European Journal of Plant Pathology **104:** 477–487, 1998.

Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia

C. Vernière[1], J.S. Hartung[2], O.P. Pruvost[3], E.L. Civerolo[4], A.M. Alvarez[5], P. Maestri[6] and J. Luisetti[6]

[1] *Station de Recherche Agronomique INRA-CIRAD, 20230 San Giuliano, Corsica, France;* [2] *USDA-ARS, Room 238, Building 010-A, BARC-West, Beltsville, MD 20705, USA;* [3] *CIRAD-FLHOR, BP 180, 97455 Saint Pierre cédex, La Réunion, France;* [4] *USDA-ARS, Department of Plant Pathology, University of California, Davis, CA, USA;* [5] *Department of Plant Pathology, University of Hawaii, Honolulu, HI 96822, USA;* [6] *INRA, BP 57, 49071 Beaucouzé, France*

Accepted 26 March 1998

Key words: citrus bacterial canker, detection, epidemiology, monoclonal antibodies, variability, taxonomy

Abstract

Strains of *Xanthomonas axonopodis* pv. *citri* were isolated from Mexican lime (*Citrus aurantifolia*) trees in several countries in southwest Asia. These strains produced typical erumpent bacterial canker lesions on Mexican lime but not on grapefruit (*C. paradisi*). Lesions on grapefruit were watersoaked and blister-like in contrast to the typical erumpent lesions seen after artificial inoculation with all described pathotypes of *X. axonopodis* pv. *citri*. This group of strains hydrolysed gelatin and casein and grew in the presence of 3% NaCl as is typical of *X. axonopodis* pv. *citri* pathotype A. RFLP analyses and DNA probe hybridization assays also gave results consistent with *X. axonopodis* pv. *citri* pathotype A. Metabolic fingerprints prepared with the Biolog® system showed similarities as well as differences to *X. axonopodis* pv. *citri* pathotype A. In spite of the physiological and genetic similarities to pathotype A of *X. axonopodis* pv. *citri*, these strains had no or very little affinity for polyclonal antiserum prepared against any of the reference strains of *X. axonopodis* pv. *citri* and also did not react with monoclonal antibody A1, an antibody that detects all strains of pathotype A of *X. axonopodis* pv. *citri*. These strains were also insensitive to bacteriophage Cp3 like *X. axonopodis* pv. *citri* pathotype A and unlike *X. axonopodis* pv. *citri* pathotype B. We conclude that these strains, designated Xcc-A*, represent a variant of *X. axonopodis* pv. *citri* pathotype-A with pathogenicity limited to *C. aurantifolia*. The existence of extensive genotypic and phenotypic variation within pathotype A of *X. axonopodis* pv. *citri* was unexpected and further complicates the systematics of this species.

Citrus bacterial canker (CBC), caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) is a widespread disease in citrus producing areas of the tropical and the subtropical world. It probably originated in Southeast Asia or India and occurs in more than 30 countries (Civerolo, 1984; Civerolo, 1994). Different forms of CBC (A-C), corresponding to different pathotypes of *Xac* have been described. The Asiatic form, CBC-A (*Xac* pathotype A; *Xac*-A), is both the most widespread and is the most economically important form. The host range of *Xac*-A strains is broader than that of the other pathotypes (Civerolo, 1984; Stall and Civerolo, 1991). The typical lesions are erumpent, callus-like, with watersoaked, oily, tan colored margins that become brown with age. ‘Cancrosis B’ or CBC-B (*Xac* pathotype B; *Xac*-B) has been found in a few countries in South America and has a more restricted host range. Lemons *(Citrus limon* (L.) Burm) are the most susceptible citrus species while grapefruit *(C. paradisi* Macf.) and sweet orange *(C. sinensis* (L.) Osb.) are little affected in the groves. The CBC-C form or Mexican lime canker (*Xac* pathotype C; *Xac*-C) affects only Mexican lime (*C. aurantifolia* (Christm.) Swingle) in Brazil. The symptoms induced by *Xac* -B

