Characterization of AvrBs3-Like Effectors from a *Brassicaceae* Pathogen Reveals Virulence and Avirulence Activities and a Protein with a Novel Repeat Architecture

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Xanthomonas campestris pv. *armoraciae* strain 5 is a *Brassicaceae* pathogen that expresses three members of the highly related *avrBs3* gene family of type III effectors. Here, we report on the isolation and characterization of these genes, designated *hax2*, *hax3*, and *hax4* (homolog of *avrBs3* in *Xanthomonas*). All three Hax proteins are translocated from *Xanthomonas* spp. into the plant cell via the type III secretion system. Hax3 and Hax4 show the typical structure of AvrBs3-like effectors and contain a repetitive region in their central part consisting of 34-amino-acid (aa) repeats. By contrast, the Hax2 repeat region is composed of 35-aa repeats that are characterized by an additional proline residue. Hax2, Hax3, and Hax4 contain 21.5, 11.5, and 14.5 repeats, respectively. Genetic studies revealed an additive effect of *hax2*, *hax3*, and *hax4* on disease symptom formation of *X. campestris* pv. *armoraciae* strain 5 on radish. The contribution of individual genes to the aggressiveness of strain 5 is quantitatively different, with *hax2* showing the strongest effect on the development of chlorosis and necrosis. In addition, *hax3* and *hax4*, but not *hax2*, have a *Bs4*-dependent avirulence activity in tomato and in transgenic *Nicotiana benthamiana* expressing the *Bs4* resistance gene.

Additional keywords: AvrBs4, *Bs3*, *Lycopersicon esculentum*, *Raphanus sativus*.

Most effector proteins of phytopathogenic bacteria are secreted by a conserved type III secretion (TTS) system (i.e., without proteolytic processing) and are translocated into the plant cell (Büttner and Bonas 2002; Cornelis and Van Gijsegem 2000; Tampakaki et al. 2004). The TTS system, encoded by the *hrp* gene cluster and a key component of bacterial pathogenicity, is present in most gram-negative plant-pathogenic bacteria. Hrp-secreted proteins contribute to virulence and fitness of the pathogen (i.e., disease symptom formation, bacterial multiplication in the plant tissue, and pathogen persistence) (Alfano and Collmer 2004; Büttner and Bonas 2003; Chang et al. 2004). So far, a virulence function was demonstrated only for a few effectors, strongly suggesting an additive effect of the injected effector proteins. In fact, as many as 40 or more type III

effectors are expressed by a given pathogenic strain (Cunnac et al. 2004; Schechter et al. 2004).

Many type III effectors were identified by their avirulence activity. These avirulence (Avr) proteins mediate specific recognition of the bacterium by a plant that carries the matching resistance gene (“gene-for-gene hypothesis”) (Flor 1971; Keen 1990). Specific recognition of an effector protein induces plant defense reactions that generally result in the induction of a rapid localized cell death, the hypersensitive response (HR), associated with an arrest of pathogen ingress (Greenberg and Yao 2004; Klement 1982).

A well-analyzed class of Avr proteins is the AvrBs3 family that shares a high degree of sequence homology and is, with one exception (Brg11 from *Ralstonia solanacearum*) (Cunnac et al. 2004), exclusively found in *Xanthomonas* spp. (Lahaye and Bonas 2001). Interestingly, some members of the AvrBs3 family not only display an avirulence activity but also contribute to the virulence of the bacteria. For instance, strong virulence effects were found for PthA from *X. axonopodis* pv. *citri* that is essential for the elicitation of citrus canker (Swarup et al. 1991), AvrB6 from *X. campestris* pv. *malvacearum*, which increases watersoaking of cotton leaves and release of bacteria to the leaf surface (Yang et al. 1996), and AvrXa7 from *X. oryzae* pv. *oryzae* that contributes to pathogen aggressiveness in rice (Bai et al. 2000). The type member of the protein family, AvrBs3 from the pepper and tomato pathogen *X. campestris* pv. *vesicatoria* (Bonas et al. 1989), elicits the HR in resistant pepper plants and hypertrophy symptoms in susceptible pepper and tomato plants as well as in other solanaceous species (Marois et al. 2002). In some cases, a particular *Xanthomonas* sp. strain expresses 10 or more AvrBs3 family members that differ in their activities or may work additively (Bai et al. 2000; Yang and White 2004; Yang et al. 1996).

All members of the AvrBs3 family share a common structure. The most striking feature is the central part of the proteins that consists of 5.5 (AvrXa5) to 28.5 (PthXo3) tandem repeats of typically 34 amino acids (aa) (accession AAQ79773) (Lahaye and Bonas 2001; Yang and White 2004). The repeat sequences are almost identical; however, there is some variation, notably at amino acid positions 12 and 13 of a given repeat (White et al. 2000). The number and order and, thus, the sequence of repeats determine both the avirulence and virulence specificity of a particular AvrBs3 family member (Bonas et al. 1993; Herbers et al. 1992; Marois et al. 2002; Yang et al. 2000; Yang et al. 1994; Yang and Gabriel 1995a). Furthermore, the highly conserved C terminus of AvrBs3-like proteins contains functional nuclear localization signals (NLSs) and an acidic activation domain

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Accession numbers for *hax2*, *hax3*, and *hax4* are AY993937, AY993938, and AY993939, respectively.

(AAD) (Szurek et al. 2001; Van den Ackerveken et al. 1996; Yang and Gabriel 1995b; Zhu et al. 1998). Both motifs are essential for activity of most AvrBs3-like proteins studied, and the NLS-dependent import of AvrBs3 into the plant cell nucleus has been shown using immunocytochemistry (Marois et al. 2002; Szurek et al. 2001, 2002; Van den Ackerveken et al. 1996; Yang et al. 2000; Zhu et al. 1998, 1999). These results suggest that AvrBs3-like proteins modulate plant gene transcription. This idea is corroborated by the fact that AvrBs3 dimerizes in the plant cell, as has been found for many transcription factors (Gürlebeck et al. 2005). Furthermore, a number of pepper genes are induced in the presence of AvrBs3 in an AAD-dependent manner (Marois et al. 2002). By contrast, recognition of AvrBs4 from *X. campestris* pv. *vesicatoria* by the tomato resistance gene *Bs4* is independent of both NLSs and AAD (Bonas et al. 1993; Schornack et al. 2004). The virulence activity of AvrBs4 as well as that of many other AvrBs3-like effectors is still enigmatic.

