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Assessment of the Genetic Diversity of *Xanthomonas axonopodis* pv. phaseoli and *Xanthomonas fuscans* subsp. *fuscans* as a Basis To Identify Putative Pathogenicity Genes and a Type III Secretion System of the SPI-1 Family by Multiple Suppression Subtractive Hybridizations[▽]

Seyed Mehdi Alavi,[1] Saeideh Sanjari,[1] Fabien Durand,[1] Chrystelle Brin,[1] Charles Manceau,[1]†* and Stéphane Poussier[2]†*

UMR PaVé, INRA, 42 rue Georges Morel, 49071 Beaucouzé Cedex 1, France,[1] and INH, 2 rue Le Nôtre, 49045 Angers Cedex 1, France[2]

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Fluorescent amplified fragment length polymorphism revealed that strains of *Xanthomonas axonopodis* pv. phaseoli and *Xanthomonas fuscans* subsp. *fuscans* are genetically distinct and can be grouped into four genetic lineages. Four suppression subtractive hybridizations were then performed to isolate DNA fragments present in these bean pathogens and absent from closely related xanthomonads. Virulence gene candidates were identified such as homologs of hemagglutinins, TonB-dependent receptors, zinc-dependent metalloproteases, type III effectors, and type IV secretion system components. Unexpectedly, homologs of the type III secretion apparatus components (SPI-1 family), usually reported in animal pathogens and insect symbionts, were also detected.

Understanding the molecular mechanisms used by plant pathogens to attack their hosts is central to the study of plant pathology. Such fundamental knowledge is essential for the development of new strategies for the control of the economically important diseases caused by these microorganisms. *Xanthomonas axonopodis* pv. phaseoli (44) and *Xanthomonas fuscans* subsp. *fuscans* (40) (also designated *Xanthomonas axonopodis* pv. phaseoli variant *fuscans* [44]) are the causative agents of common bacterial blight of bean (*Phaseolus vulgaris* L.), a disease that occurs worldwide and leads to important yield losses (5). Both pathogens have the same host range and epidemiological features (45), but it has been reported that the *X. fuscans* subsp. *fuscans* strains are generally more aggressive toward their hosts than *X. axonopodis* pv. phaseoli strains (31, 45). Both bacteria also have similar biochemical phenotypes, except that *X. fuscans* subsp. *fuscans* can produce a melanin-like pigment in culture (16). Currently, nothing is known about the virulence and host specificity determinants of these bean pathogens. To identify such determinants and to be as exhaustive as possible, we decided in this study to consider the genetic diversity of both bean pathogens as the basis for performing several suppression subtractive hybridizations (SSHs). We first report the determination of a large genetic diversity within *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains by using fluorescent amplified fragment length polymorphism (F-AFLP), which has never been used with these bean pathogens. AFLP is known to be a very powerful DNA fingerprinting technique that allows very fine discrimination and reliable determination of taxonomic and phylogenetic relationships between strains (15, 20, 33, 39). Then, we describe the results of our SSHs. We used SSH, since it is reported to be an effective approach in the identification of virulence determinants and genetic diversity in bacteria (2, 6, 18, 34, 37, 42, 47).

F-AFLP revealed that strains of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* can be grouped into four genetic lineages. In this study, we first assessed the genetic diversity of a worldwide collection of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains by F-AFLP (Table 1). We also worked with a set of selected strains with different host specificities (Table 1) in order to choose representative strains of phylogenetically closely related xanthomonads as the driver for our SSH approach. F-AFLP experiments were performed and analyzed as previously described (39).

Interestingly, this study revealed that strains of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* are genetically different and can be grouped into four distinct genetic lineages (Fig. 1). The *X. axonopodis* pv. phaseoli strains are distributed within three lineages, and the *X. fuscans* subsp. *fuscans* strains formed the remaining lineage. High bootstrap values indicated that this clustering is well supported and that the dendrogram was robust (Fig. 1). Therefore, this result provides further data to show that *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains are genetically distinct and that strains of *X. axonopodis* pv. phaseoli are more heterogeneous than those of *X. fuscans* subsp. *fuscans* (1, 3, 7, 19, 25, 26, 29, 40, 44). More-

* Corresponding author. Mailing address for Stéphane Poussier: UMR PaVé, INH, 2 rue Le Nôtre, 49045 Angers Cedex 1, France. Phone: 33 241 22 55 01. Fax: 33 241 22 54 78. E-mail: stephane.poussier@inh.fr. Mailing address for Charles Manceau: UMR PaVé, centre INRA, 42 rue Georges Morel, 49071 Beaucouzé Cedex 1, France. Phone: 33 241 22 57 40. Fax: 33 241 22 57 05. E-mail: manceau@angers.inra.fr.

† These authors contributed equally to this work.

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TABLE 1. Bacterial strains used in this study

