Four intracellular genomes direct weevil biology: Nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*

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ABSTRACT Cell physiology in the weevil *Sitophilus oryzae* is coordinated by three integrated genomes: nuclear, mitochondrial, and the "*S. oryzae* principal endosymbiont" (SOPE). SOPE, a cytoplasmic bacterium (2×10^3 bacteria per specialized bacteriocyte cell and 3×10^6 bacteria per weevil) that belongs to the proteobacteria γ3-subgroup, is present in all weevils studied. We discovered a fourth prokaryotic genome in somatic and germ tissues of 57% of weevil strains of three species, *S. oryzae, Sitophilus zeamais,* and *Sitophilus granarius,* distributed worldwide. We assigned this Gram-negative prokaryote to the *Wolbachia* group (α-proteobacteria), on the basis of 16S rDNA sequence and fluorescence *in situ* DNA–RNA hybridization (FISH). Both bacteria, SOPE and *Wolbachia*, were selectively eliminated by combined heat and antibiotic treatments. Study of bacteria involvement in this insect's genetics and physiology revealed that SOPE, which induces the specific differentiation of the bacteriocytes, increases mitochondrial oxidative phosphorylation through the supply of pantothenic acid and riboflavin. Elimination of this γ3-proteobacterium impairs many physiological traits. By contrast, neither the presence nor the absence of *Wolbachia* significantly affects the weevil's physiology. *Wolbachia*, disseminated throughout the body cells, is in particularly high density in the germ cells, where it causes nucleocytoplasmic incompatibility. The coexistence of two distinct types of intracellular proteobacteria at different levels of symbiont integration in insects illustrates the genetic complexity of animal tissue. Furthermore, evolutionary timing can be inferred: first nucleocytoplasm, then mitochondria, then SOPE, and finally *Wolbachia*. Symbiogenesis, the genetic integration of long-term associated members of different species, in the weevil appears to be a mechanism of speciation (with *Wolbachia*) and provides a means for animals to acquire new genes that permit better adaptation to the environment (with SOPE).

Intracellular symbiosis, or endosymbiosis, is the most sophisticated association between eukaryotic cells and intracellular microorganisms. It implies the coordination between associated genomes (1) forming a new entity, the symbiocosm (2). This concept encompasses the endosymbiotic theory dealing with the origin of plastids and mitochondria (3, 4). In insects, endosymbioses often display specialized adaptations such as the differentiation of host cells or bacteriocytes (5). Furthermore, endosymbionts are often intimately involved in several metabolic pathways of the host, and most cannot divide by themselves *in vitro* (6–8). The association is generally considered as mutualistic. However, the current knowledge on symbiosis shows a continuum between parasitic and mutualistic relationships. A striking example, which rather evokes parasitism, is the *Wolbachia* symbiosis. This intracellular rick-

ettsia-like organism is widespread in arthropods and is known to alter host reproduction in mainly three ways: cytoplasmic incompatibility (CI) (9, 10), parthenogenesis (11), and feminization of genetic males (12). Moreover, recent work demonstrated that *Wolbachia* may also be virulent, causing tissue degeneration and early death in *Drosophila* (13).

The rice weevil *Sitophilus oryzae* (Coleoptera, Rhynchophoridae) shares an endosymbiotic association with a so-called *Sitophilus oryzae* principal endosymbiont (SOPE), a Gram-negative bacterium belonging to the Enterobacteriaceae family (14). This symbiosis has been investigated for more than 70 years (2). Investigators have shown that SOPE supplies the weevil with vitamins, such as pantothenic acid, riboflavin, and biotin (15), and interacts with mitochondrial oxidative phosphorylation by increasing mitochondrial enzymatic activity (16), thus enhancing greatly the flight ability of adult insects (17). It also interacts with amino acid metabolism (18, 19). All these features indicate that SOPE is greatly integrated in the host physiology. Moreover, the SOPE genome apparently has undergone genetic changes throughout its intracellular life history (20), and its expression is partly controlled by the host (21). We interpret these observations to indicate a long period of microorganism–insect coadaptation. Here, we show with 16S rDNA sequencing and fluorescence *in situ* hybridization (FISH) that the weevil symbiocosm is complicated by the coexistence of four genomes: nuclear, mitochondrial, SOPE, and a fourth intracellular genome of the *Wolbachia* group (α-proteobacteria). Combined heat and antibiotic treatments resulted in a selective elimination of both types of endosymbiotic bacteria, which allowed study of their involvement in the biology of the insect. The comparison between SOPE and *Wolbachia* leads to the conclusion that they represent two different levels of organism integration in the host biology, and it illustrates the genetic complexity of eukaryotic cells.