Most plant species that are colonized by xanthomonads are not easily amenable to molecular genetic analyses; therefore, we set out to identify AvrBs3 homologs (homolog of AvrBs3 in *Xanthomonas* [Hax]) in xanthomonads that infect the model plant *Arabidopsis thaliana* and related plant species. Because *X. campestris* pv. *campestris*, the most prevalent pathovar utilized in *Xanthomonas*–*Arabidopsis* studies (Buell 2002), does not contain any *avrBs3*-like genes based on genome analysis (da Silva et al. 2002), we chose *X. campestris* pvs. *armoraciae* and *raphani*, both representing nonsystemic pathogens of *Brassicaceae* (Davis et al. 1991; Parker et al. 1993). Here, we report on the identification and analysis of three new members of the AvrBs3 family from *X. campestris* pv. *armoraciae*. Intriguingly, the *hax* genes contribute to the aggressiveness (i.e., the severity of symptom formation) of the pathogen on *Raphanus sativus*. One Hax protein, Hax2, contains an unusual repeat region consisting of 35-aa repeats. Additionally, we found that Hax3 and Hax4 are specifically recognized in tomato in a *Bs4*-dependent manner and, thus, have avirulence activity.

RESULTS

Isolation of *avrBs3*-like genes from *X. campestris* pv. *armoraciae*.

Our aim was the genetic analysis of *avrBs3*-like genes in *Brassicaceae*-specific xanthomonads to assess their role in the interaction with the host plants. Seven strains of *X. campestris* pvs. *armoraciae* and *raphani* were tested for the presence of *avrBs3* homologs by Southern analysis. Five strains, *X. campestris* pv. *armoraciae* strains 5, 28, 3838, and 7383 and *X. campestris* pv. *raphani* strain 25, were positive (data not shown). *X. campestris* pv. *armoraciae* strain 5, which contains three members of the *avrBs3* family, was chosen for further analysis because it is pathogenic on several *Arabidopsis* ecotypes and radish accessions. In addition, strain 5 can be efficiently transformed by conjugation. The *avrBs3*-related genes were isolated and designated *hax2* to *hax4* (discussed below). Southern hybridization showed that *hax2* is located on a natural plasmid of approximately 35 kb in *X. campestris* pv. *armoraciae* strain 5, whereas *hax3* and *hax4* are located on the chromosome (data not shown).

Sequence analysis revealed that *hax2*, *hax3*, and *hax4* encode proteins with high homology to AvrBs3 and predicted sizes of 140, 102, and 112 kDa, respectively. Indeed, proteins of expected sizes could be detected by Western blot analysis (discussed below). The N-terminal regions up to the first repeat (amino acids 1 to 288) are identical in the three Hax proteins, but they contain two amino acid exchanges compared with AvrBs3 (P17S and G67D). The C-terminal regions (last

278 aa) of Hax2 and Hax3 are less well conserved and harbor 28 and 12 aa exchanges compared with AvrBs3, respectively. In both cases, there are two amino acid differences in the AAD and one in the last NLS (Szurek et al. 2001). The comparison of the Hax2 and Hax3 C termini revealed 16 aa differences. Interestingly, the C terminus of Hax4 is identical to the corresponding region in AvrBs3. The repeat region of *hax2*, *hax3*, and *hax4* consists of 21.5, 11.5, and 14.5 repeats, respectively. For *hax2*, the number of repeats was confirmed by a partial digest with *MscI*, which cuts once in each repeat (Fig. 1A). Strikingly, all repeat units of Hax2 consist of 35 aa, which is in contrast to Hax3 and Hax4 and all other known AvrBs3-like proteins from *Xanthomonas* spp. (Fig. 1B). The additional amino acid in each Hax2 repeat is a proline at position 33. The last amino acid of each repeat is an aspartate or cysteine instead of the glycine that is usually present at this position. There is only one described member of the AvrBs3 family that contains 35-aa repeats, Brg11 from *Ralstonia solanacearum* (Cunnac et al. 2004). Phylogenetic analysis (Fig. 2) showed that Hax2 is closely related to AvrBs3 family members from different *Xanthomonas* spp. (group 1) and more distantly related to Brg11 from *R. solanacearum* (group 2).

hax2, *hax3*, and *hax4* probably were acquired by horizontal gene transfer.

Sequence analysis of regions flanking the *hax* genes revealed interesting features indicative of horizontal gene transfer (Fig. 3). First, imperfect 62-bp inverted repeats (IRs) that typically flank *avrBs3*-like genes but also other effector genes (Noël et al. 2003) are at least partially present at the *hax* loci. The IR upstream of *hax3* and *hax4* (IR-L) contains one base pair difference compared with the *avrBs3* IR-L and is identical to the IR-L upstream of *avrBs4* from *X. campestris* pv. *vesicatoria*, which shares 97% identity with *avrBs3* on the DNA level (Bonas et al. 1993). The IR-L upstream of *hax2* is less well conserved: there are 12 bp exchanges and 8 insertions compared with the *avrBs3* IR-L. The IR downstream of *hax3* and *hax4* (IR-R) is only partially conserved. Downstream of *hax3*, a stretch of 39 bp is conserved, including 1 bp difference compared with the *avrBs3* IR-R, and the IR-R of *hax4* consists of a conserved 22-bp stretch. Downstream of *hax2*, the IR-R is missing. Instead, this region harbors an interrupted open reading frame (ORF) that is partially homologous to the *ISxcl1* transposase gene from *X. campestris* pv. *campestris* (identity more than 90% on protein level) (da Silva et al. 2002). Mobile elements and phage-related genes also were found in the regions flanking *hax3* and *hax4* (Fig. 3). Interestingly, there is a sequence downstream of *hax3* which, on the amino acid level, is highly related to the AvrB family from *Pseudomonas syringae* (46% identity to AvrB over 289 aa) (Lindeberg et al. 2005; Tamaki et al. 1988). However, an insertion sequence (IS) element of the *IS476* type (Chen et al. 1999; Kearney and Staskawicz 1990) has inserted into the 5' region of the gene, removing the putative promoter and translation start codon. Alternative start codons but no obvious promoter or Shine-Dalgarno sequence are present in the sequence of the IS element; therefore, this ORF could be a pseudogene. Together, the sequence analysis of *hax2*, *hax3*, and *hax4* and their flanking regions suggests that the *hax* genes are located in pathogenicity islands that were acquired by horizontal gene transfer (Dobrindt et al. 2004).

Hax2, Hax3, and Hax4 are translocated by the *Xanthomonas* TTS system.

