.1–241.4, $N = 15$). Density was normalized following the same procedure as for F_1 generation, after the removal of the uninfected females (restricted to the two uninfected sublines 8.3 and 8.4) (Kolmogorov-Smirnov one sample test, $n = 348$, $D = 0.04$, NS). The nested analysis of variance showed an increase in the strain contribution to variance in bacterial density to 72.5% (from 53.1% in the F_1), 13.8% contribution of females within a strain, and 13.7 percent residual (which includes differences between eggs within females). Both the strain contribution and the female contribution were significant ($P < 0.001$).

Results are indicative of stochastic turnover of bacteria in the sublines between the F_1 and F_7 generations. For example, the line 13 sublines (13.1 and 13.12) contained four B-infected sublines and one uninfected subline at the F_7 generation, in contrast to two AB, two A and one uninfected females at the F_1 generation. Line 8, which had reasonable bacterial densities among the tested F_1 females, yielded two uninfected sublines by the F_7. This high degree of stochastic loss was no doubt influenced by maintenance of these lines through single females from the F_1 to F_7 generations. However, by the F_7 generation, the lines apparently stabilized considerably. All examined eggs from infected lines contained bacteria (in contrast to F_1 generation). All lines were stable in terms of bacterial type from the F_3 to the F_{11} generation (Table 4), except for 13.2 which went from B, based upon the Southern hybridization at generation F_3, to ∅ (no Wolbachia infection) based upon cytogenetics at generation F_7 and PCR at generation F_{11}. Further tests have confirmed the loss of bacteria in this line. The same pattern of infection was found when primers specific for A and B group 16S rDNA (WERREN *et al.* 1995a) were used (data not shown).

Effect of single and double infection on compatibility: By the F_8 generation, a number of single-infected R511D lines had been produced in which the two bacterial types (A and B) were separated. We therefore investigated the effect of single A, single B, and double AB infections on compatibility. A set of crosses were performed using the laboratory strains of known bacterial type and compatibility, LbII (AB), Ti277 (A) and AsymC (∅). One AB strain, four B strains, two A strains

TABLE 6

Percent females (compatibility) in crosses between R511D sublines and standard laboratory strains

Type	Cross	LbII	Ti277	AsymC	Self
AB	1.1 male	85 ± 20 (21)	0 ± 0.5 (21)	0 ± 0 (23)	90 ± 5 (20)
	1.1 female	87 ± 19 (24)	90 ± 4 (24)	92 ± 3 (24)	
B	13.1 male	89 ± 3 (20)	0 ± 1 (25)	1 ± 2 (22)	86 ± 7 (19)
	13.1 female	11 ± 2 (25)	16 ± 14 (25)	86 ± 8 (21)	
B	13.5 male	88 ± 3 (23)	0 ± 0.3 (23)	0 ± 0.4 (21)	77 ± 27 (23)
	13.5 female	4 ± 5 (22)	10 ± 11 (20)	90 ± 3 (23)	
B	13.12 male	86 ± 6 (21)	0 ± 0.4 (23)	0 ± 0 (19)	91 ± 3 (22)
	13.12 female	5 ± 7 (20)	7 ± 8 (15)	90 ± 4 (20)	
B	4.9 male	86 ± 5 (19)	1 ± 2 (22)	0 ± 1 (23)	87 ± 3 (24)
	4.9 female	4 ± 8 (20)	8 ± 4 (23)	83 ± 19 (24)	
A	15.12 male	89 ± 5 (22)	88 ± 4 (24)	5 ± 4 (21)	86 ± 4 (21)
	15.12 female	1 ± 2 (18)	83 ± 20 (18)	86 ± 17 (24)	
A	12.1 male	91 ± 2 (22)	82 ± 19 (23)	6 ± 6 (25)	86 ± 8 (24)
	12.1 female	0 ± 0.1 (23)	80 ± 20 (21)	86 ± 6 (21)	
∅	8.3 male	86 ± 11 (22)	86 ± 5 (25)	91 ± 4 (22)	87 ± 6 (16)
	8.3 female	0 ± 0 (14)	6 ± 22 (17)	90 ± 3 (21)	
∅	13.2 male	88 ± 3 (18)	88 ± 5 (18)	91 ± 4 (22)	82 ± 15 (25)
	13.2 female	0 ± 0 (22)	12 ± 10 (25)	88 ± 4 (23)	
	Self	87 ± 21 (20)	85 ± 7 (18)	89 ± 3 (21)	

