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Genes for the Type IV Secretion System in an Intracellular Symbiont, *Wolbachia*, a Causative Agent of Various Sexual Alterations in Arthropods

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Wolbachia **species are intracellular bacteria known to cause reproductive abnormalities in their hosts. In this study, we identified *Wolbachia* genes encoding homologs to the type IV secretion system by which many pathogenic bacteria secrete macromolecules. The genes identified encoded most of the essential components of the secretion system and were cotranscribed as an operon.**

The genus *Wolbachia* comprises a group of maternally inherited intracellular bacteria that have been identified in a variety of arthropod hosts. *Wolbachia* organisms cause various sexual alterations in their hosts, such as cytoplasmic incompatibility (CI), thelytokous parthenogenesis, feminization, and male killing (20), whose mechanisms are yet unknown. CI is the most commonly expressed phenotype in a wide range of insect species. This phenotype results in reduced egg viability in crosses between *Wolbachia*-infected males and uninfected females in the simplest case (16, 20, 23). Since *Wolbachia* organisms are not present in mature sperm (3, 12), it is believed that the bacteria modify sperm during their development, possibly by secreting some proteins. Based on this assumption, we directed our attention to the macromolecule secretion system in *Wolbachia*.

Many gram-negative bacteria have conserved macromolecule secretion systems. Type IV secretion systems have been mainly found in pathogenic bacteria, such as *Agrobacterium tumefaciens* (25), *Bordetella pertussis* (22), *Helicobacter pylori* (6), *Brucella suis* (15), *Legionella pneumophila* (21), and *Rickettsia prowazekii* (1). The secreted proteins and nucleoproteins play important roles in the virulence of these bacteria (4, 7). In this study, we tested the presence of the type IV secretion system in *Wolbachia*.

The Taiwan cricket, *Teleogryllus taiwanemma*, and the Mediterranean flour moth, *Ephestia kuehniella* (Yokohama strain), were used as the sources of *Wolbachia. Wolbachia* strains infecting arthropods are divided into A and B groups (23). The *Wolbachia* strain carried by *T. taiwanemma* (*w*Tai) and the strain carried by *E. kuehniella* (*w*KueYO) belong to the B and A group, respectively (12, 14, 18). Both *w*Tai and *w*KueYO cause CI in each host (12, 18). Tetracycline-treated strains of these insects were used as the uninfected control strains.

Genes for a type IV secretion system have often been found as an operon that consists of several *virB* genes and a *virD4* gene (4, 5). The VirD4 protein is believed to bind the molecule to be secreted and deliver it to the pore complex on the bacterial membrane formed with VirB proteins (4, 9, 10). To test the presence of genes for a type IV secretion system in *Wolbachia*, we performed Southern hybridization using the *virD4* gene of *R. prowazekii* (lambda clone F958) as a probe. Unless otherwise mentioned, standard molecular methods were used (17). As shown in Fig. 1, positive signals were observed in the lanes with samples from *Wolbachia*-infected insect hosts but not in those with samples from uninfected insect hosts. The signal from *w*KueYO was more intensive than that from *w*Tai. This was probably because *E. kuehniella* contained *Wolbachia* at a higher density than *T. taiwanemma*. *virD4* and its flanking regions of *w*Tai were cloned and sequenced by screening the genomic library of *w*Tai (13). It turned out that the sequenced region of *w*Tai contained *virB8*, *-B9*, *-B10*, *-B11*, and *-D4* (Fig. 2A) and that the predicted amino acid sequences of their products showed significant similarity to those homologs of *A. tumefaciens* and *R. prowazekii* (Table 1). This region did not contain *virB4*, which encodes the integral cytoplasmic membrane protein that plays a critical role in the type IV secretion system (2, 8). The presence of *virB4* in a different region of the *w*Tai genome was confirmed by Southern hybridization using *R. prowazekii virB4* (lambda clone P438) as a probe (data not shown). It is postulated that the minimum components of the typical type IV secretion system consist of VirB4, VirB7 (or VirB8), VirB9, VirB10, VirB11, and VirD4 homologs (7, 24). Thus, the genes identified from *w*Tai contained most of the minimum sets for the typical type IV secretion system.