Species (genetic lineage no.)	Strain[a]	Host	Geographic origin	Yr of isolation
X. fuscans subsp. *fuscans*[b]	CFBP4834[d]	*Phaseolus vulgaris*	France	1998
	CFBP6165	*Phaseolus vulgaris*	Canada	1957
	CFBP6166	*Phaseolus vulgaris*	South Africa	1963
	CFBP6167	*Phaseolus* sp.	United States	1954
	CFBP6960	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6970	Unknown	United States	1990
	CFBP6971	Unknown	Tanzania	1992
X. axonopodis pv. phaseoli (1)[c,f]	CFBP2534[g]	*Phaseolus vulgaris*	United States	Unknown
	CFBP6164[d]	*Phaseolus vulgaris*	Romania	1966
	CFBP6982	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6983	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6984	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6985	*Phaseolus vulgaris*	Reunion Island	2000
X. axonopodis pv. phaseoli (2)[f]	CFBP6989[d]	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6990	*Phaseolus vulgaris*	Reunion Island	2000
X. axonopodis pv. phaseoli (3)[f]	CFBP6992	*Phaseolus vulgaris*	Reunion Island	2000
	CFBP6994[d]	Unknown	Tanzania	1990
	CFBP6996	*Phaseolus vulgaris*	Reunion Island	2000
	JW162.16	*Phaseolus vulgaris*	Reunion Island	2000
	JW351.4	*Phaseolus vulgaris*	Reunion Island	2000
	JW352.2	*Phaseolus vulgaris*	Reunion Island	2000
X. alfalfae subsp. *alfalfae*[b]	CFBP3836	*Medicago sativa*	Sudan	Unknown
X. alfalfae subsp. *citrumelonis*[b]	CFBP3371	Unknown	Unknown	1989
X. citri subsp. *citri*[b]	CFBP2866[e]	*Citrus aurantiifolia*	Brazil	1982
X. citri subsp. *malvacearum*[b]	CFBP2530	*Gossypium hirsutum*	Sudan	1958
X. fuscans subsp. *aurantifolii*[b]	CFBP3528[e]	*Citrus limon*	Argentina	1988
X. axonopodis pv. allii[c]	CFBP6107	*Allium fistulosum*	Japan	1998
	CFBP6369[e,g]	*Allium cepa*	Reunion Island	1996
X. axonopodis pv. begoniae[c]	CFBP2524[g]	*Begonia* sp.	New Zealand	1962
X. axonopodis pv. glycines[c]	CFBP2526[g]	*Glycine hispida*	Sudan	1956
X. axonopodis pv. manihotis[c]	CFBP2603	*Manihot esculenta*	Colombia	1972
X. axonopodis pv. vesicatoria[c]	CFBP1604	*Capsicum annuum*	Guadeloupe	Unknown
	CFBP5600	*Lycopersicon esculentum*	Martinique	1993
X. axonopodis[c]	CFBP4924[h]	*Axonopus scoparius*	Colombia	1949

[a] CFBP, Collection Française des Bactéries Phytopathogènes (INRA, Angers, France); JW, bacterial collection of the Pole de Protection des Plantes (CIRAD, Reunion Island, Saint-Pierre, France).
[b] Taxonomy as proposed by Schaad et al. (40).
[c] Taxonomy as proposed by Vauterin et al. (44).
[d] Representative strains of the four genetic lineages of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans*, as determined by our F-AFLP analyses (Fig. 1) and used separately as testers in our four SSHs.
[e] Strains used as drivers in our four SSHs.
[f] Genetic lineages 1, 2, and 3 represent the three genetic lineages of *X. axonopodis* pv. phaseoli, as revealed by our F-AFLP analyses (Fig. 1).
[g] Pathotype strains.
[h] Type strain.

over, F-AFLP provides new information, since it is the first technique that allows the identification of three distinct genetic lineages for *X. axonopodis* pv. phaseoli. Interestingly, genetic lineage 1 of *X. axonopodis* pv. phaseoli appears phylogenetically distant from genetic lineages 2 and 3, which are more closely related to *X. fuscans* subsp. *fuscans*. Furthermore, it is worth noting that this F-AFLP genetic clustering was supported by our SSH results, since strains belonging to genetic lineage 1 carry numerous DNA sequences that are not present in the other genetic lineages, such as those encoding a putative type III secretion system of the *Salmonella p*athogenicity *i*sland-1 (SPI-1) protein family (Table 2). Altogether, our investigations highlight the need for a novel taxonomic study including representative strains of the four newly identified genetic lineages, since it might lead to the reclassification of these strains into new species or subspecies.

Strains of the four genetic lineages were all pathogenic in bean and appeared to be genetically distinct from strains of other xanthomonads that are pathogenic in different host plants. These results suggest not only that strains of the four genetic lineages share specific DNA sequences that may be involved in pathogenicity in bean but also that these strains possess different DNA sequences that could account for a distinct host range. Pathogenicity tests for a large host range are under way to determine whether this genetic diversity could be related to distinct host ranges. In these pathogenicity tests, it will be interesting to include strains that are closely related to bean pathogens based on F-AFLP, such as the *X. citri* subsp. *citri* CFBP2866 strain (Fig. 1), in order to determine whether these strains could also be pathogenic in bean.

SSHs confirmed the genetic heterogeneity of *X. axonopodis* pv. phaseoli and of *X. fuscans* subsp. *fuscans* and revealed DNA fragments likely acquired by horizontal gene transfers. Four SSH experiments were then performed by selecting as the tester representative strains of each genetic lineage (*X. fuscans* subsp. *fuscans* CFBP4834, *X. axonopodis* pv. phaseoli

