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Identification of New Protein-Protein Interactions Involving the Products of the Chromosome- and Plasmid-Encoded Type IV Secretion Loci of the Phytopathogen *Xanthomonas axonopodis* pv. citri

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The recently sequenced genome of the bacterial plant pathogen *Xanthomonas axonopodis* pv. citri contains two *virB* gene clusters, one on the chromosome and one on a 64-kb plasmid, each of which codes for a previously uncharacterized type IV secretion system (T4SS). Here we used a yeast two-hybrid assay to identify protein-protein interactions in these two systems. Our results revealed interactions between known T4SS components as well as previously uncharacterized interactions involving hypothetical proteins coded by open reading frames in the two *X. axonopodis* pv. citri *virB* loci. Our results indicate that both loci may code for previously unidentified VirB7 proteins, which we show interact with either VirB6 or VirB9 or with a hypothetical protein coded by the same locus. Furthermore, a set of previously uncharacterized *Xanthomonas* proteins have been found to interact with VirD4, whose gene is adjacent to the chromosomal *virB* locus. The gene for one member of this family is found within the chromosomal *virB* locus. All these uncharacterized proteins possess a conserved 120-amino-acid domain in their C termini and may represent a family of cofactors or substrates of the *Xanthomonas* T4SS.

Many gram-negative bacterial pathogens of both animals and plants inject macromolecules into their host cells during the infection process. These molecules, usually proteins, but in some cases nucleoprotein complexes, carry out key functions during infection and are required for disease development (10, 11, 13, 22, 26, 34, 38). Two highly complex multiprotein systems may mediate this type of transfer: the type III secretion system (T3SS) and the type IV secretion system (T4SS). About half of the two dozen or more components of the T3SS are evolutionarily related to the core transmembrane components of the bacterial flagellar system (38). T3SSs are responsible for the secretion of pathogenicity factors which play central roles in diseases caused by animal pathogens such as *Yersinia* spp., *Salmonella*, *Pseudomonas aeruginosa*, and enteropathogenic *Escherichia coli* and by plant pathogens such as *Ralstonia solanacearum, Pseudomonas syringae, Xanthomonas* spp., and *Erwinia* (10, 11, 26, 38).

The T4SSs, on the other hand, are made up of a superfamily of secretion machines that are apparently unrelated to the T3SSs. The best characterized members of the T4SS superfamily are those involved in bacterial conjugation in which broad-host-range plasmids are transferred between gram-negative bacteria (30). Other members of the T4SS superfamily mediate the secretion or transfer of protein or protein-DNA complexes to their hosts (6, 8, 9, 13, 14). T4SSs play fundamental roles in disease development by the animal pathogens *Bordetella pertussis* (15), *Brucella suis* (36), *Bartonella henselae* (41), *Helicobacter pylori* (46), *Rickettsia prowazekii* (3), and *Legionella pneumophila* (52). The role of T4SSs in the development of disease in plants is well established in the case of crown gall tumors induced by *Agrobacterium tumefaciens* (56). Recent genome sequencing projects have identified T4SSs in the phytopathogens *Xanthomonas* spp. (18) and *Xylella fastidiosa* (45). The functions of these T4SSs in the promotion of disease in their host plants are unknown (7, 32), and to date no proteins have been shown to be released from *Xanthomonas* cells via a T4SS.

Xanthomonas axonopodis pv. citri is the causative agent of citrus canker, a disease of significant economic importance worldwide. The sequence of the *X. axonopodis* pv. citri genome revealed that it carried two loci, both originally named *virB*, coding for putative and distinct T4SSs, one on the chromosome and one in the 64-kb megaplasmid pXAC64 (18). A similar chromosomal *virB* cluster was found in the closely related *Xanthomonas campestris* pv. campestris genome (18). The two *X. axonopodis* pv. citri *virB* loci possess extra open reading frames that code for previously uncharacterized proteins, some of which have been identified only in xanthomonad genomes. Since the molecular bases of the bacterium-plant interactions required for the development of xanthomonad-derived diseases in general and citrus canker in particular are poorly understood, the functions of the T4SSs encoded by xanthomonad genomes are of great interest. In this study, we

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used a number of *X. axonopodis* pv. citri VirB proteins as baits in two-hybrid assays against a prey library derived from *X. axonopodis* pv. citri total genomic DNA. Our results revealed interactions between known T4SS components as well as some involving hypothetical proteins encoded by the two *X. axonopodis* pv. citri *virB* loci. Furthermore, a set of previously uncharacterized proteins was found to interact with the T4SS coupling protein VirD4, whose gene is found adjacent to the chromosomal *virB* operon. Finally, we have identified genes in both operons that probably code VirB7 orthologs that were not identified in the original annotation of the *X. axonopodis* pv. citri genome (18).

MATERIALS AND METHODS

X. axonopodis* pv. citri (strain 306) genomic DNA library, cloning of baits, and two-hybrid screens.** The construction of the *X. axonopodis* pv. citri 306 genomic DNA library in the plasmid vector pOAD (50) linearized with PvuII has been described (1). The library contains over 2×10^6 independent clones derived from fragments in the size range 500 to 3,000 bp. *X. axonopodis* pv. citri DNA sequences (18) encoding proteins for use as baits were amplified by PCR using primers containing unique restriction sites (usually NcoI and XhoI) to permit cloning into the pOBD vector downstream of, and in frame with, the Gal4 DNA binding domain (50). In some cases, fragments lacking one or more putative transmembrane helices (35) were employed as baits. These cases are described in the text and in Table 2. *Saccharomyces cerevisiae* strain PJ694-a (*MATa** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*) (28) was grown at 30°C in YAPD medium or SC medium as described previously (1). SC medium was prepared lacking one or more specific components: adenine (−Ade), histidine (−His), tryptophan (−Trp), and leucine (−Leu). In the case of growth on solid medium, 1.6% Bacto-Agar and 3-aminotriazole (3AT) were added. *S. cerevisiae* was transformed with pOBD bait plasmids, selected on SC-Trp plates, and then used in high-efficiency transformations with the pOAD library, using 30 μg of plasmid DNA (1, 19), and selected on SC-Trp-Leu-His-Ade plus 3AT plates (1). The amount of 3AT used varied between 5 and 50 mM, depending on the bait, in order to eliminate false positives due to low levels of basal expression from the *GAL1-HIS3* reporter. Colonies that grew in the absence of His and Ade were transferred to fresh SC-Trp-Leu-His-Ade plus 3AT plates. Plasmids were isolated and prey DNA was sequenced as described previously by using pOAD- or pOBD-specific primers (1). Sequences were analyzed by comparison with the *X. axonopodis* pv. citri genome database (18).

The incidence of false positives in two-hybrid assays using the pOAD-*X. axonopodis* pv. citri genomic DNA library has been described previously (1). Several promiscuous preys are detected at a frequency sufficient to warrant suspicion of their physiological significance: (i) TolC, (ii) members of the large TonB-dependent receptor family, (iii) the products of the two *wapA* genes (the XAC1866 and XAC1305 genes), (iv) the XAC3515 protein, and (v) the plasmid-encoded PthA and KfrA proteins. For this reason, these preys were considered to be false positives and were not considered further.

Expression and purification of recombinant XAC2609 and XAC2610. The genes encoding XAC2609, XAC2610, and a XAC2610 fragment lacking the first 21 codons were amplified by PCR from genomic DNA of *X. axonopodis* pv. citri. The full-length XAC2609 gene was cloned in the pET11a vector (Novagen) (47) between NdeI and BamHI sites. Two forms of the XAC2610 gene ($XAC2610_{His}$ and $XAC2610_{His\text{-}22\text{-}267}$) were cloned fused with an N-terminal His tag (MG SSHHHHHHSSGLVPRGSHM) in the pET28a plasmid (Novagen) between NdeI and BamHI sites. The full-length XAC2610 gene was also cloned in pET28a between NcoI and HindIII sites for expression without a His tag. These constructs were transformed into *E. coli* strain BL21(DE3)RP, and the synthesis of recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside. XAC2609 recombinant protein was purified from the soluble fraction of the bacterial lysate by Q-Sepharose anion-exchange chromatography (Amersham Bioscience). $XAC2610_{His\text{-}22\text{-}267}$ was affinity purified by using an Ni^{2+}-chelating Sepharose resin (Amersham Bioscience).

