Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species

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Bacterial blight is an emerging disease that affects primarily onion, but also garlic and Welsh onion. The present study was undertaken to characterize the causative xanthomonad(s) by a polyphasic approach using a worldwide collection of 33 bacterial strains. Analysis of 16S rRNA gene sequence similarities indicated that the causal agent belongs to the *campestris* core in the genus *Xanthomonas*, which is in agreement with results of phenotypic characterization (analyses of carbon source utilization and fatty acid methyl esters). However, DNA–DNA hybridization, thermal stability of DNA reassociation and fluorescent amplified fragment length polymorphism analysis allowed the causal agent to be identified as a pathovar of *Xanthomonas axonopodis*.

INTRODUCTION

Bacterial blight of onion (BBO), caused by a *Xanthomonas* species, was initially observed in Barbados in 1971 (Paulraj & O'Garro, 1993). The first characterization of the pathogen was performed in Hawaii, where the disease appeared in 1975 (Alvarez *et al.*, 1978). A disease inducing similar symptoms was reported in mainland USA in the 1940s and ascribed to '*Xanthomonas striiformans*' (Thomas & Weinhold, 1953), but the biochemical characteristics of this species do not match those of the genus (Swings *et al.*, 1993). BBO has subsequently been reported in many countries in the Americas, Asia and Africa (Isakeit *et al.*, 2000; Kadota *et al.*, 2000; Neto *et al.*, 1987; Nunez *et al.*,

2002; O'Garro & Paulraj, 1997; Roumagnac *et al.*, 1997; Sanders *et al.*, 2003; Schwartz & Otto, 2000; Serfontein, 2001; Trujillo & Hernandez, 1999).

The taxonomic position of strains of *Xanthomonas* species pathogenic to onion has never been determined, neither in the reclassification of *Xanthomonas* performed by Vauterin *et al.* (1995), nor in a recent study comparing amplified fragment length polymorphism (AFLP) analysis and repetitive extragenic palindromic PCR (rep-PCR) to DNA–DNA relatedness in the genus *Xanthomonas* (Rademaker, 2000; Rademaker *et al.*, 2000). Bacteria recently isolated from Welsh onion in Japan were considered to be a novel pathovar of *Xanthomonas*, and the name *Xanthomonas campestris* pv. *allii* pv. nov. was proposed (Kadota *et al.*, 2000). This classification was, however, based only on biochemical and physiological characteristics and pathogenicity tests with a range of host plants, which is insufficient for species definition according to the recommendations of Wayne *et al.* (1987) and Stackebrandt *et al.* (2002). Kadota *et al.* (2000) have shown that the bacterium is pathogenic to onion and Welsh onion, but non-pathogenic to chive, Chinese chive and hyacinth. However, strains isolated in Barbados were pathogenic to onion, chive, leek, garlic, shallot (Bowen *et al.*, 1998), French bean, soybean,

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Abbreviations: AFLP, amplified fragment length polymorphism; BBO, bacterial blight of onion; CGM values, current genome mispairing values; FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank accession number for the partial 16S rDNA sequence of *Xanthomonas axonopodis* pv. *allii* CFBP 6369 is AY135649.

Primer/adaptor sequences, a dendrogram based on FAME analysis, Biolog results and correspondence analysis and a fuller version of Fig. 1 are available as supplementary material in IJSEM Online.

winged bean, field pea, moth bean and lima bean (O'Garro & Paulraj, 1997), whereas strains isolated in Hawaii were pathogenic to onion and Welsh onion, but not to bean (Alvarez *et al.*, 1978).

Because of the partial characterization of the bacterium associated with BBO and the incomplete definition of its host range, the present study was initiated to characterize a large worldwide collection of BBO strains by a polyphasic approach based on 16S rRNA gene sequencing, DNA–DNA hybridization, analyses of carbon source utilization, fatty acid methyl esters and fluorescent amplified fragment length polymorphisms (FAFLPs) and pathogenicity tests. Based on the results obtained, the denomination *Xanthomonas axonopodis* pv. *allii* is proposed.

METHODS

Bacterial strains and media. Thirty-three strains of *Xanthomonas* species isolated from leaves of three *Allium* species, the type strains of 21 *Xanthomonas* species (Trébaol *et al.*, 2000; Vauterin *et al.*, 1995) and some additional *Xanthomonas axonopodis* pathovars were used in this study (Tables 1 and 2). Cultures were stored as lyophilized cells and/or in a freezer at -80°C ; they were checked for purity and grown routinely on YPGA (7 g yeast extract, 7 g peptone, 7 g glucose and 15 g agar l^{-1} ; 20 μg propiconazole ml^{-1} ; pH 7.2) at 28°C , except *Xanthomonas populi*, which was grown at 19°C . All strains included in this study were deposited in the Collection Française de Bactéries Phytopathogènes (CFBP, INRA Angers, France) and strain numbers used throughout are CFBP numbers, except where noted.