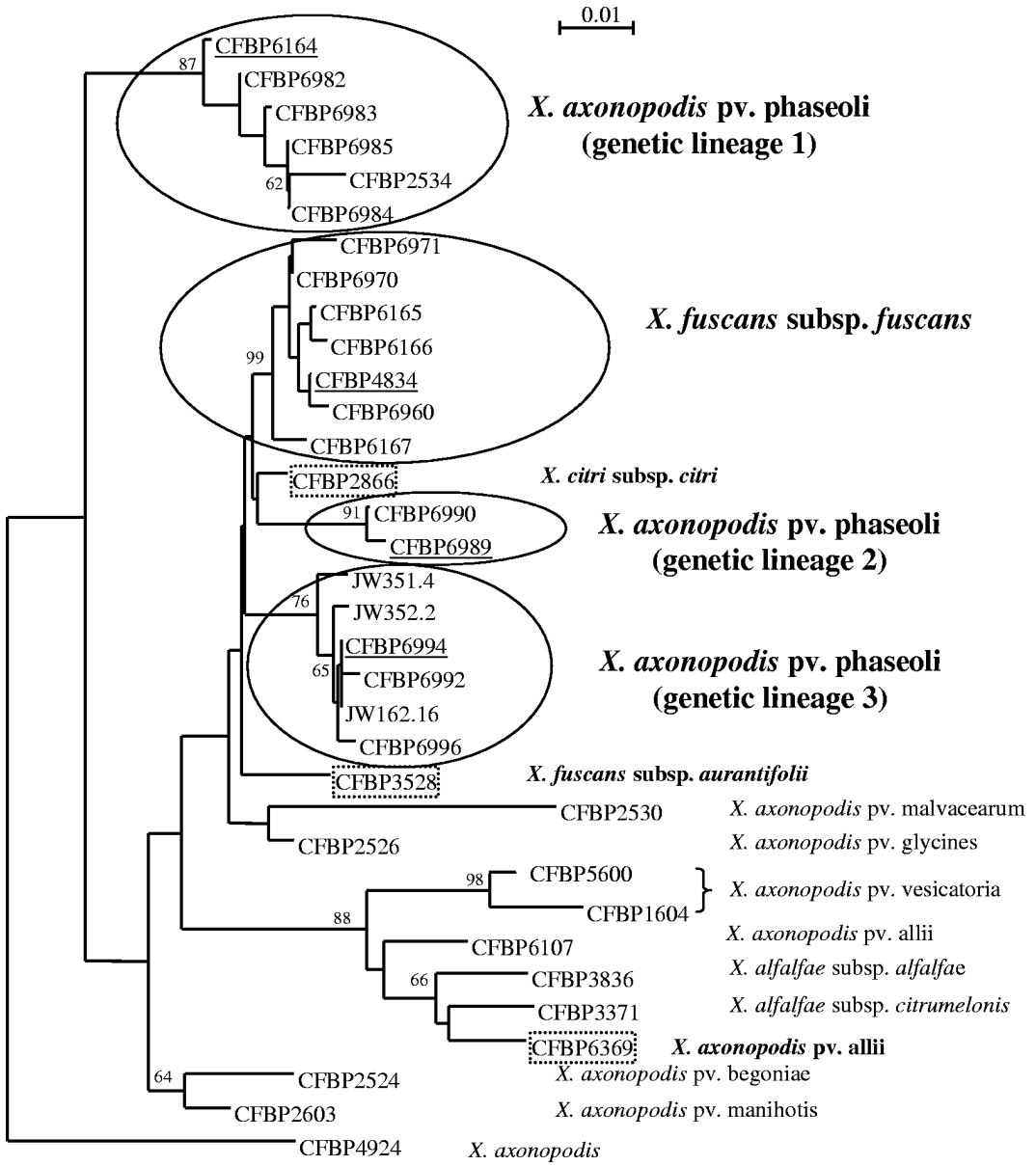

FIG. 1. A dendrogram constructed by using the neighbor-joining method shows the phylogenetic relationships of AFLP fingerprints of *X. fuscans* subsp. *fuscans* and *X. axonopodis* pv. phaseoli strains and related xanthomonads. The representative strains of the four genetic lineages of these bean pathogens (black circles), selected as testers for our four SSHs, are underlined. The three closely related xanthomonad (*X. axonopodis* pv. allii [44], *X. fuscans* subsp. *aurantifolii* [40], and *Xanthomonas citri* subsp. *citri* [40]) strains, selected as the drivers for our four SSHs, are indicated by dotted boxes. Numbers at the branch points represent the bootstrap values (1,000 replicates). Only high bootstrap values (>60) are displayed. Strain CFBP4924 is the type strain of the *X. axonopodis* species (44).

CFBP6164, *X. axonopodis* pv. phaseoli CFBP6989, and *X. axonopodis* pv. phaseoli CFBP6994) and by selecting as the driver a mixture of strains belonging to three closely related xanthomonads, as revealed by F-AFLP: *X. fuscans* subsp. *aurantifolii* (strain CFBP3528) (40) and *X. citri* subsp. *citri* (strain CFBP2866) (40), the causal agents of citrus canker, and *X. axonopodis* pv. allii (strain CFBP6369) (44), the causal agent of bacterial blight of onion. These four subtractions were achieved according to the protocol of the PCR-Select bacterial genome kit (Clontech), except that the hybridization temperature was increased from 63°C to 73°C due to the high G+C content (~65%) in the genomes of these *Xanthomonas* strains (44). For each subtractive library, the specificity of inserts from selected recombinant clones was checked by performing Southern blot hybridizations using tester and driver genomic DNAs separately as probes. Inserts from tester-specific clones were sequenced at the Station Biologique de Roscoff (France), and sequences were examined by using BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gov/BLAST/). Genomic signatures of subtracted DNA sequences were also analyzed by using Genstyle software (http://genstyle.imed.jussieu.fr/).

TABLE 2. Sequence and distribution analyses of the 39 unique DNA fragments subtracted from the four tester strains of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans*