His tag pulldown assays. Experiments were carried out at room temperature. The proteins $XAC2610_{His\text{-}22\text{-}267}$ and XAC2609 and an *E. coli* lysate were dialyzed against buffer A (25 mM Tris-HCl, 100 mM NaCl, 3 mM 2-mercaptoethanol, 5 mM imidazole [pH 8.4]). To a 0.35-ml aliquot of Ni^{2+}-chelating Sepharose resin in buffer A, 100 μM $XAC2610_{His\text{-}22\text{-}267}$ was added. At the same time either 10 μM XAC2609 or a lysate derived from 10 ml of *E. coli* BL21(DE3) culture (A_{600} = 0.8) or both were added. In control experiments, the resin was mixed with only $XAC2610_{His\text{-}22\text{-}267}$ or with a mixture of XAC2609 and the bacterial cell lysate without $XAC2610_{His\text{-}22\text{-}267}$. The mixtures were washed three times with 1 ml of wash buffer (25 mM Tris-HCl, 100 mM NaCl, 3 mM 2-mercaptoethanol, 50 mM imidazole [pH 8.6]). Bound proteins were released with 0.03 ml of elution buffer (25 mM Tris-HCl, 100 mM NaCl, 3 mM 2-mercaptoethanol, 500 mM imidazole [pH 8.9]). Samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Comparison of the two *X. axonopodis* pv. citri T4SSs. Multiple T4SSs may be found in a single bacterial genome. For example, the genome of *A. tumefaciens* codes for three T4SSs (12). One, coded by the *virB* operon on the tumor-inducing (Ti) plasmid, is necessary for the transfer of this plasmid to the host cell and the development of disease. The other two T4SSs, coded by the *trb* and *avhB* loci, situated on the Ti plasmid and pAtC58, respectively, mediate the conjugal transfer of their respective plasmids between *Agrobacterium* cells (12). *Bartonella tribocorum* requires two independent T4SSs for the establishment of intraerythrocyte infection, one of which is highly similar to classical conjugation machines (43).

X. axonopodis pv. citri has two T4SSs, both encoded by what were originally named *virB* clusters, one on the chromosome and one on the plasmid pXAC64 (Fig. 1; 18). These were the first reported cases of T4SSs found in *Xanthomonas*. In terms of organization and degree of sequence similarity of its protein products, the *virB* cluster of pXAC64 is most closely related to the mating pair formation (*mpfA* to *mpfJ*) cluster of the IncP-9 TOL plasmid pWW0 of *Pseudomonas putida* (21) (Table 1 and see below) and to the *virB* cluster of pXcB isolated from *Xanthomonas citri* (GenBank accession number gi:38639487). The pWW0-encoded T4SS probably mediates conjugative plasmid transfer between bacterial cells (21). The chromosomal *virB* locus has a different organization from that found in the plasmid (Fig. 1), and the corresponding gene products possess low degrees of sequence identity: for example, 40% identity for VirB1, 23 to 34% identity for VirB3, -B4, -B8, -B9, -B10, and -B11, and very low or no significant identity for VirB2, -B5, -B6, and -B7 (Table 1). The products of the chromosomal cluster are most similar (approximately 40% identity) to the proteins encoded by the *tra* clusters found in other broad-host-range plasmids, most notably pSB105 and pIPO2 isolated from the microbial communities residing in the alfalfa and wheat rhizospheres, respectively (42, 48). Both pSB105 and pIP02 have genetic organizations similar to plasmid pXF51 of the citrus phytopathogen *X. fastidiosa* (32).

While the functions of several T4SS proteins are well established at the phenotypic level (8, 13, 30), the mechanisms of action of many of the components of these secretion machines are not well understood at the molecular level. Furthermore, both of the *virB* loci in *X. axonopodis* pv. citri possess open reading frames that code for previously uncharacterized proteins, in some cases specific to xanthomonads (Fig. 1 and Table 1). In this study, we focused on a subset of *X. axonopodis* pv. citri *virB* locus products that have not been characterized previously or whose mechanism of action is not clear. Table 2 lists these proteins and summarizes the results obtained in two-hybrid assays in which they were used as baits. The total num-

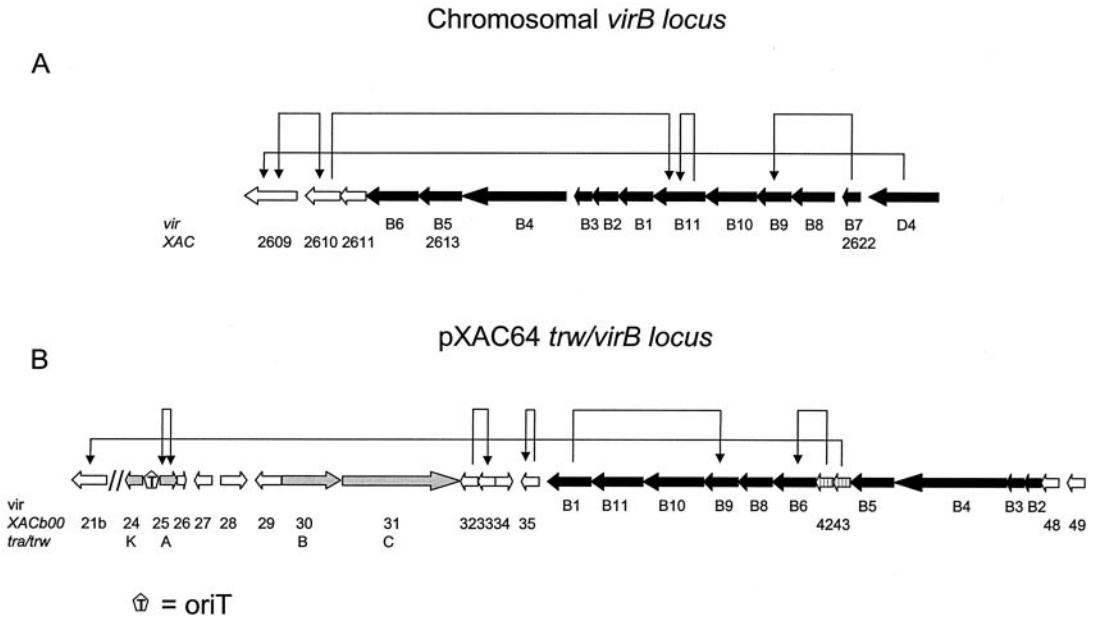


FIG. 1. *X. axonopodis* pv. citri *virB* clusters localized on the *X. axonopodis* pv. citri chromosome (A) and the pXAC64 megaplasmid (B). Genes whose products show similarity with other known VirB proteins are shown as black arrows. The XACb0042 and XACb0043 genes, one or both of whose products may exercise VirB7 functions, are shown as striped arrows. Genes coding for orthologs of the DNA processing proteins TraK and TrwA, -B, and -C in pXAC64 are shown in grey. Other genes that code for proteins of unknown function are shown in white. Arrows indicate interactions observed in yeast two-hybrid assays (bait → prey).

ber of positive interactions in which each prey was identified is presented, discounting false-positive preys (see above and Table 2). Positive interactions are those observed in assays necessitating the simultaneous activation of the *GAL1-HIS3* and *GAL2-ADE2* gene reporters. We consider interactions truly significant when they were observed in more than one prey derived from the same protein.