Pathogenicity tests. Onion (*Allium cepa* L. cv. Red Creole), chive (*Allium schoenoprasum* L. cv. Civette), garlic (*Allium sativum* L. cv. Vacoa), leek (*Allium porrum* L. cv. Gros long d'été), shallot (*Allium cepa* var. *ascalonicum* Backer cv. Ambition), bean (*Phaseolus vulgaris* L. cv. Michelet) and hyacinth (*Hyacinthus orientalis* L. cv. Carnegie) were inoculated with 21 BBO strains (Table 1). Onion was screened as a potential host of *X. axonopodis* pv. *phaseoli*, because beans have been reported to be a host of the xanthomonad that causes BBO (O'Garro & Paulraj, 1997). Onion was also inoculated with *Xanthomonas hyacinthi*, the only xanthomonad from the Liliaceae for which a taxonomic position has been clearly determined (Table 2). All bacterial suspensions used for pathogenicity tests were prepared in 0.01 M Tris/HCl buffer (Sigma; pH 7.2) and adjusted spectrophotometrically (Milton Roy Spectronic 601) to approximately 10^8 c.f.u. ml^{-1} (OD_{600} of 0.05). These suspensions were further diluted in Tris buffer to reach the bacterial densities mentioned below. The *Allium* species (ten plants per species and strain) were spray-inoculated as described previously (Roumagnac *et al.*, 2000) using bacterial suspensions containing approximately 10^7 c.f.u. ml^{-1} . Ten bean plants were inoculated per strain by finger-rubbing bacterial suspensions of approximately 10^5 c.f.u. ml^{-1} on all trifoliate leaves of each plant. After inoculation, plants were covered with a translucent plastic bag for 48 h. Four hyacinth plants were inoculated per strain by pricking the leaf with a needle and then infiltrating 0.5 ml bacterial suspensions containing approximately 10^5 c.f.u. ml^{-1} . Negative controls consisting of four (hyacinth) or ten (bean and *Allium* spp.) individuals were inoculated similarly with sterile buffer in all experiments. All plants were placed in a growth chamber at a temperature of $30 \pm 1^{\circ}\text{C}$, except for hyacinth (12 h per day at $24 \pm 1^{\circ}\text{C}$ and 12 h at $18 \pm 1^{\circ}\text{C}$), under a relative humidity of $95 \pm 5\%$ and a photoperiod of 12 h. Symptom

appearance and progress were observed periodically after inoculation for 2 weeks. All experiments were repeated once.

Phenotypic methods

Biochemical and physiological characteristics. The BBO strains were cultivated on YPGA medium at 28°C . Gram test, oxidation–fermentation glucose metabolism, cytochrome *c* oxidase activity, nitrate reduction, urease activity, indole production, hydrolysis of starch, aesculin, cellulose and Tween 80, milk proteolysis, the presence of fluorescent pigments, production of H_2S from cysteine and tolerance to NaCl were assayed as described by Vernière *et al.* (1991). Pectinolytic activity and gelatin hydrolysis were respectively assayed according to Hildebrand (1971) and Lelliott *et al.* (1966).

Fatty acid methyl ester analysis. Strains were grown on medium containing nutrient broth (Difco) and 0.8% agar for 24 h at 28°C . Cultures were then transferred to a medium containing trypticase soy broth (BBL) and 0.75% agar and were grown at 28°C for 24 h. Strains 1976, 2532 and 4691 required 48 h incubation. Strain 4924 exhibited minimal growth on nutrient agar and was grown exclusively on TSBA. *X. populi* was not included in the analysis due to its very poor growth on all media tested. Extraction of fatty acids was performed according to the procedure described in MIDI Technical Note 101 (http://www.midi-inc.com/media/pdfs/TechNote_101.pdf). Bacterial strains were identified using the Sherlock TSBA40 version 4.00 method and a library running with the MIDI system version 4.13 DOS edition software. The GC system consisted of an HP 5890 GC, an HP 7673A autosampler and an HP 3396 series II integrator. Fatty acids were separated on an Ultra2 cross-linked 5% PHME siloxane 25 M column and detected with an FID. Hydrogen was used as the carrier gas. A dendrogram edited as described by Norman *et al.* (1997) was based on data from 32 BBO strains and 20 type strains of *Xanthomonas* species. Mean fatty acid profiles were generated using the library generation software.

Utilization of carbon sources. Six BBO strains (6107, 6359, 6362, 6367, 6369 and 6383) and the type strains of 19 *Xanthomonas* species were characterized phenotypically by the Biolog GN microplate system. *Xanthomonas fragariae* and *X. populi* were not analysed because of their poor reactivity in GN microplates. Experiments were performed as described by Vauterin *et al.* (1995). Two microplates were inoculated per strain and incubated at 28°C for 48 h. Plates were then scored visually for carbon source utilization and OD_{590} were determined for the 96 wells using a microplate reader (Biotek EL 320). OD data were analysed by correspondence analysis with the software ADE-4 (Thioulouse *et al.*, 1997). Groups of strains were defined by the Ward clustering method (Ward, 1963) using the same software. Biolog GN data were also used for identification purposes using the Microlog 1 software (Biolog) release 3.50.

Molecular methods

Sequencing of the 16S rRNA gene. Total DNAs of strains 6364, 6366, 6367 and 6369 were extracted by the CTAB method (Ausubel *et al.*, 1991). Amplification of the 16S rRNA genes was performed by PCR as described by Nesme *et al.* (1995) using the specific primers FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-3') and FGPS1509' (5'-AAGGAGGGGATCCAGCCGCA-3'). The amplified 16S rDNA fragments were purified with a QIAquick PCR purification kit (Qiagen) and cloned into the pGEM-T easy plasmid as recommended by the manufacturer (Promega). Sequence data were obtained by single-pass double-stranded analysis (Genome Express) using primers T7 and SP6, which flank the cloning region in the pGEM-T easy plasmid. Sequence data were compared with those of other xanthomonads (Hauben *et al.*, 1997; Trébaol *et al.*, 2000) by alignment using the CLUSTAL method of MEGALIGN (DNASTAR).

Table 1. Levels of DNA reassociation between *X. axonopodis* pv. *allii* CFBP 6369 and strains pathogenic to *Allium* species used in this study

All strains listed in this table are currently known as *X. axonopodis* pv. *allii*.