SSH clone[a]	Size (bp)	% G+C content	Southern blot hybridization result with DNA probes from[d]:					BLASTX results			
			Driver	*X. fuscans* CFBP 4834	*X. axonopodis* CFBP 6164	*X. axonopodis* CFBP 6989	*X. axonopodis* CFBP 6994	Predicted function	E value(s)	GenBank accession no.	Organism
4834-1[b,c]	713	47	−	+	+	+	+	AvrBsT protein; IS*Xax1* transposase	5 E^{-96}; 2 E^{-37}	AAD39255; AY935340	*X. axonopodis* pv. vesicatoria; *X. axonopodis* pv. phaseoli
4834-2[b]	745	57	−	+	−	−	−	Type IV secretion system protein TraF (VirB5)	5 E^{-56}	YP_199242	*X. oryzae* pv. oryzae
4834-3	1,081	48	−	+	+	−	−	*Xanthomonas* outer protein C (XopC)	8 E^{-154}	YP_364166	*X. axonopodis* pv. vesicatoria
4834-4[b,c]	1,149	48	−	+	+	−	−	*Xanthomonas* outer protein C (XopC); IS*Xcd1* transposase	5 E^{-86}; 2 E^{-4}	YP_364166; NP_637461	*X. axonopodis* pv. vesicatoria; *X. campestris* pv. campestris
4834-5	1,118	55	−	+	−	−	−	Integral membrane protein (WxcO)	8 E^{-10}	AAK53474	*X. campestris* pv. campestris
4834-6[b]	779	62	−	+	−	+	+	Conserved hypothetical protein	2 E^{-45}	YP_342068	*Nitrosococcus oceani*
4834-7[c]	1,102	50	−	+	−	−	−	Conserved hypothetical protein; C-5 cytosine-specific DNA methylase	4.9 E^{-41}; 6 E^{-37}	ZP_00681364; ZP_00681363	*Xylella fastidiosa*; *Xylella fastidiosa*
6164-1[b]	598	40	−	−	+	−	−	Type III secretion system protein (PrgH/EprH)	3 E^{-20}	YP_001063173	*Burkholderia pseudomallei*
6164-2[b]	864	60	−	−	+	−	−	Type III secretion system protein (YscJ/HrcJ)	7 E^{-27}	ZP_01768663	*Burkholderia pseudomallei*
6164-3	602	52	−	−	+	−	−	Type III secretion system protein (BsaQ)	4 E^{-07}	YP_106131	*Burkholderia mallei*
6164-4[c]	508	65	−	+	+	+	+	IS*Xax1* transposase; L-threonine 3-dehydrogenase	5 E^{-50}; 2 E^{-17}	AY935340; YP_362783	*X. axonopodis* pv. phaseoli; *X. axonopodis* pv. vesicatoria
6164-5[c]	548	65	−	+	+	+	+	IS*Xax1* transposase; hypothetical protein	4 E^{-39}; 3 E^{-43}	AY935340; YP_364143	*X. axonopodis* pv. phaseoli; *X. axonopodis* pv. vesicatoria
6164-6	480	40	−	−	+	−	−	Putative type I restriction modification system protein	4 E^{-54}	NP_717074	*Shewanella oneidensis*
6164-7[c]	659	659	−	+	+	+	+	IS*Xac3* transposase; putative TraI protein (TrwC)	4 E^{-19}; 3 E^{-42}	NP_643556; ABA25997	*X. campestris* pv. campestris; *Pseudomonas* sp.
6164−8[b]	640	58	−	−	+	−	−	Hypothetical protein	5 E^{-59}	ZP_01365986	*Pseudomonas aeruginosa*
6164-9	759	63	−	−	+	−	−	Hypothetical protein	4 E^{-47}	AAM37073	*X. axonopodis* pv. citri
6164-10[b]	556	53	−	−	+	−	−	Hypothetical protein	1 E^{-11}	ZP_01642272	*Stenotrophomonas maltophilia*
6164-11	579	65	−	−	+	−	−	Unknown function			
6164-12	586	47	−	−	+	−	−	Unknown function			
6164-13	563	34	−	−	+	−	−	Unknown function			
6164-14[b]	553	34	−	−	+	−	−	Unknown function			
6164-15	717	47	−	−	+	−	−	Unknown function			
6164-16	666	50	−	−	+	−	−	Unknown function			
6164-17[b]	745	36	−	−	+	−	−	Unknown function			
6989-1	688	41	−	−	−	+	−	Filamentous hemagglutinin-related protein	2 E^{-51}	NP_642141	*X. axonopodis* pv. vesicatoria
6989-2	1,074	41	−	−	−	+	−	Putative surface adhesin	4 E^{-40}	YP_001098807	*Herminiimonas arsenicoxydans*
6989-3[c]	730	59	−	−	−	+	+	Site-specific DNA methyltransferase; hypothetical protein	2.1 E^{-14}; 3 E^{-07}	NP_638315 ZP_00415806	*X. campestris* pv. campestris; *Azotobacter vinelandii*
6989−4	824	55	−	+	−	+	+	Hypothetical protein	5 E^{-05}	YP_363001	*X. axonopodis* pv. vesicatoria
6989-5	587	54	−	−	−	+	−	Hypothetical protein	3 E^{-18}	NP_643587	*X. axonopodis* pv. citri
6989-6	1,087	42	−	−	−	+	−	Hypothetical protein	5 E^{-18}	ZP_01638616	*Pseudomonas putida*
6989-7	658	41	−	−	−	+	+	Unknown function			
6989-8[b]	650	50	−	−	−	+	−	Unknown function			
6994-1[c]	793	46	−	+	+	+	+	AvrBsT protein; IS*Xac2* transposase	7 E^{-97} 0.063	AAD39255 YP_363027	*X. axonopodis* pv. vesicatoria; *X. axonopodis* pv. vesicatoria
6994-2[b]	244	54	−	+	+	−	+	TonB-dependent receptor	9.6 E^{-20}	NP_779483	*Xylella fastidiosa*
6994-3	439	40	−	+	+	+	+	Putative transposase	8 E^{-67}	NP_943128	*Pseudomonas* sp.
6994-4[b]	361	61	−	+	+	+	+	Putative secreted protein (putative zinc-dependent metalloprotease)	4 E^{-56}	YP_365137	*X. axonopodis* pv. vesicatoria