Sex ratio of the progeny is given as the mean proportion of females. Sample size is the number of families scored. (See legend of Table 4 for R511D sublines labeling). Infection types are given in parentheses: LbII, AB; Ti277, A; AsymC, ∅.

and two ∅ (uninfected) strains were tested (Table 6). Results show the following general patterns. The AB-infected strains show the same compatibility relationships as the standard double-infected strains LbII and R511. Males are compatible with LbII and Ti277, and nearly completely incompatible with AsymC. The B-infected strains show quite interesting compatibility relationships. Males from B strains are compatible with LbII, incompatible with Ti277 (A) and incompatible with AsymC (∅). Females are nearly completely incompatible with LbII (AB) and Ti277 (A), and compatible with AsymC (∅).

A and B Wolbachia in *N. vitripennis* appear to be bidirectionally incompatible with each other. In addition, double-infected males are incompatible with both single A and single B-infected females, whereas the reciprocal is compatible.

To further investigate these effects, crosses were performed among the R511D sublines between AB-, A-, and B-infected sublines. Results are shown in Figure 2. The A (12.1) and B (4.9) sublines are bidirectionally incompatible, whereas the AB line (1.1) is unidirectionally incompatible with both A and B sublines. Subline 1.1 males are incompatible with both 12.1 and 4.9 females, whereas 1.1 females are compatible with both 12.1 and 4.9 males. These results have been further confirmed using other A and B sublines (data not shown). These results show rapid formation of partial to complete reproductive isolation between descendants of a single strain within a species, due to the segregation of associated symbiotic bacteria.

DISCUSSION

Since the discovery that two distinct sequences of Wolbachia 16S rDNA are found in some insect strains (BREEUWER *et al.* 1992), there have been two alternative explanations for the observation. Either the strains harbor double infections of distinct Wolbachia types or Wolbachia have two (or more) different 16S rDNA operons. Nevertheless, evidence that double infections do occur has been mounting. WERREN *et al.* (1995a) showed by PCR amplification that strains from seven different species having two different 16S rDNA types also have two distinct sequences of the protein coding gene *ftsZ*, whereas strains with single 16S rDNA also have single *ftsZ*. ROUSSET and SOLIGNAC (1995) found by PCR that some strains of *D. simulans* and *D. sechellia* contain two 16S rDNA types, whereas other strains contain only one. They further showed that the apparent double and single infections are associated with compatibility differences. Similarly, SINKINS *et al.* (1995a) found by PCR amplification, strains with single and double 16S rDNA sequences in the mosquito *Aedes albopictus*. Males from strains with two 16S rDNA sequences are incompatible with females from single 16S rDNA strains. Finally, MERCOT *et al.* (1995) succeeded in producing strains of *D. simulans* with single 16S rDNA types (based upon PCR) from a strain containing two 16S rDNA types and showed that the two types, when separated, are bidirectionally incompatible with each other. Their ability to separate the two types demonstrates that the sequences come from sep-