MATERIALS AND METHODS

Insect Rearing. Experiments were conducted with two *S. oryzae* strains: a Chinese strain (Ch) from Guangzhou which was shown to harbor both SOPE and *Wolbachia*, and a French laboratory strain (SFr) harboring only SOPE. Weevils were reared on wheat at 27.5°C and 70% relative humidity.

16S rDNA Cloning and Sequencing. Total DNA was isolated from the Chinese strain as described by Heddi *et al.* (14). Primers used for 16S rDNA amplification were the eubacterial universal primers: 27for 5′-AGAGTTTGATCATGGCT-CAG-3′ [nucleotides 8–27, *Escherichia coli* numbering (GenBank accession no. J01859)] and 1487rev 5′-TACCTTGT-TACGACTTCACC-3′ (nucleotides 1487–1506). Reaction

Abbreviations: SOPE, *Sitophilus oryzae* principal endosymbiont; FISH, fluorescence *in situ* hybridization.
Data deposition: The 16S rDNA sequence of *Wolbachia* reported in this paper has been deposited in the GenBank database (accession no. AF035160).
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mixtures for PCR amplification consisted of 1.5 units of *Taq* DNA polymerase (Appligene, Illkirch, France), 1.5 mM MgCl$_2$, 0.2 mM deoxynucleotide triphosphate, 0.3 μM primers, and 10 ng of DNA template in a final volume of 50 μl. The PCR parameters were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s. PCR products were cloned in a pMOS*Blue* vector (Amersham), and positive clones were characterized with specific endonuclease digestions. Two panels were identified and two representative clones of each panel were sequenced as described previously (14), to detect possible PCR misincorporations. The clones from each panel were found to be identical.

FISH Procedure. Insect tissues were fixed in alcoholic Bouin's solution and embedded in paraffin. Five-micrometer-thick sections were mounted on poly(L-lysine)-coated microscope slides. After toluene dewaxing and rehydration, sections were digested in 100 μg/μl pepsin in 0.01 M HCl for 10 min at 37°C. Specific oligonucleotide probes were designed by sequence alignment of *Wolbachia* and SOPE 16S rDNA. Two *Wolbachia* probes 5′ end labeled with rhodamine were used to increase the signals: W1, 5′-AATCCGGCCGARCCGACCC-3′, and W2. 5′-CTTCTGTGAGTACCGTCATTATC-3′. SOPE probe (S) was 5′ end labeled with fluorescein: S, 5′-TACCCCCCTCTACGAGACTC-3′. Hybridization was performed at 45°C in a dark moisture chamber in 0.9 M NaCl/20 mM Tris·HCl/5 mM EDTA/0.1% SDS/10× Denhardt's solution. After a 30 min preincubation period, 50 ng of each probe (W1, W2, and S) was added and incubation was continued for 3 hr. Slides were washed twice in the same buffer at 48°C for 20 min, rinsed with distilled water, and mounted in Vectashield medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI).

***Wolbachia* and SOPE Detection in *Sitophilus* Populations.** Individuals were tested for the presence or the absence of *Wolbachia* and SOPE by PCR and FISH. For PCR, specific primers were designed on the 16S gene: forward 5′-CGGGGGAAAAATTTATTGCT-3′, reverse 5′-AGCTG-TAATACAGAAAGTAAA-3′, generating a-589 bp fragment for *Wolbachia*; and forward 5′-TAATAGCGCCATCGATT-GAC-3′, reverse 5′-CCGAAGGCACCAAGGCAT-3′, generating a 530-bp fragment for SOPE. PCR parameters were

described above, except for the hybridization temperature (T_H), 50°C (30 s) for *Wolbachia* and 53°C (30 s) for SOPE.