Interactions involving proteins coded by the chromosomal *virB* locus. (i) Interactions involving the VirD4 ATPase coupling protein. VirD4 and its orthologs are thought to be membrane-associated ATPases involved in the recognition of substrates of type IV secretion. These substrates may be protein-DNA complexes (i.e., the T DNA-VirD2 complex of *A. tumefaciens* and T strand-protein complexes in bacterial conjugation) or simply proteins (i.e., VirF of *A. tumefaciens*, CagA of *H. pylori*, and DotA and RalF of *L. pneumophila*) destined for the extracellular milieu or the interior of another bacterial cell or eukaryotic host (6, 9, 12, 14, 34). As it is not clear what the actual substrates of the chromosomally encoded type IV system of *X. axonopodis* pv. citri are, it is of interest to identify proteins that interact with VirD4.

As shown in Table 2 and Fig. 2, when full-length VirD4 was used as a bait in two-hybrid assays, all of the preys sequenced were derived from a set of 12 previously uncharacterized proteins of unknown function. These proteins vary in size from 112 to 854 amino acids. Alignment of the sequences of these proteins by using the SAM-T99 server (29) revealed that all share a conserved domain of approximately 120 amino acids (Fig. 2) that is found in the C-terminal regions of the larger proteins, while the smallest proteins of this group (XAC1165 and XAC0323) are made up solely of this domain. This conserved domain was found in all 68 VirD4 preys sequenced. A consensus sequence was determined by noting residues conserved in at least 6 of 12 members of the family (residues in bold in Fig. 2B). Forty-nine positions in the approximately 120-residue domain meet this criteria. Furthermore, individual proteins in this group have identities with the consensus sequence at between 24 and 40 positions (Fig. 2B). Neither the consensus sequence nor any of the conserved domains in any of the VirD4 baits has significant sequence similarity with any proteins of known structure or function in the public databases, and the only gram-negative bacteria that code for proteins with these domains are *X. axonopodis* pv. citri (14 genes) and *X. campestris* pv. campestris (16 genes) (see the legend to Fig. 2B). We will therefore refer to these proteins as "*Xanthomonas* VirD4 interacting proteins" or XVIPs and to the conserved domains as "XVIP conserved domains" (XVIPCDs). The XVIPCD does have some notable features, including several blocks of more conserved sequences (GLxRIDHV and FAVQ GxxDPAHxRAHV, where x is any residue) and a glutamine-rich C-terminal tail present in 11 of 12 proteins (Fig. 2B). No single residue is absolutely conserved in all the XVIPCDs, though some are present in 10 or 11 of 12 domains (indicated with asterisks in Fig. 2B). A conserved C-terminal RXR sequence has been found in C termini of the secreted virulence factors VirE2 and VirF from *A. tumefaciens* (6, 44, 51). However, this sequence is not found in the conserved sequences of the XVIPCD.

The largest number of VirD4 preys (13 of 68) was derived from XAC0466, of which the smallest interacting fragment corresponds to residues 471 to 590 (Table 2). Interestingly, the XVIPCD of XAC0466 shows the least similarity to the XVIPCD consensus sequence (Fig. 2B). To confirm the VirD4-XAC0466 interaction, we used a C-terminal XAC0466

TABLE 1. Comparison of *Xanthomonas* and pWW0 T4SS components

Protein	*Xanthomonas* chromosomal *vir* locus: gene no., no. of amino acids	Identity chromosome vs plasmid (%)[d]	Similarity chromosome vs plasmid (%)	*Xanthomonas* pXAC64 *trw* or *vir locus*: gene no., no. of amino acids	Identity, pXAC64 vs. pWW0 (%)	Similarity, pXAC64 vs pWW0 (%)	pWW0 *tra* or *mpf* locus: gene name, no. of amino acids
HP[a]	N[b]			XACb0021b[g], 129	45	63	orf176, 130
TraK/TraD	N			XACb0024, 148	42	59	*traD*, 151
TrwA/TraA	N			XACb0025, 131	30	53	*traA*, 127
HP	N			XACb0026, 57			N
HP	N			XACb0027, 90			N
HP	N			XACb0028, 151			N
HP	N			XACb0029, 124			N
VirD4/TrwB/TraB	XAC2623, 557	20	39	XACb0030, 524	63	74	*traB*, 516
TrwC/TraC	N			XACb0031, 991	64	79	*traC*, 978
HP	N			XACb0032, 107			N
HP	N			XACb0033, 72			N
HP	N			XACb0034, 140			N
HP	N			XACb0035, 139			N
VirB1	XAC2617, 280	40	49	XACb0036, 292	46	60	*mpfJ*, 315
VirB2	XAC2616, 136	19	52	XACb0047, 125	32	55	*mpfA*, 153
VirB3	XAC2615, 103	20	32	XACb0046, 99	48	65	*mpfB*, 98
VirB4	XAC2614, 817	26	42	XACb0045, 877	58	74	*mpfC*, 894
VirB5	XAC2613, 275	(15[e])	(54[e])	XACb0044, 220	60	73	*mpfD*, 229
VirB6	XAC2612, 350	18	56	XACb0041, 288	44	58	*mpfE*, 287
VirB7	XAC2622, 139	—[c]	—	XACb0042[f], 131	34	50	orf199, 130
	**	—	—	XACb0043[f], 82	38	54	orf200, 70
VirB8	XAC2621, 348	25	40	XACb0040, 224	36	55	*mpfF*, 226
VirB9	XAC2620, 255	23	42	XACb0039, 261	55	73	*mpfG*, 261
VirB10	XAC2619, 389	24	39	XACb0038, 406	45	60	*mpfH*, 421
VirB11	XAC2618, 346	34	51	XACb0037, 340	46	63	*mpfI*, 343

[a] HP, hypothetical protein.
[b] N, no corresponding protein identified in this locus.
[c] —, no significant similarity identified.
[d] All identity and similarity statistics obtained using the BlastP program except for comparisons between *Xanthomonas* VirB2, VirB6, and VirB5 paralogs, for which ClustalW (24) was used.
[e] Not identified as VirB5 in the *X. axonopodis* pv. citri genome (18). Statistics from alignment in Fig. 3A.
[f] Not identified as VirB7 in the *X. axonopodis* pv. citri genome (18).
[g] This gene was not annotated in the *X. axonopodis* pv. citri genome. See the text.

fragment (residues 450 to 590) as bait in two-hybrid assays against the *X. axonopodis* pv. citri genomic DNA prey library. Of 47 preys sequenced, 46 were derived from the TolC protein, a known false-positive prey in these assays (1). However, the single remaining clone was derived from the *virD4* gene and coded for residues 136 to 558 of the VirD4 protein (Table 2).

Eight of the preys found to interact with VirD4 were derived from the XAC2609 gene, located on the other side of the chromosomal *virB* locus (Fig. 1). When the 431-residue XAC2609 protein was used as a bait in the two-hybrid assay, all 11 preys sequenced were derived from the XAC2610 protein, coded by the gene adjacent to that of XAC2609 (Fig. 1 and Table 2). Furthermore, when full-length XAC2610 was used as bait, one of nine preys sequenced was found to be derived from residues 9 to 431 of XAC2609. XAC2610 is a 267-residue protein of unknown function. Residues 76 to 267 define the smallest fragment of XAC2610 found to interact with XAC2609 (Table 2). The only known XAC2610 orthologs are proteins of unknown function found in *P. syringae* and *Nitrosomonas europaea*, and the sequence similarity is restricted to the C-terminal halves of these proteins (24 to 32% identity).