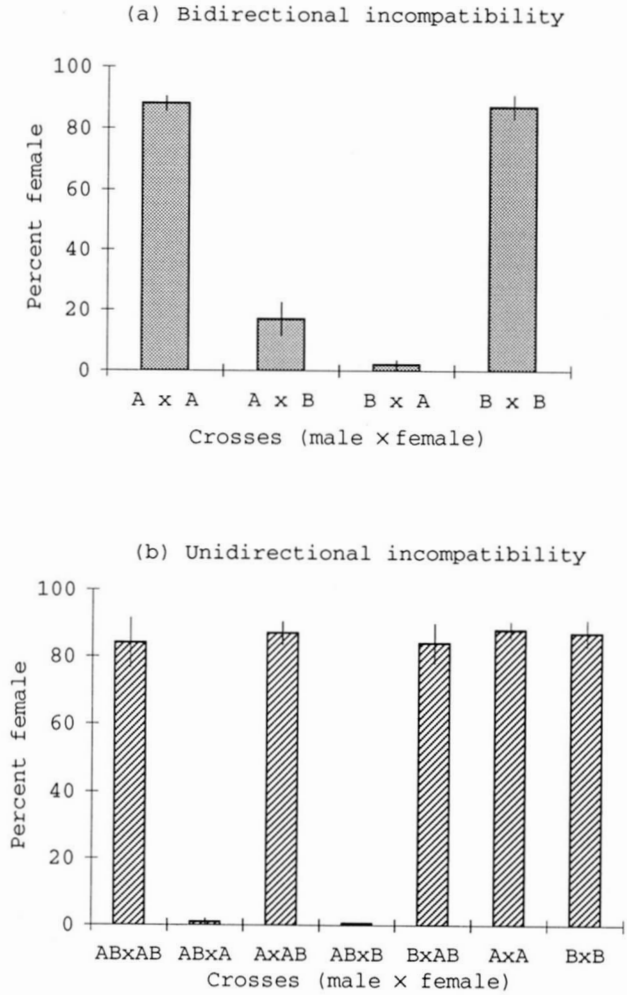

FIGURE 2.—Crosses between AB, A and B type R511D sublines. Percent females is shown for crosses between sublines 1.1 (AB), 15.12 (A) and 13.1 (B) along with the standard deviation. The A and B lines are bidirectionally incompatible, whereas the AB line males are unidirectionally incompatible with A and B subline females.

arate bacterial strains, rather than from two different operons within a single bacterial strain.

Our results establish the existence of double infections in the parasitic wasp *N. vitripennis*. First we show the presence of two different bacterial sequence types in several standard laboratory strains, but single sequence type in one laboratory strain (Ti277). Compatibility differences between these strains are associated with occurrence of double and single infections. Second, we show that the two types of Wolbachia can be separated from each other (after diapause), producing sublines (R511D) infected with one or the other type (A or B) from a double-infected strain (R511). These two bacterial types are bidirectionally incompatible with each other. Separation of the two types shows that they are not merely different gene sequences within a single bacterial strain, but represent single gene sequences within different bacterial strains. Finally, we confirm by Southern hybridization of genomic DNA the occurrence of double and single infections. This establishes that the two types of Wolbachia are indeed present within infected females and are not due to PCR contamination. Given the sensitivity of PCR, confirmation of PCR results by Southern hybridization is worthwhile.

How do double infections become established within a species? Presumably, double infections typically arise from the horizontal transfer of a Wolbachia into a cytoplasm previously infected with a different Wolbachia. It appears that the double infection can become established because double infection creates a "new" incompatibility type cytoplasm that has an advantage over single-infected cytoplasm. In all cases of double infection reported so far (including this study), complete or partial incompatibility is found between double-infected males and single-infected females (MERCOT *et al.* 1995; ROUSSET and SOLIGNAC 1995; SINKINS *et al.* 1995a,b). The double-infected cytoplasm has a selective advantage and will typically increase because double-infected eggs are compatible with both double- and single-infected sperm, whereas single-infected eggs are incompatible with double-infected sperm. Thus, double infection will increase under the same general conditions that favor increase of single infection in an uninfected population (CASPARI and WATSON 1959; TURELLI 1994). Consistent with the advantage of double infections, WERREN *et al.* (1995b) found among a sample of neotropical insect species that double infections occurred significantly more frequently than expected by chance, indicating that these infections are more easily established or maintained.