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TABLE 2—*Continued*

SSH clone[a]	Size (bp)	% G+C content	Southern blot hybridization result with DNA probes from[d]: Driver	*X. fuscans* CFBP 4834	*X. axonopodis* CFBP 6164	*X. axonopodis* CFBP 6989	*X. axonopodis* CFBP 6994	BLASTX results: Predicted function	E value(s)	GenBank accession no.	Organism
6994-5	297	57	−	+	+	+	+	Conserved hypothetical protein	$3\ E^{-14}$	YP_452829	*X. oryzae* pv. oryzae
6994-6	347	56	−	+	+	+	+	Conserved hypothetical protein	$4\ E^{-11}$	YP_342069	*Nitrosococcus oceani*
6994-7	166	64	−	+	+	−	+	Unknown function			

[a] The first number of each SSH clone indicates the tester strain (*X. fuscans* subsp. *fuscans* CFBP4834, *X. axonopodis* pv. phaseoli CFBP6164, *X. axonopodis* pv. phaseoli CFBP6989, or *X. axonopodis* pv. phaseoli CFBP6994) used in each SSH experiment.
[b] The insert sequences from these clones were found in more than one clone.
[c] Clones contained sequences from two genes (based on BLASTX results).
[d] The distribution of each clone was analyzed by Southern blot hybridization experiments with genomic DNA from driver strains (CFBP3528, CFBP6369, and CFBP2866) or tester strains (*X. fuscans* subsp. *fuscans* CFBP4834, *X. axonopodis* pv. phaseoli CFBP6164, *X. axonopodis* pv. phaseoli CFBP6989, or *X. axonopodis* pv. phaseoli CFBP6994) as probes.

First, we analyzed 353 clones obtained from four subtractive libraries by Southern blot hybridizations, as described above. This analysis showed that only 75 out of the 353 subtracted DNA fragments could be considered specific to the tester strains, since they were not detected in the driver strains. Then, sequencing these 75 DNA fragments allowed the identification of 39 unique DNA sequences, as redundant sequences were revealed (Table 2). Therefore, our investigation revealed that *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* tester strains contained at least 39 DNA sequences that are not shared by driver strains belonging to closely related xanthomonads (*X. axonopodis* pv. allii, *X. citri* subsp. *citri*, and *X. fuscans* subsp. *aurantifolii*). Interestingly, almost half (17) of these sequences were from the *X. axonopodis* CFBP6164 strain. This result is consistent with that of our F-AFLP analysis (Fig. 1), since the *X. axonopodis* CFBP6164 strain is less closely related to the driver strains than to the three other tester strains. Another point of interest is that by performing further Southern blot hybridizations using tester-genomic DNA as probes, only 9 out of the 39 subtracted DNA fragments were shown to be shared by the four tester strains (Table 2). This result demonstrates that these bean pathogens are genetically heterogeneous, further confirming our F-AFLP analysis. It would now be interesting to extend this study in order to know the distribution of the 39 subtracted DNA fragments, not only in the many strains belonging to the four newly identified genetic lineages of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* but also in other xanthomonads strains that are pathogenic for different host plants. Such a study would determine whether these DNA fragments could be considered bean-specific pathogens or lineage-specific pathogens. These distribution studies combined with pathogenicity tests, as described above, should impact future functional studies of genes that could play a role in the *X. axonopodis* pv. phaseoli-bean and *X. fuscans* subsp. *fuscans*-bean interactions.

Sequencing of the subtracted DNA fragments revealed that the G+C content of the majority of these sequences was considerably lower (average value, 51%) (Table 2) than the average value of total DNA for *X. axonopodis* pv. phaseoli (~65%) (44). This result confirms the tendency of SSH in the identification of A+T-rich regions (47) and suggests that many of the subtracted DNA fragments may have been acquired from other organisms by horizontal gene transfers. Sequence analyses showed that the DNA fragments subtracted from *X. axonopodis* pv. phaseoli and from *X. fuscans* subsp. *fuscans* have high identities with sequences from other *Gammaproteobacteria* (*Xanthomonas* sp., *Xylella* sp., *Pseudomonas* sp., *Azotobacter* sp., *Shewanella* sp., *Nitrosococcus* sp., *Stenotrophomonas* sp.) and more strikingly from *Betaproteobacteria* (*Burkholderia* sp., *Herminiimonas* sp.) (Table 2). Altogether, these observations support the idea that *Xanthomonas* genomes have been subjected to numerous horizontal transfer events during evolution and sometimes from phylogenetically distant bacteria (9, 23).

Pathogenicity gene candidates of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* are identified by SSHs. Sequence analyses revealed that the 39 different subtracted DNA fragments can be assigned to diverse functional classes: metabolism, transposase, membrane structure, adhesion, secretion, and unknown functions (Table 2). An interesting feature is that the IS*Xax1* element was identified. This result demonstrates the effectiveness of our SSHs, as we recently reported that this IS element is carried only by the *X. axonopodis* pv. phaseoli, *X. fuscans* subsp. *fuscans* and *X. axonopodis* pv. *vesicatoria* strains (1).

Another interesting feature of our SSHs is the identification of several homologs to known genes encoding proteins involved in the pathogenicity and/or host specificity of bacteria (13/39 subtracted DNA fragments). For instance, two subtracted DNA fragments have significant similarities to putative filamentous hemagglutinins and surface adhesins (Table 2). Such proteins have already been shown to contribute to the virulence of several proteobacteria, and they could be considered good candidates for identifying determinants that control host specificity, since bacterial attachment to host tissues by these proteins is a first step in pathogenesis (14, 24, 36). Another subtracted DNA fragment is a TonB-dependent receptor homolog (Table 2). TonB-dependent receptors are outer membrane proteins known mainly for the active transport of iron-siderophore complexes in gram-negative bacteria, but some of them have been shown to play a major role in plant-*X. campestris* pv. campestris interactions (4, 46). We also identified a homolog of a putative secreted protein harboring a putative zinc-dependent metalloprotease motif (Table 2). Similar metalloproteases have been reported to account for the

virulence or the host specificity of several gram-negative bacteria (22, 28). The identification of TraF and TraI homologs (Table 2) strongly suggests that the *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains harbor a putative type IV secretion system, like other *Xanthomonas* strains (13, 35, 41), even though its contribution to virulence was demonstrated only with the *X. campestris* pv. campestris strain 8004 (35). We are currently investigating the roles of all of these genes in the virulence or host specificity of *X. fuscans* subsp. *fuscans* and *X. axonopodis* pv. phaseoli strains.