The high homology of Hax2, Hax3, and Hax4 to AvrBs3 strongly suggests that these proteins are effectors that are translocated through the Hrp TTS system. To test this hypothesis,

Fig. 1. The Hax2 repeat region consists of 21.5 repeats of 35 amino acids (aa). **A**, Determination of the repeat number in *hax2* by partial *MscI* cleavage. DNA of pBSH2ps was partially digested with *MscI* and separated on an 0.8% agarose gel. Sizes of molecular weight markers and repeat numbers are indicated on the left and right, respectively. **B**, Amino acid sequence of the Hax2 repeat region in the single letter code. In contrast to most AvrBs3-like proteins, each repeat unit of Hax2 consists of 35 aa. The additional amino acid (P) is marked by a gray box. Compared with the sequence of the first repeat unit, only the differences in the following repeats are indicated.

Fig. 2. Phylogenetic tree of members of the AvrBs3 protein family. The phylogenetic tree was created using the neighbor-joining method. Numbers above the nodes refer to bootstrap scores larger than 60. Grouping was based on Lindeberg and associates (2005). Protein accession numbers are: *Xanthomonas campestris* pv. *vesicatoria* AvrBs3, P41727; AvrBs4, S34809; *X. axonopodis* pv. *citri* (Xac) Apl1, BAA37119; Apl2, BAA37120; Apl3, BAA37121; PthA, AAC43587; PthA1, NP_644708; PthA2, NP_644725; PthA3, NP_644743; PthA4, NP_644793; PthB, NP_942641; *X. oryzae* pv. *oryzae* AvrXa3, AAN01357; AvrXa5, AAQ79773; AvrXa7, AAF98343; AvrXa7-1M, AAT46122; AvrXa7-2M, AAT46127; AvrXa7-3M, AAT46128; AvrXa7-4M, AAT46125; AvrXa10, AAA92974; *X. campestris* pv. *malvacearum* Avrb6, AAB00675; PthN, AAB69865; *X. campestris* pv. *manihotis* (Xcm) PthB, AAD01494; *Ralstonia solanacearum* Brg11, NP_519936.

we took advantage of the fact that the repeat region of AvrBs3 determines the specificity of recognition in pepper plants containing the resistance gene *Bs3* (Bonas et al. 1989; Herbers et al. 1992). Therefore, chimeric genes were constructed by replacing the repeat regions in *hax2*, *hax3*, and *hax4* with the corresponding region of *avrBs3* (Fig. 4A). The resulting hybrid genes were expressed in *X. campestris* pv. *vesicatoria* strain 85-10, an *avrBs3*-lacking *Xanthomonas* strain that is patho-

genic on pepper Early Cal Wonder (ECW)–30R and ECW. All transconjugants elicited a specific HR in the pepper line ECW–30R (*Bs3*) which recognizes AvrBs3, but not in the near-isogenic line ECW (*bs3*) (Fig. 4C; data not shown). The plant reactions to inoculation with *X. campestris* pv. *vesicatoria* strain 85-10 expressing the *hax3-avrBs3* or *hax4-avrBs3* hybrids were similar to the HR elicited by 85-10 expressing *avrBs3*, whereas the strain expressing the *hax2-avrBs3* chi-

Fig. 3. Schematic overview of the *hax* loci in *Xanthomonas campestris* pv. *armoraciae* strain 5. The *hax* genes are indicated by white boxes, black boxes correspond to transposase or phage-related genes, gray boxes to part of an insertion element, the dotted box indicates a putative pseudogene which on amino acid level is homologous to the AvrB-family, and the hatched box represents a gene encoding a conserved hypothetical protein. Arrowheads indicate orientation of transcription. White triangles correspond to the conserved 62-bp inverted repeats (IRs) flanking *avrBs3*. Truncated open reading frames and IRs are indicated by a zigzag border. The zigzag line within an arrow corresponds to a frameshift. Only the outermost restriction sites used for cloning of the *hax* genes are shown.

Fig. 4. Hax2, Hax3, and Hax4 are translocated into the plant cell. **A**, Strategy to generate chimeric genes between *hax2* to *hax4* and *avrBs3*, here exemplified for *hax2* (see text for details). **B**, Western blot analysis of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) 85-10 and the type III secretion system-deficient mutant 85-10ΔA-C expressing the *hax2-avrBs3* (pLRAH2), *hax3-avrBs3* (pLRAH3), and *hax4-avrBs3* (pLRAH4) hybrids, respectively, under control of the respective *hax* gene promoter. Strain 85-10 expressing wild-type *avrBs3* (pL6Δ622) or carrying the empty vector (pLAFR6) was used as control. Approximately 40 μg of total protein extract were separated on an 8% polyacrylamide gel and analyzed by immunoblot using an AvrBs3-specific antibody. **C**, Reactions of pepper Early Cal Wonder–30R (*Bs3*) 3 days after inoculation with bacterial suspensions of 5×10^8 CFU/ml. Strains were identical to those in B. The leaf was bleached with ethanol for better visualization of the hypersensitive response.

mera caused a weaker HR. The latter might be due to the amino acid differences in the C terminus of Hax2. It should be noted that the Hax2-specific amino acid differences are located outside of the NLSs and the AAD, suggesting that additional regions in the protein contribute to its function. When the chimeric constructs were expressed in a TTS system-deficient mutant, no HR was observed. This indicates that translocation of the Hax-AvrBs3 hybrid proteins depends on a functional TTS system (Fig. 4C).

It has been shown previously that NLSs and AAD in the C terminus of AvrBs3 are necessary for its *Bs3*-specific recognition (Szurek et al. 2001; Van den Ackerveken et al. 1996); therefore, our experimental data also show the functionality of both NLS and AAD in the C termini of Hax2, Hax3, and Hax4.

***hax2*, *hax3*, and *hax4* modulate the aggressiveness of *X. campestris* pv. *armoraciae* strain 5 on its host plant *Raphanus sativus*.**

To investigate whether *hax2*, *hax3*, and *hax4* play a role in the *X. campestris* pv. *armoraciae* strain 5 interaction with its host plants, deletion mutants were constructed. For this, fragments of 2.2 kb in *hax2* and 1.4 kb in *hax4*, each encompassing the repeat region, were deleted. In *hax3*, a 1.7-kb internal deletion was generated, removing the repeat region and part of the 3' region of the gene (discussed below). The mutated *hax* alleles were introduced individually into the genome of *X. campestris* pv.

armoraciae strain 5 using the suicide plasmid pOK1 and replacing the wild-type *hax* alleles by double recombination events, creating $\Delta hax2$, $\Delta hax3$, and $\Delta hax4$ mutant strains. Stepwise introduction of different deletion constructs resulted in all possible combinations of double mutants in *X. campestris* pv. *armoraciae* strain 5 ($\Delta hax23$, $\Delta hax24$, $\Delta hax34$) and a triple mutant ($\Delta hax234$). All mutations were confirmed by Southern blot analysis of *Bam*HI-digested DNA (Fig. 5A). Because *Bam*HI cuts each *hax* gene twice outside of the repeat region, the *hax2* and *hax4* deletion derivatives that both lack the repeat region are of the same size. Therefore, the mutants also were verified by Southern blot analysis of genomic DNA digested with *Sal*II, which cuts outside of the *hax* genes (data not shown), and Western blot analysis using an α -AvrBs3 antibody (Fig. 5B). The bacteria were grown in complex medium; therefore, detection of an AvrBs3-homologous protein suggests that the *hax* genes are expressed constitutively. The same is true for *avrBs3* and *avrBs4* (Bonas et al. 1993; Knoop et al. 1991).