Cytogenetically, cytoplasmic incompatibility results from the dysfunction of paternal chromatin in the first mitotic division after fertilization (RYAN and SAUL 1968; JOST 1971; BEEUWER and WERREN 1990; O'NEILL and KARR 1990; REED and WERREN 1995). A combination of genetic and cytogenetic studies in *N. vitripennis* have established that it is the paternal chromosomes that improperly condense in incompatible crosses (RYAN and SAUL 1968; BREEUWER and WERREN 1990; REED and WERREN 1995). REED and WERREN (1995) further showed that the paternal chromatin mass can either be lost in the first mitosis or fragmented with segregation of some paternal chromatin to daughter nuclei. These results are supported by studies showing production of centric fragments in incompatible crosses (RYAN *et al.* 1985; BEUKEBOOM *et al.* 1993). Based upon genetic and cytogenetic studies, Wolbachia presumably cause cytoplasmic incompatibility by modification of sperm (probably sperm chromatin) in the male and rescue of that modification within the egg.

Our results have implications on the mechanism of action of Wolbachia. We show nearly complete bidirectional incompatibility between single-infected lines harboring different Wolbachia strains (A and B). These

data indicate that A and B Wolbachia of *N. vitripennis* constitute two independent and cross incompatible CI factors and suggest that the two bacteria types differ in the mechanisms of paternal chromatin modification and rescue, or in the targets associated with a common mechanism. In addition, the observation that double-infected males are incompatible with singly infected females of either type (A or B) indicate that the paternal chromosomes are specifically, separately and distinctly imprinted in the spermatocytes and rescued in the egg cytoplasm by each strain of Wolbachia.

The questions arise as to how many separate incompatibility types (modification and rescue mechanisms) exist and how quickly do new ones evolve. The evidence demonstrates that *Nasonia* A and B group Wolbachia differ in their mechanisms of action. Many strains of A and B group Wolbachia are found in a diverse range of insects (WERREN *et al.* 1995b), and these two groups have diverged 58–67 mya (based upon synonymous substitution rates), allowing ample time for the evolution of divergent mechanisms. In contrast, the H (Hawaii) and R (Riverside) strains of *D. simulans* are bidirectionally incompatible (O'NEILL and KARR 1990), but both belong to the A group of Wolbachia and are estimated to have diverged from each other only 0–2.5 mya (WERREN *et al.* 1995a). This suggests that the divergent mechanisms of action of Wolbachia can evolve in a reasonably short period of time.

As well as bacterial strain, bacterial infection levels may be important in determining compatibility relationships. BREEUWER and WERREN (1993b) proposed that both level of incompatibility and direction of incompatibility can be influenced by bacterial dosage. This "bacterial dosage model" was based upon two lines of evidence: lower bacterial numbers in some strains (notably Ti277) correlated with direction of incompatibility and changes in incompatibility of the standard laboratory strain LbII occurred after partial curing antibiotic treatments. We now know that Ti277 is singly infected with A bacteria whereas LbII is doubly infected with A and B bacteria. Hence, the unidirectional incompatibility between LbII males and Ti277 females is likely to be due to differences in bacteria type rather than just density. This conclusion is further supported by bidirectional incompatibility between A and B R511D sublines with similar bacterial densities.

However, other effects may still be explained by bacterial infection levels. The partial compatibility of Ti277 with AsymC could be due to its intermediate bacterial density. If there is a threshold level of bacteria necessary for sperm modification, then variability in bacterial density among spermatocytes could explain the production of some female progeny (partial incompatibility) in the Ti277 × AsymC crosses. Alternatively, partial compatibility of Ti277 with AsymC could result from a weak potential of the A-Wolbachia for CI induction. This issue is best resolved by determining whether increased density of the A-Wolbachia in this strain leads to complete incompatibility. We have selected for increased incompatibility in Ti277 and found a correlated increase in bacterial density (M.-J. PERROT-MINNOT and J. H. WERREN, data not shown), supporting the role of infection level in this case.

In terms of potential bacterial strain effects, GIORDANO *et al* (1995) present evidence that the *D. mauritiana* Wolbachia strain does not express incompatibility. Similarly, the *D. melanogaster* Wolbachia may be a weakly expressing CI strain (HOFFMANN 1988). Both are A-Wolbachia based on *ftsz* sequencing (WERREN *et al.* 1995a). Thus, there may be a spectrum of CI expression within A-Wolbachia. But, interactions with bacterial density and host genotype must, of course, also be considered.