Insect Treatment and Genetic Analysis. Female fertility and development time of *S. oryzae* progeny were measured from crosses between naturally symbiotic Chinese strain (Ch), or its SOPE-aposymbiotic heat-treated relative (ChH), and their tetracycline-treated derivatives (ChT and ChHT). ChH insects were obtained by heat treatment of adults: 35°C and 90% relative humidity for 1 month, followed by female oviposition under normal conditions (22). Tetracycline-treated insects were fed two generations on wheat flour pellets mixed with tetracycline (1 mg/g of flour). To avoid possible direct effects of treatments, virgin pairs of adults were fed for 2 weeks on wheat grains before crossing. Progenies from a 14-day egg-laying were counted to estimate fertility per female and from a 2-day egg-laying to measure the development time from egg-laying to adult emergence. *Wolbachia* and SOPE were detected by FISH and PCR.

Mitochondrial Enzymatic Assays. Mitochondria-specific enzymatic activities were assayed by using the procedure of Heddi *et al.* (16). For vitamin supplementation experiments, insects were reared during one generation on wheat flour artificial pellets supplemented with pantothenic acid (2.38 mg/kg of wheat flour) and with riboflavin (1.88 mg/kg of wheat flour).

RESULTS

Phylogenetic Position of *S. oryzae* Endosymbionts. The cloning and sequencing of PCR products from reactions using 16S rDNA universal primers on total DNA from a Chinese strain of *S. oryzae* (Ch) revealed two different sequences. One sequence was identical to SOPE 16S rDNA (14) and the other belongs to the α-proteobacteria. Fig. 1 positions these sequences relative to several representatives of the α and γ subdivisions of proteobacteria. Whereas SOPE is found within the Enterobacteriaceae, the other sequence (GenBank accession no. AF035160) falls into the B-group of *Wolbachia* (23), with which it forms a monophyletic group relative to the other α-proteobacteria. The 16S rDNA sequence of *S. oryzae Wolbachia* is over 97% identical to *Wolbachia* known from other insects.
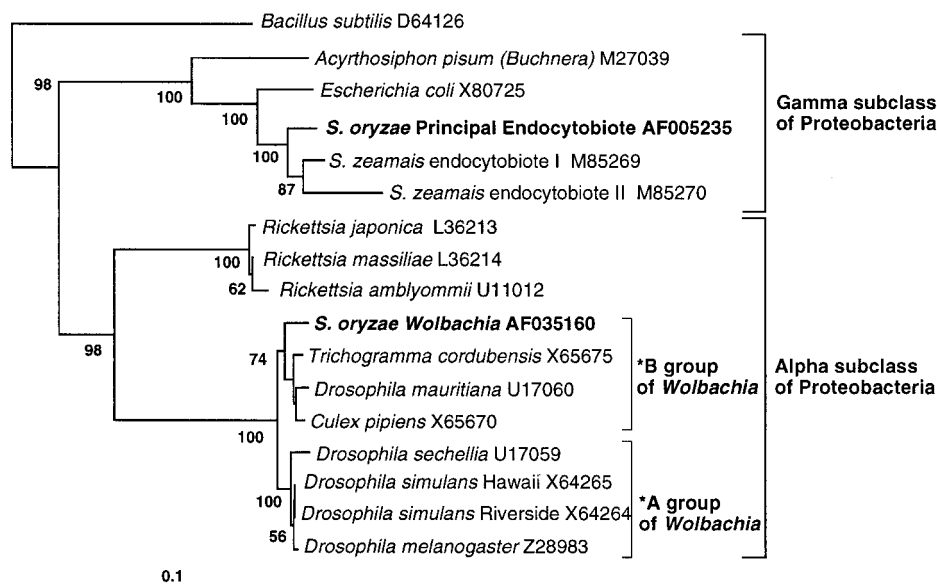

FIG. 1. Phylogenetic tree of the SOPE and *Wolbachia* based on sequences of 16S rDNA genes. The other rDNA sequences were searched at the National Center for Biotechnology Information by using the BLAST network service. Alignments were performed and the phylogenetic tree was created as in ref. 14. *Bacillus subtilis*, a Gram-positive bacterium, was used as the out-group. The name of the bacteria or the host insect species is followed by the GenBank accession number. Scale bar, 0.1 substitution per site. *According to the classification of Werren *et al.* (23).