The XAC2609-XAC2610 interaction was studied further in pulldown assays using recombinant XAC2609 and $XAC2610_{His\text{-}22\text{-}267}$. While full-length forms of recombinant XAC2610, with and without His tags, are only partially soluble upon bacterial cell lysis (results not shown), $XAC2610_{His\text{-}22\text{-}267}$ was found to be soluble under the conditions tested. This protein has an N-terminal His tag fused to a XAC2610 fragment lacking the first 21 residues. The deleted portion (MLT RELARAVAGVIYLICVVS), which may be part of a signal sequence (35), is rich in hydrophobic residues that may contribute to the insolubility of the full-length protein. Results of pulldown assays demonstrate that $XAC2610_{His\text{-}22\text{-}267}$ immobilized on a nickel chelating resin binds specifically to purified XAC2609, as well as to XAC2609 mixed with the soluble lysate of BL21(DE3) cells (Fig. 3).

The remaining eight preys that interacted with XAC2610 were derived from VirB11, FliK (flagellar hook-length protein), and BfeA (Table 2). When VirB11 was used as a bait, none of the preys were derived from XAC2610. Instead, 55 of 63 preys were derived from VirB11 (Table 2). One of these preys corresponded to residues 11 to 347, while all the others were the full-length VirB11 protein. Sequencing of these full-length clones demonstrated that they were all derived from recombination events between the pOBD prey vector and a pOAD-library-derived vector (1). These homotropic interactions are not surprising in light of the hexameric ring structure of an ADP-bound VirB11 homolog in *H. pylori* (55). In *A. tumefaciens*, VirB11 is a putative ATPase localized to the cytosolic side of the inner membrane that may be involved in pilus morphogenesis and/or substrate transfer through the T4SS (9, 39). It has been suggested that VirB4, VirB7, VirB8,

TABLE 2. Summary of protein-protein interactions involving *X. axonopodis* pv. citri T4SS components observed in this study

Bait name (residues in bait)	Bait gene no.	Total positive preys sequenced[a]	Specific preys [name or gene no: initial codons of preys (no. of times observed)]
Chromosomal T4SS			
VirD4 (1–557)	XAC2623	68	XAC0151: 11(1), 22(1), 25(1), 40(1), 120(1) XAC1918: 120(1), 222(1), 229(1), 306(1), 360(1), 369(1), 470(2) XAC3266: 301(1), 318(1), 320(2), 416(1), 514(1), 658(1), 663(1), 1) XAC2609: 30(2), 90(1), 127(1), 144(1), 170(1), 187(1), 314(1) XAC0096: 329(1), 330(1), 355(1), 402(1), 463(1), 478(1), 500(1) XAC0574: 10(1), 75(1), 80(1), 82(1), 88(1), 96(1), 134(1) XAC1165: 1(1), 6(1) XAC0466: 40(1), 124(1), 158(1), 195(1), 227(1), 280(1), 308(1), 352(1), 400(2), 436(1), 456(1), 471(1) XAC3634: 1(1), 13(1), 18(1), 20(1), 29(1), 38(1) XAC4264: 94(1) XAC0323: 30(1) XAC2885: 82(1)
XAC0466 (450–589)	XAC0466	1[c]	VirD4/XAC2623: 136(1)
XAC2609 (1–431)	XAC2609	11	XAC2610: 19(1), 28(1), 39(1), 41(1), 49(2), 51(4), 76(1)
XAC2610 (1–267)	XAC2610	9	FliK/XAC1949: 255(2), 265(3), 266(1) XAC2609: 9(1) VirB11/XAC2618: −14(1) BfeA/XAC3207: 596(1)
VirB11 (1–346)	XAC2618	60	VirB11/XAC2618: R(54),[b] 11(1) YhjX/XAC2488: 343(2), 348(1) PoxF/XAC4066: 80(1) XAC3517: 651(1)
XAC2622/VirB7 (1–139)	XAC2622	6	VirB9/XAC2620: 19(4), 101(1), 130(1)
pXAC64 T4SS			
VirB1 (1–292)	XACb0036	6	VirB9/XACb0039: 28(1), 30(3), 32(2)
XACb0042 (33–131)	XACb0042	13	VirB6/XACb0041: −4(2), −3(4), −2(1), 2(1), 3(1), 4(1), 8(3)
XACb0043 (1–82)	XACb0043	14	XACb0021b: 17(1), 20(1), 21(1), 25(1), 31(2), 33(1), 34(1) TatC/XAC4216: 1(4) FabB/XAC3625: 1(1) RhlB/XAC3829: 359(1)
XACb0035 (1–139)	XACb0035	27	XACb0035: R(11), −133(2), −99(1), −65(1), −55(1), 1(3), 2(4), 3(4)
XACb0032 (1–107)	XACb0032	11	XACb0033: −46(1), −43(1), −4(1), 3(1), 5(2), 6(1), 8(1), 16(1), 18(1) XAC1696: −50(1)
XACb0025/TrwA (1–131)	XACb0025	9	XACb0025: R(5), −4(2), 2(2)

[a] Not including false-positive preys mentioned in the text and described in reference 1.
[b] R indicates preys derived from recombination events between the bait and prey vectors as described in text and in reference 1.
[c] Forty-six other preys were derived from TolC, a false-positive prey, when using this library (1).

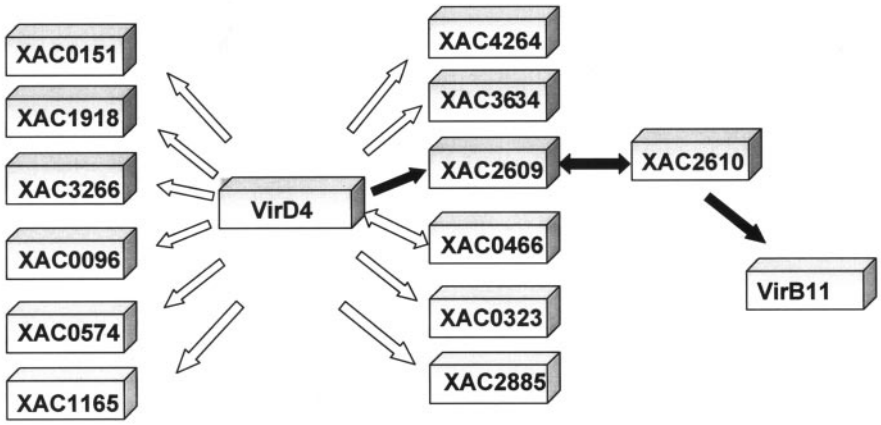
VirB9, VirB10, VirB11, and VirD4 make up the minimal core structure necessary for T4SS transport (16).

The above results are evidence for a small network of interactions involving VirD4, VirB11, and two other previously uncharacterized proteins (XAC2609 and XAC2610) which are also products of the chromosomal *virB* locus (Fig. 1 and 2A). The VirD4-like proteins of *H. pylori* and the R388, RP4, and F plasmids all have been shown to bind DNA and/or ATP (6, 23, 25). In *A. tumefaciens*, VirD4 has been shown to interact with VirB10, a protein predicted to be localized to the inner membrane (20, 31) with the C-terminal region of the exported virulence protein VirE2 (44). We did not detect interactions between *X. axonopodis* pv. citri VirD4 and VirB10 and did not detect extensive sequence similarity between *A. tumefaciens* VirE2 and the *X. axonopodis* pv. citri XVIPs or XVIPCDs. We note that none of the protein substrates of the T4SSs of *H. pylori*, *L. pneumophila*, or *B. pertussis* (see above) possess significant sequence similarity with VirE2.

(ii) Interactions between XAC2622 and VirB9 may mimic VirB7-VirB9 interactions observed in other T4SSs. VirB9 is one of the principal periplasmic components of the T4SS transport pore, probably associated with the bacterial outer membrane (9, 13). In *A. tumefaciens*, interactions between VirB9 and subunits VirB7, VirB8, and VirB10 have been detected by a number of techniques, including two-hybrid assays (2, 17, 53). VirB7 proteins from a variety of sources are small lipoproteins (only 55 residues in *A. tumefaciens*) with high sequence variability and are considered essential for T4SS function (2, 16). No *virB7* gene was found beside *virB8* in the *X. axonopodis* pv. citri chromosome, its usual location in the *virB* loci of other bacteria (6, 13). In this position is a gene encoding a small (139-residue) hypothetical protein (XAC2622) whose only ortholog is found in the equivalent position within the *X. campestris* pv. campestris genome (18).