Strain	Other designation/source*	Host	Geographical origin	Year of isolation	Relative binding with labelled DNA from CFBP 6369 (%)
CFBP 6107†	MAFF 311173/I. Kadota	<i>A. fistulosum</i> L.	Japan	1998	83
CFBP 6108†	MAFF 311179/I. Kadota	<i>A. fistulosum</i> L.	Japan	1998	89
CFBP 6357		<i>A. cepa</i> L.	Réunion, France	1995	97
CFBP 6358†		<i>A. sativum</i> L.	Réunion, France	1995	100
CFBP 6359†	LMG 580/A. Alvarez	<i>A. cepa</i> L.	Hawaii	1980	82
CFBP 6360	LMG 9490/A. Alvarez	<i>A. cepa</i> L.	Hawaii	1989	83
CFBP 6361	LMG 9493/A. Alvarez	<i>A. cepa</i> L.	Hawaii	1989	74
CFBP 6362†	IBSBF 594/J. R. Neto	<i>A. cepa</i> L.	Brazil	1986	88
CFBP 6363	IBSBF 595/J. R. Neto	<i>A. cepa</i> L.	Brazil	1986	78
CFBP 6364†	A1/Z. Amat Novo	<i>A. sativum</i> L.	Cuba	1986	92
CFBP 6365	A6/Z. Amat Novo	<i>A. sativum</i> L.	Cuba	1986	78
CFBP 6366†		<i>A. cepa</i> L.	Réunion, France	1996	89
CFBP 6367†	P360/D. Stead	<i>A. cepa</i> L.	Barbados	?	80
CFBP 6368	P372/D. Stead	<i>A. cepa</i> L.	Barbados	?	78
CFBP 6369 ^{PT} †		<i>A. cepa</i> L.	Réunion, France	1996	100
CFBP 6371		<i>A. cepa</i> L.	Réunion, France	1996	97
CFBP 6372		<i>A. cepa</i> L.	Réunion, France	1996	100
CFBP 6373		<i>A. cepa</i> L.	Réunion, France	1996	81
CFBP 6374		<i>A. cepa</i> L.	Mauritius	1996	100
CFBP 6375		<i>A. cepa</i> L.	Réunion, France	1996	80
CFBP 6376†	3582/S. Saumtally	<i>A. cepa</i> L.	Mauritius	1997	97
CFBP 6377	IBSBF 989/L. Beriam	<i>A. cepa</i> L.	Brazil	1993	88
CFBP 6378†	IBSBF 1393/I. Almeida	<i>A. cepa</i> L.	Brazil	1998	80
CFBP 6379†	0153/A. Schwartz	<i>A. cepa</i> L.	Colorado, USA	1996	91
CFBP 6380†	0177/A. Schwartz	<i>A. cepa</i> L.	Colorado, USA	1997	90
CFBP 6381†	19110/J. B. Jones	<i>A. cepa</i> L.	Texas, USA	1998	81
CFBP 6382†	19112/J. B. Jones	<i>A. cepa</i> L.	Texas, USA	1998	74
CFBP 6383†	19117/J. B. Jones	<i>A. cepa</i> L.	Texas, USA	1998	81
CFBP 6384†	BD142-1/L. Mabena	<i>A. cepa</i> L.	South Africa	?	79
CFBP 6385†	BD142-2/L. Mabena	<i>A. cepa</i> L.	South Africa	?	77
CFBP 6386†	BD211/L. Mabena	<i>A. cepa</i> L.	South Africa	?	78
CFBP 6387†	?/G. Trujillo	<i>A. cepa</i> L.	Venezuela	1997	79
CFBP 6388†	?/G. Trujillo	<i>A. cepa</i> L.	Venezuela	2001	80

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†Strains used for pathogenicity tests on onion, garlic, chive, shallot, leek, bean and hyacinth. All strains listed in Table 1 were pathogenic to onion when inoculated according to the method described by Roumagnac *et al.* (2000).

DNA–DNA hybridization. DNA was extracted and purified according to Brenner *et al.* (1982). Native DNA of strain 6369 was labelled *in vitro* with tritium-labelled nucleotides by random priming using Megaprime DNA labelling system RPM 1604 (Amersham International). The S1 nuclease–trichloroacetic acid method used for DNA–DNA hybridization experiments was described by Crosa *et al.* (1973) and modified by Grimont *et al.* (1980). The reassociation

temperature used was 70 °C. DNA–DNA hybridization experiments with tritium-labelled DNA of strain 6369 were performed at least twice.

Thermal stability of DNA reassociation. For strains that had DNA–DNA hybridization values of between 55 and 70 % (Grimont, 1988) with strain 6369, the temperature at which 50 % reassociated

Table 2. Levels of DNA reassociation between *X. axonopodis* pv. *allii* CFBP 6369, the type strains of 21 *Xanthomonas* species and the pathotype strains of three pathovars of *X. axonopodis*