Table 1. Strains of *Xanthomonas axonopodis* pv. *citri* used in this study

strains	CBCD group	Origin	year	host
Xc269	A*[1]	Saudi Arabia	1988	*C. aurantifolia*
Xc270	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc271	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc272	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc273	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc274	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc275	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc276	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc277	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc278	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc279	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc280	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc281	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc282	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc283	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc289	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc290	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc291	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc292	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc293	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc322	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc323	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc328	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc329	A*	Saudi Arabia	1988	*C. aurantifolia*
Xc164	A*	India	1988	*Citrus* sp.
Xc165	A	India	1988	*C. aurantifolia*
Xc166	A*	India	1988	*C. aurantifola*
Xc167	A	India	1988	*C. limon*
Xc168	A	India	1988	*Poncirus trifoliata*
Xc169	A*	India	1988	*C. aurantifola*
Xc170	A	India	1988	*C. sinensis*
CFBP2851	A	India	1988	*Citrus* sp.
JF90-2	A*	Oman	1986	*C. aurantifola*
JF90-3	A*	Oman	1986	*C. aurantifola*
JF90-5	A	Oman	1986	*C. aurantifola*
JF90-8	A	Oman	1986	*C. aurantifola*
JF90-12	A	Oman	1986	*C. aurantifola*
JM47-2	A*	Iran	1991	*C. aurantifola*
Xc100	A	Pakistan	1984	*Citrus* sp.
Xc158	A	Pakistan	1988	*C. sinensis*
Xc98	A	Yemen	1982	*C. aurantifolia*
Xc251	A	Yemen	1988	*Citrus* sp.
Xc252	A	Yemen	1988	*Citrus* sp.
Xc62	A	Japan	1978	*Citrus* sp.
Xc64	B	Argentina	1979	*C. limon*
Xc69	B	Argentina	1979	*C. limon*
Xc84	B	Uruguay	1984	*C. limon*
Xc90	D	Mexico		*C. aurantifolia*
Xc70	C	Brazil		*C. aurantifolia*

[1]: * initially suspected as *Xac* -A strains but with a unique phenotype.

and -C are very similar to those induced by *Xac*-A on hosts where symptoms are induced (Civerolo, 1994; Stall and Civerolo, 1991). Another disease named bacteriosis (*Xac* pathotype D) was reported on Mexican lime in Mexico. The validity of 'pathotype D' is problematic since only one pathogenic bacterial strain was isolated. The disease with which 'pathotype D' was originally associated is now called *'mancha foliar de los citricos'* and is attributed to *Alternaria limicola* (Becerra et al., 1988; Palm and Civerolo, 1994).

A novel bacterial disease of citrus was described in 1984 in Florida. Foliar symptoms include flat necrotic lesions with watersoaked margins and are found principally on the rootstock 'Swingle' citrumelo (*C. paradisi* X *Poncirus trifoliata*) in citrus nurseries (Graham and Gottwald, 1991; Stall and Civerolo, 1991). This disease, called citrus bacterial spot (CBS) is caused by strains of *X. axonopodis* and is of little economic importance compared to CBC-A. Many studies have made possible a thorough characterization of both CBC and CBS strains and have revealed variability among the different pathotypes of *Xac* and among *X. axonopodis* strains causing CBS as well as extensive differences between all CBC- and CBS-inducing strains (see reviews Civerolo, 1984; Graham and Gottwald, 1991; Stall and Civerolo, 1991).

These conclusions are confirmed by accumulated data from physiological tests (Vernière et al., 1991; Vernière et al., 1993), phage typing (Wu et al., 1993), total protein profiles after SDS-PAGE, DNA-DNA solution hybridizations (Vauterin et al., 1991; Egel et al., 1991), plasmid DNA fingerprints (Pruvost et al., 1992), plasmid-based hybridization probes (Hartung, 1992) and polymerase chain reaction-based assays (Hartung et al., 1993), and restriction enzyme analysis of amplified DNA fragments of an *hrp*-related DNA sequence (Leite et al., 1994). Moreover, a pathogenicity gene *pth*A which is required to elicit typical symptoms of CBC was isolated from a pathotype A strain of *Xac* (Swarup et al., 1991; Swarup et al., 1992). Hybridizations of total DNA with a *pth*A fragment revealed different profiles between CBC-A strains and cancrosis B and canker C strains. No hybridization was observed with *X. a.* pv. *citrumelo* strains (Swarup et al., 1992).

Based on these studies one may distinguish three groups of strains of *X. axonopodis* involved in citrus diseases: *Xac*-A, *Xac*-B (includes pathotypes C and D) and *X. axonopodis* strains associated with CBS (Stall and Civerolo, 1991). However, the taxonomy of these strains has been controversial (Gabriel et al., 1990; Vauterin et al., 1990; Young et al., 1990; Young et al., 1991). A recent reclassification of the genus *Xanthomonas* based on DNA-DNA hybridization and metabolic activity studies confirmed this interpretation (Vauterin et al., 1995). At that time, xanthomonads associated with citrus were moved from *X. campestris* into the species *X. axonopodis*. Pathotype A, and pathotypes B and C and the *X. axonopodis* CBS strains may now be referred to respectively as *X. axonopodis* pv. *citri*, *X. a.* pv. *aurantifolii* and *X. a.* pv. *citrumelo* (Vauterin et al., 1995). However, this proposal was not validated by the sub-committee on taxonomy of plant pathogenic bacteria (Young et al., 1996).

During the last decade, CBC was reported in southwest Asia including Saudi Arabia, Oman, Iraq (Ibrahim and Bayaa, 1989), the United Arab Emirates (El Goorani, 1989) and Iran (Alizadeh and Rahimian, 1990). We describe here the characterization of phenotypically atypical *Xac* strains isolated from Mexican lime in Saudi Arabia, Oman, Iran, and India. Although closely related to *Xac*-A, these strains can be readily distinguished from previously known strains of *Xac*-A based on their atypical pathogenicity on *Citrus* and *Poncirus* species as well as on some hybrids.