When we used XAC2622 as bait in two-hybrid assays against the genomic DNA library, six of seven preys were derived from VirB9 (Table 2). The smallest VirB9 fragment observed to mediate this interaction corresponds to the C-terminal half of the protein (residues 130 to 255, Table 2). As VirB7-VirB9 interactions have been observed before (2, 4, 27), the interaction between XAC2622 and VirB9 detected here suggests that XAC2622 may fulfill the same role in *X. axonopodis* pv. citri

A



B

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XAC0466 (488) HPDHA------LHNAIRSKLP----------SMISNETAAHVTL-QAKQN---------------
XAC3266 (735) HPDHS------TYQQIHSWVR----GTGNWNVEESKNVTASLYKQQTEDP--------------
XAC2609 (315) HPDNA------MYNGAVSKLEALGERGGFANRKELEQAAGQIVF-ESKVS---------------
XAC0096 (506) HPDHR------MLEQIRSGVRKIDESVGKPYDDMSERVSRSLLA-VCKDNREAYPAAGDRSLADN
XAC1918 (477) LSQDP------LHSQAEDAVRRLEQGLGREYDDNSARLAASSAY-LAKEN---------------
XAC0574 (317) HVGNG------LFQDAQRGVHAQDARAGRTPDQHSAQLSGSLAS-EMHAA---------------
XAC3634 (189) NPDHP------LFNQALNKLEQLGPN-AFANRQQLVNAAGHMTF-GAKVS---------------
XAC0151 (120) HPDHA------MHQQIRSKVEQLDAANGRTFDATSERMTASLLT-LAKDN---------------
XAC1165 (  1) ------------------------MGRASDAHSERMSASLAH-LAKEH---------------
XAC0323 (191) GPDHPEHPDHYLFAQIREAVGVLDAELGKPVDEASERMAARLLP-LAKQH---------------
XAC2885 (271) ARGNALQPGSQLFNDAVVKLDGQRDRLGLRDDTAFLNTAASVAA-RAGND---------------
XAC4264 (165) PPERAEHPNNELLEKIRSGVRGLDQQAGKSWDESSDRLSASLLL-MATEK---------------
Cons.         HPDH       L  Q    V       G   D  S    ASL     K
              6876       7  6    7       *   8  9    887     8

XAC0466 GIDSADKLQNVT---------VQDGKAFVMGTT---PG--FRAAVDLNQPAPT-LEQTSAQL-LAGQS
XAC3266 LLQRVDKVT-------GGLGKDGAENVFAVYAPFGDKGPFFHAHVDGREASQEPAQQNLQQAEVIKQD
XAC2609 GLQRIDHVVPNK----------SGDGFFAVQGELTDPA-MQRVFVDRNQAQNQPLENSSRQAAEESQR
XAC0096 ALSRVDHVVMGK-----------TGNMFAVEGRLDDPA-HRRVHVEIDQAIRTPVEQSDQKLLAANQA
XAC1918 GLSRIDHVVLSE----NTKSVRQGENVFVVEGALNDPA-HKMAHMKTSDAIAQPVEQSLAQLQALGET
XAC0574 GGRRIDAVMMSP----------DAARTFAVQGRVDDPA-QLRVSVDTMTAMNTPLEQSSQRVAENAAR
XAC3634 GMQRIDMVAQSK----------DGNGLFAVQGQPTDPA-HQRIYTEKATAAERPLEQSSNAVRQDAQT
XAC0151 GLTRVDHVLLSE----KTRDSPAAQTLFVVQGDPKDPA-MLRAHMPTADAAQRPVQESFTQLESVNQR
XAC1165 GLERIDHVMLSN----QTPRAAAAATVFVVQGEPSNPA-HLRASMPTDVAVTTPVEQSLAKLQELDAR
XAC0323 GFDQVDYVVLSRHLGE------VGENVFLVRGELSDPA-HLRAHITTQEAMETSVDASLAQLDEINRR
XAC2885 GLQRIDHLVPNRD----------GDSLIAVQGRMDDPA-HLRSHVQTASAANEPAQTNVSQLQQHNQQ
XAC4264 GFTARDDLKFAFNTPTEKLAGGEILHMWREGHHSPDPAAH-RAHMTTQEALAVPADQRLAQMEVMQQT
Cons.   GL RIDHV  S                FAVQG   DPA H RAHV T  A   P EQS  QL    Q
        *7 96*69  6                *6*69   *** 7 *776 7  *   * 788  87    8