To determine whether *hax2* to *hax4* contribute to the virulence of *X. campestris* pv. *armoraciae* strain 5, bacterial growth and symptom development of wild-type and mutant strains were compared in the host plant red radish (*Raphanus sativus* L. var. *sativus*). On radish accession RA40/76, *X. campestris* pv. *armoraciae* strain 5 causes chlorotic lesions 2 to 3 days postinoculation (dpi) that later become necrotic. Because the plants used were not isogenic, there was some variation in the severity of disease symptoms and the timing of symptom development. To get reliable data, chlorosis and necrosis symptoms caused by the *hax* mutants always were compared with the wild-type symptoms observed on the same leaf and at the time point at which greatest differences between all inoculations on this leaf were visible (3 to 10 dpi) (Table 1). The $\Delta hax2$ and $\Delta hax4$ single mutants showed slightly reduced disease symptoms on approximately 50% of the inoculated leaves, whereas the $\Delta hax3$ strain behaved as the wild type. The $\Delta hax23$ and $\Delta hax24$ double mutants were reduced in their aggressiveness on almost all leaves tested, whereas the $\Delta hax34$ mutant behaved similarly to the $\Delta hax2$ and $\Delta hax4$ strains (Fig.

Table 1. Disease symptoms of *hax* mutants and complemented mutants on radish compared with *Xanthomonas campestris* pv. *armoraciae* wild-type strain 5^a

Mutant strain	Reduced symptoms ^b	Complementation studies	
		Restored symptoms ^c	Plasmid used ^d
$\Delta hax2$	10/21	n.d.	...
$\Delta hax3$	3/21	n.d.	...
$\Delta hax4$	10/21	n.d.	...
$\Delta hax23$	23/25	8 (5)/10	pLH23
$\Delta hax24$	23/25	9 (7)/10	pLH24
$\Delta hax34$	16/24	9 (7)/10	pLH34
$\Delta hax234$	37/39	12 (12)/16	pLH23
		11 (9)/16	pLH24
		9 (3)/14	pLH34

^a Symptoms on radish accession RA40/76 were analyzed when leaves showed the clearest differences between wild-type, mutants and complemented strains (3 to 10 days postinoculation). Data are combined results of at least three independent experiments.

^b Number of leaves with reduced symptoms compared with wild-type symptoms on the same leaf, relating to total numbers of leaves inoculated.

^c Number of leaves where *hax* mutant transconjugants carrying the wild-type genes showed complementation compared with wild-type and mutant symptoms on the same leaf, relating to the total number of leaves inoculated. Numbers in brackets correspond to full restoration of wild-type symptoms; n.d. indicates not determined. The triple mutant was complemented by introduction of pLAFR6 constructs containing two *hax* genes.

^d pLAFR6 derivative introduced for complementation, carrying *hax2* and *hax3* (pLH23), *hax2* and *hax4* (pLH24), and *hax3* and *hax4* (pLH34) under control of their own promoters.

Fig. 5. Analysis of *hax2*, *hax3*, and *hax4* in *Xanthomonas campestris* pv. *armoraciae* strain 5 and mutant strains. **A**, Genomic Southern blot of total DNA isolated from strain 5 and *hax* deletion mutants. Numbers in the mutant names correspond to the mutated *hax* gene or genes. Approximately 2 μ g of DNA were digested with *Bam*HI and separated on an 0.8% agarose gel. The blot was hybridized to the internal 3.38-kb *Pst*II/*Sac*I fragment of *avrBs3*. **B**, Western blot of total protein extracts of strain 5 and *hax* deletion mutants. Bacteria were grown in complex medium. Approximately 60 μ g of total protein extract was separated on a 10% polyacrylamide gel and analyzed by immunoblot using an AvrBs3-specific antibody.

6A; Table 1). The $\Delta hax234$ triple mutant showed reduced disease symptoms compared with the wild type in almost all cases. The ability of the double mutants to cause wild-type symptoms could be restored at least partially by introducing pLAFR6 derivatives carrying the genes that were deleted in the respective mutant (Fig. 6A; Table 1). Unfortunately, cloning all three *hax* genes into the same plasmid was unsuccessful. Therefore, the $\Delta hax234$ mutant could be complemented only partially by introduction of plasmids pLH23, pLH24, and pLH34, each containing two different *hax* genes (Fig. 6B; Table 1).

In addition to scoring macroscopic symptoms, the growth of *X. campestris* pv. *armoraciae* strain 5 and the $\Delta hax234$ mutant was determined in radish RA40/76 for a period of 8 days (data not shown). Surprisingly, the triple mutant grew slightly better than the wild type (approximately threefold) at the beginning of the infection (i.e., between 2 and 4 dpi). At later time points, no significant differences were found. In conclusion, *hax2*, *hax3*, and *hax4* modulate the aggressiveness of *X. campestris* pv. *armoraciae* strain 5 on radish to a different extent, but the symptom formation does not correlate with the bacterial growth behavior in the plant.

Hax3 and Hax4 are recognized by the tomato *Bs4* gene.

To test whether one of the *hax* genes isolated in this study has avirulence activity, the *X. campestris* pv. *armoraciae* strain 5 wild-type strains and *hax* mutants were inoculated into plants that are known to recognize members of the AvrBs3 family. For example, AvrBs3 elicits the HR in pepper plants of cultivar ECW-30R (which contains the resistance gene *Bs3*) (Bonas et al. 1989), and AvrBs4 is recognized in tomato (*Lycopersicon esculentum*) cv. MoneyMaker (MM) plants containing the resistance gene *Bs4* (Ballvora et al. 2001). In pepper ECW-30R plants, neither *X. campestris* pv. *armoraciae* strain 5 nor the *hax* mutants induced the HR (data not shown). When strain 5 and its *hax* mutant derivatives were inoculated into tomato cv. MM (*Bs4*), all strains caused a rapid HR 1 dpi (Fig. 7A; data not shown), suggesting that avirulence determi-