Expression of the genes identified above was examined by reverse transcription (RT)-PCR. Total RNA from the testis of *T. taiwanemma* was reverse transcribed by SuperScript

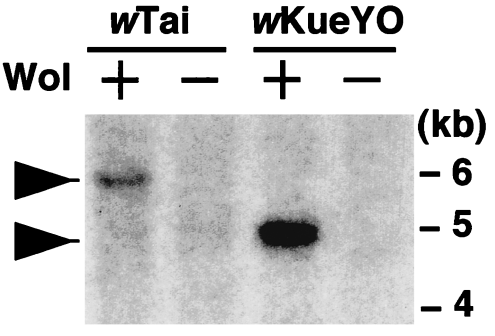

FIG. 1. Detection of the *virD4* gene in two *Wolbachia* strains by Southern hybridization using *virD4* of *R. prowazekii* as a probe. Wol+, *Wolbachia*-infected insects; Wol−, uninfected insects. Total DNA samples from insects were digested with *Eco*RI prior to agarose gel electrophoresis.

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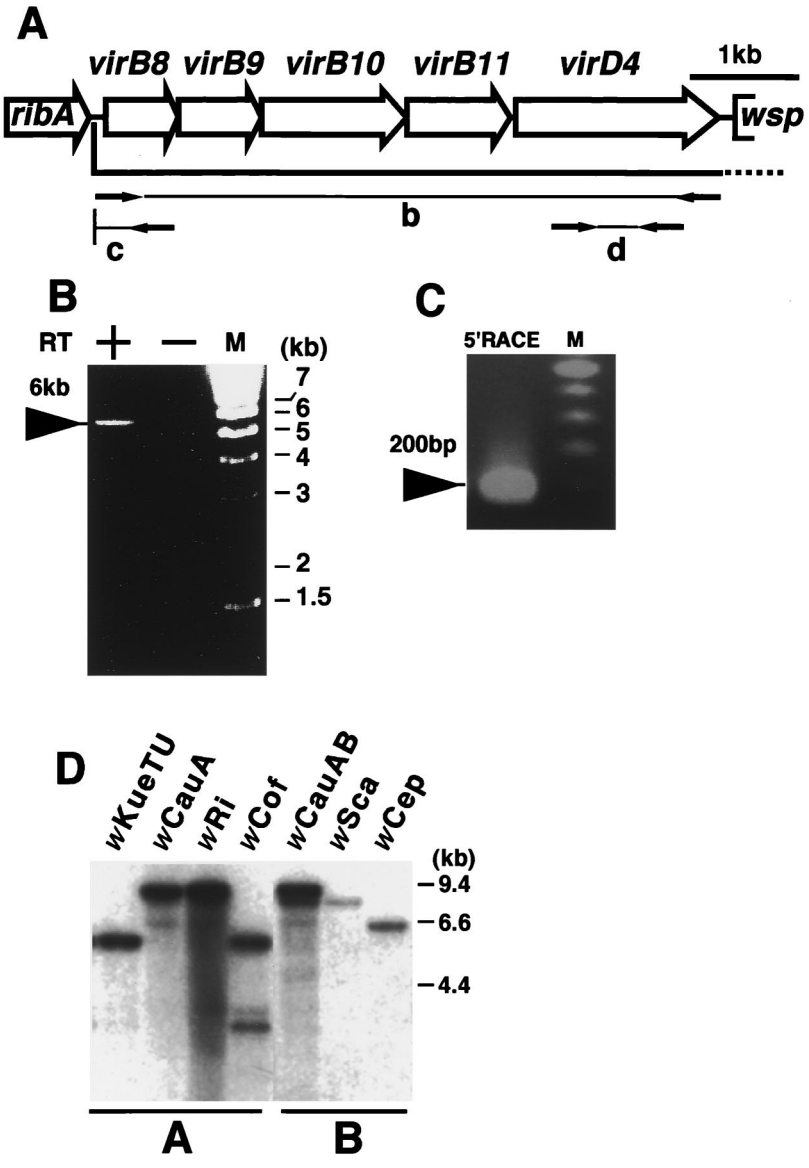

