Isolation, Pure Culture, and Characterization of "*Candidatus* Arsenophonus arthropodicus," an Intracellular Secondary Endosymbiont from the Hippoboscid Louse Fly *Pseudolynchia canariensis*

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Members of the genus *Arsenophonus* comprise a large group of bacterial endosymbionts that are widely distributed in arthropods of medical, veterinary, and agricultural importance. At present, little is known about the role of these bacteria in arthropods, because few representatives have been isolated and cultured in the laboratory. In the current study, we describe the isolation and pure culture of an *Arsenophonus* endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis*. We propose provisional nomenclature for this bacterium in the genus *Arsenophonus* as "*Candidatus* Arsenophonus arthropodicus." Phylogenetic analyses indicate that "*Candidatus* Arsenophonus arthropodicus" is closely related to the *Arsenophonus* endosymbionts found in psyllids, whiteflies, aphids, and mealybugs. The pure culture of this endosymbiont offers new opportunities to examine the role of *Arsenophonus* in insects. To this end, we describe methods for the culture of "*Candidatus* Arsenophonus arthropodicus" in an insect cell line and the transformation of this bacterium with a broad-host-range plasmid.

Many members of the class *Insecta* maintain mutualistic associations with one or more specialized symbiotic bacteria (2). Bacteria that participate in these associations are classified either as primary (P) or secondary (S) endosymbionts, because they often coexist in a single insect host. The P-endosymbionts are predicted to be ancient in origin because their phylogenies are concordant with those of their host insects over a substantial period of evolutionary time, indicating long-term coevolution. On the other hand, the S-endosymbionts are predicted to be recent in origin because their phylogenies show little or no concordance with their insect hosts, indicating recent acquisition.

While the ancient P-endosymbionts are known to have defined mutualistic functions in their insect hosts, the role of the S-endosymbionts is not yet well understood. From an evolutionary standpoint it seems likely that S-endosymbionts have beneficial (mutualistic) roles in their insect hosts because they are maintained predominantly through a maternal (vertical) transmission strategy. Several recent studies have provided experimental evidence for a number of beneficial effects conferred by the S-endosymbionts of aphids, which recently received new nomenclature (20). These benefits include host plant specialization (17, 27), increased resistance to hymenopteran parasitoids (21, 22), and increased tolerance to heat stress (5, 19). In addition, there is evidence indicating that S-endosymbionts can provide some level of functional compensation for the loss of P-endosymbionts in a laboratory population of aphids (16). While these studies are both exciting and encouraging, the ability to perform experimentation in these systems would be greatly enhanced with the opportunity to genetically manipulate S-endosymbionts. The application of recombinant DNA technology would permit functional analysis of individual genes in the endosymbiont genomes, providing a platform to explore the molecular mechanisms involved in symbiotic interactions. Ultimately, it is the lack of availability of culture systems for insect endosymbionts that limits the application of these techniques in the laboratory.

To date, several S-endosymbionts have been cultured successfully outside of their natural hosts in insect cell lines (9, 15, 29). These laboratory cell lines have proved extremely useful for the establishment of monoseptic S-endosymbiont cultures. Since S-endosymbionts are not readily isolated in large numbers from their host insects, the establishment of these insect cell cultures provides a source of material that is useful both for genome studies and for the subsequent development of pure culture systems. The isolation of bacteria in pure culture is important because it facilitates the application of recombinant DNA technology through the isolation of clones. To date only two insect endosymbionts, *Arsenophonus nasoniae* and *Sodalis glossinidius*, have been isolated in pure culture (7, 11). *S. glossinidius* currently serves as a model system for the investigation of insect-symbiont interactions because tools are available to manipulate the bacterial genome (6, 8).

In the current study we describe the isolation, culture, and characterization of an S-endosymbiont that resides in the tissues of a pigeon louse fly, *Pseudolynchia canariensis*. We propose the provisional name "*Candidatus* Arsenophonus arthropodicus" for this newly discovered S-endosymbiont based on the fact that it shows a close phylogenetic relationship to other members of the genus *Arsenophonus*. Since close relatives of "*Candidatus* Arsenophonus arthropodicus" are found in a wide range of arthropod taxa, the isolation, pure culture, and genetic transformation of this bacterium should prove

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useful in future efforts directed towards elucidating the role of this group of insect S-endosymbionts.

MATERIALS AND METHODS

PCR amplification, cloning, and sequencing of 16S rRNA gene sequences from *P. canariensis.* Universal bacterial 16S rRNA gene primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) were used to establish an inventory of 16S rRNA gene sequences in DNA isolated from newly deposited *P. canariensis* pupae, obtained from a colony of hippoboscid flies maintained at the University of Utah. Five pupae were washed in 0.5% Triton X-100 to remove any surface contamination prior to DNA extraction. For this and all other total DNA extractions we used the DNeasy tissue extraction kit (QIAGEN), following the supplier's instructions. PCR was performed on isolated DNA using primers 27F and 1492R under standard reaction conditions (14) with 10 ng of template DNA, 300 nM of each primer, 200 μM of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, and 0.02 U of *Taq* DNA polymerase (Promega, Madison, Wis.) per microliter of reaction mix. Cycling conditions consisted of an initial denaturation step (95°C, 4 min) followed by 30 rounds of amplification involving denaturation (95°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 2 min), followed by a final extension step at 72°C for 4 min. The PCR product was analyzed by gel electrophoresis. Gels were stained with SYBR Gold (Molecular Probes, Invitrogen, Carlsbad, Calif.) and visualized on a Dark Reader (Clare Chemical Research, Delores, Colo.) to prevent DNA damage. The 1.5-kbp 16S rRNA gene PCR product was excised and purified with a QIAGEN MinElute gel extraction kit (QIAGEN, Valencia, Calif.). The PCR product was then cloned into the pTOPO 2.1 vector (Invitrogen) according to the supplier's instructions. Forty-eight recombinant clones were sequenced to completion at the University of Utah sequencing facility using vector-specific primers and internal sequencing primers that were designed as sequence information became available.

