Journal of Invertebrate Pathology **77,** 251–257 (2001)
doi:10.1006/jipa.2001.5026, available online at http://www.idealibrary.com on IDEAL®

Wolbachia Bacteria Effects after Experimental Interspecific Transfers in Terrestrial Isopods

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Received February 15, 2001, accepted April 20, 2001

***Wolbachia* bacteria are intracellular parasites, vertically transmitted from mothers to offspring through the cytoplasm of the eggs. They manipulate the reproduction of their hosts to increase in frequency in host populations. In terrestrial isopods for example, *Wolbachia* are responsible for the full feminization of putative males, therefore increasing the proportion of females, the sex by which they are transmitted. Vertical transmission, however, is not the only means for *Wolbachia* propagation. Infectious (i.e., horizontal) transmission between different host species or taxa is required to explain the fact that the phylogeny of *Wolbachia* does not parallel that of their hosts. The aim of this study was to investigate, by experimental transinfections, whether *Wolbachia* strains could be successfully transferred to a different, previously uninfected isopod host. While *Wolbachia* survived in all the studied recipient species, vertical transmission was efficient only in cases where donor and recipient species were closely related. Even in this case, *Wolbachia* strains did not always keep their ability to entirely feminize their host, a deficiency that can be link to a low bacterial density in the host tissues. In addition, *Wolbachia* infection was associated with a decrease in host fertility, except when the bacterial strain came from the same host population as the recipient animals. This suggest that *Wolbachia* could be adapted to local host populations. It therefore seems that isopod *Wolbachia* are highly adapted to their host and can hardly infect another species of hosts. The successful infection of a given *Wolbachia* strain into a new isopod host species therefore probably requires a strong selection on bacterial variants.**

***Key Words: Wolbachia*; crustaceans; endosymbionts; reproductive parasites; sex ratio; specificity.**

INTRODUCTION

Wolbachia α-proteobacteria are a striking and intriguing case of intracellular parasites. *Wolbachia* are probably the most widespread bacteria in invertebrates, infecting 20–80 % of all insects (e.g., Werren and Windsor, 2000; Jeyaprakash and Hoy, 2000), some mites (e.g., Johanowicz and Hoy, 1995), numerous terrestrial isopods (Bouchon *et al.*, 1998), and filarial worms (Bandi *et al.*, 1998). *Wolbachia* are maternally transmitted, through the cytoplasm of the eggs. Instead of increasing their hosts' reproduction or survival, as expected from classical theory (Fine, 1975; Yamamura 1993), they evolved toward "reproductive parasitism," i.e., they alter hosts' reproduction to facilitate their spread in host populations (O'Neill *et al.*, 1997). Some *Wolbachia* strains bias the sex ratio of their host toward females, i.e., the transmitting sex (reviewed in Bandi *et al.*, 2001). In terrestrial isopods particularly, they are responsible for the feminization of genetic males (Martin *et al.*, 1973; Bouchon *et al.*, 1998). Other *Wolbachia* strains induce parthenogenesis and male-killing in insects (Bandi *et al.*, 2001). Another *Wolbachia*-induced alteration of reproduction is the cytoplasmic incompatibility (CI) expressed in numerous (if not all) host taxa infected by this symbiont (O'Neill *et al.*, 1997).

The repartition of *Wolbachia* strains across hosts and the diversity of their effects cannot been explained by strict vertical transmission only. Infectious (i.e., horizontal) transmission between different host species or taxa is required to explain the overall noncongruence between host and symbiont phylogenies and the widespread *Wolbachia* infection among arthropods (Rousset *et al.*, 1992; Rigaud and Rousset, 1996; Vavre *et al.*, 1999).

Several studies using experimental infections revealed that *Wolbachia* can often infect a foreign indi-

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0022-2011/01 $35.00

vidual host. However, a stable implantation of the infection in lineages after vertical transmission through generations is not always achieved. Failures often occur when *Wolbachia* are transinfected in a host phylogenetically distant from their native host (Rigaud and Juchault, 1995; Van Meer and Stouthamer, 1999; Heath *et al.,* 1999; but see Pintureau *et al.,* 2000), whereas successes are generally found between closely related species (Boyle *et al.,* 1993; Clancy and Hoffmann, 1997; but see Braig *et al.,* 1994). Furthermore, *Wolbachia* implantation in a foreign host lineage often leads to loss of strength of their phenotypic expression. Differences in these cases were found to be due to different *Wolbachia* density or distribution in their new host's tissues (Breeuwer and Werren, 1993; Clancy and Hoffmann, 1997; Poinsot *et al.,* 1998; Pintureau *et al.,* 2000). All these data suggest that only a few attempts of *Wolbachia* horizontal transfers in the wild have led to permanent establishment in a new host species.