nants in *X. campestris* pv. *armoraciae* strain 5 other than Hax proteins are recognized by tomato. Recognition is not dependent on the *Bs4* gene because the HR also was triggered in the tomato line MM^{*bs4*}-BC4 that is homozygous for the *bs4* allele (Fig. 7A) (Ballvora et al. 2001). To test whether any of the Hax proteins has avirulence activity, the plasmids pLH2, pLH3, and pLH4 carrying *hax2*, *hax3*, and *hax4*, respectively, under control of their respective promoters, were conjugated into the tomato-pathogenic *X. campestris* pv. *vesicatoria* 75-3. The transconjugants were inoculated into tomato cv. MM (*Bs4*) and MM^{*bs4*}-BC4 (*bs4*) plants. Strains expressing *hax3* or *hax4* elicited a similar HR in MM as the *avrBs4*-expressing strain (Fig. 7A). By contrast, inoculation of 75-3 expressing *hax2* did not induce the HR. No HR was observed in *bs4* plants, indicating that the recognition of Hax3 and Hax4 is *Bs4*-dependent. In addition, both Hax3 and Hax4, but not Hax2, also were recognized in a *Bs4*-dependent manner when they were transiently expressed in tomato using *Agrobacterium*-mediated transformation (data not shown).

To confirm that the observed HR induced by Hax3 and Hax4 is due to *Bs4*, we also analyzed the interaction in the heterologous *Nicotiana benthamiana* system using *Agrobacterium*-mediated transformation. This is an ideal test system because *N. benthamiana* does not harbor *Bs4* activity, but co-expression of *Bs4* and *avrBs4* triggers a *Bs4*-mediated defense response (Schornack et al. 2004). Co-expression of *Bs4* with *hax3* or *hax4* triggered a defense reaction 8 dpi (Fig. 7B). Agroinfiltration using *Bs4* in combination with *hax2* did not induce a plant response. These data confirm that both Hax3 and Hax4 are recognized by the tomato resistance gene *Bs4* and, thus, represent new avirulence proteins.

DISCUSSION

Here, we report on the isolation and characterization of three new *avrBs3*-like genes, *hax2*, *hax3*, and *hax4*, from the *Brassicaceae*-pathogenic *X. campestris* pv. *armoraciae* strain

Fig. 6. *hax2*, *hax3*, and *hax4* have an additive effect on the aggressiveness of *Xanthomonas campestris* pv. *armoraciae* strain 5 on radish. **A**, Symptoms on radish accession RA40/76 after inoculation of the following strains: *X. campestris* pv. *armoraciae* strain 5 (wt); *hax* double ($\Delta hax23$, $\Delta hax24$, and $\Delta hax34$) and triple ($\Delta hax234$) mutants in strain 5 background; and double mutants complemented with pLAFR6 derivatives carrying *hax2* and *hax3* (pLH23), *hax2* and *hax4* (pLH24), or *hax3* and *hax4* (pLH34), respectively, under control of their own promoters. Inoculation density was 5×10^7 CFU/ml. **B**, Complementation of the $\Delta hax234$ triple mutant. Transconjugants of $\Delta hax234$ carrying pLH23, pLH24, and pLH34 were inoculated. As controls, $\Delta hax234$ carrying the empty vector (pLAFR6) and the *X. campestris* pv. *armoraciae* wild-type strain 5 were used. The photographs were taken 4 days postinoculation.

5. The corresponding proteins were shown to be type III effectors (i.e., they are translocated into the plant cell). The AvrBs3 protein family is one of the largest and best-analyzed classes of bacterial type III effector proteins. Sequences of more than 20 family members were found in the GenBank database by BLASTX search (Altschul et al. 1997), and even more AvrBs3-like proteins have been described for which the se-

quence is not yet known. Common features of these effectors are a central repeat region and the presence of NLSs and AAD, both of which were shown to be required for activity (Marois et al. 2002; Szurek et al. 2001; Van den Ackerveken et al. 1996; Yang et al. 2000; Zhu et al. 1998, 1999).

Based on our phylogenetic analysis to integrate the newly identified Hax proteins into the AvrBs3 family, the family members form two subgroups (Fig. 2). Group 1 comprises all fully sequenced AvrBs3-like proteins from different *Xanthomonas* spp., whereas the Brg11 protein from *Ralstonia solanacearum* represents a separate group (group 2). Hax3 and Hax4 clearly belong to group 1. The same is true for Hax2, although this protein shares the unusual repeat architecture with Brg11. Each of the 21.5 repeats of Hax2 is 35 aa long instead of the typical 34-aa repeats of most other AvrBs3 family members (Lahaye and Bonas 2001). Brg11 from *R. solanacearum* is the only other AvrBs3-like protein besides Hax2 with repeats of 35 aa (Cunnac et al. 2004). In both proteins, the additional amino acid is a proline at position 33 of each repeat. Interestingly, in Hax2, the last amino acid of each repeat is a cysteine or aspartate instead of glycine, which usually is present at this position in AvrBs3-like proteins. In Brg11, this amino acid is highly variable: arginine, valine, alanine, glycine, or aspartate. Moreover, the Brg11 repeats themselves are exceptionally variable in sequence compared with each other as well as compared with the repeats of other AvrBs3-like proteins, including Hax2 (Genin and Boucher 2004). In contrast to Hax2, a contribution of Brg11 to bacterial virulence is not known (Cunnac et al. 2004).

We have identified another AvrBs3-like protein with 35-aa repeats, Hax1 from *X. campestris* pv. *armoraciae* 3838, which is similar in repeat architecture to Hax2 (E. Marois, G. Van den Ackerveken, and U. Bonas, *unpublished data*). It is interesting to note that all three AvrBs3-like proteins with 35-aa repeats were found in *Brassicaceae*-pathogenic bacteria. It remains to be seen whether there is a correlation between repeat architecture of AvrBs3-like effectors and host range of the bacteria.

The 105-bp repeats of *hax2* and the typical 102-bp repeats of other *avrBs3* family members are highly homologous and probably have evolved from each other by insertion or deletion of one codon, respectively. The new sequence subsequently may have been amplified by tandem duplication and spread by recombination events. Intragenic and intergenic recombinations have been suggested to be common mechanisms for the rearrangement of the repeat region in *avrBs3*-like genes (De Feyter et al. 1993; Yang and Gabriel 1995a). First emergence of the 102- or 105-bp repeat in a gene that already contained several repeat sequences would result in "mixed" repeat regions. Indeed, *avrXa7* and *pthN* contain three and one, respectively, unusual 99-bp repeats among their normal 102-bp repeats (Yang et al. 2000). Interestingly, *hax2* and *brg11* are solely composed of 105-bp repeats. In the latter cases, structural or functional requirements on the protein level might not allow mixed repeat regions. Sequence analysis of additional *avrBs3* family members and structural analysis of AvrBs3-like proteins may shed more light on this question.