PCR detection of "*Candidatus* Arsenophonus arthropodicus." Specific PCR primers CAIF (5-GCC TGA TGC AGC CAT GCC GCG TGT ATG-3′) and CAIR (5-GTC ATC CCC ACC TTC C-3′) were designed to amplify a 500-bp fragment of the "*Candidatus* Arsenophonus arthropodicus" 16S rRNA gene sequence. These primers were then used to detect "*Candidatus* Arsenophonus arthropodicus" both in tissues isolated from *P. canariensis* and in samples of medium collected throughout the culturing procedure. For tissue-specific PCR, insect tissues (hemocytes, gut, fat body, and reproductive tissues) were isolated by dissection and rinsed three times in sterile 0.85% (wt/vol) saline. DNA was isolated from individual tissues using the QIAGEN DNeasy tissue kit (QIAGEN). For both liquid and solid-phase cultures, DNA was extracted directly from culture material using the same procedure. The detection of "*Candidatus* Arsenophonus arthropodicus" was performed in PCRs with 10 ng of template DNA, 300 nM of each primer, 200 μM of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, and 0.02 U of *Taq* DNA polymerase (Promega) per microliter of reaction mix. Cycling conditions consisted of an initial denaturation step (95°C, 4 min) followed by 30 rounds of amplification involving denaturation (95°C, 30 s), annealing (65°C, 1 min), and extension (72°C, 1 min), followed by a final extension step at 72°C for 4 min.

Phylogenetic methods. We analyzed the 16S rRNA gene sequence of "*Candidatus* Arsenophonus arthropodicus" alongside 16S rRNA gene sequences from a number of other arthropod endosymbionts and free-living members of the family *Enterobacteriaceae* obtained from the GenBank database. Sequences were aligned using ClustalX, and the alignments were checked manually and adjusted for accuracy. The phylogenetic analysis was conducted using the PAUP³ 4.0 package (25). Initially, a neighbor-joining tree was constructed based on the F84 model of sequence evolution. Maximum likelihood (ML) parameters were then estimated from the neighbor-joining tree and used to search for the best ML tree with the heuristic tree-bisection-reconnection (TBR) branch-swapping algorithm. Once the ML tree was obtained, ML parameters were reestimated from the tree and used to search again using the TBR algorithm. This process was repeated until there was no significant improvement in the log likelihood score of the best ML tree. ML bootstrap analysis was then conducted using the same parameters and TBR algorithm.

Isolation and culture of bacteria from *P. canariensis.* Initially, bacteria were isolated from newly deposited pupae derived from the laboratory colony of *P. canariensis.* All steps in the isolation and culture procedure were carried out in a sterile cabinet under laminar flow conditions. In preparation for culture, five pupae were rinsed for 5 min in at least 20 volumes of sterile water containing 0.5% Triton X-100, with vigorous shaking to remove surface contaminants. Pupae were then washed for 5 min in 20 volumes of 5% sodium hypochlorite in order to kill any remaining surface contaminants. Following surface sterilization,

pupae were rinsed five times in sterile water and twice in sterile Mitushashi and Maramorosch basal medium without fetal calf serum (MM medium) (7). Pupae were then homogenized in 10 ml MM medium supplemented with antibiotics (100 μg/ml polymyxin B and 500 μg/ml vancomycin) to prevent growth of contaminating bacteria. The 10-ml culture was maintained in a 25-cm² ventilated tissue culture flask at 25°C and checked at daily intervals for growth.

Pure culture isolation of "*Candidatus* Arsenophonus arthropodicus." For pure culture isolation, we used methods described previously for the solid-phase culture of *S. glossinidius* (7). Briefly, 100 to 200 μl of liquid from the 5-day-old pupal culture (described above) was streaked onto sterile agar plates containing MM medium supplemented with 0.7% agar. Inoculated plates were quickly transferred to a microbiological gas jar and flushed with at least 20 volumes of a commercially prepared gas mixture comprising 5% oxygen, 10% carbon dioxide, and 85% nitrogen. The gas jar was sealed to maintain microaerophilic conditions and transferred to a 25°C incubator. Single colonies were picked and grown in MM medium to establish pure liquid cultures. Pure liquid cultures were cryopreserved following the addition of a cryoprotectant (15% [vol/vol] glycerol final concentration) by snap-freezing in a dry ice-ethanol bath, prior to storage at −80°C.

Culture of "*Candidatus* Arsenophonus arthropodicus" in an *Aedes albopictus* cell line. *A. albopictus* cell line C6/36 was obtained from the American Type Culture Collection. The cell line was maintained according to the supplier's instructions and established procedures (29). Aliquots of "*Candidatus* Arsenophonus arthropodicus" were inoculated into synchronized confluent monolayers of insect cells at a low multiplicity of infection (<50). Insect cells were gently removed at intervals following infection using a cell scraper to provide material for microscopic examination.