Most of these data concerned CI or parthenogenesis-inducing *Wolbachia* in insects and only a few feminizing *Wolbachia* in crustaceans. The aim of this study was to follow the behavior of isopod feminizing *Wolbachia* transferred into several foreign host species, to answer the following questions: Can a feminizing *Wolbachia* be established in foreign host lineages after a horizontal transfer? Do closely related *Wolbachia* induce different effects when transferred into the same foreign hosts? And if so are the differences explained by differences in bacterial load?

MATERIAL AND METHODS

Wolbachia and Their Hosts

Wolbachia came from infected host lineages maintained in the laboratory for several years. Two *Wolbachia* strains were used; one named *w*Av from *Armadillidium vulgare,* collected in 1990 at Cells sur Belles, France, and one strain named *w*An from *A. nasatum*, collected in 1994 at Mignaloux, France. These strains are phylogenetically closely related; their 16S rDNA sequences are identical (Bouchon *et al.,* 1998), whereas sequences of the variable *wsp* gene show a divergence of 0.48% (Cordaux *et al.,* 2001). Both strains induce feminization in their original host in the wild and in the lab (i.e., genetic males infected with *Wolbachia* generally develop into females; when feminization is incomplete, they can develop an intersex phenotype). Infected females thus produce highly female-biased progenies: 95% of daughters are generally obtained on average (e.g., Juchault and Legrand, 1979; Rigaud and Juchault, 1993).

Wolbachia were transferred into uninfected strains of the following species: *A. vulgare* (from Nice, France), *A. nasatum* (from Mignaloux) (both Armadillididae family), *Oniscus asellus* (Oniscidae family, from Quinçay, France), and *Porcellio scaber* (Porcellionidae, from Celles sur Belle, France). In these uninfected lineages, there was no bias in sex ratio and sex is genetically determined by sex chromosomes. In *A. vulgare* and *O. asellus* WZ individuals are female and ZZ individuals are male. In *A. nasatum,* XX individuals are female, XY are male. The heterogamety system is unknown in *P. scaber*. The woodlice that were used have been maintained by outbreeding in the lab for at least 5 years; they have a generation time of approximately 1 year. The different host families used were phylogenetically well differentiated (Michel-Salzat and Bouchon, 2000).

Inoculations

The transfer was made by the injection of a homogenate of infected tissues into the general cavity of adult females, as described in Rigaud and Juchault (1995). Per combination of host species and *Wolbachia* strain, between 10 and 20 females were injected. Between 12 and 20 females were kept as negative controls. No control consisting of injection of uninfected extract was made, since a previous experiment concluded that the presence of *Wolbachia* in an homogenate was required to affect female reproduction (Juchault and Mocquard, 1989). Injected females were kept for 250 days before crosses were performed, a delay allowing *Wolbachia* to infect host tissues and express a feminizing effect (if any) (Rigaud and Juchault, 1995; Bouchon *et al.,* 1998). Injected and control females were crossed with uninfected males. Their offspring were reared in separate boxes and were sorted by sex around 3 months later, after sexual differentiation. During all the experiment, animals were fed *ad libidum* with carrot slices and dead leaves. The same procedure was followed to maintain strains for further generations, 1 generation being obtained per year.

PCR Assays

The success of the transfer was controlled by PCR screening of the females injected (after they released the young) and in young 6 months after their birth, except those kept for crossing, which were tested later. Total DNA was extracted and PCR amplifications were performed following Bouchon *et al.* (1998). The *Wolbachia*-specific primer set for the 16S rDNA was used (99f–994r, O'Neill *et al.,* 1992). In samples testing negative for *Wolbachia,* a mitochondrial primer set was used in a separate reaction to ensure that the host's cytoplasmic DNA was accessible as described in Bouchon *et al.* (1998).