Materials and methods

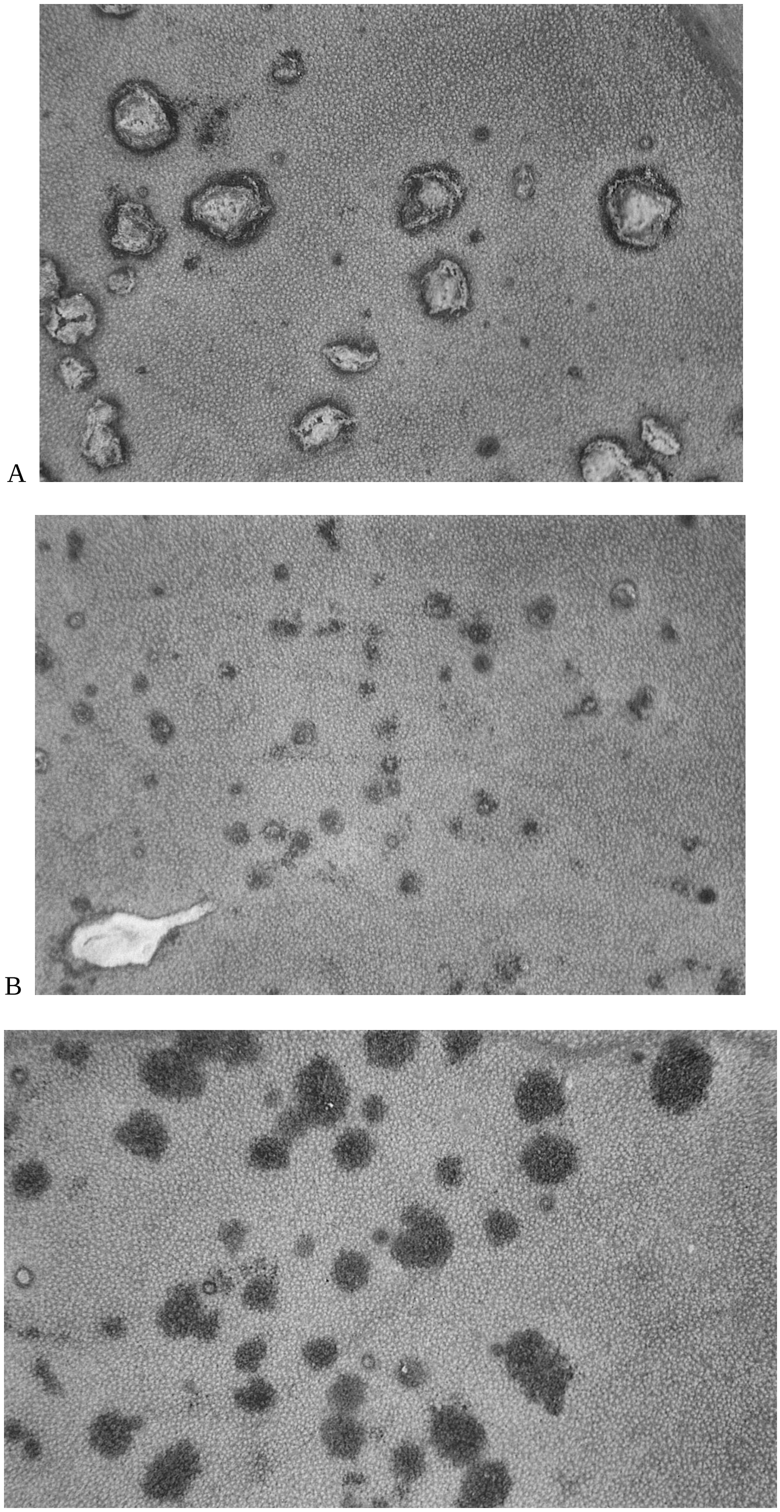
Bacterial strains

Strains from southwest Asia presenting a distinct combination of host range and symptomatology, referred to below as *Xac* -A*, were compared to reference strains of *Xac* -A and *Xac* -B (Table 1). Strains from Saudi Arabia and Oman were isolated in 1988 and 1986 respectively. Additional strains from Iran and India were also included. All of these strains were isolated from Mexican lime (Table 1).

Pathogenicity tests

Attached leaf assay

Immature fully expanded 'Mexican' lime and 'Marsh' grapefruit seedling leaves were infiltrated by pressing the opening of a syringe without a needle gently against the abaxial leaf surface supported by one finger. Inoculum was prepared from 24 h cultures grown on PYDAC medium (Vernière et al., 1991). Cell suspensions were adjusted turbidimetrically and diluted to contain approximately 10^5 CFU ml^{-1}. Plants were maintained in the greenhouse at 28-30 °C.



A B C

Figure 1. Differential symptomatology of *Xac*-A and *Xac*-A* strains of *Xac* on attached leaves of ‘Duncan’ grapefruit. a) – typical callus-like lesions with watersoaked margins (*Xac*-A strain Xc62); b) – blister-like lesion with more or less water soaked margin (*Xac*-A* type 1). c) – flat and watersoaked lesions (*Xac*-A* type 2).