Microscopy. The deconvolution microscope was used to visualize "*Candidatus* Arsenophonus arthropodicus" in samples of hemolymph isolated from teneral adult hippoboscids, in cultured insect cell lines, and in pure culture. For hemolymph and pure culture samples, freshly isolated material was stained in a three-step procedure using a lipophilic styryl dye, FM4-64 (Molecular Probes/Invitrogen), in combination with 4′,6′-diamidino-2-phenylindole (DAPI). In this procedure material was first stained with FM4-64 (10 μg/ml in MM medium) for 30 min, then stained with DAPI (6 μg/ml in MM medium) for 20 min, and finally destained in MM medium for 20 min. Material was pelleted at 2,000 × g for 5 min between each staining step to permit removal and replacement of stains. Material from the insect cell lines was stained using the same procedure, except in this case DAPI was omitted to permit visualization of bacteria in sections throughout the insect cells. Following staining, specimens were examined on an Applied Precision Deltavision inverted deconvolution microscope under the DAPI and rhodamine channels. Three-dimensional *Z*-projections were collected using automated procedures and deconvolved using the Softworx package (Applied Precision, Issaquah, Wash.).

Determination of genome size and structure. We used a CHEF DR-II pulsed-field gel electrophoresis (PFGE) system (Bio-Rad, Hercules, Calif.) to determine the size and organization of the genome of "*Candidatus* Arsenophonus arthropodicus." Chromosomal DNA plugs were prepared from 3-day-old cultures of "*Candidatus* Arsenophonus arthropodicus" (optical density at 600 nm [OD₆₀₀], ≈0.1) using the Bio-Rad bacterial genomic DNA plug kit (Bio-Rad), following the supplier's instructions. At 4 h prior to the harvesting of cells, 12.5 μg/ml chloramphenicol was added to the symbiont cultures to ensure synchronization of chromosome replication. To obtain a suitable concentration of chromosomal DNA for restriction digestion and PFGE, each 100 μl of agarose plug contained bacteria from 2 ml of symbiont culture (OD₆₀₀, 0.2). When we checked the integrity of the chromosomal DNA by performing PFGE on undigested plug DNA, we observed multiple small DNA species representing extrachromosomal elements. Subsequent to further analyses these extrachromosomal elements were removed by preparative PFGE according to previously described methods (1). For chromosome size determination, 1- to 2-mm slices of chromosomal DNA plugs were digested in separate reactions with restriction enzymes that cut infrequently in the genomes of enteric bacteria. Prior to digestion, chromosomal plugs were incubated overnight in 250-μl aliquots of 1× restriction buffer. Following equilibration, restriction buffers were replaced and supplemented with 20 U of restriction enzyme. The digestion reaction mixtures were stored at 4°C for 1 h to permit diffusion of restriction enzymes into the agarose plugs. The reactions were then maintained at the appropriate temperature for optimal digestion of each enzyme overnight. Digested plugs and appropriate size standards (New England Biolabs) were subjected to PFGE using a range of separation conditions. The resulting gels were stained and analyzed using the Quantity One software package (Bio-Rad).

Plasmid transformation of "*Candidatus* Arsenophonus arthropodicus." The broad-host-range plasmid pCM66 (18) was used for the transformation of "*Can-*

didatus Arsenophonus arthropodicus" using standard procedures. Briefly, 100-ml aliquots of bacteria from a 5-day-old culture (OD$_{600}$, 0.2) were made competent for heat shock transformation using the CaCl$_2$ method (13). Approximately 50 ng of plasmid DNA was then added to a 1-ml aliquot of competent cells (approximately 10^8 to 10^9 cells/ml). The cells were maintained on ice for 30 min and then subjected to a brief heat shock (42°C, 90 s). Following heat shock the bacteria were chilled on ice for 90 seconds and then transferred to 20 ml of MM medium for overnight recovery at 25°C. Antibiotic selection was applied the following morning by supplementing the culture with 20 µg/ml kanamycin to select for bacteria harboring pCM66. Kanamycin-resistant transformants were maintained by serial passage in the laboratory using standard procedures.

Restriction enzyme analysis of extrachromosomal DNA isolated from wild-type and transformed symbionts. Circular extrachromosomal DNA was isolated from 25-ml cultures (OD$_{600}$, 0.2) of wild-type and pCM66-transformed "*Candidatus* Arsenophonus arthropodicus," using the Promega Wizard miniprep system (Promega), according to the supplier's instructions. One-microgram aliquots of the resulting extrachromosomal DNA samples were digested with a range of restriction enzymes (Promega and New England BioLabs) under appropriate reaction conditions. The digested extrachromosomal DNA samples were then analyzed by conventional agarose gel electrophoresis.

Nucleotide sequence accession numbers. The sequences of PC1 to PC4 have been submitted to GenBank and their accession numbers are DQ115535 to -8.

TABLE 1. Sequences in the GenBank database that share the highest identity with 16S rRNA gene sequences from *P. canariensis* (PC1 to -4)

Query	Accession no.	% Identity	Description
PC1	AY861701	94	*Sodalis glossinidius* (tsetse fly symbiont)
	AF476100	94	Symbiont of *Amonostherium lichtensiodes*
	AF476109	93	Symbiont of *Paracoccus nothofagicola*
PC2	AY264665	99	Symbiont of *Aleuroplatus gelatinosus*
	AB038366	99	Symbiont of *Diaphorina citri*
	AY264673	99	Symbiont of *Australiococcus greville*
PC3	M85267	99	*Wolbachia* sp. from *Rhinocyllus conicus*
	AY833061	98	*Wolbachia* sp. from *Culex pipiens*
	AY007548	98	*Wolbachia* sp. from *Acalymma blandulum*
PC4	M84646	99	*Wolbachia* sp. from *Nasonia vitripennis*
	U83096	99	*Wolbachia* sp. from *Gryllus integer*
	AY754820	99	*Wolbachia* sp. from *Metaseiulus occidentalis*