Fluorescence in Situ Hybridization

Wolbachia density was compared using fluorescence *in situ* hybridization (FISH) adapted from the tech-

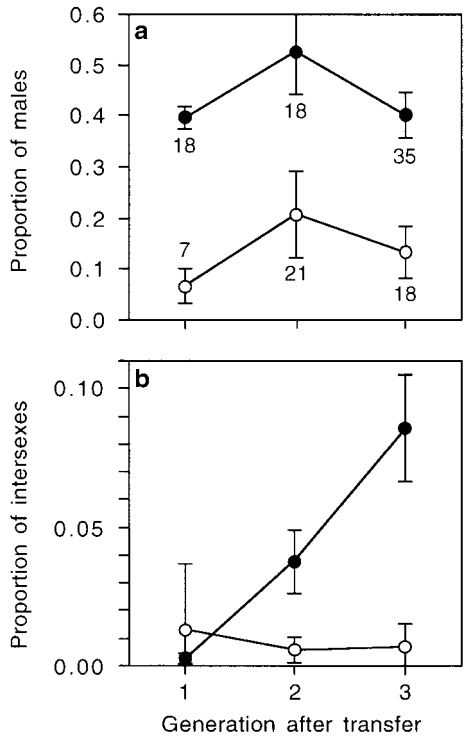

FIG. 1. Proportion of males (a) and proportion of intersexes (b) in broods of *A. vulgare* females infected with *w*An (black symbols) and *A. nasatum* females infected with *w*Av (white symbols) during the 3 generations following the transfer. The number of broods tested at each generation is indicated in a.

nique developed by Heddi *et al.* (1999). Quantitative results on *Wolbachia* load are very hard to obtain from this technique when observations were made on entire tissues or semifine sections, mainly because *Wolbachia* form aggregates within the host cells, which do not allow accurate counting (Pintureau *et al.*, 2000; Pennings, results not shown). To obtain a homogenous solution of *Wolbachia* from host cells, tissues of interest were dissected and homogenized in 200 μl of PBS. The homogenate was then subjected to 10 s of ultrasound, allowing destruction of the cell wall, but preserving nuclei (see Fig. 2). The solution was then fixed for 5 min by adding 200 μl 4% paraformaldehyde and 100 μl was spread on a polylysine-coated slide and air dried. The FISH technique was then performed, as described in Heddi *et al.* (1999). The *Wolbachia*-specific probe W2 was used (Heddi *et al.*, 1999), labelled with rhodamine at the 5′ end (Eurogentec). After hybridisation, slides were mounted in 50μl Fluoprep medium (BioMérieux) containing DAPI (1μg/ml). Observations were made under Zeiss Axioplan microscope equipped for fluorescence, using a x60 oil objective. For each individual, 10 objective fields were sampled randomly on the slide, and all *Wolbachia* (stained with Rhodamine) and nuclei (stained with DAPI) were counted. For each slide, there was no significant difference in the number of nuclei and bacteria between the ten fields (χ^2 for homogeneity N.S. in all cases, data not shown). The total number of *Wolbachia* and nuclei were therefore pooled to estimate the ratio *Wolbachia*/nuclei for a given individual. This method provides a measure of relative bacteria load, not an absolute number per individual.

RESULTS

All injected females that were tested were positive for *Wolbachia* infection, confirming the efficiency of the injection technique (Table 1). A decrease in fertility was observed in transinfected females (Table 2), except in *A. nasatum* infected with *wAn*, the only case in which the hosts were infected with a *Wolbachia* strain that came not only from the same species but also from the same population. In *A. vulgare*, the *wAv* bacteria and the transinfected host were between two sets of host populations distant by 800 km. The decrease of fertility was very strong in *O. asellus*, whatever the

TABLE 1

Wolbachia Infection Status of Females Injected with Symbionts from *A. vulgare* (*w*Av) and Symbionts from *A. nasatum* (*w*An) and of Their Female Offspring, as Revealed by PCR Assays

		Mothers[a]		F1 daughters[b]	
Recipient species	*Wolbachia* type	Examined	Infected	Examined	Infected
P. scaber	*w*Av	4	4	19	0
	*w*An	4	4	22	2
O. asellus	*w*Av	5	5	14	1
	*w*An	5	5	12	0
A. vulgare	*w*Av	5	5	20	19
	*w*An	5	5	32	28
A. nasatum	*w*Av	5	5	49	41
	*w*An	5	5	20	19

[a] Females sampled at random among injected females.
[b] Offspring sampled at random in four broods of mothers controlled positive for *Wolbachia* infection.