XAC0466 -QQQQAQQEQQKVAMGGR                  (24)
XAC3266 -QTRQQALEETQQQTARQDQARPSRSL         (28)
XAC2609 -QAIQVQTQESASRSMSM                  (29)
XAC0096 -IAQERALTQQQEVARGMNEPNQGSLSR        (38)
XAC1918 -QRQQQSQQQEQQREQLIAPQHRMV           (39)
XAC0574 -QSAVLEQQQSQTQQQQQGARALG            (34)
XAC3634 -QTQVQDQHEQQRSQTRSVG                (32)
XAC0151 -LAQERTQDLAVEQQRSQEQQHRGPVPSR       (40)
XAC1165 TLAQAQSLAQERSMAQGEAVKPRSIG          (33)
XAC0323 -LMLRLPRR                           (31)
XAC2885 -HTQQPPQQQQEQRRVIQQ                 (31)
XAC4264 -KAQEIMQAQQQETMQTQSTQARSM           (28)
Cons.    Q Q   Q Q Q                        (49)
         6 8   7 7 6
```

FIG. 2. Interactions involving VirD4 and XVIPCDs. (A) Schematic representation of the extended web of interactions involving VirD4. Arrows show interactions observed in two-hybrid assays. Black arrows indicate interactions between components whose genes are in the chromosomal *virB* locus. (B) Sequence alignment of the XVIPCDs from each protein found to interact with VirD4. Residues indicated in bold are those encountered in at least 6 of 12 XVIPCDs. These conserved residues are indicated below the alignment (Cons.). Numbers below the alignment indicate the total number of XVIPCDs that present the conserved residue at that position (*, highly conserved residues observed in 10 or more XVIPCDs). Following the sequence of each XVIPCD, in parentheses, is the total number of residues in that XVIPCD that match the consensus sequence. The number of the first residue in each XVIPCD is indicated in parentheses at the beginning of each sequence. In addition to the above 12 proteins, the *X. axonopodis* pv. citri genome codes for two more proteins with XVIPCDs: XAC1062 and XAC3404. The closely related *X. campestris* pv. campestris genome (18) codes for 16 proteins with XVIPCDs: XCC0065, -0066, -0068, -0309, -0573, -1056, -1115, -1138, -1898, -1902, -2722, -3145, -3567, -3623, -3802, and -4193.

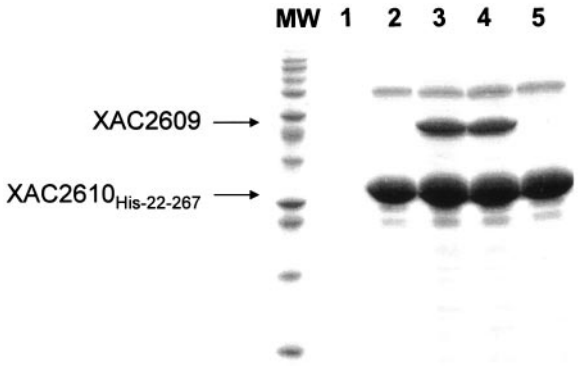

FIG. 3. Pulldown assays for the XAC2609-XAC2610 interaction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples bound to the Ni^{2+}-chelating Sepharose resin in the presence of 50 mM imidazole and released with 500 mM imidazole (see Materials and Methods) is shown. Resin was initially loaded with the following samples in the presence of 5 mM imidazole: 10 μM XAC2609 and the soluble fraction of lysate from a 10-ml culture (A_{600} = 0.8) of *E. coli* BL21(DE3) (lane 1), 100 μM $XAC2610_{His\text{-}22\text{-}267}$ (lane 2), 100 μM $XAC2610_{His\text{-}22\text{-}267}$, 10 μM XAC2609, and *E. coli* lysate (lane 3), 100 μM $XAC2610_{His\text{-}22\text{-}267}$ and 10 μM XAC2609 (lane 4), and 100 μM $XAC2610_{His\text{-}22\text{-}267}$ and *E. coli* lysate (lane 5). MW, molecular weight markers. Arrows indicate the proteins $XAC2610_{His\text{-}22\text{-}267}$ (29 kDa) and XAC2609 (47 kDa).

(and *X. campestris*) as VirB7 in other T4SSs. This hypothesis is supported by analysis of the XAC2622 primary sequence, using the Psort program (35), which predicts its localization to the outer membrane due to the presence of a CATK sequence (residues 21 to 24) in which the conserved Cys residue is modified in lipoproteins. We note that the *X. axonopodis* pv. citri genome (18) codes for LolA, LolB, LolC, LolD, and LolE orthologs, which comprise a system that regulates the sorting of lipoproteins in a manner that depends on the sequence surrounding the modified Cys residue (49).

(iii) The chromosomal *virB* locus seems to code for a complete T4SS. The *virB5* gene in the chromosomal locus was not identified in the initial annotation of the *X. axonopodis* pv. citri genome (18), and some well-characterized T4SSs function without VirB5 orthologs (14). However, *virB5* genes are normally encountered between *virB4* and *virB6* in *loci* coding for T4SSs (13). In this position in the chromosomal *virB* locus is an open reading frame, XAC2613 (Fig. 1), whose product has some similarity (40%) but very low identity (15%) with the VirB5 protein coded by the pXAC64 *virB* locus (Table 1). An alignment between these two proteins is shown in Fig. 4A. Little is known about VirB5 except that it may be incorporated as a minor pilus subunit (40). If this is the case, *Xanthomonas* VirB5 may be expected to be at the front lines of a host-pathogen "arms race" characterized by accelerated evolution of proteins at extracellular interspecies contact interfaces. Similar ideas have been put forward to explain striking differences in sequence conservation between the five paralogs of TrwJ, TrwI, and TrwH (orthologs of VirB5, VirB6, and VirB7, respectively) produced by tandem gene duplication in *B. tribocorum* (43).

The above results suggest that XAC2622 and XAC2613 may correspond to VirB7 and VirB5 proteins in other T4SSs. Therefore, the chromosomal *virB* locus may code for all the components of a functional T4SS. The role of the chromosome-encoded T4SS in *X. axonopodis* pv. citri pathogenesis remains to be elucidated. The *X. axonopodis* pv. citri chromosomal T4SS locus lacks genes coding for the DNA-processing components normally found in T4SSs associated with bacterial conjugation. This points to the possibility of unidentified protein substrates for this system. A hypothesis that awaits more rigorous testing is that a set of substrates may in fact be the VirD4-interacting proteins (XVIPs).