X. campestris pv. *armoraciae* strain 5 is pathogenic in *Arabidopsis thaliana* and red radish. Although a virulence activity of *hax2*, *hax3*, and *hax4* on *Arabidopsis* could not be identified thus far (data not shown), the *hax* genes are important for disease symptom formation on radish, albeit with different impact. The *hax2* gene has the strongest influence because its deletion led to a symptom reduction on 50% of all inoculated leaves (Table 1). The deletion of *hax4* alone also resulted in symptom reduction, in contrast to a single *hax3* mutant, suggesting a stronger contribution of *hax4* to virulence. When *hax3* and *hax4* were deleted in addition to *hax2*, symptom formation

Fig. 7. Hax3 and Hax4 have avirulence activity in tomato and *Nicotiana benthamiana* (Bs4). **A**, Hypersensitive response (HR) induction in tomato (*Lycopersicon esculentum*) cv. MoneyMaker (Bs4) and the line MM^{bs4}-BC4 (bs4) by *Xanthomonas campestris* pv. *armoraciae* (Xca) 5, Δ hax234, and *X. campestris* pv. *vesicatoria* 75-3 derivatives. The following constructs were introduced into strain 75-3: pLH2, pLH3, and pLH4 carrying *hax2*, *hax3*, and *hax4*, respectively, under control of their own promoters. *X. campestris* pv. *vesicatoria* 75-3 expressing *avrBs4* (pDS200F) or carrying the empty vector (pLAFR6) was used as control. Inoculation density was 5×10^8 CFU/ml. The leaf was harvested 3 days postinoculation and bleached with ethanol. **B**, Transient expression of *hax2*, *hax3*, and *hax4* under control of the 35S promoter in *Nicotiana benthamiana* using *Agrobacterium*-mediated delivery. The leaf was harvested 9 days after overlapping inoculation of *A. tumefaciens* strain GV3101 delivering Bs4 (continuous line) and strain GV3101 delivering *hax2* (pAGH2), *hax3* (pAGH3), and *hax4* (pAGH4) (dashed lines). Bacterial suspensions of an optical density at 600 nm = 0.8 were used for infiltration. Strain GV3101 delivering *avrBs4* (pVSF200) was inoculated as positive control; as negative control, strain GV3101 carrying the empty vector (pAG35S) was used. The leaf was bleached with ethanol for better visualization of the HR.

was reduced further. This suggests that the *hax* genes have an additive effect on virulence. Strains containing several *avrBs3*-like genes that act in concert to achieve full virulence were described before in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *malvacearum* (Bai et al. 2000; Yang and White 2004; Yang et al. 1996). At least 5 of 10 *avrBs3*-like genes in *X. campestris* pv. *malvacearum* H1005 were suggested to work additively in the induction of watersoaking symptoms on cotton (Yang et al. 1996). In contrast, the functions of *avrXa7*, *avrxa5*, and *aB4.5* from *X. oryzae* pv. *oryzae* PXO86 are specific, because the genes were not able to complement each other for wild-type symptoms and growth of the bacteria in rice (Bai et al. 2000). Interestingly, *pthXo1*, *pthXo2*, *pthXo3*, *pthXo1-2*, *pthXo2-2*, and *avrXa7-2* isolated from other *X. oryzae* pv. *oryzae* strains also show *avrXa7*-like virulence activities (Yang and White 2004). These genes, as well as *pthA* from *X. axonopodis* pv. *citri*, are major virulence determinants that are essential for full virulence of the respective bacteria. They enhance not only plant macroscopic symptoms but also bacterial growth in the plant tissue (Bai et al. 2000; Swarup et al. 1991; Yang and White 2004). In contrast, the *avrB6* gene from *X. campestris* pv. *malvacearum* does not affect bacterial growth, but is involved in bacterial spreading by increasing the release of bacteria to the leaf surface (Yang et al. 1994).

AvrBs3 is thought to act as transcriptional regulator in the plant cell nucleus. Its nuclear localization has been shown directly using immunocytochemistry (Szurek et al. 2002). Additionally, AvrBs3 dimerizes in the plant cell cytoplasm prior to nuclear import (Gürlebeck et al. 2005). Using cDNA amplified fragment length polymorphism, 13 pepper genes were identified that are induced by AvrBs3 in an AAD-dependent manner (Marois et al. 2002). Four of these genes also were induced when plant protein synthesis was blocked by cycloheximide, suggesting a direct function of AvrBs3 in transcriptional activation via the AAD or indirectly by recruitment of plant transcription factors (Marois et al. 2002). A direct binding to double-stranded DNA has been shown for AvrXa7 supporting the hypothesis of a direct role of AvrBs3-like effectors in transcriptional activation of target genes, but no specific DNA sequence was identified (Yang et al. 2000). The isolation of *hax2*, *hax3*, and *hax4* from a *Brassicaceae* pathogen now may allow the analysis of the transcriptional modulation of host genes in *Arabidopsis*, a plant that is more amenable for molecular studies. Although a contribution of the *hax* genes to the virulence of *X. campestris* pv. *armoraciae* on *Arabidopsis* has not yet been demonstrated, it is probable that these virulence factors have similar targets in both host plants of strain 5.

The repeat region of AvrBs3 family members is essential not only for their virulence activity but also for their specific recognition by resistant plants. In addition, the recognition typically depends on functional NLS and AAD motifs in the C termini of the Avr proteins (Szurek et al. 2001; Van den Ackerveken et al. 1996; Yang et al. 2000; Zhu et al. 1998, 1999). Here, we used the pepper *Bs3* resistance reaction to show the translocation of Hax-AvrBs3 hybrid proteins by the *Xanthomonas* TTS system and to confirm that Hax2, Hax3, and Hax4 contain functional NLSs and AAD (Fig. 4). We noticed, however, that the Hax2-AvrBs3 hybrid protein was less well recognized by *Bs3* pepper plants than the Hax3-AvrBs3 and Hax4-AvrBs3 chimeras. To identify amino acid differences that might be responsible for the reduction in *Bs3* recognition efficiency, we compared the N- and C-terminal sequences of Hax2 with the corresponding sequences of Hax3, the closest Hax2 homolog with respect to these regions, and found 16 aa differences in the C termini of the proteins. Besides one glutamine-to-histidine exchange at the very end of Hax2, all other sequence differences are located within 85 aa downstream of the repeat region. This is in

accordance with recent data demonstrating the contribution of the 3' region to virulence and avirulence activities of AvrBs3-like proteins from *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri* in rice and citrus, respectively (Ishihara et al. 2003). Interestingly, a putative leucine zipper motif has been identified in this region that might be involved in the interaction with plant proteins (Gabriel 1999). It is very well possible that the amino acid exchanges in Hax2 result in a weaker interaction with target pepper proteins and, hence, account for the attenuated HR induced by the Hax2-AvrBs3 hybrid protein.