Detached leaf assay

Immature fully expanded 'Mexican' lime and 'Marsh' grapefruit leaves were sterilized by soaking for 2 min in 1% sodium hypochlorite followed by rinsing in sterile distilled water. Leaves were placed on the surface of 1% water agar with their abaxial surfaces facing upwards. Ten wounds were made per leaf with a needle and droplets (10 μl) of bacterial suspensions containing approximately 10^6 CFU ml^{-1} were placed on each wound. Leaves were incubated in a growth chamber at 28 °C with a photoperiod of 12 h light and 12 h dark for 3 weeks.

Growth in planta

Attached 'Mexican' lime and 'Marsh' grapefruit leaves were inoculated as described above with the strains Xc328 and Xc329 (*Xac*-A*, from Saudi Arabia) and strain Xc62 (*Xac*-A, from Japan) as a reference strain. Leaf disks (1 cm) were removed using a corkborer 0, 1, 2, 3, 6 and 8 days after inoculation and ground individually in phosphate buffered saline (PBS (NaCl: 8 g; KCl: 0.2 g; Na_2HPO_4: 1.44 g; KH_2PO_4: 0.24 g; dH_2O: 1000 ml; pH: 7.2)). Drops (10 μl) of appropriate dilutions in PBS were deposited on PYDAC plates and incubated for 3-4 days at 28 °C. Three replicate disks per strain and per host were taken from different plants at each date. Bacterial populations were expressed as the log CFU cm^{-2}. Data were subjected to an analysis of variance (ANOVA) with time after inoculation as a repeated measure (Stat View 4.02, Abacus Concept, Montclair, California, USA).

Biochemical, physiological and genetic tests

Physiological tests

Tests were performed as described by Vernière et al. (1991) and included hydrolysis of gelatin and casein and growth in the presence of 3% NaCl.

Metabolic fingerprinting

Characterization of the strains was carried out using Biolog® GN microplates (Biolog Inc., Hayward, CA). Absorbance was measured with a Dynatech MR-700 microtiter plate reader using Microlog 2N®software (Biolog). Carbon oxidation profiles were generated and identification of strains was done with the same software using the commercial database supplemented with data from our laboratory strains as described previously (Vernière et al.,1993).

Phage sensitivity

Bacteriophages Cp1, Cp2, Cp3 were deposited separately in 10 μl drops on the surface of *Xac*-seeded soft agar overlays. Plaque formation was observed at the routine test dilution (RTD), 10 x RTD and 1/10 x RTD (Civerolo, 1990).

Serological tests

Indirect ELISA tests were carried out using rabbit polyclonal antisera against strains Xc62 (*Xac* pathotype-A), Xc69 (*Xac* pathotype-B), and Xc70 (*Xac* pathotype-C) (Civerolo and Fan, 1982).

Monoclonal antibodies (mabs) X1, A1, A2, B1, B2, B3, C1 and CBS1 (Alvarez et al., 1991) were also used in indirect ELISA tests. Two new mabs were prepared against strain Xc274 from Saudi Arabia. These two clones were designated A3 and A4, respectively. Mab preparation and tests were performed as described by Alvarez et al. (1985).

DNA analyses

Restriction Fragment Length Polymorphism (RFLP) analyses were performed using seven cosmid clones obtained from strain Xc62 as described previously (Hartung and Civerolo, 1989). Probes pFL62.42 and pFL1 derived from indigenous plasmids of strain Xc62 were used in dot-blot hybridizations as described by Hartung (1992). Polymerase chain reaction assays were performed using primer pair 4/7 as described previously (Hartung et al., 1993)

Results

Pathogenicity tests

Results from preliminary virulence studies prompted a more detailed characterization of *Xac* strains from southwest Asia, designated *Xac*-A*. All of the strains (24/24) from Saudi Arabia as well as the strain from Iran and two strains each from India and Oman displayed altered pathogenic reactions compared to that of reference *Xac*-A strains. In attached leaf assays, this group of strains induced leaf lesions on Mexican lime that were raised and erumpent, with callus-like tissues, narrow watersoaked margins, and light yellow chlorotic haloes (Figure 1a) as are typical CBC-A lesions. However, in attached grapefruit leaf assays, a species highly susceptible to *Xac* -A, lesions were morphologically different from typical CBC-A lesions. Two types of atypical lesions were observed on