Strain*	Current nomenclature	Relative binding (%) with labelled DNA from CFBP 6369 (ΔT_m)
<i>Xanthomonas</i> type strains		
CFBP 1156 ^{T*}	<i>X. hyacinthi</i>	19
CFBP 1817 ^T	<i>X. populi</i>	37
CFBP 1976 ^T	<i>X. bromi</i>	43
CFBP 2054 ^T	<i>X. translucens</i> pv. <i>translucens</i>	17
CFBP 2157 ^T	<i>X. fragariae</i>	27
CFBP 2523 ^T	<i>X. albilineans</i>	15
CFBP 2528 ^T	<i>X. arboricola</i> pv. <i>juglandis</i>	37
CFBP 2532 ^T	<i>X. oryzae</i> pv. <i>oryzae</i>	46
CFBP 2533 ^T	<i>X. hortorum</i> pv. <i>pelargonii</i>	37
CFBP 2542 ^T	<i>X. cucurbitae</i>	18
CFBP 2543 ^T	<i>X. vasicola</i> pv. <i>holcicola</i>	49
CFBP 4188 ^T	<i>X. cynarae</i>	41
CFBP 4641 ^T	<i>X. sacchari</i>	21
CFBP 4642 ^T	<i>X. cassavae</i>	40
CFBP 4643 ^T	<i>X. pisi</i>	40
CFBP 4644 ^T	<i>X. melonis</i>	34
CFBP 4645 ^T	<i>X. vesicatoria</i>	44
CFBP 4690 ^T	<i>X. codiae</i>	44
CFBP 4691 ^T	<i>X. theicola</i>	11
CFBP 4924 ^T	<i>X. axonopodis</i> pv. <i>axonopodis</i>	55 (0.5)
CFBP 5251 ^T	<i>X. campestris</i> pv. <i>campestris</i>	39
Strains of <i>X. axonopodis</i> pathovars		
CFBP 1815*	<i>X. axonopodis</i> pv. <i>phaseoli</i> var. <i>fuscans</i>	ND
CFBP 1816	<i>X. axonopodis</i> pv. <i>phaseoli</i> var. <i>fuscans</i>	ND
CFBP 2534 ^{PT*}	<i>X. axonopodis</i> pv. <i>phaseoli</i>	65 (0.2)
CFBP 2524 ^{PT}	<i>X. axonopodis</i> pv. <i>begoniae</i>	67
CFBP 2525 ^{PT}	<i>X. axonopodis</i> pv. <i>citri</i>	ND
CFBP 2526 ^{PT}	<i>X. axonopodis</i> pv. <i>glycines</i>	ND
CFBP 2530 ^{PT}	<i>X. axonopodis</i> pv. <i>malvacearum</i>	ND
CFBP 2603 ^{PT}	<i>X. axonopodis</i> pv. <i>manihotis</i>	70
CFBP 2866	<i>X. axonopodis</i> pv. <i>aurantifolii</i>	ND
CFBP 3371 ^{PT}	<i>X. axonopodis</i> pv. <i>citrumelo</i>	ND
CFBP 3528	<i>X. axonopodis</i> pv. <i>aurantifolii</i>	ND
CFBP 3836 ^{PT}	<i>X. axonopodis</i> pv. <i>alfalfae</i>	ND
CFBP 5600	<i>X. axonopodis</i> pv. <i>vesicatoria</i>	ND

ND, Not determined; ^T, type strain; ^{PT}, pathotype strain.

*Strains used to inoculate onion.

DNA became hydrolysable by nuclease S1 (T_m) was determined using the method of Crosa *et al.* (1973). The ΔT_m value corresponds to the T_m value of the homoduplex (strain 6369) minus the T_m value of the heteroduplex.

DNA base composition. The G+C contents (mol%) of strains 6107, 6369, 6381 and 6385 were determined by the thermal denaturation method (Marmur & Doty, 1962) and calculated using the equation of Owen & Lapage (1976).

Fluorescent AFLP (FAFLP) analysis. Six BBO strains (6107, 6359, 6362, 6367, 6369 and 6383), all type strains of *Xanthomonas* species, except that of *X. populi*, and 12 strains of *X. axonopodis* (Table 2) were submitted to FAFLP analysis. Genomic DNA was extracted and

purified as described by Brenner *et al.* (1982) and/or using the DNeasy tissue kit (Qiagen). To test the reproducibility of the FAFLP technique, strain 6369 was used as a control in each independent AFLP experiment (15 runs). To test the consistency of the two extraction methods, DNA of strain 6369 extracted by both methods was submitted to the FAFLP protocol in three independent experiments. Two reaction mixtures were prepared for digestion and ligation. For the first mixture, 5 µl genomic DNA (2 ng µl⁻¹) was mixed in a total volume of 15 µl with 3.6 U of each restriction enzyme *EcoRI* and *MspI* (Eurogentec), 2 µl 0.5 M NaCl, 0.2 µl 100 × BSA solution (Biolabs), 1.5 µl 10 × T4 ligase buffer (Biolabs), 1 µl 2 µM *EcoRI* adaptor (Cybergène) and 1 µl 20 µM *MspI* adaptor (Cybergène). The second mixture was prepared in a total volume of 5 µl, with 0.5 µl 10 × T4 ligase buffer and 100 U T4 DNA ligase

(Biolabs). The two reaction mixtures were combined and incubated for 3 h at 37 °C. After incubation, the reaction mixture was diluted tenfold with distilled water.

Two successive amplification reactions were then performed. Firstly, amplification was performed in 20 µl containing 4 µl diluted DNA from the restriction–ligation step, 2 µl 10× Goldstar buffer (Eurogentec), 5 mM MgCl₂, 2 mM dNTPs, 0.25 µM each of *Eco*RI and *Msp*I primer and 0.5 U *Taq* DNA polymerase (Goldstar red; Eurogentec). This pre-selective amplification consisted of incubation of the reaction mixture for 2 min at 72 °C followed by 20 cycles with the following cycle profile: a 20 s DNA denaturation step at 94 °C, 30 s annealing step at 56 °C and 2 min extension step at 72 °C. PCR products were then diluted tenfold with distilled water. Secondly, a selective amplification using a 5'-JOE-labelled selective *Eco*RI primer (*Eco*RI + G primer) and the unlabelled selective *Msp*I primer (*Msp*I + TG primer) was performed in a 20 µl reaction mixture containing 1.5 µl diluted pre-amplified DNA from the previous PCR, 2 µl 10× Goldstar buffer (Eurogentec), 2.5 mM MgCl₂, 2 mM dNTPs, 50 nM labelled selective *Eco*RI primer, 250 nM selective *Msp*I primer and 0.5 U *Taq* DNA polymerase (Goldstar red; Eurogentec). Sequences of the adaptors, primers and selective primers used in FAFLP are available in Supplementary Table A in IJSEM Online. The selective amplification consisted of a 2 min denaturation step at 94 °C, followed by 33 cycles of a DNA denaturation step at 94 °C for 20 s, a 30 s annealing step (see below) and a 2 min extension step at 72 °C. The annealing temperature was 66 °C for the first cycle and was then decreased by 1 °C per cycle for the next 9 cycles and was 56 °C for the last 23 cycles. A final extension step was performed at 60 °C for 30 min. Amplification reactions were performed in a PE-9700 thermocycler (Applied Biosystems). One microlitre from the selective amplification was added to 18.2 µl formamide and 0.3 µl GENESCAN TAMRA-500 ladder as an internal standard (Perkin Elmer) and denatured at 95 °C for 3 min. Samples were then submitted to capillary electrophoresis for 35 min (15 kV) in an ABI-310 Genetic Analyzer (Applied Biosystems) with performance optimized polymers POP-4. The FAFLP fingerprints were superimposed and compared visually using GENESCAN software (Applied Biosystems). The threshold for assigning a peak was set to 100 relative fluorescence. The presence and absence of fragments were scored in a binary matrix. A similarity matrix using the Jaccard coefficient was calculated (NTSYS 2.0) and an unweighted neighbour-joining tree (Gascuel, 1997) was built using DARWIN 4.0 software (CIRAD). The robustness of the tree was assessed by bootstrap analysis using the same software (1000 repeated samplings). Current genome mispairing (CGM) values were calculated as described by Mougél *et al.* (2002). CGMs are obtained from the mathematical transformation of Jaccard similarity coefficients into genetic distances, which takes into account the number of nucleotide sites that constrain the AFLP technique (Mougél *et al.*, 2002).