TABLE 2

Mean Number of Young per Brood and Proportion of Males Produced by Isopod Species after Transinfection by *Wolbachia* from *A. vulgare* (*w*Av) or *Wolbachia* from *A. nasatum* (*w*An)

Recipient species	Infection	*N*	Young/brood (mean ± SEM)	P^a	Percentage of males (mean ± SEM)	P^b
P. scaber	None	12	52.01 ± 3.84[a]	**	46.72 ± 1.85	n.s.
	*w*Av	8	33.54 ± 3.97[b]		46.20 ± 2.91	
	*w*An	14	35.64 ± 4.19[b]		46.71 ± 1.72	
O. asellus	None	11	36.83 ± 2.17[a]	***	52.63 ± 1.29	n.s.
	*w*Av	14	5.44 ± 1.16[b]		43.51 ± 3.47	
	*w*An	11	6.81 ± 2.87[b]		42.79 ± 5.80	
A. vulgare	None	17	111.26 ± 6.95[a]	***	50.47 ± 0.80[a]	***
	*w*Av	14	73.82 ± 7.84[b]		5.41 ± 3.17[b]	
	*w*An	18	73.22 ± 6.03[b]		39.95 ± 2.73[c]	
A. nasatum	None	14	38.95 ± 3.48	n.s.	47.43 ± 1.41[a]	***
	*w*Av	7	28.86 ± 2.69		7.36 ± 3.88[b]	
	*w*An	10	38.43 ± 5.62		4.03 ± 2.50[b]	

Note. For each recipient species, values followed by different letters were significantly different ($P < 0.05$) after Tukey–Kramer posthoc test.

[a] One-way ANOVA test comparing the three groups for each recipient species.

[b] Kruskal–Wallis nonparametric test comparing the three groups for each recipient species; n.s. $P > 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

symbiont they received, and more moderate in *P. scaber* and *A. vulgare*.

The *Wolbachia* vertical transmission to offspring differed greatly between species. Transmission was weak in daughters from *P. scaber* and *O. asellus*, while transmission was strong in *A. vulgare* and *A. nasatum*, independent of the bacterial strain they received (Table 1). In line with the poor transmission to offspring, no significant bias in offspring sex ratio was observed in *P. scaber* and *O. asellus* (Table 2). Strong excesses of females were observed in broods of *A. nasatum* females, whatever the symbiont they received (Table 2). A similar excess of females was observed in *A. vulgare* females infected with their native *Wolbachia*, while *w*An induced moderate sex ratio biases, intermediate between those of control and *w*Av-infected females (Table 2).

To follow the infection over time, F2 generations were obtained from F1 females in *P. scaber* infected with *w*An and *A. vulgare* and *A. nasatum* infected with *w*An and *w*Av symbionts, respectively (only broods from females that were tested positive for *Wolbachia* infection were kept for further investigation). In *P. scaber*, one infected F1 daughter produced 21 males and 25 females; all of 10 females that were tested were PCR negative for *Wolbachia* infection. No subsequent generation was continued.

For *A. vulgare* infected with *w*An and *A. nasatum* infected with *w*Av the evolution of the proportion of males and the proportion of intersexes (i.e., individuals infected by *Wolbachia* but imperfectly feminized, see Rigaud and Juchault, 1998) are given in Fig. 1. During the 3 generations following the transfer, there was no significant change in the proportion of males, neither in *A. vulgare* (Kruskal-Wallis test: $\chi^2_2 = 2.03$; $P > 0.35$) nor in *A. nasatum* (K-W test:$\chi^2_2 = 0.97$; $P > 0.60$). The proportion of intersexes was very low and stable in *A. nasatum* infected with *w*Av (K-W test: $\chi^2_2 = 0.01$, $P > 0.99$), a situation commonly found in naturally infected lineages (Juchault and Legrand, 1979; Rigaud and Juchault, 1998). However, the proportion of intersexes changed with generations in *A. vulgare* (K-W test: $\chi^2_2 = 16.95$; $P < 0.001$), reaching unusually high values (near 9%) in the third generation following transfer.