Interactions involving proteins encoded by the plasmid *virB* locus. Similarity searches against the public sequence databases indicate that most members of the *X. axonopodis* pv. citri plasmid *virB* cluster are most closely related to and ordered in the same manner as the corresponding mating pair formation (*mpfA* to *mpfJ*) genes from the IncP-9 TOL plasmid pWW0 of *P. putida* (21) (Table 1). *mpf* genes code for the T4SS (mating channel) required for bacterial conjugation/plasmid mobilization. The proteins of these two conjugation systems are also very similar to those encountered in the 37-kbp pXcB plasmid from *X. citri* (gi:38639487; Q. Yuan, A. M. Brunings, B. El-Yacoubi, S. Shanker, and D. Gabriel, unpublished results; 7). Adjacent to the *virB* locus in pXAC64 are 12 genes for proteins XACb0024 to XACb0035, of which 8 (XACb0026 to XACb0029 and XACb0032 to XACb0035) have no orthologs in pWW0 or pXcB (Table 1), while the protein products of the remaining 4 genes (XACb0024, XACb0025, XACb0030, and XACb0031) are similar to the pWW0-encoded proteins TraD, TraA, TraB, and TraC, respectively (Table 1 and see below).

(i) Interactions between XACb0042 and VirB6. The original annotation of the pXAC64 *virB* cluster did not identify any genes coding for VirB7 orthologs (18). However, between genes *virB6* and *virB5* in pXAC64 there are two open reading frames (XACb0042, 131 amino acids; and XACb0043, 82 amino acids), whose positions make them candidates for coding VirB7 orthologs. The only orthologs of these two proteins in the public databases are hypothetical proteins encoded by orf199 and orf200, respectively, within the *mpfA* to *mpfJ* cluster of the pWW0 plasmid of *P. putida* (21; Table 1) and two proteins encoded by the plasmid pXcB of *X. citri* (7). While the functions of these proteins in *Xanthomonas* or *P. putida* are not yet known, limited sequence similarities have led to the suggestion that XACb0043 and its orthologs may be VirB7 proteins (7, 21; see below). Since VirB7 has been shown to interact with VirB6, VirB7, VirB8, and VirB9 (2, 27, 53), we decided to identify protein-protein interactions involving XACb0042 and XACb0043 to determine whether one or both exhibit behavior similar to that observed for VirB7 in other systems.

When XACb0042 (residues 33 to 131) was used as bait in the two-hybrid assay, 100% of the preys encountered (13 of 13) were found to be derived from the *virB6* gene (XACb0041), located immediately upstream from the XACb0042 gene (Table 2, Fig. 1). The smallest VirB6 fragment started at residue 8, suggesting that the N terminus of this 288-residue protein is required for interaction with the XACb0042 protein. The plasmid-encoded VirB6 protein is 44% identical to MpfE of pWW0 (Table 1) and shows much less similarity (20 to 25%) to TrwI of *E. coli*, TraH of plasmids pIPO2T and pSB102, and VirB6 from *A. tumefaciens* and *Legionella*. VirB6 proteins are

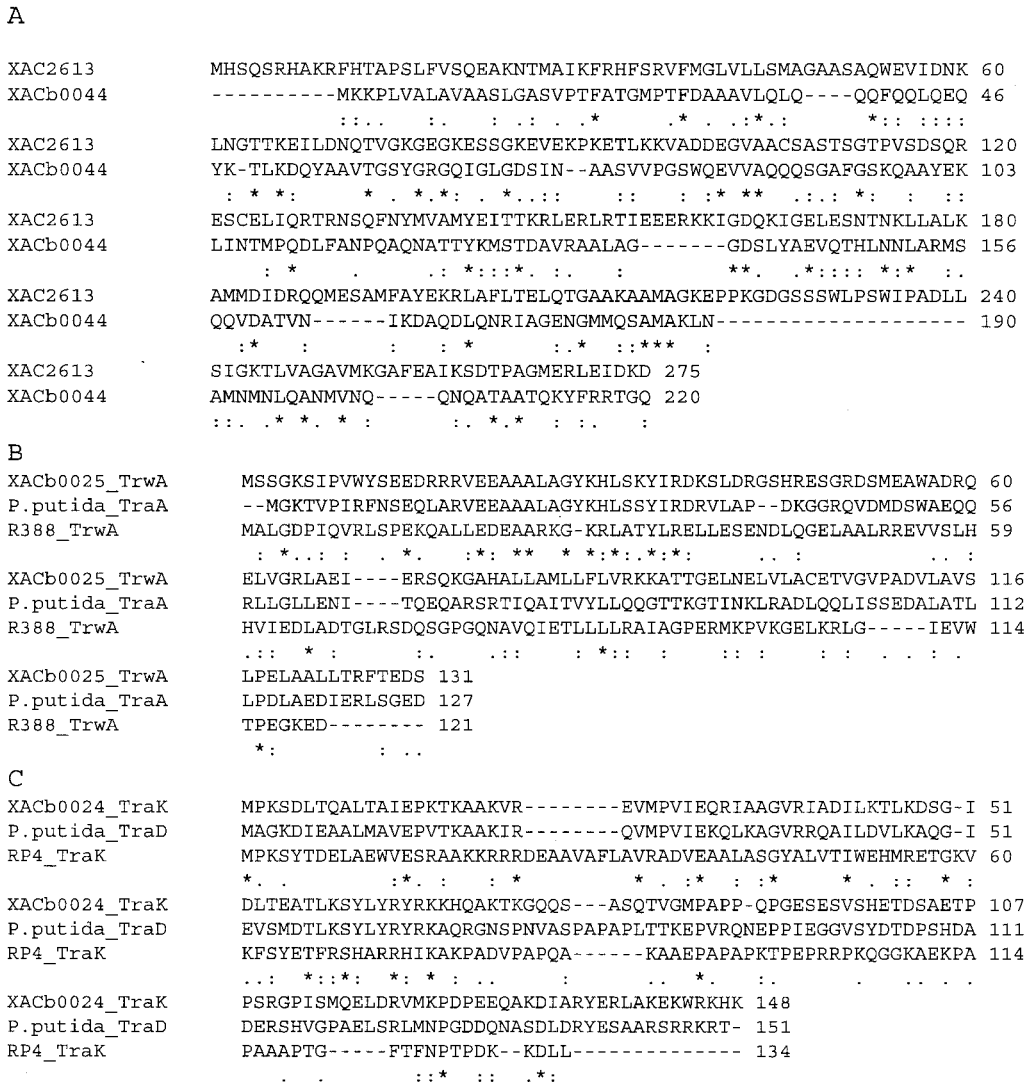

FIG. 4. (A) Alignment of VirB5 orthologs in *X. axonopodis* pv. citri. XAC2613 and XACb0044 are VirB5 orthologs encoded by the chromosomal and pXAC64 *virB loci*, respectively. (B) Alignment of *X. axonopodis* pv. citri TrwA (XACb0025) with its orthologs TrwA from the broad-host-range plasmid R388 and TraA from the pWW0 plasmid of *P. putida*. (C) Alignment of *X. axonopodis* pv. citri TraK (XACb0024) with its orthologs TraK from the broad-host-range plasmid RP4 and TraD from the pWW0 plasmid of *P. putida*. Alignments were obtained with the ClustalW program (24). Identical (*), highly conserved (:), and conserved (.) residues are indicated.

predicted to possess several transmembrane helices (54) and may form part of the type IV channel at the cytoplasmic membrane and/or may mediate formation of VirB7 and VirB9 polymeric complexes required for biogenesis of the secretion channel and pilus (14, 27). Our results suggest that the association of VirB6 with the product of the XACb0042 gene may be mimicking that previously observed between VirB6 and VirB7 (27). We note that XACb0042 does not possess any detectable similarity with VirB7, nor does it possess a lipobox consensus sequence found in other VirB7 proteins (7). However, VirB7 proteins from different species show very little sequence similarity and XACb0042 does possess two cysteine residues (at positions 76 and 94), similar to that in other VirB7 proteins.

(ii) Interactions between XACb0043 and the product of a previously unidentified gene adjacent to the pXAC64 *virB* locus. The XACb0043 gene codes for a hypothetical protein whose only orthologs are the product of orf200 in pWW0 (21) and VirB7, encoded by the *X. citri* pv. aurantifolii plasmid pXcB (7). The proposal that XACb0043 and its orthologs may in fact code for VirB7 homologues (7, 21) is supported by the position of the gene in the cluster as well as the presence of a putative lipobox within XACb0043 (7).

When XACb0043 was employed as bait in the two-hybrid assay, no interactions were observed with known VirB7-interacting proteins (VirB6, VirB8, VirB9, and VirB10). Instead, 8 of the 14 preys were derived from plasmid DNA sequences whose open reading frame overlaps but does not match that of the XA*C*b0021 gene, adjacent to the DNA processing section of the plasmid *virB* cluster (Fig. 1). The product of this new pXAC64 open reading frame (Fig. 5), which we name