RESULTS

Pathogenicity tests

All *Xanthomonas* strains isolated from *Allium* spp. induced typical symptoms, i.e. water-soaked leaf spots that turned into dry chlorotic lesions, on four out of the five *Allium* spp. assayed (onion, garlic, shallot and leek). On chive, water-soaked lesions developed further into a generalized chlorosis between the lesion and the top of the leaf. No symptoms appeared on hyacinths inoculated with the BBO strains. The type strain of *X. hyacinthi* and *X. axonopodis* pv. *phaseoli* induced typical lesions on their respective hosts, but were not pathogenic to onion. Small, water-soaked lesions that

became necrotic in about 7 days were sometimes observed on bean after inoculation with BBO strains. In contrast, *X. axonopodis* pv. *phaseoli* incited typical symptoms of common blight, with development of large, translucent lesions that resulted in a progressive disease with little or no early necrosis. Consequently, BBO strains were considered to be non-pathogenic to bean.

Phenotypic traits

Biochemical and physiological characteristics. The BBO strains formed single colonies on YPGA medium after 3 days incubation at 28 °C. Colonies were yellow, convex, round and mucoid. Cells were Gram-negative rods. Strains were obligate aerobes and glucose was utilized oxidatively. Starch, gelatin, aesculin, cellulose and Tween 80 were hydrolysed. Proteolysis of milk occurred. Cytochrome *c* oxidase and urease were not detected. Indole was not produced and nitrates were not reduced. Fluorescent pigments were not produced. Strains produced H₂S from cysteine and showed pectinolytic activity at pH values of 5.0, 7.0 and 8.5. Cells grew on YPGA medium containing up to 3 % NaCl.

Analysis of fatty acid methyl ester composition. A dendrogram based on fatty acid methyl ester profiles of 32 BBO strains and 20 type strains of *Xanthomonas* species is available as Supplementary Fig. A in IJSEM Online. Thirty of the 32 BBO strains formed a cluster with a Euclidian distance of less than 6.3; strain groupings were unrelated to geographical origin or host. Type strains of *Xanthomonas codiae*, *Xanthomonas cynarae* and *Xanthomonas translucens* also fell into this same cluster. Removing these strains from the dendrogram did not change the Euclidian distance between the onion strains (data not shown). There were two outliers (6107 and 6108 from Japan), with a Euclidian distance of approximately 9.8.

The onion strains and the two outliers each had over 20 fatty acids (data not shown). In both groups, three fatty acids, 15:0 iso, 15:0 anteiso and summed feature 3 (16:1 ω7c/15 iso 2-OH) accounted for >50 % of each profile. Beyond that, the relative occurrence of other fatty acids differed in the two profiles (Table 3).

Utilization of carbon sources. Percentages of metabolic activities of 33 BBO strains with 95 carbon sources of the Biolog GN microplate are shown in Supplementary Table B in IJSEM Online. The 33 BBO strains were able to metabolize 32 of 95 carbon sources and all strains were identified as *Xanthomonas* spp. Phenotypic relationships between BBO strains and other xanthomonads based on correspondence analysis of metabolic fingerprints are shown in Supplementary Fig. B in IJSEM Online.

According to the correspondence analysis, the first three principal components respectively accounted for 38, 13 and 11 % of total variance. Ward clustering analysis revealed

Table 3. Relative occurrence of the most common fatty acids in 30 BBO strains and two outlier strains (6107 and 6108)Values are mean percentages $\pm$ SD.

Fatty acid	BBO strains	Outliers
15:0 iso	26.45 $\pm$ 2.20	32.46 $\pm$ 0.22
Summed feature 3*	18.24 $\pm$ 1.14	18.64 $\pm$ 0.09
15:0 anteiso	9.43 $\pm$ 1.53	9.18 $\pm$ 0.48
17:0 iso	8.45 $\pm$ 1.55	3.05 $\pm$ 0.31
iso17:1 ω 9c	5.83 $\pm$ 1.23	2.82 $\pm$ 0.17
16:0	5.70 $\pm$ 0.94	4.65 $\pm$ 0.38
13:0 iso 3-OH	3.93 $\pm$ 0.33	4.42 $\pm$ 0.20
11:0 iso	3.75 $\pm$ 0.37	4.43 $\pm$ 0.13
12:0 3-OH	3.03 $\pm$ 0.34	2.92 $\pm$ 0.00
16:0 iso	2.13 $\pm$ 0.84	1.51 $\pm$ 0.19
16:1 ω 9c	1.84 $\pm$ 0.36	2.11 $\pm$ 0.13
11:0 iso 3-OH	1.76 $\pm$ 0.18	1.94 $\pm$ 0.15
Unknown 11:799	1.49 $\pm$ 0.15	1.48 $\pm$ 0.05
14:0	1.32 $\pm$ 0.37	3.33 $\pm$ 0.39
15:0	1.16 $\pm$ 0.39	1.51 $\pm$ 0.13