Results show that prolonged diapause (and/or associated cold temperatures) causes a reduction in bacterial density and can cause bacterial loss. In addition, bacteria tend to rebound and increase in density after diapause if still present in a line. A likely explanation is that cold temperature during diapause could reduces survival and replication rate of the bacteria in the germ cells arrested in development. We do not know whether loss of Wolbachia in females maintained in prolonged diapause under laboratory conditions is relevant to natural populations. Although it is generally assumed that most females undergo diapause for one winter period, field data on the proportion of females that stay in prolonged diapause are lacking. The specific dynamics of bacterial loss as a function of time in diapause has not been studied.

In addition to diapause, other physiological and environmental factors known to lead to incomplete maternal transmission include high temperatures (STEVENS 1989) and natural antibiotic curing (STEVENS and WICKLOW 1992). In addition, a decrease in bacterial density has been reported in the spermatocytes of ageing males of *D. simulans* (HOFFMANN *et al.* 1986; BRESSAC and ROUSSET 1993), resulting in incomplete CI expression.

The changes in bacterial density in double-infected females during diapause have resulted in the production of eggs heterogeneous for bacterial type (A, B, AB or none) and bacterial density. Stochastic loss of Wolbachia strain(s) among F_1 progenies would result from unequal assortment of bacteria between oocytes and low bacterial density. Unequal segregation of bacteria among cysts has also been suspected in females of *D. simulans* and in males of *D. melanogaster* (ROUSSET and DE STORDEUR 1994; SOLIGNAC *et al.* 1994). MERCOT *et al.* (1995) have shown assortment of two bacterial types among progeny of double-infected females in *D. simulans*. In addition, reports of compatibility changes in *N. vitripennis* laboratory strains (CONNER and SAUL

1986) could be due to stochastic changes in bacterial composition and density after diapause.

An intriguing part of our study is the rapid development of nearly complete reproductive isolation between sublines of a strain due to the segregation of Wolbachia types. In natural populations though, this is unlikely to lead to the establishment of reproductively isolated populations because of the selective advantage to the surviving double-infected cytoplasms, which should quickly eliminate the single infection types. Indeed, diapause "curing," if it is common in field populations, could facilitate hybridization between *N. vitripennis* and *N. girault*i, two sibling species that have been found to be reproductively incompatible due to associated Wolbachia infections (BREEUWER and WERREN 1990).

In our opinion, Wolbachia are most likely to promote rapid reproductive isolation by one of the two following mechanisms: (1) geographically isolated populations independently acquire and fix different Wolbachia. When they come into sympatry, bidirectional incompatibility results, (2) Wolbachia are acquired and fixed in a species. Subsequently, the Wolbachia diverge in geographically isolated populations into new bidirectionally incompatible types. Again, reproductive isolation would occur if they come back into sympatry. Even unidirectional cytoplasmic incompatibility can, in principle, accelerate speciation as a result of gain of a new Wolbachia strain in one of the populations. Complete unidirectional incompatibility alone does not prevent gene flow between species and therefore is insufficient (CASPARI and WATSON 1959). However, when it is coupled with other forms of genetic incompatibility in the reciprocal direction (COYNE 1992) (*e.g.*, negative epistatic interactions between nuclear genes), then reproductive isolation could result. It is still unknown how quickly new compatibility mechanisms can evolve or how frequently Wolbachia are involved in reproductive isolation between incipient species.

We thank FRANK CÉZILLY for critical review of the manuscript and V. KATJU and K. MYLER for technical assistance. This work was supported by National Science Foundation funding to J.H.W. and a Lavoisier postdoctoral grant from the French Ministry of Foreign Affairs to M.-J.P.M.

LITERATURE CITED

AITKIN, M., D. ANDERSON, B. FRANCIS and J. HINDE, 1988 *Statistical Modelling in GLIM.* Clarendon, Oxford, UK.