Intersexes reflect a lower efficiency of feminization of *Wolbachia* (Rigaud and Juchault, 1998). The dissection of 218 intersexes revealed that 82.6% were sterile. They possess both male and female external characteristics and have nonfunctional gonads that have a structure between male and female. The remaining intersexes (17.4%) had female gonads and a tiny male *appendix masculina*. To test if the intersex phenotype could be related to a lower bacterial level, the FISH technique was used to compare *Wolbachia* amount in cells of *A. vulgare* females and intersexes. The *Wolbachia*-specific probe previously used in weevils and *Trichogramma* (Heddi *et al.*, 1999; Pintureau *et al.*, 2000) allows a clear detection of isopod *Wolbachia* (Fig. 2). A comparison of the bacterial load was impossible between female and intersex gonads because of their dissimilarity, which could lead to differences in bacterial number due to differences in cytoplasm amount (there is more cytoplasm present in oocytes than in undifferentiated cells). We therefore compared the bacterial load in a somatic organ: the nerve chord and associated fat tissues. The counting of host nuclei was used as a reference to standardize the *Wolbachia* num-

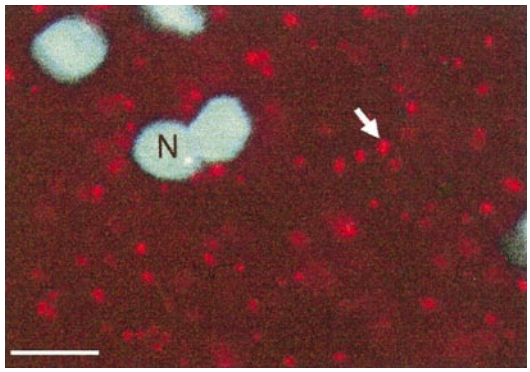

FIG. 2. *Wolbachia* (arrow) revealed in *A. vulgare* by the FISH procedure after tissue homogenization. Host nuclei (N) were stained with DAPI. Bar, 10 μm.

ber estimate (Fig. 2). The *Wolbachia* number was significantly higher in female tissues than in intersex tissues (Fig. 3, Wilcoxon rank sum test: $Z = 3.34$; $P < 0.001$).

DISCUSSION

Wolbachia could survive in their new hosts, but were vertically transmitted only in species closely related to the donors. A general resistance to *Wolbachia* cannot be involved since *P. scaber* and *O. asellus* are known to harbor *Wolbachia* in the wild (Bouchon *et al.*, 1998). But *Wolbachia* naturally hosted by these species are phylogenetically divergent from *w*Av and *w*An (Bouchon *et al.*, 1998; Cordaux *et al.*, 2001). Therefore a physiological incompatibility could be present between these *Wolbachia* and their new hosts. This hypothesis is strengthened by the fact that, in most cases, *Wolbachia* have a deleterious effect on their new hosts' fertility after transinfection, evidencing a physiological cost of their presence. Such an effect was also found in *A. vulgare* females that were infected with *Wolbachia* from another *A. vulgare* population. This could be due to a local adaptation of the *Wolbachia*. To test this the same experiment should be repeated with hosts and bacteria from each of the two populations. From our experiment it is not clear whether the decrease in offspring number is due to a direct or an indirect cost, i.e., if the decrease in fertility was due to a lower growth rate in mothers (fertility is correlated to size in isopods; Sutton *et al.*, 1984) or if it was due to a more direct effect, such as disrupting vitellogenesis or inducing a higher mortality in young. A decrease in growth rate has already been observed after intraspecific (but interpopulation) *Wolbachia* transfer in *A. vulgare* (Juchault and Mocquard, 1989). But the very strong decrease in young number observed in *O. aselus* nevertheless cannot be due to an effect on mother size only (the difference would suppose a difference in size on the same order as the decrease in fertility, which was not detected by eye) and is more likely to be due to a direct pathogenic effect of the symbiont.

Results obtained within the *Armadillidium* genus showed that there was no major incompatibility between *Wolbachia* and recipient species: symbionts were transmitted to almost all offspring (as in naturally infected lineages) and kept their feminizing effect. However, a dissymetry in the capacity of *Wolbachia* to disturb host sexuality was observed. While *w*Av have a similar feminizing effect on *A. nasatum* as *w*An, the reverse is not true. The *w*An symbiont induced a moderate sex ratio bias and a strong proportion of intersexes in *A. vulgare*, which revealed a weaker capacity to induce feminization in its new host. This dissymetry highlights the fact that these closely related symbionts adapted to their hosts in a different manner. The *w*An symbiont could have adapted more closely to its host than *w*Av. We can therefore suggest that *w*Av has kept a wider potential for infecting new hosts with success than *w*An.