*Summed feature 3 contains 16:1 ω 7c/15 iso 2-OH.

the presence of four groups of strains. Superimposition of groups on the graph showing the first two components allowed discriminating carbon sources to be easily identified. The six BBO strains studied clustered in group 1, together with *Xanthomonas sacchari*, *Xanthomonas arboricola* pv. *juglandis* and *Xanthomonas pisi*; *X. campestris* pv. *campestris*, *X. codiae*, *Xanthomonas melonis* and *Xanthomonas cassavae* formed group 2. *Xanthomonas albilineans* was the only species of group 3; and group 4 included the other 11 type strains of *Xanthomonas* species. The most characteristic substrates were D-saccharic acid for group 2 and D-mannitol and D-gluconic acid for group 3. Strains that belonged to groups 1 and 4 did not utilize these three carbon sources. Group 1 strains utilized cellobiose, D-galactose, gentiobiose, lactulose and α -ketoglutaric acid, whereas strains of group 4 did not. Within group 1, BBO strains could be differentiated from *X. sacchari* by the utilization of quinic acid, from *X. arboricola* pv. *juglandis* by the utilization of dextrin, glycogen and maltose and from *X. pisi* by the utilization of *cis*-aconitic acid.

Molecular characterization

Sequencing of the 16S rRNA gene. A fragment of 1499 bp corresponding to positions 28–1529 of the 16S rDNA of *Escherichia coli* was amplified by PCR and sequenced for each of the four BBO strains. The 16S rDNA sequences of the four BBO strains analysed were 100% identical, but this sequence differed by at least one base from the equivalent sequence of each of the 21 species of *Xanthomonas* studied. The 16S rDNA sequence of the BBO strains showed similarities to sequences of the other xanthomonads ranging from 96.7% (*X. hyacinthi*

and *X. translucens*) to more than 99% (*Xanthomonas vasicola*, *X. arboricola*, *Xanthomonas hortorum*, *X. campestris*, *X. cynarae*, *Xanthomonas oryzae*, *X. pisi*, *X. cassavae*, *Xanthomonas vesicatoria*, *Xanthomonas cucurbitae*, *Xanthomonas bromi*, *X. codiae*, *X. populi*, *X. fragariae* and *X. axonopodis*).

DNA–DNA hybridization and ΔT_m values. DNA–DNA hybridization values between strain 6369 and the other BBO strains were higher than 70% (Table 1). In contrast, DNA–DNA hybridization values between strain 6369 and the 21 type strains of *Xanthomonas* species and the type strains of three pathovars of *X. axonopodis* were lower than 70% (Table 2). The percentage reassociation of strain 6369 was 55% with *X. axonopodis* pv. *axonopodis* strain 4924 and 11–49% with the other 20 type strains of *Xanthomonas*. The pathotype strains of three pathovars of *X. axonopodis* (pv. *manihotis*, pv. *begoniae* and pv. *phaseoli*) were 65–70% related to strain 6369. The ΔT_m values were 0.2 °C between strains 6369 and 2534 (pv. *phaseoli*) and 0.5 °C between strains 6369 and 4924 (pv. *axonopodis*).

DNA base composition. The DNA G+C contents of strains 6107, 6369, 6381 and 6385 were respectively 62.7, 62.7, 62.3 and 62.3 mol%.

FAFLP analysis. Data derived from 15 different runs performed with strain 6369 allowed non-reproducible band positions to be identified; these were not considered for band scoring of all runs and strains. The composition of FAFLP patterns was not influenced by the DNA extraction method and the different runs differed only by one or two bands among 43 bands. The high reproducibility of FAFLP was further supported by maximal bootstrap values when data from DNA samples extracted by both techniques were included in the data matrix (results not shown).

A total of 147 fragments was generated and all these fragments were polymorphic. FAFLP analysis generated 62 fragments for the BBO strains; 63% of these fragments were polymorphic. All BBO strains clustered with pathotype strains of *X. axonopodis* pv. *vesicatoria*, pv. *citrumelo* and pv. *alfalfae* (Fig. 1). A fuller phylogenetic tree is available as Supplementary Fig. C in IJSEM Online.

The bootstrap value of the node grouping these strains was 63%. Some pathotype strains of *X. axonopodis* pathovars were closely related (e.g. pv. *citri* and pv. *malvacearum*, pv. *alfalfae* and pv. *citrumelo*). Among all *Xanthomonas* species, *X. axonopodis* was the most closely related to the BBO strains. Furthermore, the type strains of *Xanthomonas* species were weakly related to each other.

CGM values between strains belonging to different *Xanthomonas* species ranged from 6.0 (between *X. oryzae* 2532^T and *X. axonopodis* pv. *alfalfae* 3836) to 13.8% (between *X. fragariae* 2157^T and *X. axonopodis* pv. *begoniae* 2524). The mean CGM values were 3.6 $\pm$ 0.6% between BBO strains and

Fig. 1. Simplified unweighted neighbour-joining tree showing the relationships between type strains of *Xanthomonas* species and BBO strains based on 147 FAFLP fragments.

5.5 ± 1.5 % between strains of *X. axonopodis*. Comparison of CGMs and DNA–DNA reassociation values showed that genome mispairing was negatively and linearly correlated to DNA–DNA hybridization values ($r^2 = 0.80$).