FIG. 2. Transcriptional analysis of the *vir* operon and its distribution in *Wolbachia* strains. M, DNA size marker. (A) Open reading frame map of the *w*Tai *vir* operon. Open arrows show open reading frames. Arrows in the regions b, c, and d indicate the primers used in the experiments shown in panels B, C, and D, respectively. *ribA*, GTP cyclohydrolase II; *wsp*, a surface protein. (B) RT-PCR of the entire region of the *vir* operon. RT+ and RT− indicate the presence and absence of reverse transcriptase in the reaction mixture, respectively. (C) 5′RACE analysis. (D) Southern hybridization of different *Wolbachia* strains using *virD4* of *w*Tai. *w*KueTU, *w*CauA, *w*Ri, and *w*Cof are A group strains, and their insect hosts are *E. kuehniella* (Tsuchiura strain), the almond moth *E. cautella*, the fruit fly *Drosophila simulans* Riverside, and *D. simulans* Coffs, respectively. *w*CauAB indicates A and B group strains (*w*CauA and *w*CauB, respectively) doubly infecting *E. cautella*. Two bands were observed in *w*CauAB, at about 9.4 and 9 kb, while only a single band was observed in *w*CauA, at 9.4 kb, indicating that *w*CauB contained *virD4* distinct from that of *w*CauA. *w*Sca and *w*Cep are B group strains infecting the moth *Ostrinia scapulalis* and *Corcyra cepharonica*, respectively. All DNA samples were digested with *Eco*RI.

II RT (GIBCO BRL) using the primer virR1 (5′-TTAACC TCTATCCTCGAT-3′). PCR was performed using LA *Taq* (TAKARA) with the primer set of virLF1 (5′-ATTGGAATT CAAGTCGCTATAGCACAGTTG-3′) and virRL1 (5′-TCCT CATCGTCAAATTCATCCTTACTGTC-3′). A positive signal at about 6 kb was detected only in the lane RT+ (Fig. 2B), indicating that *vir* genes of *Wolbachia* were cotranscribed as an operon. The transcription start site of the *vir* operon was then determined by 5′RACE analysis using the 5′/3′ RACE kit (Boehringer). The transcript of the *vir* operon was reverse transcribed with the specific primer virR11 (5′-CCCTTGCTT TTATATACTCTG-3′), and a polymeric dA tail was added to the 3′ end of the cDNA by terminal transferase. A DNA fragment containing the poly(dA) addition site was amplified

TABLE 1. Identities of amino acid sequences of *w*Tai *vir* gene products to those of *w*KueYO and other known type IV secretion systems[a]

*w*Tai (AB045234)	*w*KueYO (AB045235)	*R. prowazekii* (RPXX02)	*A. tumefaciens* Ti plasmid (AF242881)	*E. coli* pKM101 (U09868, AF109305)
VirB8	86	38	31	33
VirB9	88	40	28	29
VirB10	75	35	30	29
VirB11	95	67	33	36
VirD4	89	59	35	26

[a] Numbers are percent identities calculated by DNASIS-MAC (Hitachi). GenBank accession numbers are in parentheses.

by nested PCR. Primers used were the oligo(dT) anchor primer and vir5R1 (5′-CGTTCAGCGTTTCATTTGCAG-3′) in the first PCR and the anchor primer and vir5R2 (5′-CAAATGGCTCAATAGTGCTACTTGTGC-3′) in the second PCR. As a result, a PCR product of about 200 bp was obtained (Fig. 2C). Sequence analysis of the PCR product revealed that the cDNA was extended to about 50 bp upstream from the *virB8* start codon. Thus, the transcription of the *vir* operon was started from *virB8* and extended to *virD4*, though we could not exclude the possibility that the operon may include additional downstream genes. The *vir* operon of *w*KueYO was also cloned and sequenced. This operon contained the same 5 *vir* homologs as those of *w*Tai (Table 1) and was transcribed in the same manner as the operon of *w*Tai (data not shown).

To estimate the distribution of the type IV secretion system among *Wolbachia* strains, the presence of *virD4* was examined by Southern hybridization for seven other strains, four from group A and three from group B. The probe used was the *virD4* fragment amplified from the lambda clone of *w*Tai by PCR using the primers virD4F (5′-ATCAGAGAAAGACATACGAAAAGCAGG-3′) and virD4R (5′-CAATGGCTTACCCCATCTGGC-3′). Positive signals were detected in all seven strains tested (Fig. 2D).