Table 1. SOPE and *Wolbachia* distribution in *Sitophilus* (weevil) populations

Species	Strain (collection date)	Source of weevil	Presence of *Wolbachia*	No. of *Wolbachia*, positive weevils per 10 tested
S. oryzae	GuadI (83)	Guadeloupe	−	0
	GuadII (83)	Guadeloupe	+	9
	ChI (83)	China	+	7
	ChII (83)	China	−	0
	Ind (85)	India	−	0
	Aust (90)	Australia	+	10
	TunI (81)	Tunisia	+	10
	TunII (81)	Tunisia	+	10
	Ben (92)	Benin	+	10
	Hai (85)	Haiti	+	5
S. zeamais	Syr (85)	Syria	−	0
	AzoI (90)	Azores	−	0
	AzoII (90)	Azores	−	0
	AzoIII (90)	Azores	−	0
	MexI (84)	Mexico	−	0
	MexII (84)	Mexico	−	0
	Cong (84)	Congo	+	10
	Thai (94)	Thailand	+	10
	Peru (90)	Peru	+	10
	Reun (79)	Réunion	+	10
S. granarius	FrI (82)	France	−	0
	FrII (82)	France	+	10
	FrIII (82)	France	+	2

SOPE was found in all studied *Sitophilus* strains. SOPE and *Wolbachia* were detected by PCR and FISH experiments.

SOPE and *Wolbachia* Distribution in *Sitophilus* Populations. Twenty-three *Sitophilus* strains, 10 individuals of each, belonging to three species (*S. oryzae*, *S. zeamais,* and *S. granarius*), were collected from different locations worldwide and tested by using specific PCR primers and FISH experiments for the presence of SOPE and *Wolbachia* (Table 1). While all strains and individuals were SOPE-symbiotic, only 13 of 23 strains were *Wolbachia*-symbiotic, constituting 9 strains totally infected and 4 strains partially infected.

Tissue Distribution of SOPE and *Wolbachia*. The distribution of *Wolbachia* and SOPE in weevil tissues was investigated with the FISH approach (24) (see Fig. 2). Oligonucleotide probes (19–23 nucleotides) were designed by sequence alignment of the *Wolbachia* and the SOPE 16S rDNA sequences. These probes were shown to be highly specific when applied

together on sections: (*i*) devoid of SOPE and *Wolbachia* (Fig. 2 *1*, oocyte negative control, no signal), (*ii*) containing *Wolbachia* only [*2*, oocyte (o) and follicular cells (f) of a SOPE-aposymbiotic strain, red spots only], and (*iii*) containing both *Wolbachia* and SOPE (*6*, red spots and green color, respectively). The principal endosymbiont is limited to specialized tissue: the larval bacteriome (specialized bacterial organ, lb) (*6* and *7*), the ovarian bacteriome (ob) (*8*), and to female germ cells. It is absent from all other tissue, including male germ cells (*3*). Bacteriomes fail to differentiate in the absence of the principal endosymbiont even when *Wolbachia* is present (Fig. 2 *5* shows the apex of the ovary lacking the bacteriome). Bacteriome differentiation apparently is induced by the integrated endosymbiotic bacterium of the weevils.

Wolbachia (red spots of about 0.5 μm), in contrast, is disseminated throughout the whole body of the insect and particularly is in high density in the male and female germ cells. It is located around the periplasmic membrane of the oocyte and in the surrounding follicular cells (Fig. 2 *2*, follicular cells are packed full of *Wolbachia* ►). In testis, *Wolbachia* is near many spermatid nuclei (*3*, see arrows; 4′,6-diamidino-2-phenylindole blue dye indicates the spermatid nuclei), suggesting its interaction with the male chromosomes. Everywhere else, *Wolbachia* is present only at low density and exhibits a weak signal intensity. It can be seen in the cuticle (c) and muscles (m) (*4*), in adipocytes (a) and the intestine (i) (*6*), and in the bacteriocytes, coexisting with SOPE (*6*) and representing less than 5% of the total bacterial population. In the FISH experiment conditions, the fluorescent signal is proportional to the concentration of 16S rRNA in cells. These transcripts represent the FISH probe targets, and their quantity may indicate the level of the bacterial cell metabolic activity. We interpret high levels of fluorescence of *Wolbachia* within germ cells (*2, 3,* and *5*), relative to soma line (*4* and *6*), to suggest a high metabolic activity of this bacterium in the male and female germ line.