```
                                                    tcaagtgatgaggggacgtc
ATGGGGAGTAACGTATCGACGGCCCGCGAAGCGCTCATGGCCGAGCTGCTGCAAGATGCC  60
M  G  S  N  V  S  T  A  R  E  A  L  M  A  E  L  L  Q  D  A    20
GACGCCCTCGTACGTCGCTTCGAGCAGGCAGACGAGGCACTGACAGGAAAGATCGAGAAG 120
D  A  L  V  R  R  F  E  Q  A  D  E  A  L  T  G  K  I  E  K   40
GCGACAACGGACGCGGCCGGAAAGGCGTTCCTGGCCGCCAAGCTCAATTTCGAGTCGGTC 180
A  T  T  D  A  A  G  K  A  F  L  A  A  K  L  N  F  E  S  V   60
ATCGACAAGAACGCCGAGAAGCTCACTGAAGCTGGCCGACATGCAGCCGCGCAGATCGGA 240
I  D  K  N  A  E  K  L  T  E  A  G  R  H  A  A  A  Q  I  G   80
AATCAGCTCAATAGCGGCGCGGCGCAGGTCGTCGCCGCGAATGCGGCGTTTGAGAGCAAG 300
N  Q  L  N  S  G  A  A  Q  V  V  A  A  N  A  A  F  E  S  K  100
GCGCGGCGGTTCGTGCTGCTGCTGGCTGGGTTCGCGTTTGTCGCCGGCGTGGTGGGCGGC 360
A  R  R  F  V  L  L  L  A  G  F  A  F  V  A  G  V  V  G  G  120
TTCGTTGGCGCCAAGCTGGCCGGGATGTAA                              390
F  V  G  A  K  L  A  G  M                                   129
```

FIG. 5. Nucleotide and translated protein sequences of the XACb0021b gene, adjacent to the pXAC64 *virB* locus. The smallest fragment found in this study to interact with XACb0043 is underlined. Twenty nucleotides upstream of the initiation codon are indicated in lowercase letters. A putative purine-rich ribosome binding site 5 to 11 nucleotides upstream of the initiation codon is indicated in lowercase italics. The initiation and termination codons are indicated in bold. Identical DNA sequences are found in both *X. axonopodis* pv. citri plasmids: positions 22841 to 22452 of pXAC64 and positions 8086 to 8475 of pXAC33 (18).

XACb0021b, is 129 residues long, and residues 34 to 129 were observed in all fragments that interacted with XACb0043 (Table 2). XACb0021b has sequence similarity to the product of orf176 of pWW0 (Table 1), and both genes are located in equivalent positions relative the *tra* and *mpf* loci of their respective plasmids (21). Thus, it seems that XACb0042 and XACb0043 are both candidates for carrying out functions attributed to VirB7 in other T4SSs: XACb0042 interacts with a protein (VirB6) previously shown to interact with VirB7, while XACb0043 has the highest sequence similarity to VirB7 and interacts with an uncharacterized protein encoded by the *trw* or *virB* locus. The precise functions of these two proteins remain to be elucidated.

(iii) Interactions between VirB1 and VirB9. When we used full-length VirB1 as a bait in two-hybrid assays, all six preys were derived from the pXAC64-encoded VirB9 protein, the smallest of which initiated at residue 32 (Table 2). This result is consistent with studies on *Agrobacterium* VirB1 which identified interactions with several other VirB proteins including VirB9 (5, 53). *Agrobacterium* VirB1 has been localized to two cellular compartments (5): first, the full-length protein is found in the periplasm due to a putative N-terminal signal sequence; second, further processing produces a C-terminal fragment (VirB1*, beginning at residue 173) that is transported to outside the cell, where a significant fraction remains loosely associated with the T4SS via interactions with VirB9 (5, 53). The N-terminus of VirB1 has similarity with lytic transglycosidases (5), and the VirB1 proteins coded by the two *X. axonopodis* pv. citri *vir* loci share a high degree of sequence identity in this region (55% identity for the region corresponding to residues 40 to 185 of *X. axonopodis* pv. citri VirB1) as well as >50% identities with the N-terminal domains of VirB1 orthologs from other sources, including pSB102, pIPO2T, pWW0, *Brucella melitensis*, and *Burkholderia cepacia*. While the C-terminal domain of the chromosome-encoded VirB1 has low but significant sequence identity with the C-terminal domain of *Agrobacterium* pTi-encoded VirB1, the C-terminal domain of pXAC64-encoded VirB1 is extremely rich in alanine (25%), proline (12%), valine (13%), and glutamine (11%) residues and shows no significant similarity with the chromosome-encoded VirB1 nor with any other VirB1 proteins except that encoded by plasmid pXcB from *X. citri* (gi:38639487).

(iv) Interactions involving the hypothetical proteins XACb0032, XACb0033, and XACb0035. Between the *trwC* and *virB1* genes in pXAC64, there are four genes which code for hypothetical proteins (XACb0032 to XACb0035, Fig. 1). These proteins have no orthologs in the set of pWW0-encoded products. XACb0032, a 106-residue protein has orthologs coded by the genomes of *Azotobacter vinelandi*i, *Chlorobium tepidum*, *Sinorhizobium meliloti*, *B. melitensis*, *Rickettsia conorii*, *Nitrosomonas europaea*, *Rhodospirillum rubrum*, and *X. fastidiosa*. XACb0033 (a 72-residue protein) has orthologs found only in *Nitrosomonas*, *Rhodospirillum*, and *Xylella*. XACb0034 and XACb0035 have no known orthologs.

The use of XACb0032 as bait in the two-hybrid assays gave rise to 11 positive preys, 10 of which mapped to the XACb0033 protein. The minimal domain required for XACb0032 interaction corresponds to residues 18 to 72 of XACb0033 (Table 2). When the 139-residue XACb0035 was used as bait in the two-hybrid assays, all 27 preys were found to be derived from the actual XACb0035 protein (Table 2). Of these, 11 corresponded to the full-length protein. Sequencing analysis of these pOAD full-length prey clones showed that they were derived from homologous recombination events of the pOBD full-length bait vector with a library-derived vector (1). The remaining 16 preys, derived from library clones, correspond to fragments of the XACb0035 protein and demonstrate that the minimal domain required for these interactions is contained between residues 4 and 139 (Table 2).

(v) Homotropic interactions involving XACb0025, a TrwA ortholog. In the conjugative process, Trw or Tra (transfer) proteins are involved in the processing of the T-strand substrate (*oriT* site cleavage, strand separation, and single-strand stabilization) and its preparation for transfer to the mating channel (37). XACb0025 is a 131-residue ortholog of the TraA and TrwA proteins from the *P. putida* plasmid pWW0 and the *E. coli* broad-host-range plasmid R388, respectively (Fig. 4C). R388 TrwA binds to *oriT*, and this binding results in the repression of transcription of the R388 *trwABC* operon (33). TrwA also locally unwinds the DNA, thereby facilitating the nickase activity of TrwC (33). XACb0024 is an ortholog of the TraD and TraK proteins from pWW0 and RP4, respectively (Fig. 4B). TraK binds to *oriT* and facilitates relaxosome formation (57). Due to these sequence similarities, we therefore name the products of the XACb0024 and XACb0025 genes TraK and TrwA, respectively (Table 1; Fig. 1).

We have identified a putative *oriT* site (5′ GGTGCGTGA TGTGTATTGA 3′) between residues 24202 and 24182 of pXAC64, based on its similarity (19 of 20 bases) with the *oriT* site of pWW0 (21). This *oriT* site, flanked on both sides by characteristic inverted repeats, is located between the XACb0024 and XACb0025 genes (Fig. 1). Another equivalent *oriT* site is found on a second *X. axonopodis* pv. citri plasmid, pXAC33 (18), between residues 6725 and 6745. This *oriT* site, as well as a second set of open reading frames coding for TraK and TrwA orthologs, is part of a completely conserved sequence in both pXAC33 (nucleotide positions 5469 to 14059) and pXAC64 (positions 25458 to 16868). These observations suggest that even though pXAC33 does not possess its own

T4SS, the T4SS encoded by pXAC64 may function as a conjugative transfer system for both plasmids. Mobilization of these plasmids may be particularly important for *X. axonopodis* pv. citri virulence as pXAC33 and pXAC64 each contain two *pthA* genes which code for AvrBs3 family avirulence proteins, substrates of the *Xanthomonas* T3SS system (18).

When using *X. axonopodis* pv. *citri* XACb0025/TrwA as a bait against the genomic bait library, all nine preys sequenced were derived from the XACb0025/TrwA protein, five from recombination events. The smallest XACb0025/TrwA fragment encoded by the preys initiated at residue 2, suggesting that the N-terminal domain is required for protein-protein contacts. This result is consistent with the tetrameric form of TrwA observed by Moncalian et al. (33).

Concluding remarks. In this report, we present evidence for specific protein-protein interactions involving several components of the two *Xanthomonas* T4SSs, encoded by the chromosomal and plasmid *virB* loci. Many of these proteins have not been functionally characterized before, and their interactions are of great interest. The assays were performed using specific T4SS components as baits and a prey library derived from whole *Xanthomonas* genomic DNA. Therefore, any interactions observed between two T4SS components are highly unlikely to have occurred by chance (i.e., the genes encoded by each *virB* locus represent less than 0.5% of the *Xanthomonas* genome). We have obtained similar, internally consistent results among components of the T3SS whose components are encoded by the *X. axonopodis* pv. citri *hrp* locus (1).

We did not observe any interactions between a bait derived from one T4SS with a prey derived from another T4SS. The present evidence then seems to point to distinct and independent functions for the two *X. axonopodis* pv. citri T4SSs: classical conjugative functions for the plasmid-encoded T4SS and as yet unknown functions for the chromosome-encoded T4SS. This hypothesis is supported by the observation that the plasmid *virB* locus is flanked by genes coding for TrwA, TrwB, TrwC, and TraK DNA processing proteins as well as an *oriT* site (Fig. 1). The chromosomal *virB* locus, on the other hand, does not contain nor is it flanked by any *trw* or *tra* orthologs.

The observed protein-protein interactions could be divided into three categories. First, we detected interactions observed previously for components of other T4SSs: TrwA-TrwA, VirB7(XAC2622)-VirB9, VirB1-VirB9, and VirB11-VirB11. The second category is made up of interactions between orthologs of known T4SS components and previously uncharacterized proteins encoded by the same locus. For the plasmid-encoded T4SS, we observed VirB6-XACb0042 and XACb0043-XACb0021b interactions. Here, the identity of the true VirB7 ortholog (XACb0042 or XACb0043) is not clear at the moment. In the chromosomal T4SS, we observed interactions between XAC2609 and VirD4 and between XAC2610 and VirB11. These interactions can in turn be seen to be part of a larger web of interactions involving VirD4, XAC2609, XAC2610, and VirB11 from the same locus as well as other XVIPs encoded by genes from other chromosomal locations (Fig. 2A). In the third category, we find interactions between uncharacterized and in some cases novel proteins encoded by the same locus: XAC2609-XAC2610, XACb0032-XACb0033, and XACb0035-XACb0035. The interactions in the second and third categories are of clear biological interest. They point to gaps in our knowledge regarding the molecular functioning of T4SSs involved in plasmid mobility or in alternative secretion functions.

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