DISCUSSION

BBO is an emerging disease that may have an increasing economic impact in future. The causal agent, recently isolated from Welsh onion in Japan, has been described as *Xanthomonas campestris* pv. *allii* pv. nov. (Kadota *et al.*, 2000). The species allocation made by these authors, based solely on a biochemical and physiological characterization, does not meet the recommendations of Wayne *et al.* (1987) and Stackebrandt *et al.* (2002). Our study was performed to assess the accurate taxonomic position of the bacterium. To achieve this, a worldwide collection of strains of xanthomonads pathogenic to *Allium* species was examined by appropriate methods recommended for the taxonomic description of bacterial species (Stackebrandt *et al.*, 2002). Moreover, international standards for naming pathovars of phytopathogenic bacteria were also used (Dye *et al.*, 1980).

In accordance with the results of phenotypic characterization, 16S rDNA sequence similarities indicated that the BBO strains belong to the genus *Xanthomonas* and should be added to the *X. campestris* core described previously by Hauben *et al.* (1997). However, 16S rDNA sequence analysis is known to be limited in terms of taxonomic resolution at the species level for members of the genus *Xanthomonas* (Moore *et al.*, 1997).

Taxonomic relationships at the species level were assessed by DNA–DNA hybridization, thermal stability of DNA reassociation and FAFLP analysis. All BBO strains belonged to a single genomospecies, as indicated by DNA reassociation values with BBO strain 6369, which were 74–100 %. Several reports indicated that xanthomonads associated with a single disease phenotype can belong to more than one *Xanthomonas* genomospecies (Jones *et al.*, 2000; Vauterin *et al.*, 1995).

The type strains of *Xanthomonas* species were only 11–55 % related to strain 6369. The most closely related type strain was *X. axonopodis* (with 55 % DNA reassociation). This is below the 70 % threshold allowed for species definition (Wayne *et al.*, 1987). However, three other pathovars belonging to *X. axonopodis* included in our study showed 65–70 % DNA relatedness to strain 6369. The close relationship between the BBO strains and *X. axonopodis* was confirmed by the low ΔT_m values (0.2 and 0.5 °C) obtained with strains 2534 (*X. axonopodis* pv. *phaseoli*) and 4924 (*X. axonopodis* pv. *axonopodis*), respectively. These values, which are much lower than the 5 °C ΔT_m threshold (Wayne *et al.*, 1987), indicate that the BBO causal agent should be included in the species *X. axonopodis* [i.e. group 9 defined by Vauterin *et al.* (1995)]. Other xanthomonads (e.g. pv. *coracanae*, pv. *phaseoli*, pv. *sesbaniae*, pv. *vitians*) with less than 70 % DNA relatedness to *X. axonopodis* have previously been allocated to this species (Trébaol *et al.*, 2000; Vauterin *et al.*, 1995). Thus, our data further confirm that *X. axonopodis* has a wider internal genetic variability than is commonly found in *Xanthomonas* species. Results from FAFLP analysis further confirmed that BBO strains were

more similar to *X. axonopodis* than to any other *Xanthomonas* species. The unweighted neighbour-joining tree inferred from FAFLP patterns showed that BBO strains clustered with three pathovars of *X. axonopodis*, pv. *vesicatoria*, pv. *citrumelo* and pv. *alfalfae*. This is in full agreement with the results of Janssen *et al.* (1996), who used a different set of restriction enzymes and selective primers for AFLP and showed that *X. axonopodis* formed two clusters. One of them, cluster I, consisted of three subdivisions, α , β and γ ; the γ subdivision contained the two pathovars *alfalfae* and *citrumelo* (pv. *vesicatoria* was not included in their study) with a linkage level of $71.0 \pm 3.7\%$. In our case, the linkage level, calculated with the same index as Janssen *et al.* (1996), between strains of the 'alfalfae-citrumelo-vesicatoria-BBO' group was 67.6% (data not shown). Similarly, Rademaker *et al.* (2000) analysed 80 AFLP and 178 rep-PCR (rep-, BOX- and ERIC-PCR) fingerprints of *Xanthomonas* strains. *X. axonopodis* consisted of six genetic clusters. Pathovars *alfalfae*, *citrumelo* and *vesicatoria* all belonged to the same group, designated 9.2 (Rademaker, 2000). In addition to the results of Rademaker *et al.* (2000), our study indicates that AFLP data are well correlated with DNA-DNA hybridization data at the species level and, therefore, the allocation of BBO strains to the species *X. axonopodis* was confirmed.

The CGM value provides an accurate measure of the genetic distance between genomes (Mougel *et al.*, 2002). From data obtained on *Agrobacterium* (Mougel *et al.*, 2002), a CGM of 13.6% corresponded to the usual 70% hybridization threshold above which strains are considered to belong to a single species (Wayne *et al.*, 1987). In our study, the maximal CGM value obtained between *Xanthomonas* type strains and the BBO strain used as a probe for DNA-DNA hybridization was 9.1% , indicating that AFLP profiles of distinct species of *Xanthomonas* may be closer than those of distinct species of *Agrobacterium*. Furthermore, a highly significant linear relationship between CGM and DNA-DNA hybridization values was found over the whole range of values. Our data further confirm the conclusions of Mougel *et al.* (2002), who stated that the correspondence between CGM and the threshold of 70% DNA relatedness must be established with care for different bacterial genera.