Interestingly, we also isolated two homologs of genes encoding type III secretion system (T3SS) effector genes *avrBsT* and *xopC* (Table 2). Both genes have been reported for only a few strains of *X. axonopodis* pv. vesicatoria, the causal agent of the bacterial spot disease of pepper and tomato (8, 27, 30, 38; our unpublished data). The *avrBsT* homolog may play a significant role in the pathogenicity of *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans*, since further Southern blot hybridizations and PCRs showed that this gene is carried by all strains belonging to these bean pathogens (Fig. 1 and Table 2; data not shown). AvrBsT is a member of the YopJ/AvrRxv protein family that is widely distributed in proteobacteria and is predicted to encode a Cys protease that targets intracellular host proteins (11, 32). AvrBsT from *X. axonopodis* pv. vesicatoria triggers the hypersensitive response from pepper plants, but its virulence contribution was not demonstrated (11). Regarding the *xopC* homolog, it appeared to be harbored only by strains belonging to *X. fuscans* subsp. *fuscans* and to the genetic lineage 1 of *X. axonopodis* pv. phaseoli, based on Southern blot hybridizations and PCRs (Fig. 1, Table 2, data not shown). The biochemical function of XopC remains unknown (30, 38). Studies to determine the roles of *avrBsT* and *xopC* homologs in the interactions between the *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains and bean plants are in progress.

A putative type III secretion system of the SPI-1 family is detected in one *X. axonopodis* pv. phaseoli genetic lineage. Strikingly, we found significant similarities between putative proteins encoded by three subtracted DNA fragments and components of a type III secretion system belonging to the SPI-1 family (10) (Table 2). This T3SS family is usually found in animal pathogens and insect symbionts and is required for host cell invasion (10, 43). These subtracted sequences were detected only in the *X. axonopodis* pv. phaseoli CFBP6164 strain which belongs to the *X. axonopodis* pv. phaseoli genetic lineage 1 (Fig. 1). Further Southern blot hybridizations and PCRs revealed that only strains belonging to this genetic lineage carry these DNA sequences (Table 1 and Table 2; data not shown). Moreover, by using specific PCR primers (12), we tried to detect in strains belonging to the four genetic lineages the T3SS of the Hrp2 family that has been identified in many xanthomonads (10, 13, 17, 35, 41, 43). Our data strongly suggest that genetic lineage 1 strains of *X. axonopodis* pv. phaseoli have two T3SSs (types Hrp2 and SPI-1), whereas those of the three others genetic lineages have only one T3SS (the Hrp2 type). What could be the contribution of the putative SPI-1 T3SS in this particular group of *X. axonopodis* pv. phaseoli strains? It is worth noting that a T3SS of the SPI-1 family was recently disclosed by using SSH with *Erwinia amylovora*, the causal agent of fire blight of apple and pear (42). It has been speculated that this SPI-1 T3SS may allow interactions of *E. amylovora* and insects involved in the pathogen spread (42). Interestingly, the transmission of *X. axonopodis* pv. phaseoli by insects was reported, but the precise interaction between the bacterium and the insect host remains poorly understood (21). Does this T3SS play a role in the *X. axonopodis* pv. phaseoli life cycle? Our main objectives are now to get the complete sequence of this T3SS, to study its distribution and evolution in xanthomonads as well as in other plant pathogenic bacteria, and to study its functionality in interactions with plant and animal cells.

In conclusion, this study helped us to identify pathogenicity gene candidates for *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains and a putative type III secretion apparatus that is usually not found in plant pathogenic bacteria. It also provides new insights into the diversity and evolution of these plant pathogenic bacteria. Finally, this work provides an excellent basis for further exploration of the specific interaction between the *X. axonopodis* pv. phaseoli or *X. fuscans* subsp. *fuscans* strain and bean and before the forthcoming genome sequencing of our model strain, *X. fuscans* subsp. *fuscans* CFBP4834.