AKAIKE, H., 1973 Information theory and an extension of the maximum likelihood principle, pp. 267–281 in *International Symposium on Information Theory,* Ed. 2, edited by B. N. PETRAN and F. CSAKI. Akademiai Kiadi, Budapest, Hungary.

BARR, A. R., 1980 Cytoplasmic incompatibility in natural populations of a mosquito, *Culex pipiens.* Nature **283:** 71–72.

BEUKEBOOM, L. W., K. M. REED and J. H. WERREN, 1993 Effects of deletions on mitotic stability of the paternal sex ratio (PSR) chromosome from Nasonia. Chromosoma **102:** 20–26.

BRAIG, H. R., H. GUZMAN, R. B. TESH and S. L. O'NEILL, 1994 Replacement of the natural Wolbachia symbiont of *Drosophila simulans* with a mosquito counterpart. Nature **367:** 453–455.

BREEUWER, J. A. J., and J. H. WERREN, 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature **346:** 558–560.

BREEUWER, J. A. J., and J. H. WERREN, 1993a Effect of genotype on cytoplasmic incompatibility between two species of Nasonia. Heredity **70:** 428–436.

BREEUWER, J. A. J., and J. H. WERREN, 1993b Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis* Genetics **135:** 565–574.

BREEUWER, J. A. J., R. STOUTHAMER, S. M. BARNS, D. A. PELLETIER, W. G. WEISBURG *et al.,* 1992 Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Mol. Biol. **1:** 25–36.

BRESSAC, C., and F. ROUSSET, 1993 The reproductive incompatibility system in *Drosophila simulans:* Dapi-analysis of the Wolbachia symbionts in sperm cysts. J. Invertebr. Pathol. **61:** 226–230.

CASPARI, E., and G. S. WATSON, 1959 On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution **13:** 568–570.

CONNER, G. W., and G. B. SAUL, 1986 Acquisition of incompatibility by inbred wild-type stocks of *Mormoniella.* J. Hered. **77:** 211–213.

COYNE, J. A., 1992 Genetics and speciation. Nature **355:** 511–515.

GIORDANO, R., S. L. ONEILL and H. M. ROBERTSON, 1995 Wolbachia infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *Drosophila mauritiana.* Genetics **140:** 1307–1317.

HOFFMANN, A. A., 1988 Partial incompatibility between two australian populations of *Drosophila melanogaster.* Entomol. Exp. Appl. **48:** 61–67.

HOFFMANN, A. A., M. TURELLI and G. M. SIMMONS, 1986 Unidirectional incompatibility between populations of *Drosophila simulans.* Evolution **40:** 692–701.

JOST, E., 1971 Meiosis in the male of *Culex pipiens* and *Aedes albopictus* and fertilization in the *Culex pipiens* complex. Can. J. Genet. Cytol. **13:** 237–250.

MERCOT, H., B. LLORENTE, M. JACQUES, A. ATLAN and C. MONTCHAMPS-MOREAU, 1995 Variability within the seychelles cytoplasmic incompatibility system in *Drosophila simulans.* Genetics **141:** 1015–1023.

O'NEILL, S. L., and T. L. KARR, 1990 Bidirectional incompatibility between conspecific populations of *Drosophila simulans.* Nature **348:** 178–180.

O'NEILL, S. L., R. GIORDANO, A. M. E. COLBERT, T. L. KARR and H. M. ROBERTSON, 1992 16S rDNA phylogenetic analysis of the bacterial endosymbiont associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:** 2699–2702.

REED, K. M., and J. H. WERREN, 1995 Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (Wolbachia): a comparative study of early embryonic events. Mol. Reprod. Dev. **40:** 408–418.

RIGAUD, T., C. SOUTY-GROSSET, R. RAIMOND, J. P. MOCQUARD and P. JUCHAULT, 1991 Feminizing endocytobiosis in the terrestrial crustacean *Armadillium vulgare* Latr. (Isopoda): recent acquisitions. Endocytobiosis Cell Res. **7:** 259–273.

ROUSSET, F., and E. DE STORDEUR, 1994 Properties of *Drosophila simulans* strains experimentally infected by different clones of the bacterium Wolbachia. Heredity **72:** 325–331.