Interestingly, Hax3 and Hax4 are recognized in a *Bs4*-dependent manner in tomato plants when they are delivered by *X. campestris* pv. *vesicatoria*. The avirulence activity of Hax3 and Hax4 was confirmed by transient expression in tomato and *Bs4*-transgenic *N. benthamiana* plants. Hence, the resistance protein Bs4 (Schornack et al. 2004) is able to recognize three different effectors under natural conditions: AvrBs4, Hax3, and Hax4. Additionally, the AvrBs3 protein also triggers a *Bs4*-dependent HR when it is overexpressed in planta (Schornack et al. 2004). Resistance proteins with dual recognition capacity have been described before; for example, the *Arabidopsis* RPM1 protein which recognizes AvrRpm1 and AvrB from *Pseudomonas syringae* (Mackey et al. 2002). In this case, both effector proteins are sequence unrelated but interact with the same plant target, the *Arabidopsis* protein RIN4. By contrast, AvrBs4, Hax3, and Hax4 are highly related members of the same effector family. Although AvrBs3-like proteins are highly homologous, most of them are specifically recognized. The specificity depends on the repeat region and, therefore, is based on the sequence of the repeats and their specific order (Lahaye and Bonas 2001). Interestingly, the AvrBs4 N terminus and its first 3.5 repeats are sufficient to specifically elicit the *Bs4*-dependent HR in tomato. This indicates that the C terminus of AvrBs4 containing NLSs and AAD and a major part of the repeat region are not necessary for recognition by Bs4 (Bonas et al. 1993; Schornack et al. 2004). Intriguingly, truncated Hax3 and Hax4 proteins containing the N terminus and 2.5 or 4.5 repeats, respectively, also are recognized by Bs4 (S. Kay and U. Bonas, unpublished data). It is conceivable that Bs4 recognizes a conserved motif within AvrBs4, Hax3, and Hax4. However, such a motif could not be identified on the primary sequence level. Alternatively, AvrBs4, Hax3, and Hax4 might interact with the same target protein in the plant cell. Following the guard model (Van der Biezen and Jones 1998), this interaction might be detected by Bs4, which then triggers the plant defense reaction. So far, the only reported plant target proteins of AvrBs3 family members are AvrBs3-interactors from pepper, including importin α and several uncharacterized proteins (Szurek et al. 2001). The identification of new plant targets may help to unravel the molecular basis of the activities of different AvrBs3 family members.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study were *Escherichia coli* strains DH5 α , DH10B, DH5 α (λ pir), and HB101 (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) (Boyer and Roulland-Dussoix 1969; Ménard et al. 1993); *Agrobacterium tumefaciens* GV3101 (Van Larebeke et al. 1974); *X. campestris* pv. *raphani* strain 25; *X. campestris* pv. *armoraciae* strains 5, 27, 28, 30, 3838, and LMG 7383 (supplied by J. Parker, Sainsbury Laboratory, Norwich, U.K.); and *X. campestris* pv. *vesicatoria* strains 85-10, 85-10 Δ A-C, and 75-3 (Bonas et al. 1989; Minsavage et al. 1990; Noël et al. 2003). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium, *Xanthomonas* spp. at 30°C in NYG broth (Daniels et al. 1984), and *A. tu-*

mefaciens at 30°C in YEB (yeast extract broth) medium. Plasmids were introduced into *E. coli* and *A. tumefaciens* by electroporation and into *Xanthomonas* spp. using pRK2013 as a helper plasmid in triparental mating (Figurski and Helinski 1979).

Plant material and inoculations.

Radish (*Raphanus sativus* L. var. *sativus*) plants of accession RA40/76 "Feuerkugel" (IPK Genebank Gatersleben, Germany) were grown in a walk-in growth chamber (Vötsch Industrietechnik, Balingen-Frommern, Germany) at day and night temperatures of 25 and 22°C, respectively, with 16 h of light. For *X. campestris* pv. *armoraciae* inoculations, 5- to 7-week-old plants were used. Bacterial suspensions of 10⁷ to 10⁸ CFU/ml were infiltrated into leaves using a syringe with a 27-gauge needle. Plants of pepper (*Capsicum annuum*) cv. ECW and the near-isogenic line ECW-30R containing the resistance gene *Bs3* (Minsavage et al. 1990), tomato (*L. esculentum*) cv. MM containing the resistance gene *Bs4* and the line MM^{bs4}-BC4 containing the *bs4* allele (Ballvora et al. 2001), and *N. benthamiana* were grown in the greenhouse under standard conditions (day and night temperatures of 25 and 19°C, respectively, for pepper and 23 and 19°C for tomato and *N. benthamiana*, with 16 h of light and 60 to 40% humidity). Six-week-old plants were used for *Xanthomonas* inoculations at 5 × 10⁸ CFU/ml using a needleless syringe for pepper and a syringe with a 27-gauge needle for tomato plants. Infiltrations with *A. tumefaciens* were performed as described previously (Van den Ackerveken et al. 1996). Inoculated pepper plants were transferred to a Percival growth chamber (Percival Scientific, Perry, IA, U.S.A.) at day and night temperatures of 28 and 24°C, respectively, with 16 h of light; inoculated *N. benthamiana* and tomato plants were kept in the greenhouse.

Isolation of *hax2*, *hax3*, and *hax4*.

Because we were unable to clone the *hax2* gene on one fragment, *hax2* was isolated in three parts. First, the internal 3.9-kb *PstI/SacI* fragment was cloned from the natural plasmid in *X. campestris* pv. *armoraciae* strain 5 into pBluescript-KS II (pB-KS; Stratagene, Heidelberg, Germany), resulting in plasmid pBSH2ps. Two *MlsI* fragments of 2.1 and 1.3 kb containing the 5' and the 3' terminal part of the gene, respectively, were cloned into *Eco32I*-digested pB-KS. The *hax2* parts were combined in plasmid pUCH2 bearing the complete *hax2* gene and 183 bp of its upstream region in pUC18. Details on clonings are available upon request. For the isolation of *hax3* and *hax4*, a partial genomic library of *X. campestris* pv. *armoraciae* strain 5 was constructed. Genomic *SallI*-fragments between 5 and 15 kb were cloned into pB-KS. Colony hybridization using the digoxigenin-labeled (Roche GmbH, Mannheim, Germany) 3.4-kb *PstI/SacI* fragment of *avrBs3* as a probe identified four positive clones, which were confirmed by Southern blot analysis following standard protocols (Ausubel et al. 1996). Two clones, pGBH3 and pGBH3-2, carry *hax3* on a 6.5-kb insert; the other two, pGBH4 and pGBH4-2, carry *hax4* on a 8.3-kb fragment. For further analysis, we chose pGBH3 and pGBH4.

