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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▽

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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-

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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* pathogenicity island-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

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