RESULTS

Analysis of 16S rRNA gene sequences amplified from *P. canariensis* pupal DNA. The sequencing of 48 clones from the *P. canariensis* 16S rRNA gene library led to the identification of four distinct 16S rRNA gene sequences, represented approximately equally within the library. The four 1.5-kbp consensus 16S rRNA gene sequences were submitted for BLAST search with the NCBI database to establish their identity. The results are presented in Table 1. The first sequence (PC1) was unique among the four identified because it did not share a high level of sequence identity with any 16S rRNA gene sequences in the GenBank database. Instead, it shared a relatively low level of sequence identity (<95%) with the 16S rRNA genes of a number of insect endosymbionts, including *Sodalis glossinidius*. This result suggests that PC1 is derived from the primary endosymbiont of *P. canariensis*. P-endosymbionts typically have 16S rRNA gene sequences that are substantially diverged from those of other insect endosymbionts and related free-living bacteria because they have evolved in isolation for long periods of time in their hosts. The second 16S rRNA gene sequence, PC2, shared the highest level of identity (99%) with a number of 16S rRNA gene sequences from the *Arsenophonus* group of S-endosymbionts that are found in a number of unrelated insect hosts. The remaining two *P. canariensis* 16S rRNA gene sequences, PC3 and PC4, each shared a high level of sequence identity (99%) with 16S rRNA gene sequences reported for *Wolbachia* spp. Notably, PC3 and PC4 shared only 97% sequence identity with one another, indicating that *P. canariensis* harbors two distinct strains of *Wolbachia*. For the remainder of the current study we focus on the *Arsenophonus* endosymbiont that harbors 16S rRNA gene sequence PC2. The details relating to the characterization of the putative P-endosymbiont and *Wolbachia* spp. will be published in a separate manuscript.

Tissue-specific distribution of "*Candidatus* Arsenophonus arthropodicus." Primers CAIF and CAIR were used in PCRs to specifically detect the *Arsenophonus* 16S rRNA gene sequence in host insect tissues. We detected the *Arsenophonus* 16S rRNA gene sequence in hemocytes, gut, fat body, and reproductive tissues isolated from newly emerged *P. canarien-sis* flies. The widespread distribution of this bacterium in tissues of the host insect is not unusual given that other S-endosymbionts are known to share similar widespread tissue distributions (7, 15, 29).

Phylogenetic analysis. According to the maximum likelihood analysis presented in the current study (Fig. 1), the *Arsenophonus* group of arthropod endosymbionts forms a robust clade supported by 100% of bootstrap resamples. Only one member of this clade, "*Candidatus* Phlomobacter betae," a pathogen of sugar beets closely related to "*Candidatus* Phlomobacter fragariae" (31), is not known to be associated with an arthropod host. The PC2 sequence, derived from the 16S rRNA gene library of *P. canariensis*, is tightly clustered within the *Arsenophonus* clade. Although some phylogenetic structure does exist within the *Arsenophonus* clade, it is not well resolved by analysis of the 16S rRNA gene, which evolves relatively slowly. The important point to note is that very closely related *Arsenophonus* endosymbionts are found in distantly related arthropod hosts. This can only be explained by the recent lateral transfer of *Arsenophonus* between distantly related arthropod hosts.

Establishment of a primary *Arsenophonus* culture from *P. canariensis* pupae. The primary culture of the *P. canariensis* *Arsenophonus* endosymbiont was obtained from surface-sterilized *P. canariensis* pupae. Initially, cultures were obtained from homogenates derived from five pupae, although we subsequently found that cultures could be established from single pupae. To restrict the growth of contaminating bacteria prior to pure culture isolation, the primary culture medium was supplemented with vancomycin and polymyxin B. Whereas vancomycin prevents the growth of a wide range of gram-positive bacteria, polymyxin B restricts the growth of gram-negative bacteria that are sensitive to the bactericidal effects of antibacterial cationic peptides (10). Given that cationic peptides are an important component of the insect innate immune system (3), it is logical to assume that many insect S-endosymbionts, especially those residing in hemolymph, would display resistance to these peptides. Bacterial growth was first ob-
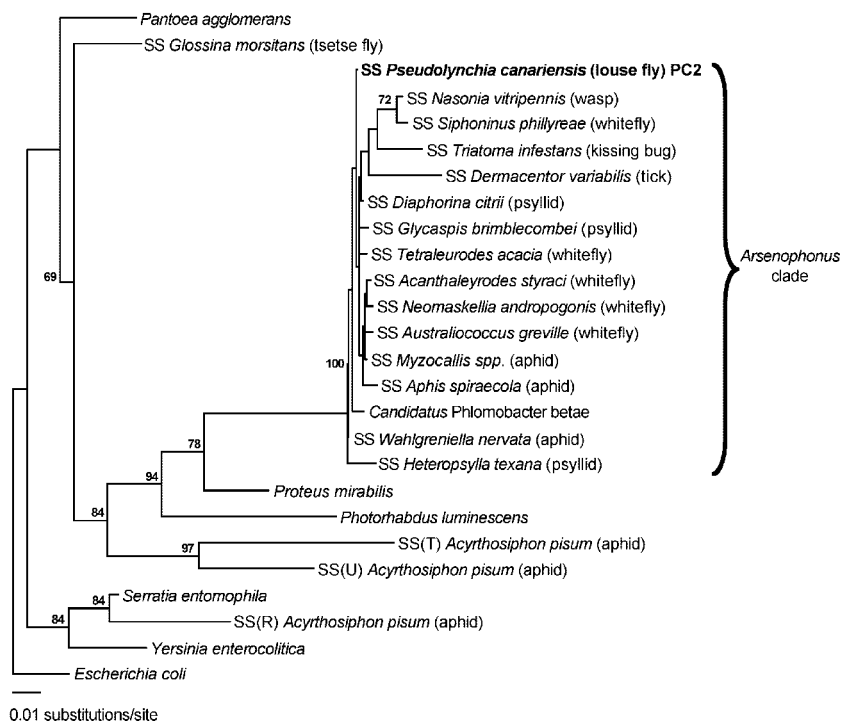
FIG. 1. 16S rRNA gene phylogeny based on S-endosymbionts of arthropods and some closely related free-living bacteria. The S-endosymbionts have the prefix SS followed by the proper name of their host, whereas free-living bacteria have proper binomial nomenclature. Only bootstrap values greater than 60% are shown adjacent to each node. The GenBank accession numbers for the 16S rRNA gene sequences are as follows: *P. agglomerans*, AF373196; *E. coli*, U00096; *S. entomophila*, AJ233427; *Y. enterocolitica*, AF366378; "*Candidatus* Phlomobacter betae," AY057392; *P. mirabilis*, AF008582; *P. luminescens*, BX571859; SS *Glossina morsitans*, AY861704; SS(R) *Acyrthosiphon pisum*, AY620432; SS *Pseudolynchia canariensis*, DQ115536; SS *Glycaspis brimblecombei*, AF263561; SS *Nasonia vitripennis*, M90801; SS *Triatoma infestans*, U91786; SS *Dermacentor variabilis*, AY265347; SS *Diaphorina citrii*, AB038366; SS *Tetraleurodes acacia*, AY264670; SS *Acanthaleyrodes styraci*, AY264663; SS *Neomaskellia andropogonis*, AY264668; SS *Australiococcus greville*, AY264673; SS *Heteropsylla texana*, AF263562; SS *Wahlgreniella nervata*, AY136168; SS *Myzocallis* spp., AY136153; SS *Siphoninus phillyreae*, AY264669; SS *Aphis spiraecola*, AY136142; SS(T) *Acyrthosiphon pisum*, AY462101; SS(U) *Acyrthosiphon pisum*, AY462102.