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REFERENCES

1. **Alavi, S. M., S. Poussier, and C. Manceau.** 2007. Characterization of IS*Xax1*, a novel insertion sequence restricted to *Xanthomonas axonopodis* pv. phaseoli (variants fuscans and non-fuscans) and *Xanthomonas axonopodis* pv. vesicatoria. Appl. Environ. Microbiol. **73:**1678–1682.
2. **Bernier, S. P., and P. A. Sokol.** 2005. Use of suppression-subtractive hybridization to identify genes in the *Burkholderia cepacia* complex that are unique to *Burkholderia cenocepacia*. J. Bacteriol. **187:**5278–5291.
3. **Birch, P. R. J., L. J. Hyman, R. Taylor, A. F. Opio, C. Bragard, and I. K. Toth.** 1997. RAPD PCR-based differentiation of *Xanthomonas campestris* pv. phaseoli from *Xanthomonas campestris* pv. phaseoli var. fuscans. Eur. J. Plant Pathol. **103:**809–814.
4. **Blanvillain, S., D. Meyer, A. Boulanger, M. Lautier, C. Guynet, N. Denance, J. Vasse, E. Lauber, and M. Arlat.** 2007. Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. PLoS ONE **21:**e224.
5. **Broughton, W. J., G. Hernandez, M. Blair, S. Beebe, P. Gepts, and J. Vanderleyen.** 2003. Beans (*Phaseolus* spp.)-model food legumes. Plant Soil **252:**55–128.
6. **Castaneda, A., J. D. Reddy, B. El-Yacoubi, and D. W. Gabriel.** 2005. Mutagenesis of all eight *avr* genes in *Xanthomonas campestris* pv. campestris had no detected effect on pathogenicity but one *avr* gene affected race specificity. Mol. Plant Microbe Interact. **18:**1306–1317.
7. **Chan, J. W. Y. F., and P. H. Goodwin.** 1999. Differentiation of *Xanthomonas campestris* pv. phaseoli from *Xanthomonas campestris* pv. phaseoli var. fuscans by PFGE and RFLP. Eur. J. Plant Pathol. **105:**867–878.
8. **Ciesiolka, L. D., T. Hwin, J. D. Gearlds, G. V. Minsavage, R. Saenz, M. Bravo, V. Handley, S. M. Conover, H. Zhang, J. Caporgno, N. B. Phengrasamy, A. O. Toms, R. E. Stall, and M. C. Whalen.** 1999. Regulation of expression of avirulence gene avrRxv and identification of a family of host interaction factors by sequence analysis of avrBsT. Mol. Plant Microbe Interact. **12:**35–44.
9. **Comas, I., A. Moya, R. K. Azad, J. G. Lawrence, and F. Gonzalez-Candelas.** 2006. The evolutionary origin of Xanthomonadales genomes and the nature of the horizontal gene transfer process. Mol. Biol. Evol. **23:**2049–2057.
10. **Cornelis, G. R.** 2006. The type III secretion injectisome. Nat. Rev. Microbiol. **4:**811–825.
11. **Cunnac, S., A. Wilson, J. Nuwer, A. Kirik, G. Baranage, and M. B. Mudgett.** 2007. A conserved carboxylesterase is a suppressor of AvrBsT-elicited resistance in *Arabidopsis*. Plant Cell **19:**688–705.
12. **Darsonval, A., A. Darrasse, D. Meyer, M. Demarty, K. Durand, C. Bureau, C. Manceau, and M.-A. Jacques.** 2008. The type III secretion system of *Xanthomonas fuscans* subsp. *fuscans* is involved in the phyllosphere colonization process and in transmission to seeds of susceptible beans. Appl. Environ. Microbiol. **74:**2669–2678.
13. **da Silva, A. C., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B.**

Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, L. M. Alves, A. M. do Amaral, M. C. Bertolini, L. E. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L. P. Ciapina, R. M. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. Ferreira, R. C. Ferreira, M. I. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, E. G. Lemos, M. V. Lemos, E. C. Locali, M. A. Machado, A. M. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, A. Rossi, J. A. Sena, C. Silva, R. F. de Souza, L. A. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature **417:**459–463.