Differential Influence of the Two Symbionts. To investigate separately the influence of both SOPE and *Wolbachia*, genetic analysis was conducted with different crosses between individuals treated or not with heat and/or tetracycline (Table 2). Heat treatment disrupted the SOPE symbiosis in *S. oryzae*. Tetracycline treatment for two generations removes both *Wolbachia* and SOPE. Relative to other insects such as *Drosophila* or *Trichogramma*, *Wolbachia* of weevils are resistant to the heat treatment (evidence given by PCR and FISH experiments). Table 2 displays four salient results. First, *Wolbachia* seems not to be much involved in weevil physiology: development times in crosses 6 to 8 differ only slightly from the development time of cross 5 (perhaps because of a secondary effect of the antibiotic). Second, unidirectional cytoplasmic incompatibility is seen clearly when females but not males are

Table 2. Female fertility and development time of *S. oryzae* progeny from crosses between naturally symbiotic Chinese strain (Ch), or its SOPE-aposymbiotic heat-treated relative (ChH), and their tetracycline-treated derivatives (ChT and ChHT)

Parents						Progeny			
Cross		Female		Male		Mean fertility, 14-d progeny		Mean development time, days	
No.	F × M	W	S	W	S		nb1		nb2
1	Ch × Ch	+	+	+	+	57.60 ± 6.37	10	34.81 ± 0.27	159
2	Ch × ChT	+	+	−	−	51.71 ± 4.35	7	33.99 ± 0.35	46
3	ChT × Ch	−	−	+	+	4.80 ± 2.29*	5	48.00*	1
4	ChT × ChT	−	−	−	−	18.40 ± 4.37*	5	35.66 ± 0.26	92
5	ChH × ChH	+	−	+	−	28.85 ± 1.05	20	38.17 ± 0.43	78
6	ChH × ChHT	+	−	−	−	25.51 ± 1.47	21	41.25 ± 0.49*	51
7	ChHT × ChH	−	−	+	−	6.84 ± 1.15*	17	40.04 ± 0.76*	26
8	ChHT × ChHT	−	−	−	−	21.15 ± 2.32*	20	44.41 ± 0.30*	138