served in the *P. canariensis* pupal cultures at 5 days following establishment. Microscopic examination revealed the presence of uniform gram-negative rods in all cultures. The identity of the cultured bacterium was then established by 16S rRNA gene sequencing. All 16 clones examined from a library of 16S rRNA gene PCR products derived from the bacteria in culture had sequences that were 100% identical to the PC2 (*Arsenophonus*) sequence from *P. canariensis*. All subsequent culturing was performed without antibiotics.

Establishment of a pure culture on solid medium. Pure cultures were established from the primary liquid cultures on MM agar plates under an artificial microaerobic atmosphere in a sealed gas jar. Colonies were first visible on the agar plates after 5 days of incubation at 25°C. After 10 days, colonies had reached 2 to 3 mm in diameter and plates were removed from the gas jar. All colonies were irregular and raised with an undulate margin, light brown pigmentation, and a dull, moist surface. Ten colonies were picked at random from plates and inoculated into MM medium to establish pure liquid cultures. Following growth, we examined the morphology of cells from these pure cultures under the deconvolution microscope (Fig. 2). We also examined hemocytes collected from newly emerged adult flies for comparison. All of the pure cultures contained uniform rod-shaped cells, measuring 2 to 5 μm in length and 0.3 μm in diameter. Morphologically similar forms

were found in the insect hemocytes, although many of these intracellular forms did not stain well with FM4-64. To confirm the identity of bacteria in the pure cultures, DNA was isolated from bacterial cells in each culture and subjected to PCR analysis. PCR was performed using both universal 16S rRNA gene primers and primers CAIF and CAIR, designed to specifically amplify the *Arsenophonus* 16S rRNA gene sequence obtained initially from the *P. canariensis* clone library. The 16S rRNA gene sequences obtained from each pure culture were found to be identical to the PC2 (*Arsenophonus*) sequence from *P. canariensis*. DNA from each culture also tested positive with the CAIF and CAIR primers in PCRs. We therefore conclude that we obtained pure cultures of the *Arsenophonus* S-endosymbiont of *P. canariensis*. Pure cultures were cryopreserved, stored at −80°C for several weeks, and successfully resuscitated into culture in MM medium.

Provisional nomenclature. Following pure culture isolation, we propose the provisional name "*Candidatus* Arsenophonus arthropodicus" for the S-endosymbiont of *P. canariensis*. Provisional placement in the genus *Arsenophonus* is proposed based on the high level of 16S rRNA gene sequence identity between the bacterium obtained in pure culture in this study and other members of the genus *Arsenophonus*. Since it is likely that the majority of the *Arsenophonus* S-endosymbionts found in arthropods will belong to the same species (based
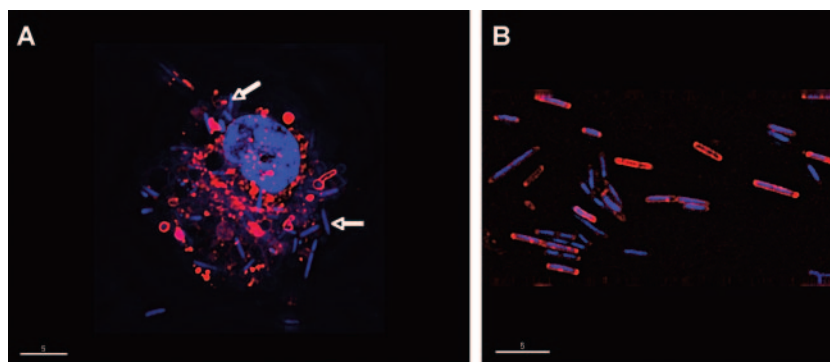
FIG. 2. "*Candidatus* Arsenophonus arthropodicus" in a *P. canariensis* hemocyte (A) and in pure culture in the laboratory (B). The deconvolved images were generated from material stained with FM4-64 and DAPI. The majority of bacteria (indicated by arrows) in the hemocyte were not stained with FM4-64, indicating that they lack cell walls in this intracellular form. Bars, 5 μm.

on the fact that many share 99% sequence identity in the 16S rRNA gene), we propose the use of the species epithet *arthropodicus* (Latin for belonging to arthropods) Formal nomenclature will be proposed for "*Candidatus* Arsenophonus arthropodicus" following the application of phenotypic tests to establish the biochemical properties of this bacterium. Attempts will also be made to obtain pure cultures of related bacteria harbored by other arthropods.