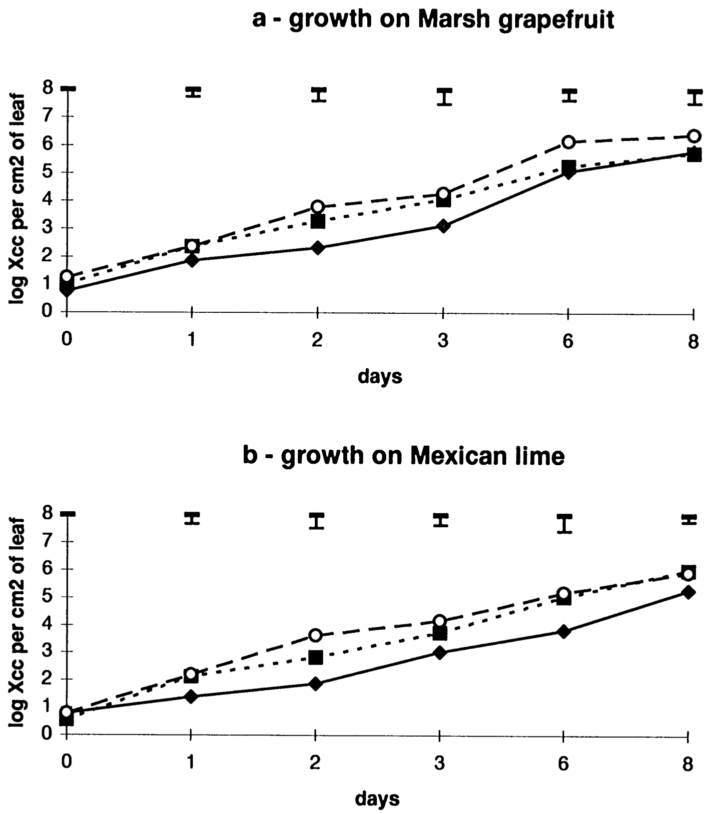


Figure 2. Growth *in vivo* of *X. axonopodis* pv. *citri* A* strains Xc328 (■) and Xc329 (♦) with a reference *Xac* A strain Xc62 (○) on (a) grapefruit and (b) Mexican lime. Bars represent the LSD at a 5% level.

attached grapefruit leaves. The first type was characterized by slightly raised, blister-like lesions. These lesions were more or less watersoaked, but never erumpent (Figure 1b). The second type was flat and watersoaked. Sometimes the center of the lesion was necrotic (Figure 1c). Other strains from India, Oman, Pakistan and Yemen induced lesions typical of CBC-A on grapefruit and other citrus varieties. Attached leaf inoculations with the *Xac*-A* strains on 'Swingle' citrumelo, *Citrus volkameriana* and 'Carrizo' citrange (*P. trifoliata* x*C. sinensis*) showed a similar atypical symptomatology. Because of the atypical leaf lesions observed in attached leaf assays, bacterial growth *in vivo* was observed in grapefruit and 'Mexican' lime leaves for both *Xac*-A and *Xac*-A* (Figure 2). No statistically significant differences were observed between the growth rate of the *Xac*-A* strains and that of the reference *Xac*-A strain in either grapefruit or Mexican lime leaves.

In detached leaf assays the different symptoms induced by *Xac*-A and *Xac*-A* were very clear-cut and somewhat different from symptoms observed in attached leaf inoculations. All strains produced erumpent callus-like tissue on detached 'Mexican' lime leaves. In contrast, the *Xac*-A* strains caused no or limited watersoaking on detached grapefruit leaves, while the typical strains caused the erumpent tissue reactions (Figure 3).

Biochemical, physiological and genetic tests

In contrast with the atypical pathogenicity of the *Xac*-A* strains, results of biochemical tests, bacteriophage typing, and DNA analyses were generally consistent with those of *Xac*-A strains. The observed hydrolysis of gelatin and casein and the growth on 3% NaCl were characteristic of *Xac*-A strains. The insensitivity to bacteriophage Cp3 separated the *Xac*-A* strains from the *Xac*-B group of strains (Table 2). The coefficients of similarity (Nei and Li, 1979) to strain *Xac*-A reference strain Xc62 obtained from RFLP analysis clearly grouped the *Xac*-A* strains with typical *Xac*-A strains, and separated them from *Xac*-B and *Xac*-C strains (Table 2). DNA from the *Xac*-A* strains also

Table 2. Characterization of the southwest Asian type *X. axonopodis* pv. *citri Xac*-A* strains by phage typing, biochemical tests, DNA analyses and serological tests

	Xac-A	*Xac*-A*	*Xac*-B [1]	*Xac*-C[1]
phage sensitivity				
- Cp1	v	-	-	-
- Cp2	v	-	-	-
- Cp3	-	-	+	-
biochemical tests				
- hydrolysis of gelatin	+	+	-	-
- hydrolysis of casein	+	+	-	+
- growth on NaCl 3%	+	+	-	-
Dot blot hybridizations[2]				
- pFL1	++	++	+	+
- pFL62.42	++	++	+	+
RFLP (similarity coefficients F)				
- to CBC-A (Xc62)	0.83–0.97	0.76–1.00	0.61–0.62	0.62
- to CBC-B (Xc69)	0.40	0.41–0.44	0.89–0.96	0.85
- to CBC-B (Xc84)	0.49	0.41–0.43	ND	ND
- to CBC-C (Xc70)	0.53	0.46–0.47	0.82–0.89	ND
PCR identification (primer 4/7)	+	+	v	v
ELISA with polyclonal antibodies				
- anti Xc62	1.00[3]	0.10–0.28	ND	ND
- anti Xc69	0.57[3]	0.36–0.64	1.00[3]	ND
- anti Xc70	0.46[3]	0.19–0.36	ND	1.00[3]
ELISA with monoclonal antibodies				
- X1	+	+	+	+
- A1	+	-	-	-
- A2	v	-	-	-
- B1, B2, B3	-	-	v[4]	-
- C	-	-	-	v
- CBS1	-	-	-	-