Wolbachia (W) and SOPE (S) were detected by FISH and PCR using respective specific 16S probes and primers. 14-d progeny, mean progeny per female from a 14-day egg-laying of 2- to 3-week-old females (results are mean ± SE); nb1, pair number; nb2, progeny number from a 2-day egg-laying; +, presence; −, absence. *, Significant differences compared with the control crosses of each group—i.e., 1 for 2, 3, 4, and 5 for 6, 7, 8 (bilateral Dunnett test, α = 0.05).
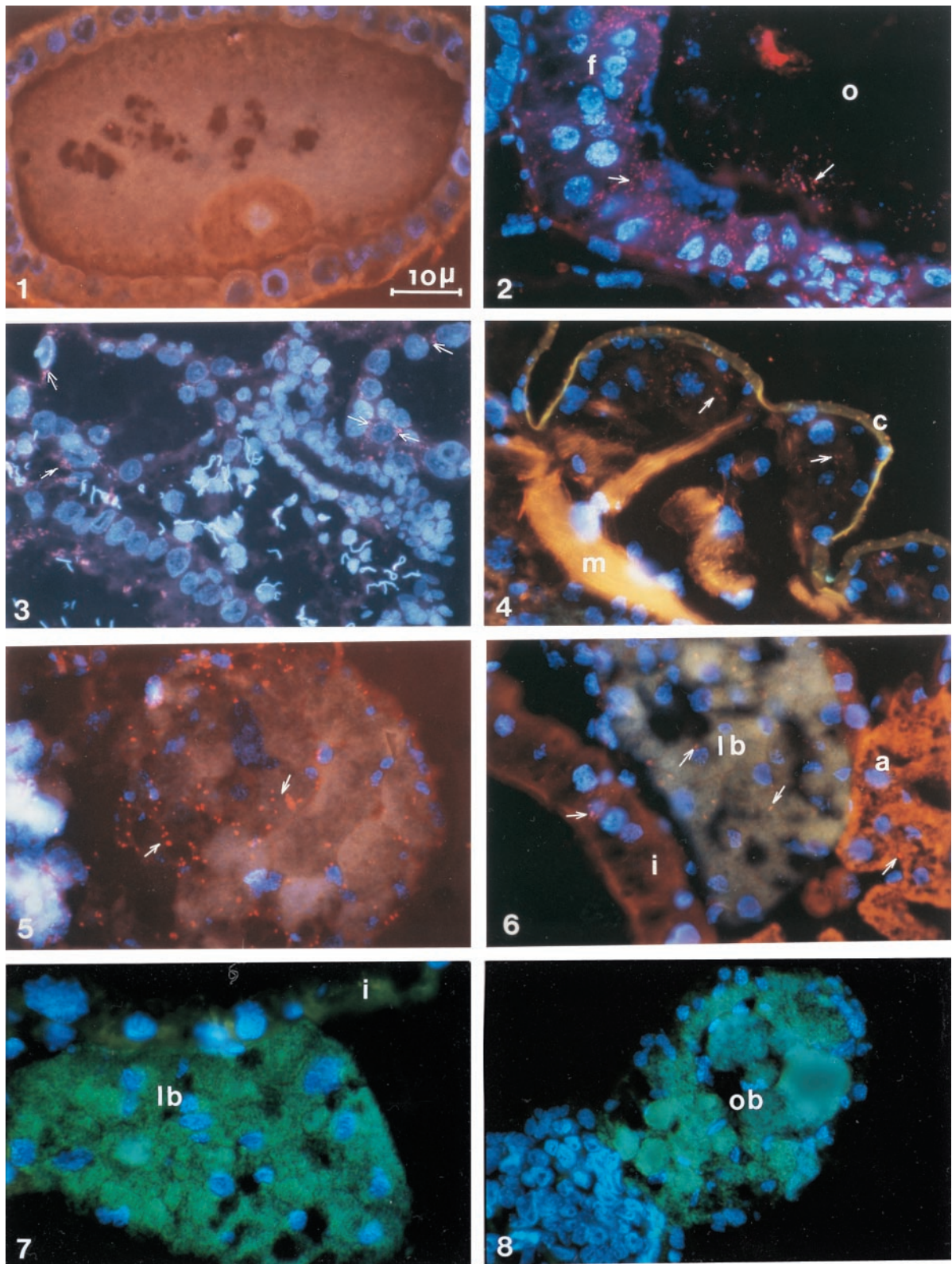
FIG. 2.   FISH of *Wolbachia* (red) and SOPE (green) in different weevil tissues. (*1* and *2*) Comparison between the *Wolbachia*-negative control (*1*) [oocyte from the SOPE-aposymbiotic Chinese strain (ChH) treated with tetracycline (ChHT)] and the *Wolbachia*-positive control (*2*) [oocyte (o) and follicular cells (f) of the SOPE-aposymbiotic strain (ChH)]. (*3*) Testes of the SOPE- and *Wolbachia*-symbiotic strain (Ch) (see arrows, *Wolbachia* near spermatid nuclei). (*4*) Cuticle (c) and muscle (m) of the SOPE- and *Wolbachia*-symbiotic strain (Ch). (*5*) SOPE-aposymbiotic apex of ovary (ChH). (6) Intestine (i), the larval bacteriome (lb) in green and the adipocytes (a) of the Ch strain: both SOPE (green) and *Wolbachia* (red spots, arrows) are visible in the bacteriocytes. (*7* and *8*) Larval bacteriome (lb) and the ovarian bacteriome (ob) of the French *Wolbachia*-free strain (SFr). All slides were hybridized with the three probes (W1, W2, and S; see *Materials and Methods*). (Scale bar, 10 μm.)

treated with tetracycline, whatever the SOPE status of the strain (crosses 3 and 7). Third, tetracycline treatment of males

reestablishes high levels of fertility, which are almost restored to normal in ChHT × ChHT (as compared with the corre-

sponding untreated cross, ChH × ChH), but which are only partly restored in the cross ChT × ChT, because of the concomitant lack of SOPE in the ChT strain. Finally, heat treatment decreases female fertility and increases the insect development time (compare crosses 1 and 5). The most harmful cross is when Ch females are tetracycline treated and males are not. This generates the weakest fertility. In the experiment to measure the development time only one weevil with a development time of 48 days has emerged, indicating the action of both *Wolbachia* (cytoplasmic incompatibility) and SOPE (decreased fertility).

Metabolic Interaction Between Nucleus, SOPE, and Mitochondria. In a previous experiment (16), we have shown that SOPE improves mitochondrial enzymatic activity. Since SOPE supplies the host with vitamins (pantothenic acid and riboflavin), we have investigated in the present study the effect of vitamin supplementation on mitochondrial enzymatic activities of the weevils. Thus, vitamin supplementation experiments were conducted on wheat flour artificial pellets. Fig. 3 shows clearly that mitochondrial enzymatic activities of SOPE-aposymbiotic individuals are much more affected than SOPE-symbiotic ones, when reared on vitamin-supplemented pellets. As a result, differences between the strains are significantly attenuated for three enzymes of four (ANOVA2 test, $P >$ 0.05), suggesting that SOPE influences the weevil energy metabolism principally through nutritional supply. However, we do not exclude that vitamin supplementation could influence other metabolic pathways of the host.