Culture of "*Candidatus* Arsenophonus arthropodicus" in an insect cell line. The pure culture isolate of "*Candidatus* Arsenophonus arthropodicus" was inoculated at a low multiplicity of infection into a confluent *A. albopictus* C6/36 cell culture. We then used a deconvolution microscope to monitor the course of the infection process. The deconvolution microscope was used to visualize sections (Z-projections) of insect cells at 4 h and 48 h postinfection. At 4 hours postinfection, bacteria were observed adhering to the surface of insect cells (Fig. 3A and C, showing the Z-projections corresponding to the cell surface) but not in the intracellular section (Fig. 3B). At 48 h postinfection, bacteria were observed in the cytoplasm of insect cells (Fig. 3E, showing the intracellular Z-projection) but not on the surfaces of insect cells (Fig. 3D and F). These results indicate that "*Candidatus* Arsenophonus arthropodicus" is capable of establishing an intracellular infection in insect cells in vitro.

"*Candidatus* Arsenophonus arthropodicus" genome size and composition. The genome of "*Candidatus* Arsenophonus arthropodicus" is comprised of a single chromosome and multiple extrachromosomal elements. To obtain an accurate estimate of chromosome size, it was necessary to remove extrachromosomal DNA from agarose plug preparations of chromosomal DNA prior to restriction analysis and PFGE. In the absence of extrachromosomal DNA, we analyzed intact chromosomal DNA using two rare-cutting restriction enzymes, NotI and AscI. Each of these enzymes yielded a reasonable number of fragments for chromosome size estimation (Fig. 4). The chromosome size estimates based on the NotI and AscI digests were 3,457 kbp and 3,561 kbp, respectively. The stoichiometry of fragments resolved on the pulsed-field gels was consistent with the presence of a single chromosome. Following the estimation of chromosome size, we attempted to estimate the size and composition of extrachromosomal elements

in the genome of "*Candidatus* Arsenophonus arthropodicus." Extrachromosomal DNA was isolated from cultures by using an alkaline lysis procedure and digested with a range of restriction enzymes. Restriction fragments were then separated by conventional agarose gel electrophoresis (Fig. 5A). The stoichiometry of restriction fragments indicated that at least two species of extrachromosomal DNA are present in "*Candidatus* Arsenophonus arthropodicus." The smallest of these species (designated pARS1) is a circular plasmid with a size of 9.9 kbp with a single EcoRI site and at least three XbaI sites. One or more additional, larger plasmids are also present. At this stage we cannot provide an accurate estimate of the number and sizes of the larger extrachromosomal elements in "*Candidatus* Arsenophonus arthropodicus."

Plasmid transformation of "*Candidatus* Arsenophonus arthropodicus." We used the broad-host-range plasmid pCM66 (18) to transform the pure culture isolate of "*Candidatus* Arsenophonus arthropodicus." Transformation was achieved with high efficiency after rendering bacteria competent for plasmid transformation by treatment with $CaCl_2$. Following selection for pCM66 transformants, extrachromosomal DNA was isolated from a cloned transformant and analyzed by digestion with the restriction enzyme XbaI. The restriction profile (Fig. 5B) confirmed the presence of both pCM66 and endogenous extrachromosomal DNAs in the transformed bacteria. Plasmid pCM66-transformed bacteria have now been maintained for over 3 months in continuous culture in the laboratory.