+ = positive, - = negative, v = variable responses among the strains.
[1] data from Vernière et al., 1991; Hartung and Civerolo, 1989; Hartung et al., 1993; Alvarez et al., 1991. Some of these data were rechecked in the present work.
[2] : ++ = intense spot, + = weak spot.
[3] absorbance values are given relative to the homologous strains Xc62 (A), Xc69 (B), Xc70 (C).
[4] *Xac*-B isolates are positive for at least one 'B' mab.
ND: no data.

hybridized with two probes specific for *Xac*, pFL1 and pFL62.42 and was detected by PCR as is typical for *Xac* -A (Table 2).

However, metabolic fingerprints based on carbon source oxidation usually did not identify the *Xac*-A* strains as *Xac*-A. An identification of *Xac*-A was given in only 4.3% of the observations while 53.4% of the observations resulted in an identification as *X. campestris* pv. *dieffenbachiae* 'B'. The *Xac*-A* strains were also never identified as *Xac*-B or as strains of *X. axonopodis* that cause CBS. Based on the G-test (Sokal and Rohlf, 1969), the *Xac*-A* strains as a group were statistically distinguishable from *Xac*-A strains as a group by differential utilization of 5 carbon sources out of the 95 tested with the Microlog GN plate (Table 3). Nevertheless, the *Xac*-A* strains shared the typical *Xac*-A group profile of assimilation of L-Fucose, D-Galactose and Alaninamide (Vernière et al., 1993) (Table 4).

Serological tests

In spite of the physiological, bacteriophage typing, RFLP and PCR data that placed the *Xac*-A* strains with *Xac*-A, no strong affinity of *Xac*-A* strains for polyclonal antibodies raised against any *Xac* patho-

Table 3. Oxidation of carbon sources by southwest Asian (*Xac*-A*) and reference strains of *X. axonopodis* pv. *citri* pathotype A using the Biolog® GN plate system

Xac -strains	α-D-Lactose lactulose	Propionic acid	D,L-α-Glycerol phosphate	Glucose-1-phosphate	Glucose-6-phosphate
Reference *Xac* -A strains n=141[1]	83[2] S***[3]	77 S***	9 S***	24 S***	10 S***
Xac-A* strains n=29	42	43	66	69	42

[1] n: number of strains tested. Data are combined from this study and from that of Vernière et al., 1993.
[2] Data are expressed as percentage of strains tested that oxidized the carbon sources indicated.
[3] S: significant statistical differences in each column using G-test statistic between the two groups of strains (*** = p <0.001, ** = p < 0.01) (Sokal and Rohlf, 1969).

Table 4. Oxidation of three carbon sources by reference strains of *X. axonopodis* pv. *citri* pathotypes A, B and C and *X. axonopodis* pv. *citri* -A* strains isolated from southwest Asia using the Biolog® GN plate system

Xac -Pathotypes	D-Galactose	Alaninamide	L-Fucose
A (n = 141)[1]	95.7[2] (0.7)[3]	100	76.6 (11.3)
A* (n = 29)	100	100	96.5 (3.5)
B (n = 9)	0	100	0
C (n = 3)	100	0	100

[1] n = number of strains tested. Data are combined from this study and from that of Vernière et al., 1993.
[2] per cent of strains with positive results.
[3] per cent of strains with variable results for the four sets of data (two replicates and two readings).

types was detected. Antisera raised against reference strains Xc62, Xc69, and Xc70 (*Xac* -A, -B, -C, respectively) reacted very weakly with these strains (Table 2). The range of absorbance values obtained with the *Xac*-A* strains using the polyclonal antibody anti Xc69 (0.36-0.64) is higher than that showed using the antibody developed against the reference *Xac*-A strain Xc62 (0.10-0.28). Monoclonal antibody X1 (Alvarez et al., 1991) identified the *Xac*-A* strains as *X. axonopodis*. Reactions with mabs B1, B2, B3, C and CBS1 were negative and thus uninformative except that strains belonging to *Xac*-B reacted positively for at least one of the mabs B1, B2 or B3 (Table 2). The *Xac*-A* strains did not react with either mabs A1 or A2 while the *Xac*-A strains showed an affinity for mab A1 and variable responses for A2. Also, 90% of the *Xac*-A* strains reacted with mab A3 as compared to only 14.8% of *Xac*-A strains (Table 5). All *Xac*-A* strains which did not react with mab A3 also did not react with mab A4 as was the case for strains of *Xac* - B and C.