DISCUSSION

Discovered by Pierantoni in 1927 (25) and studied by several authors (2), *S. oryzae* symbiosis remains poorly described at the molecular level. Using two complementary approaches (i.e., PCR–cloning–sequencing and FISH), we demonstrate clearly in this work that some *Sitophilus* strains harbor two separate endosymbionts: *S. oryzae* principal endosymbiont (SOPE), a Gram-negative bacterium belonging to the γ-proteobacteria, and a second intracellular bacterium identified as *Wolbachia* (α-proteobacteria) (Fig. 1). SOPE, in the Enterobacteriaceae family, shares 95% sequence homology with *Escherichia coli* (14). The closest intracellular symbiotic bacterium is the primary endosymbiont of *S. zeamais* (97.8% homology), the

sibling species of *S. oryzae*. The *Wolbachia* of *S. oryzae* belongs to the B-group of *Wolbachia* (23), which causes cytoplasmic incompatibility in several insect genera such as *Aedes, Drosophila,* and *Culex* (9, 10, 26).

Histologically, *Wolbachia* exhibits different features from SOPE (Fig. 2). It is disseminated in tissues throughout the whole body of *S. oryzae,* including follicular cells, oocytes, testis, adipocytes, and intestine, and even within the bacteriocytes, where it coexists with SOPE. However, the density of *Wolbachia* is very tissue variable. It is highly abundant in the germ cells, but it is present at low density in other tissues. In line with this finding is the weak fluorescent signal observed in these tissues compared with the germ cells, where it is well known that *Wolbachia* interacts actively with the host nuclei, modifying the developing sperm in testis (possibly by means of chromatin-binding proteins) (10, 26, 27). In contrast, SOPE is tolerated only by the bacteriocytes and the female germ cells, by an as-yet-unknown mechanism. Bacteriocytes differentiate only in the presence of SOPE. SOPE-aposymbiotic insects completely lack bacteriomes, even when they are *Wolbachia*-symbiotic (see Fig. 2 *5*). The differentiation of the bacteriocytes suggests, therefore, the existence of a specific molecular signal from the SOPE to the host genome during embryogenesis. Moreover, the number of SOPE cells is controlled by the host genome (28). Therefore, a long period of SOPE–host coadaptation relative to *Wolbachia* seems necessary to establish such a specific interacting system. These observations tempt us to consider *Wolbachia* as a more recent and less integrated endosymbiont than SOPE. Paillot (29) considered parasitism a first step toward symbiosis, as exhibited by the *Amoeba* model (30). *Wolbachia* might be an attenuated parasite that only recently infected the weevils.

The coexistence of both proteobacteria in the weevil shows the metabolic and the genetic complexity existing in such eukaryotic cells. Four genomes interact, with each playing a different role in the insect's biology. The α-proteobacterium *Wolbachia* seems much less involved in weevil physiology, since its elimination from the insect cells by the antibiotic treatment only slightly affects the larval development time. Reproduction is greatly impaired by the presence of *Wolbachia* because of cytoplasmic incompatibility between *Wolbachia* and the weevil nuclear genome, independent of the γ-proteobacteria principal endosymbiont (Table 2). The *Wolbachia*-correlated repro-
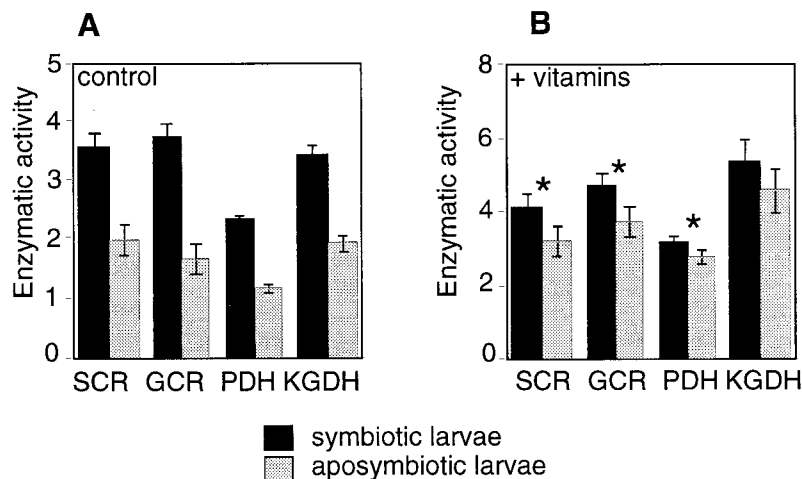