Differences were found in the number of symbionts within cells of adult *A. vulgare* females and intersexes. This result suggest that a weaker capacity of *Wolbachia* to infect host tissues can be at the origin of a weaker feminizing effect in their hosts. This dose effect for *Wolbachia*-induced feminization would be similar to some cases of cytoplasmic incompatibility (Breeuwer and Werren, 1993). However, we must acknowledge that it could be possible that the observation of bacterial density in adults does not reflect the density at the time of sex differentiation (i.e., the moment when the bacterial effect is expressed; in the adults we have tested, the damage has already been done). So more studies are needed to confirm these first observations.

To be established in a new host population after infection, *Wolbachia* symbionts must select three keys: (i) a "compatibility" key, (ii) a "transmission" key, to infect a novel host's generations, and (iii) a "disruption" key, to turn the host's reproduction at their advantage

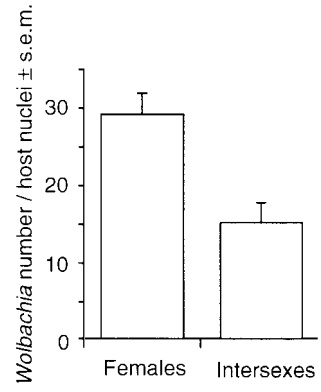

FIG. 3. Mean number of *Wolbachia* per host nuclei in the nerve chord of *A. vulgare* females ($n = 13$) and intersexes ($n = 19$).

to spread in hosts populations. A previous study (Bouchon *et al.*, 1998) showed that *Wolbachia* often kept their feminizing effect when transferred into adult male of different species, which could be perceived as a conserved ability to disrupt host sexuality (key iii). Particularly, *P. scaber* adult males were feminized by *w*An. In the present study, *w*An transferred in *P. scaber* females were vertically transmitted in one case but without feminization. This difference may be due to a number of vertically transmitted bacteria too low to induce feminization in embryos. This is supported by the fact that F2 *P. scaber* daughters were not infected by *w*An. Overall, however, our results suggested that feminizing *Wolbachia* did not keep strong potentialities to propagate by contagion, mainly because they lack the "transmission" key. Also, the reduction of fertility following host changes would not help to successfully infect a new host species. Finally, the moderate feminization expressed by *w*An in *A. vulgare* would probably not allow its persistence in this species. The absence of an important excess of females added to the high proportion of sterile intersexes and the reduction of fertility would not allow this *Wolbachia* strain to spread deterministically in an uninfected population, because infected females do not produce more daughters than uninfected females (Taylor, 1990; Hatcher and Dunn, 1995). The infection of *A. nasatum* females with *w*Av symbionts was more successful. There was a decrease in fertility, but thanks to the high female-biased sex ratio they induce, *w*Av symbionts would have a good chance to persist and spread in *A. nasatum* populations.

In conclusion, successful horizontal transmission of *Wolbachia* between two distantly related terrestrial isopods species would probably require a strong selection on a new *Wolbachia* variant. This does not mean that such transfers are impossible, as suggested by phylogenetic analyses (Bouchon *et al.*, 1998; Cordaux *et al.*, 2001), but these events will only rarely be successful. Maybe several million years ago *Wolbachia* had more potentialities (plasticity) to infect several host species than at present, and they may have lost these potentialities following a selection and a coevolution with the peculiar hosts that are isopods. There is a possibility, however, that some *Wolbachia* lineages are less specialized and can infect other host species more easily. A way to test this hypothesis would be to try to transfer *Wolbachia* from species where recent phylogenetic work has revealed recent horizontal transfer (Cordaux *et al.*, 2001). Of course it is also possible that chances of horizontal transfers happening have always been small, but in evolutionary history during millions of generations even small chances may have left enough opportunities for horizontal transfers to happen. The present only shows the successes of evolution. Our experiment may have been just one of the many unsuccessful attempts.

ACKNOWLEDGMENTS

Microscopic observations were made at the Service Interdisciplinaire de Microscopie et d'Imagerie Scientifique, Poitiers. We thank A. Heddi and C. Katchadourian for help with developing the FISH technique, R. Comte for efficient help with rearing the animals, and T. van Opijnen for useful comments on the manuscript.

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