In this study, it was demonstrated that *w*Tai has the genes for the type IV secretion system and that the *vir* operon containing these genes is expressed, suggesting that this system is functional in *w*Tai. The *vir* operon was highly conserved between A and B group *Wolbachia*. In addition, many *Wolbachia* strains were shown to share *virD4*. These observations imply that the type IV secretion system is ubiquitous and may have important roles in *Wolbachia*. Although the function of this system in *Wolbachia* is not clear, the type IV secretion systems in other pathogenic bacteria are known to secrete various macromolecules that affect the physiology of the host cells. Examples include the T-DNA complex of *A. tumefaciens* for inducing tumors in plant cells (5, 25), pertussis toxin of *B. pertussis* for ADP-ribosylation of the G protein of various cells (11), and CagA of *H. pylori* for pseudopodium formation to facilitate phagocytosis of epithelial cells (19). It is conceivable that *Wolbachia* organisms secrete certain molecules that may participate in the expression of CI through the type IV secretion system.

Nucleotide sequence accession numbers. The sequence of *virD4* and its flanking regions in *w*Tai obtained in this study was submitted to GenBank and assigned accession no. AB045234. The sequence of the *vir* operon of *w*KueYO obtained in this study was also submitted to GenBank and was assigned accession no. AB045235.

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REFERENCES

1. **Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland.** 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature **396:**133–140.
2. **Berger, B. R., and P. J. Christie.** 1993. The *Agrobacterium tumefaciens virB4* gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. J. Bacteriol. **175:**1723–1734.
3. **Bressac, C., and F. Rousset.** 1992. The reproductive incompatibility system in *D. simulans*: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. J. Invertebr. Pathol. **61:**226–230.
4. **Burns, D. L.** 1999. Biochemistry of type IV secretion. Curr. Opin. Microbiol. **2:**25–29.
5. **Christie, P. J.** 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J. Bacteriol. **179:**3085–3094.
6. **Covacci, A., S. Falkow, D. E. Berg, and R. Rappuoli.** 1997. Did the inheritance of a pathogenicity island modify the virulence of *Helicobacter pylori*? Trends Microbiol. **5:**205–208.
7. **Covacci, A., J. L. Telford, G. D. Giudice, J. Parsonnet, and R. Rappuoli.** 1999. *Helicobacter pylori* virulence and genetic geography. Science **284:**1328–1333.
8. **Dang, T. A. T., and P. J. Christie.** 1997. The VirB4 ATPase of *Agrobacterium tumefaciens* is a cytoplasmic membrane protein exposed at the periplasmic surface. J. Bacteriol. **179:**453–462.
9. **Das, A., and Y.-H. Xie.** 1998. Construction of transposon Tn3*phoA*: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. Mol. Microbiol. **27:**405–414.
10. **Disque-Kochem, C., and B. Dreiseikelmann.** 1997. The cytoplasmic DNA-binding protein TraM binds to the inner membrane protein TraD in vitro. J. Bacteriol. **179:**6133–6137.
11. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136–169.
12. **Kamoda, S., S. Masui, H. Ishikawa, and T. Sasaki.** 2000. *Wolbachia* infection and cytoplasmic incompatibility in the cricket, *Teleogryllus taiwanemma*. J. Exp. Biol. **203:**2503–2509.
13. **Masui, S., S. Kamoda, T. Sasaki, and H. Ishikawa.** 1999. The first detection of the insertion sequence ISW*1* in the intracellular reproductive parasite *Wolbachia*. Plasmid **42:**13–19.
14. **Masui, S., T. Sasaki, and H. Ishikawa.** 1997. *GroE*-Homologous operon of *Wolbachia*, an intracellular symbiont of arthropods. Zool. Sci. **14:**701–706.
15. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
16. **O'Neill, S. L., A. A. Hoffmann, and J. H. Werren (ed.).** 1997. Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, United Kingdom.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Sasaki, T., and H. Ishikawa.** 1999. *Wolbachia* infections and cytoplasmic incompatibility in the almond moth and the Mediterranean flour moth. Zool. Sci. **16:**739–744.
19. **Stein, M., R. Rappuoli, and A. Covacci.** 2000. Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. Proc. Natl. Acad. Sci. USA **97:**1263–1268.
20. **Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst.** 1999. *Wolbachia pipientis*: microbial manipulation of arthropod reproduction. Annu. Rev. Microbiol. **53:**71–102.
21. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
22. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90:**2970–2974.
23. **Werren, J. H.** 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. **42:**587–609.
24. **Winans, S. C., D. L. Burns, and P. J. Christie.** 1996. Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. Trends Microbiol. **4:**64–68.
25. **Zambryski, P.** 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. **22:**1–30.