FIG. 3.   Specific enzymatic activities of mitochondrial suspensions isolated from SOPE-symbiotic and SOPE-aposymbiotic larvae of a *Wolbachia*-free French strain (SFr). (*A*) Control wheat flour artificial pellets, not supplemented with vitamins. (*B*) Pellets supplemented with pantothenic acid and riboflavin. Data are expressed as the mean of at least 10 repeats ±SD. SCR, succinate cytochrome *c* reductase (y unit = 1 μmol·mg^{-1}·s^{-1}); GCR, glycerol-3-phosphate cytochrome *c* reductase (y = 0.5 μmol·mg^{-1}·s^{-1}); PDH, pyruvate dehydrogenase (y = 0.1 μmol·mg^{-1}·s^{-1}); KGDH, α-ketoglutarate dehydrogenase (y = 0.2 μmol·mg^{-1}·s^{-1}). The effect of vitamins on mitochondrial activities (i.e., interaction between vitamin and symbiosis factors) was found to be significant (∗, $P < 0.05$) in three of the four independent ANOVA2 tests performed.

ductive abnormality may be the result of an asynchronous condensation of parental chromatin, which leads to the formation of embryos with aneuploid or haploid nuclei (27).

SOPE greatly enhances female fertility and decreases larval development time. Furthermore, SOPE influences the behavior of weevil populations by enhancing the flight ability of the host (17). These energy-dependent performances were mediated by the SOPE effect on mitochondrial oxidative metabolism (16). Mitochondrial enzymatic complexes that form the phosphorylation chain are encoded by mitochondrial DNA (13 polypeptides) and nuclear genome (over 60 other polypeptides) (31). Nuclear proteins are imported into mitochondria, where they interact with mitochondrial polypeptides and with coenzymes, partly originating from vitamins, to construct enzymatic complexes involved in ATP synthesis. Coordination between associated genomes in the cytosol is thus crucial for aerobic energy production. This study shows that the improvement of weevil mitochondrial enzymatic activities by SOPE is essentially due to the pantothenic acid and riboflavin supply (Fig. 3). SOPE does not exhibit any effect on mitochondrial DNA replication or expression (unpublished data). Pantothenic acid and riboflavin, vitamins in low quantity in wheat, are directly involved in biosynthesis of CoA and NAD^+–FAD, respectively. Both are required cofactors for mitochondrial enzyme function. We interpret SOPE to be a third genome implicated in ATP production in weevil cells.

Wolbachia and SOPE are subjected to different evolutionary constraints: SOPE (like mitochondria) is transmitted vertically only, and is strictly located in bacteriocytes and ovaries. It is present in all strains of *Sitophilus* and in all individuals. *Wolbachia,* on the other hand, is transmitted both vertically and horizontally (32), and it is disseminated throughout body cells and is at particularly high density in the ovaries and testes. It does not infect all populations.

The *Sitophilus oryzae* model of symbiosis is consistent with "serial endosymbiotic theory" (3). Endosymbiosis did not occur just once in eukaryotic evolution with nucleus origin (33), or even twice when an anaerobic protist acquired a respiring bacterium to give rise to the mitochondrion or hydrogenosome (34). Nor did bacterial endosymbiont acquisition occur only three times when plants acquired plastid ancestors (35). Acquisition of bacterial genomes by eukaryotic cells indeed continues "today" in the multicellular organism. Two types of acquired microbial genomes may be present in a single animal cell in addition to mitochondria. In *Sitophilus*, after mitochondria established in the protist ancestor, the next endosymbiont was SOPE and the third was *Wolbachia*. Endosymbionts contribute to the emergence of a new symbiocosm or type of cell where different genomes interact. The most important consequence is production of more viable offspring, indicating better adaptation to the environment. The colonization of cereal plants by *Sitophilus* was facilitated by the acquisition of SOPE, a vitamin supplier. We consider symbiosis in the rice weevil a sophisticated mechanism for acquiring new sets of genes.

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