Constructs for functional tests in *Xanthomonas* spp.

To test for expression in *Xanthomonas* spp., the *hax* genes were cloned into the broad host range vector pLAFR6 (Bonas et al. 1989). For this, the 3.4-kb *XmnI* fragment of pGBH3 was cloned into *HincII*-cleaved pUC18 in both orientations, resulting in pUCH3 and pUCH3-1, respectively. Plasmid pUCH4 was constructed by inserting the 3.8-kb *XmnI/NheI* fragment of pGBH4 into *HincII/XbaI*-cut pUC18. The *EcoRI/HindIII* fragments from pUCH2, pUCH3, and pUCH4 carrying *hax2*,

hax3, and *hax4*, respectively, behind their own promoters were cloned into pLAFR6, resulting in pLH2, pLH3, and pLH4. As positive control for HR tests on tomato plants, plasmid pDS200F was used that expresses *avrBs4* from the *lac* promoter (Schornack et al. 2004). For complementation analysis of *hax* double and triple mutants, two *hax* genes were cloned into pUC18 and a fragment containing both genes then was ligated into pLAFR6. This resulted in plasmids pLH23 (carrying *hax2* and *hax3*), pLH24 (carrying *hax2* and *hax4*), and pLH34 (carrying *hax3* and *hax4*). Details on clonings are available upon request.

To replace the *hax* repeat regions with that of *avrBs3*, the 2.5-, 1.4-, and 1.7-kb *Eco147I/HincII* fragments from pUCH2, pUCH3, and pUCH4 were replaced by the 2.0-kb *Eco147I/HincII* fragment from pBS300F (Van den Ackerveken et al. 1996), resulting in plasmids pRAH2, pRAH3, and pRAH4, respectively. The *EcoRI/HindIII* inserts of these plasmids subsequently were cloned into pLAFR6, resulting in pLRAH2, pLRAH3, and pLRAH4, respectively. Plasmid pL6A622 carries *avrBs3* on a 4.3-kb *EcoRI/HindIII* fragment from pUXV1922 (Bonas et al. 1989) in pLAFR6.

Constructs for in planta expression.

For *Agrobacterium*-mediated expression in the plant, the *hax* genes were cloned into the binary vector pAG35S (K. Peter and T. Lahaye, unpublished data) that allows expression under control of the 35S promoter, using GATEWAY technology (Invitrogen GmbH, Karlsruhe, Germany). To optimize expression in the plant, a Kozak sequence was introduced. Details on clonings are available upon request. As control for inoculation of tomato and *N. benthamiana* plants, plasmid pVS200F was used, which carries *avrBs4* under control of the 35S promoter (Ballvora et al. 2001).

Sequence analysis and confirmation of repeat numbers.

The DNA sequence of the *hax* genes and flanking regions was determined using the Sanger method and vector- and insert-specific oligonucleotides. The *hax2* repeat region was sequenced using 5' deletion constructs (generated by exonuclease III) and oligonucleotide M13F. The number of repeats in *hax2* was determined by partial digest of pBSH2ps with *MscI* as described (De Feyter et al. 1993). To sequence the *hax3* and *hax4* repeat regions, plasmids pUCH3 and pUCH4 were cut with *SmaI* and partially cleaved with *MscI*. Fragments containing the vector part and different amounts of repeats were gel purified, religated, and sequenced using the vector primer pUC18R (CACTCATTAGGCACCCAG). Sequences were run on an ABI PRISM 310 Genetic Analyzer and analyzed using SEQUENCHER (version 4.1.2; Gene Codes Corporation, Ann Arbor, MI, U.S.A.) and BLASTX (Altschul et al. 1997). The sequences of *hax2*, *hax3*, and *hax4* have been submitted to GenBank and assigned accession numbers AY993937, AY993938, and AY993939, respectively.

For phylogenetic analysis, the sequences were aligned using Clustal_X (Thompson et al. 1997) and analyzed using MEGA2 ver2.1 (Kumar et al. 2001). The neighbor-joining method was chosen to create a phylogenetic tree using the gamma model with the gamma parameter set at 2.25. Phylogenetic confidence was determined by bootstrapping, using 1,000 replicates.

Construction of *hax* mutant strains.

For the mutation of the *hax* genes in *X. campestris* pv. *armoraciae* strain 5, the suicide vector pOK1 was used, which allows the generation of nonpolar mutants in *Xanthomonas* spp. (Huguet et al. 1998). In *hax2*, a 2,205-bp fragment con-

taining the repeat region was deleted by *MscI*-digest of pBSH2ps. The remaining 1.5-kb *PstI/BamHI* insert was filled-in at the *PstI* site and cloned into *SmaI/BamHI*-cut pOK1 giving plasmid pOD2. By *NsiI* cleavage and religation of pGBH3, a 1,677-bp deletion was introduced into *hax3* encompassing the repeat region and part of the adjacent 3' region. The 4.4-kb *SmaI/NheI* fragment containing the *hax3* deletion derivative then was cloned into *SmaI/XbaI*-cut pOK1, resulting in plasmid pOD3. To mutate *hax4*, first, the 3.0-kb *BamHI* fragment from pGBH4 was subcloned into pB-KS, giving plasmid pBSH4b. *MslI* cleavage of pBSH4b and subsequent religation introduced a 1,428-bp deletion encompassing the repeat region of *hax4*. The remaining 1.6-kb *BamHI* fragment was cloned into pOK1, yielding pOD4.

Plasmids pOD2, pOD3, and pOD4 were introduced into *X. campestris* pv. *armoraciae* strain 5, thus replacing the wild-type copy of the gene with the deletion construct by a double recombination event. This resulted in $\Delta hax2$, $\Delta hax3$, and $\Delta hax4$ mutant strains, respectively. By introduction of pOD3 and pOD4 into the $\Delta hax2$ strain, $\Delta hax23$ and $\Delta hax24$ mutants respectively, were generated; and, by introduction of pOD4 into the $\Delta hax3$ strain, a $\Delta hax34$ mutant was made. To generate a $\Delta hax234$ triple mutant, pOD4 was introduced into the $\Delta hax23$ strain.

Immunoblot analysis.

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose following standard protocols (Ausubel et al. 1996). Western blots were reacted with polyclonal anti-AvrBs3 antibody (Knoop et al. 1991) and anti-rabbit immunoglobulin secondary antibody which was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

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