Discussion

Strains of *Xac* originating from southwest Asia, including Saudi Arabia, Oman, Iran as well as India exhibited a pathogenicity distinctive within *X. axonopodis* pv. *citri*. They elicited typical CBC symptoms when inoculated to Mexican lime, their host of origin. These strains failed however to incite any erumpent lesions typical of CBC on grapefruit, a species very susceptible to CBC-A, and which typically produces erumpent lesions when inoculated with *Xac*. Erumpent lesions also were not induced on the other citrus varieties tested. Only atypical blister-like or watersoaked lesions have been induced on species other than Mexican lime. These lesions never developed further to give a typical canker. This symptomatology is different from *Xac*-C strains which also are specific to Mexican lime, since in contrast with the symptoms induced by *Xac*-A*, no symptoms developed after inoculation of grapefruit with *Xac*-C (Malavolta et al., 1987; Namekata and Ball, 1977; Stall et

Table 5. Serotypes of *X. axonopodis* pv. *citri* pathotype A and *X. axonopodis* pv. *citri* pathotype A* defined by monoclonal antibodies (mabs) A1, A3 and A4

	Xac -A strains (n = 81)[1]				*Xac*-A* strains (n = 29)			
mab A1	+	+	+	+	-	-	-	-
mab A3	+	-	-	+	+	-	-	+
mab A4	+	-	+	-	+	-	+	-
%	9.9	29.6	55.6	4.9	62.1	10.3	0	27.6

[1] : n = number of strains tested. Includes 14 *Xac*-A strains listed in Table 1 and 67 additional *Xac*-A strains from different origins. All the strains are A1 positive.

Figure 3. Responses on Marsh grapefruit in a detached leaf assay after two weeks (right: *Xac*-A strain, left: *Xac-A** strain.

al., 1982). *Xac*-B strains are also only pathogenic on a narrow host range in the field, but unlike the *Xac*-A* strains, when artificially inoculated on grapefruit and some other varieties, they give typical erumpent symptoms (Malavolta et al., 1987; Namekata and Ball, 1977; Stall et al., 1982). Although symptom expression is different for *Xac*-A and *Xac*-A* in grapefruit leaves, there was no measureable difference in multiplication of the bacteria in inoculated leaves (Figure 2).

Hybridizations with specific DNA probes and PCR amplification using primers specific for *Xac* all confirmed these *Xac*A* strains as *Xac* but were not conclusive criteria for assigning the strains to a pathotype. The hydrolysis of gelatin and casein and growth on medium containing 3% NaCl, as well as the RFLP profiles were, however, typical of *Xac*-A strains. Surprisingly, the *Xac*-A* strains did not react with either the mab A1 which is highly specific to *Xac*-A strains (Alvarez et al., 1991) or with polyclonal antiserum raised against *Xac*-A reference strain Xc62. Although sufficient variation within *X. axonopodis* pv. *citri* has been observed to describe pathotypes designated *Xac*-A and *Xac*-B, little variation among *Xac*-A strains has been reported previously (Gabriel et al., 1988; Hartung and Civerolo 1989; Vernière et al., 1993). Sensitivity to bacteriophage Cp1 has been associated with strains that assimilated mannitol (Goto, 1992) and with strains reacting with mab A2 (Alvarez et al., 1991). However, these phenotypes were not correlated with a specific host or region of origin. Recently, other *Xac*-A strains originating from the Mascarenes, a tiny archipelago in Indian ocean, were differentiated from other strains of *Xac*-A by their resistance to ß-lactam antibiotics (Vernière et al., 1994). Here we have characterized *Xac* strains closely related to *Xac*-A but which show a novel phenotype consisting of both a distinctive pathogenicity (host range and symptomatology) and the absence of the epitopes reacting with mab A1 and polyclonal antibodies raised against Xc62. All of these strains are from 'Mexican' lime and originated from southwest Asia and appear to be unique. Although the *Xac*-A* strains were not detected serologically, they were easily detected by the PCR assay designed to detect *Xac* (Table 2).

It is interesting to note that some strains isolated in India show the same novel phenotype (Table 1). CBC was described in India at the end of the last century (Civerolo, 1984) and one can speculate that this was the region of origin for the strains of *Xac* in this study. CBC was probably introduced into the southwest Asian region via infected plant or propagative material. 'Mexican lime' is currently the major citrus species grown in Saudi Arabia and presumably was a factor in the development of these novel strains.

The classification of heterogenous *Xac* strains in pathotypes A-C now appears to be more complicated. The consensus has been that strains of *Xac*-A had the widest host range (Goto, 1992; Stall and Civerolo, 1991) and constituted a clonal population (Gabriel et al., 1988; Hartung and Civerolo, 1989) within *Xac*, but the *Xac*-A* strains described herein do not correspond clearly to the *Xac* pathotype classification. They belong to the clonal *Xac*-A group defined by RFLP analyses (Hartung and Civerolo, 1989), bacteriophage typing and physiological tests (Vernière et al., 1993) but their pathogenicity brings them closer to the B/C group. They also are not serologically related to other *Xac* strains. Although complete DNA-DNA hybridization studies should be done, this study complicates the recent reclassification *Xanthomonas* associated with citrus (Gabriel et al., 1989; Vauterin et al., 1995). We have now described strains of *Xac*-A that, unexpectedly, induce typical symptomatology only on *Citrus aurantifolia* and which do not react with polyclonal or monoclonal antibodies prepared against reference *Xac*-A strains.