DISCUSSION

Members of the provisional genus *Arsenophonus* have been identified in a wide range of arthropods, including parasitoid wasps (11), triatomine bugs (15), ticks (12), whiteflies (26), aphids (23, 28), and psyllids (24). In the current study, we demonstrated that *P. canariensis*, a blood-feeding hippoboscid louse fly, also maintains an endosymbiotic bacterium closely allied to the *Arsenophonus* group. Based on the 16S rRNA gene phylogeny, the *Arsenophonus* group forms a distinct and robust monophyletic clade of arthropod endosymbionts in the gamma subdivision of *Proteobacteria*. Only one member of the
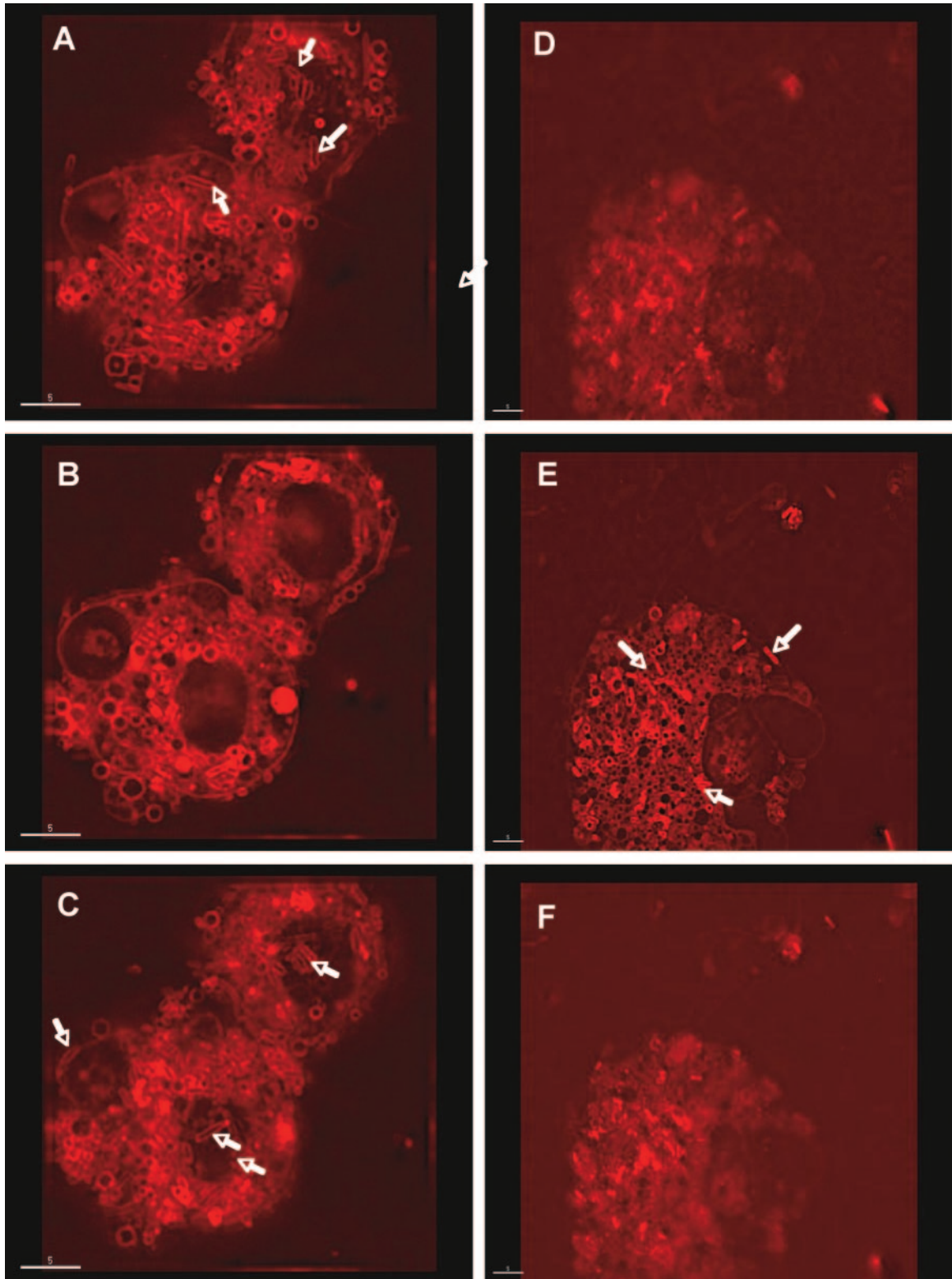
FIG. 3. "*Candidatus* Arsenophonus arthropodicus" in the *Aedes albopictus* cell line C6/36, stained with FM4-64. Micrographs were obtained at 4 h (A, B, and C) and 48 h (D, E, and F) following infection of the cell line. Plates A and D are sections obtained from the top surface of insect cells, plates B and E are intracellular sections, and plates C and F are sections obtained from the bottom surface of insect cells. Note that bacteria (indicated by arrows) adhered to the top and bottom surfaces of insect cells at 4 h postinfection (A and C) but are absent in the intracellular section (B). At 48 h postinfection, bacteria are visible in the intracellular section as they divide in the cytoplasm of insect cells (E).
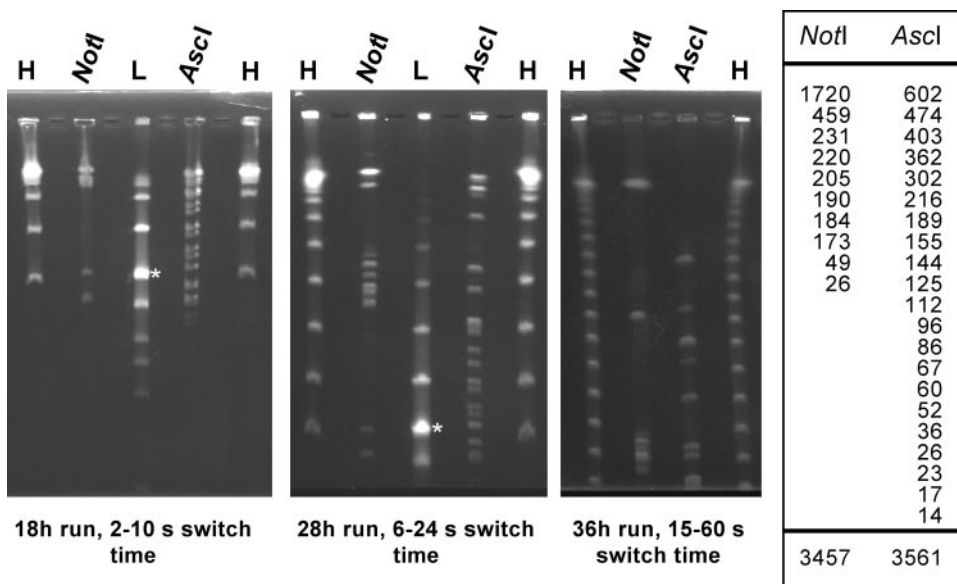
NotI	AscI
1720	602
459	474
231	403
220	362
205	302
190	216
184	189
173	155
49	144
26	125
	112
	96
	86
	67
	60
	52
	36
	26
	23
	17
	14
3457	3561

18h run, 2-10 s switch time   **28h run, 6-24 s switch time**   **36h run, 15-60 s switch time**

FIG. 4. PFGE analysis of the "*Candidatus* Arsenophonus arthropodicus" genome following removal of extrachromosomal elements. Restriction fragments and markers were resolved under three distinct sets of electrophoresis conditions, as indicated below each gel photograph. The lanes are labeled as follows: H, high-range PFGE marker (New England BioLabs), a concatemer of a 48.5-kbp λ DNA fragment; L, low-range PFGE marker (New England BioLabs), a HindIII digest of λ DNA mixed with 48.5-kbp λ DNA concatemers (asterisk marks the 48.5-kbp λ DNA fragment); N and A, NotI and AscI digests, respectively, of chromosomal DNA isolated from "*Candidatus* Arsenophonus arthropodicus." The smallest λ concatemer visible in lane H on the gel from the 36-h run is the 145.5-kbp fragment. The sizes of restriction fragments from the NotI and AscI digests are presented in the adjacent table. The total sizes of the NotI and AscI fragments were estimated to be 3,457 kbp and 3,561 kbp, respectively.