14. **Feil, H., W. S. Feil, and S. E. Lindow.** 2007. Contribution of fimbrial and afimbrial adhesins of *Xylella fastidiosa* to attachment to surfaces and virulence to grape. Phytopathology **97:**318–324.
15. **Gonzalez, C., B. Szurek, C. Manceau, T. Mathieu, Y. Séré, and V. Verdier.** 2007. Molecular and pathotypic characterization of new *Xanthomonas oryzae* strains from West Africa. Mol. Plant-Microbe Interact. **20:**534–546.
16. **Goodwin, P. H., and C. R. Sopher.** 1994. Brown pigmentation of *Xanthomonas campestris* pv. phaseoli associated with homogentistic acid. Can. J. Microbiol. **40:**28–34.
17. **Gürlebeck, D., F. Thieme, and U. Bonas.** 2006. Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. J. Plant Physiol. **163:**233–255.
18. **Harakava, R., and D. W. Gabriel.** 2003. Genetic differences between two strains of *Xylella fastidiosa* revealed by suppression subtractive hybridization. Appl. Environ. Microbiol. **69:**1315–1319.
19. **Hildebrand, D. C., N. J. Palleroni, and M. N. Schroth.** 1990. Deoxyribonucleic acid relatedness of 24 xanthomonad strains representing 23 *Xanthomonas campestris* pathovars and *Xanthomonas fragariae*. J. Appl. Bacteriol. **68:**263–269.
20. **Janssen, P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters.** 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology **142:**1881–1893.
21. **Kaiser, W. J., and N. G. Vakili.** 1978. Insect transmission of pathogenic xanthomonads to bean and cowpea in Puerto Rico. Phytopathology **68:**1057–1063.
22. **Kooi, C., B. Subsin, R. Chen, B. Pohorelic, and P. A. Sokol.** 2006. *Burkholderia cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in virulence. Infect. Immun. **74:**4083–4093.
23. **Lima, W., M. A. Van Sluys, and C. F. Menck.** 2005. Non-gamma Proteobacteria gene islands contribute to the *Xanthomonas* genome. OMICS **9:**160–172.
24. **Locht, C., R. Antoine, and F. Jacob-Dubuisson.** 2001. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. Curr. Opin. Microbiol. **4:**82–89.
25. **López, R., C. Asensio, and R. L. Gilbertson.** 2006. Phenotypic and genetic diversity in strains of common blight bacteria (*Xanthomonas campestris* pv. phaseoli and *X. campestris* pv. phaseoli var. fuscans) in a secondary center of diversity of the common bean host suggests multiple introduction events. Phytopathology **96:**1204–1213.
26. **Mahuku, G. S., C. Jara, M. A. Henriquez, G. Castellanos, and J. Cuasquer.** 2006. Genotypic characterization of the common bean bacterial blight pathogens, Xanthomonas axonopodis pv. phaseoli and *Xanthomonas axonopodis* pv. phaseoli var. fuscans by rep-PCR and PCR-RFLP of the ribosomal genes. J. Phytopathol. **154:**35–44.
27. **Minsavage, G. V., D. Dahlbeck, M. C. Whalen, B. Kearney, U. Bonas, B. J. Staskawicz, and R. E. Stall.** 1990. Gene-for-gene relationship specifying disease resistance in *Xanthomonas campestris* pv. vesicatoria-pepper interaction. Mol. Plant-Microbe Interact. **3:**41–47.
28. **Miyoshi, S. I., and S. Shinoda.** 2000. Microbial metalloproteases and pathogenesis. Microbes Infect. **2:**91–98.
29. **Mkandawire, A. B. C., R. B. Mabagala, P. Guzman, P. Gepts, and R. L. Gilbertson.** 2004. Genetic diversity and pathogenic variation of common blight bacteria (*Xanthomonas campestris* pv. phaseoli and *X. campestris* pv. phaseoli var. fuscans) suggests pathogen coevolution with the common bean. Phytopathology **94:**593–603.
30. **Noël, L., F. Thieme, J. Gabler, D. Buttner, and U. Bonas.** 2003. XopC and XopJ, two novel type III effector proteins from *Xanthomonas campestris* pv. vesicatoria. J. Bacteriol. **185:**7092–7102.
31. **Opio, A. F., D. J. Allen, and J. M. Teri.** 1996. Pathogenic variation in *Xanthomonas campestris* pv. phaseoli, the causal agent of common bacterial blight in *Phaseolus* beans. Plant Pathol. **45:**1126–1133.
32. **Orth, K., Z. Xu, M. B. Mudgett, Z. Q. Bao, L. E. Palmer, J. B. Bliska, W. F. Mangel, B. J. Staskawicz, and J. E. Dixon.** 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. Science **290:**1594–1597.
33. **Poussier, S., D. Trigalet-Demery, P. Vandewalle, B. Goffinet, J. Luisetti, and A. Trigalet.** 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology **146:**1679–1692.
34. **Qi, M., K. E. Nelson, S. C. Daugherty, W. C. Nelson, I. R. Hance, M. Morrison, and C. W. Forsberg.** 2005. Novel molecular features of the fibrolytic intestinal bacterium *Fibrobacter intestinalis* not shared with *Fibrobacter succinogenes* as determined by suppressive subtractive hybridization. J. Bacteriol. **187:**3739–3751.
35. **Qian, W., Y. Jia, S. X. Ren, Y. Q. He, J. X. Feng, L. F. Lu, Q. Sun, G. Ying, D. J. Tang, H. Tang, W. Wu, P. Hao, L. Wang, B. L. Jiang, S. Zeng, W. Y. Gu, G. Lu, L. Rong, Y. Tian, Z. Yao, G. Fu, B. Chen, R. Fang, B. Qiang, Z. Chen, G. P. Zhao, J. L. Tang, and C. He.** 2005. Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. Genome Res. **15:**757–767.
36. **Ray, S., R. Rajeshwari, Y. Sharmaand, and R. V. Sonti.** 2002. A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. oryzae exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence. Mol. Microbiol. **46:**637–647.
37. **Reickseidler, S. L., D. DeShazer, P. A. Sokol, and D. E. Woods.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. Infect. Immun. **69:**34–44.
38. **Roden, J. A., B. Belt, J. B. Ross, T. Tachibana, J. Vargas, and M. B. Mudgett.** 2004. A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. Proc. Natl. Acad. Sci. USA **101:**16624–16629.
39. **Roumagnac, P., L. Gagnevin, L. Gardan, L. Sutra, C. Manceau, E. R. Dickstein, J. B. Jones, P. Rott, and O. Pruvost.** 2004. Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. Int. J. Syst. Evol. Microbiol. **54:**15–24.
40. **Schaad, N. W., E. Postnikova, G. H. Lacy, A. Sechler, I. Agarkova, P. E. Stromberg, V. K. Stromberg, and A. K. Vidaver.** 2006. Emended classification of xanthomonad pathogens on citrus. Syst. Appl. Microbiol. **29:**690–695.
41. **Thieme, F., R. Koebnik, T. Bekel, C. Berger, J. Boch, D. Büttner, C. Caldana, L. Gaigalat, A. Goesmann, S. Kay, O. Kirchner, C. Lanz, B. Linke, A. C. McHardy, F. Meyer, G. Mittenhuber, D. H. Nies, U. Niesbach-Klösgen, T. Patschkowski, C. Rückert, O. Rupp, S. Schneiker, S. C. Schuster, F. J. Vorhölter, E. Weber, A. Pühler, U. Bonas, D. Bartels, and O. Kaiser.** 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. vesicatoria revealed by the complete genome sequence. J. Bacteriol. **187:**7254–7266.
42. **Triplett, L. R., Y. Zhao, and G. W. Sundin.** 2006. Genetic differences between blight-causing *Erwinia* species with differing host specificities, identified by suppression subtractive hybridization. Appl. Environ. Microbiol. **72:**7359–7364.
43. **Troisfontaines, P., and G. R. Cornelis.** 2005. Type III secretion: more systems than you think. Physiology **20:**326–339.
44. **Vauterin, L., B. Hoste, K. Kersters, and J. Swings.** 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. **45:**472–489.
45. **Vidaver, A. K.** 1993. *Xanthomonas campestris* pv. phaseoli: cause of common bacterial blight of bean, p. 40–44. *In* J. G. Swings and E. L. Civerolo (ed.), *Xanthomonas*. Chapman and Hall, London, United Kingdom.
46. **Wiggerich, H. G., and A. Pühler.** 2000. The *exbD2* gene as well as the iron-uptake genes *tonB*, exbB and *exbD1* of *Xanthomonas campestris* pv. campestris are essential for the induction of a hypersensitive response on pepper (*Capsicum annuum*). Microbiology **146:**1053–1060.
47. **Winstanley, C.** 2002. Spot the difference: applications of subtractive hybridization to the study of bacterial pathogens. J. Med. Microbiol. **51:**459–467.