ROUSSET, F., and M. SOLIGNAC, 1995 Evolution of single and double Wolbachia symbioses during speciation in the *Drosophila simulans* complex. Proc. Natl. Acad. Sci. USA **92:** 6389–6393.

ROUSSET, F., D. BOUCHON, B. PINTUREAU, P. JUCHAULT and M. SOLIGNAC, 1992 Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. **250:** 91–98.

RYAN, S. L., and G. B. SAUL, 1968 Post-fertilization effect of incompatibility factors in *Mormoniella.* Mol. Gen. Genet. **103:** 29–36.

RYAN, S. L., G. B. SAUL and G. W. CONNER, 1985 Aberrant segregation of R-locus genes in male progeny from incompatible crosses in *Mormoniella.* J. Hered. **76:** 21–26.

SAUL, G. G., 1961 An analysis of non-reciprocal cross incompatibility in *Mormoniella vitripennis* (Walker). Z. Vererbungsl. **92:** 28–33.

SIEGEL, S., and N. J. CASTELLAN, 1988 *Nonparametric Statistics for the Behavioral Sciences,* Ed. 2. McGraw-Hill, New York.

SINKINS, S. P., H. R. BRAIG and S. L. O'NEILL, 1995a Wolbachia *pipientis:* bacterial density and unidirectional cytoplasmic incom-

patibility between infected populations of *Aedes albopictus*. Exp. Parasit. **81:** 284–291.

SINKINS, S. P., H. R. BRAIG and S. L. O'NEILL, 1995b Wolbachia superinfections and expression of cytoplasmic incompatibility. Proc. R. Soc. Lond. B Biol. Sci. **261:** 325–330.

SOKAL, R. R., and F. J. ROLF, 1981 *Biometry*, Ed 2. Freeman, New York.

SOLIGNAC, M., D. VAUTRIN and F. ROUSSET, 1994 Widespread occurence of the proteobacteria Wolbachia and partial cytoplasmic incompatibility in *Drosophila melanogaster*. C. R. Acad. Sci. Paris **317:** 461–470.

STEVENS, L., 1988 Environmental factors affecting reproductive incompatibility in flour beetles, genus *Tribolium*. J. Invert. Pathol. **53:** 78–84.

STEVENS, L., and D. T. WICKLOW, 1992 Multispecies interactions affect cytoplasmic incompatibility in *Tribolium* flour beetles. Am. Nat. **140:** 642–653.

STOUTHAMER, R., J. A. L. BREEUWER, R. F. LUCK and J. H. WERREN, 1993 Molecular identification of microorganisms associated with parthenogenesis. Nature **361:** 66–68.

TURELLI, M., 1994 Evolution of incompatibility-inducing microbes and their hosts. Evolution **48:** 1500–1513.

WADE, M. J., and N. W. CHANG, 1994 Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite Wolbachia. Nature **373:** 72–74.

WADE, M. J., and L. STEVENS, 1994 Microorganism mediated reproductive isolation in flour beetles (Genus *Tribolium*). Science **278:** 527–528.

WALSCH, P. S., D. A. METZGER and R. HIGUCHI, 1991 Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:** 506–513.

WERREN, J. H., L. R. GUO and W. ZHANG, 1995a Evolution and phylogeny of Wolbachia: reproductive parasites of Arthropods. Proc. R. Soc. Lond. B Biol. Sci. **251:** 55–63.

WERREN, J. H., D. W. WINDSOR and L. R. GUO, 1995b Distribution of Wolbachia in neotropical arthropods. Proc. R. Soc. Lond. B Biol. Sci. **262:** 197–204.

WHITING, A. R., 1967 The biology of the parasitic wasp *Mormoniella vitripennis*. Q. Rev. Biol. **42:** 333–406.

YEN, J. H., and A. R. BARR, 1971 New hypothesis of the cause for cytoplasmic incompatibility in *Culex pipiens*. Nature **232:** 657–658.

Communicating editor: A. G. CLARK