Arsenophonus clade is not formally known to be associated with an arthropod host (31).

Although many arthropod taxa are known to maintain intimate symbiotic relationships with bacteria, in most cases there
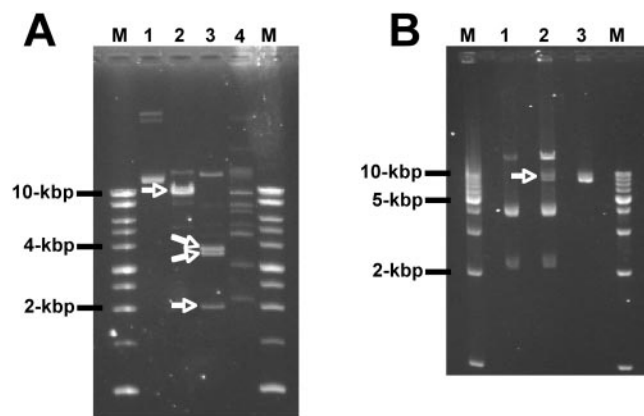

FIG. 5. Restriction enzyme digestion of extrachromosomal elements in untransformed and pCM66-transformed pure culture isolates of "*Candidatus* Arsenophonus arthropodicus." (A) Extrachromosomal DNA from untransformed bacteria before digestion (lane 1) and after digestion with EcoRI (lane 2), XbaI (lane 3), and CspCI (lane 4). Arrows indicate EcoRI and XbaI fragments of the 10-kbp plasmid pARS1. (B) Extrachromosomal DNA from untransformed bacteria digested with XbaI (lane 1), extrachromosomal DNA from pCM66-transformed bacteria digested with XbaI (lane 2), and plasmid pCM66 DNA digested with XbaI (lane 3). The XbaI-linearized fragment of pCM66 (7.6 kbp) is highlighted with an arrow. Lanes labeled M contain the Promega 1-kbp benchtop ladder (A) and Promega 1-kbp step ladder (B), with fragment sizes indicated adjacent to the gel photographs.

is a high degree of specialization between hosts and their endosymbionts. In other words, it is unusual to find extremely closely related endosymbionts in phylogenetically unrelated hosts. This holds true for both the primary (long-established) and secondary (facultative) endosymbionts of arthropods. One obvious exception to this rule occurs with *Wolbachia*, the most widely distributed symbiont found in association with arthropods and nematodes (4). Aside from *Wolbachia*, the only other group of closely related arthropod endosymbionts that shares a wide host distribution is the *Arsenophonus* group.

Although members of the *Arsenophonus* group have been identified in a wide range of arthropod species, little is known about their role in the context of symbiosis. In the parasitoid wasp *Nasonia vitripennis*, *Arsenophonus nasoniae* is known to induce a female-biased sex ratio distortion, similar to that observed with some strains of *Wolbachia* spp. (11, 30). However, in *Triatoma infestans*, a blood-sucking bug that harbors a closely related member of the *Arsenophonus* group ("*Candidatus* Arsenophonus triatominarum"), no sex ratio distortion effects have been detected in laboratory animal colonies (15). Similarly, there was no sex ratio distortion in our laboratory colony of *P. canariensis*; we recently determined the sex of a sample of 200 newly emerged flies and found 100 males and 100 females. Given that members of the *Arsenophonus* group are found in arthropod hosts that persist on a variety of diets (including vertebrate blood and plant sap), it seems unlikely that symbioses involving *Arsenophonus* have a nutritional basis. However, it is likely that they serve some important role in insects because they are so widely distributed.

In the current study, we obtained a pure culture isolate of "*Candidatus* Arsenophonus arthropodicus," a newly described

species from the louse fly *P. canariensis*. According to the phylogenetic analysis, the pure culture isolate of "*Candidatus* Arsenophonus arthropodicus" is most closely related to the *Arsenophonus* spp. described from aphids, whiteflies, and psyllids (23, 24, 26). Together, these bacteria share >99% sequence identity in their 16S rRNA genes, most likely indicating recent acquisition of *Arsenophonus* among these distantly related insects.

The pure culture isolation of "*Candidatus* Arsenophonus arthropodicus" provides new opportunities to explore the role of *Arsenophonus* spp. in insects. In this study we demonstrated pure culture isolation, cryopreservation, and artificial infection of an insect cell line with "*Candidatus* Arsenophonus arthropodicus." We also determined the composition and size of the "*Candidatus* Arsenophonus arthropodicus" genome. The size of the "*Candidatus* Arsenophonus arthropodicus" chromosome was estimated to be 3.51 Mbp, only slightly reduced relative to the 4.06-Mbp chromosome of the closest free-living relative, *Proteus mirabilis* (whole genome sequence currently undergoing annotation at the Sanger Center). We also discovered a broad-host-range plasmid vector suitable for the high-frequency transformation of "*Candidatus* Arsenophonus arthropodicus." Together, the availability of these culture and transformation systems provides a new platform to explore the associations between *Arsenophonus* and insects.

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