Correspondence analysis was used to compare metabolic fingerprints obtained from the type strains of *Xanthomonas* species and six BBO strains. This statistical method has already been used for phenotypic characterization of bacterial populations, and its major advantages for eliminating bias related to the Biolog technique have already been discussed (Frey *et al.*, 1997; Garland, 1996). BBO strains grouped with *X. sacchari*, *X. arboricola* pv. *juglandis* and *X. pisi*. Correspondence analysis resulted in determination of the most useful carbon sources for identification purposes. However, metabolic fingerprints of type strains of *Xanthomonas* species obtained in this study differed from those reported by Trébaol *et al.* (2000) and Vauterin

et al. (1995), which also differed from each other. Scortichini & Rossi (1995) reported that subculturing influenced carbohydrate utilization profiles of *X. campestris* pv. *campestris*. Recently, Riley *et al.* (2001) reported that carbon utilization profiles were variable among *Ralstonia* populations evolved in defined environments for 1000 generations. Most of the type strains of *Xanthomonas* species were isolated more than 30 years ago, and changes in phenotypic traits could have occurred. Such changes could be related to the mode and duration of conservation, the number of successive strain subcultures and occurrence of stresses during exchanges of cultures, etc. The lack of stability of phenotypic traits probably represents a bias when xanthomonad strains from culture collections are compared, and emphasizes the difficulty of comparing metabolic fingerprints obtained in a laboratory with previously published information. As a consequence, the lack of reproducibility questions the power of the Biolog technique for locating unknown xanthomonads in the classification described by Vauterin *et al.* (1995).

Although data from the fatty acid analysis were not in agreement with results from DNA-DNA hybridization and FAFLP, this analysis was useful in showing that most of the BBO strains form a tight group. All but two BBO strains formed a cluster with a Euclidian distance of less than 6.3 . According to MIDI, strains with a Euclidian distance of approximately less than 6.0 are considered to be the same subspecies or biotype. The two strains originating from Japan (6107 and 6108) were outliers. Strain 6107 has been chosen by Kadota *et al.* (2000) as the pathotype strain. Based on fatty acid methyl ester analysis, this strain is not typical of the pathovar. Therefore, strain 6369, which was shown to be typical of the pathovar by all techniques used in this study, was chosen as the probe for DNA-DNA hybridization. It is proposed that strain 6369 should be established as the neopathotype strain for pv. *allii*.

The present study was also initiated to clarify the host range of BBO strains. All BBO strains were pathogenic to all *Allium* species assayed. This result is in agreement with those reported by Bowen *et al.* (1998), but diverged from those described by Kadota *et al.* (2000). The latter authors determined that BBO strains were not pathogenic to chive using *Allium schoenoprasum* L. var. *foliosum*. In the present work, *A. schoenoprasum* L. cv. Civette, one of the most common cultivars worldwide, was used. *A. schoenoprasum* L. grows in a variety of habitats, and many local ecotypes have arisen (Poulsen, 1990). This could explain why BBO strains are pathogenic to *A. schoenoprasum* L. but not to the variant *foliosum*. O'Garro & Paulraj (1997) listed bean and other plant species of the family Fabaceae as hosts of BBO strains in Barbados. In the present study, bean leaves inoculated with BBO strains (including isolates from Barbados) sometimes exhibited small, water-soaked lesions that remained for a few days before turning necrotic, without further expansion of the lesions (data not shown). Our results are consistent with those reported by Alvarez

et al. (1978). Furthermore, several xanthomonads were reported to induce small, water-soaked lesions when inoculated onto bean (Schnathorst, 1966). Such lesions, which clearly differed from those observed during the compatible interaction between *X. axonopodis* pv. *phaseoli* and bean, were regarded as non-host interactions. Based on all the above results, the name *Xanthomonas axonopodis* pv. *allii* is proposed as the causal agent of BBO.

Description of *Xanthomonas axonopodis* pv. *allii*

Xanthomonas axonopodis pv. *allii* [al'i.i. N.L. adj. *allii* from *Allium*, the genus of onion (*Allium cepa* L.), Welsh onion (*Allium fistulosum* L.) and garlic (*Allium sativum* L.)].

Gram-negative rods, motile with one polar flagellum; obligate aerobes with oxidative metabolism of glucose and catalase-positive. Does not produce cytochrome *c* oxidase, nitrate reductase, arginine dihydrolase, urease, acetoin or indole. Colonies grown on YPGA after 2–3 days at 28 °C are yellow, convex, round and mucoid. Hydrolyses gelatin, casein, aesculin, starch, cellulose and Tween 80, produces H₂S from cysteine and shows pectinolytic activity at pH 5.0, 7.0 and 8.5. Cells tolerate up to 3% NaCl and grow on YPGA at 35 °C but not at 40 °C. Fluorescent pigments are not observed. Produces acid from xylose and ribose, but not from melezitose on ARJ medium. Metabolic activity (as assessed by Biolog GN microplates) is shown on the following carbon substrates: dextrin, glycogen, Tween 40, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, lactulose, maltose, D-mannose, D-psicose, sucrose, D-trehalose, methyl pyruvate, mono-methyl succinate, *cis*-aconitic acid, α -ketobutyric acid, α -ketoglutaric acid, malonic acid, succinic acid, bromo-succinic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-proline, L-serine and L-threonine. The following carbon substrates are not utilized: α -cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, m-inositol, α -D-lactose, D-mannitol, methyl β -D-glucoside, L-rhamnose, D-sorbitol, xylitol, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ -hydroxybutyric acid, *p*-hydroxyphenyl-acetic acid, itaconic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-histidine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol and glucose 6-phosphate.

Induces typical BBO symptoms on leaves of onion (*Allium cepa* L.), garlic (*Allium sativum* L.), chive (*Allium schoenoprasum* L.), leek (*Allium porrum* L.), shallot (*Allium cepa* var. *ascalonicum* Backer) and Welsh onion (*Allium fistulosum* L.). Characteristic leaf lesions start as lenticular, water-soaked spots, which extend and eventually coalesce. Lesions progress into dry, chlorotic spots with tissue collapse and holes. Severe infections induce leaf dieback, resulting in a reduction in bulb size. Not pathogenic to

Allium schoenoprasum L. var. *foliosum* Rgl., Chinese chive (*Allium tuberosum* Rottler), hyacinth (*Hyacinthus orientalis* L.) or bean (*Phaseolus vulgaris* L.). The DNA G + C content is 62.3–62.7 mol%. The proposed neopathotype strain is CFBP 6369 = LMG 21894.

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