An examination of the genetic mechanisms that underly pathogen/host interactions is of interest. Many such interactions are controlled by gene-for-gene complementarity (Flor, 1955; Daniels and Leach, 1993), where gene-specific resistance is controlled by an avirulence *(avr)* gene in the bacterial genome and a corresponding resistance gene in the plant genome. It has been shown that for the *avr*Bs3 family of avirulence genes, which includes *pth*A from *Xac*-A (Swarup et al., 1991; Swarup et al., 1992) that the central region of such *avr* genes is composed of a number of 102 bp direct repeats and that the number and organization of the repeats are key factors determining the interaction with plant resistance genes (Herbers et al., 1992; Bonas et al., 1993). Novel host specificities have been reported based on *pth*A constructs engineered with altered numbers of 102 bp repeats (Yang and Gabriel, 1995). Such a rearrangement, occurring spontaneously in a variant clonal subgroup of *Xac*-A, could account for the origin of the *Xac*-A* group of strains.

Acknowledgements

This work was supported in part by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur and the Ministère des Affaires Etrangères (France).

References

Alizadeh A and Rahimian H (1990) Citrus canker in Kerman province. Iran J Plant Pathol 26: 42

Alvarez AM, Benedict AA and Mizymoto CY (1985) Identification of Xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75: 722–728

Alvarez AM, Benedict AA, Mizumoto CY, Pollard LW and Civerolo EL (1991) Analysis of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* with monoclonal antibodies. Phytopathology 81: 857–865

Becerra S, Medina VM, Garza JG and Orozco M (1988) Citrus leaf spot, a new mexican lime disease: a review. In: R. Goren and K. Mendel (eds) Proc 6th Int Citrus Cong Vol 2 (pp 795–800). Balaban, Rehovot

Bonas U, Conrads-Strauch J and Balbo I (1993) Resistance in tomato to *Xanthomonas campestris* pv. *vesicatoria* is determined by alleles of the pepper-specific avirulence gene avrBs3. Mol Gen Genet 238: 261–269

Civerolo EL (1984) Bacterial canker disease of citrus. J Rio Grande Val Hort Soc 37: 127–145

Civerolo EL (1990) Bacteriophages. In: Klement Z, Rudolph K and Sands DC (eds) Methods in phytobacteriology. (pp 205–213). Akademiai Kiado, Budapest

Civerolo EL (1994) Citrus bacterial canker disease in tropical regions. In: Lemattre M, Freigoun S, Rudolph K and Swings JG (eds) Proc 8th Int Conf Plant Path Bacteria (pp 45–50). ORSTOM / INRA, Paris

Civerolo EL and Fan F (1982) *Xanthomonas campestris* pv. *citri* detection and identification by enzyme-linked immunosorbent assay. Plant Disease 66: 231–236

Daniels MJ and Leach JE (1993) Genetics of *Xanthomonas*. In: Swings JG and Civerolo EL (eds) *Xanthomonas*. (pp 301–339). Chapman and Hall, London

Egel DS, Graham JH and Stall RE (1991) Genomic relatedness of *Xanthomonas campestris* strains causing diseases of citrus. Appl Environ Microbiol 57: 2724–2730

El Goorani MA (1989) The occurrence of citrus canker disease in United Arab Emirates (U.A.E.). J Phytopathol 125: 257–264

Flor HH (1955) Host-parasite interaction in flax rust - its genetics and other implications. Phytopathology 45: 680–685

Gabriel DW, Hunter JE, Kingsley MT, Miller JW and Lazo GR (1988) Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol Plant-Microbe Interact 1: 59–65

Gabriel DW, Kingsley MT, Hunter JE and Gottwald TR (1989) Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. *citri* strains. Int J Syst Bacteriol 39: 14–22

Goto M (1992) Citrus canker. In: Kumar J, Chaube HS, Singh US, Mukhopadhyay AN. (eds) Diseases of fruit crops-Plant Diseases of International Importance. Vol III (pp 170–208). Prentice Hall, New Jersey

Graham JH and Gottwald TR (1991) Research perspectives on eradication of citrus bacterial canker diseases in Florida. Plant Disease 75: 1193–1200

Hartung JS (1992) Plasmid-based hybridization probes for detection and identification of *Xanthomonas campestris* pv. *citri*.. Plant Disease 76: 889–893

Hartung JS and Civerolo EL (1989) Restriction fragment length polymorphism distinguish *Xanthomonas campestris* strains isolated from Florida citrus nurseries from*X. c.* pv. *citri*.. Phytopathology 79: 793–799

Hartung JS, Daniel JF and Pruvost OP (1993) Detection of *Xanthomonas campestris* pv. *citri* by the polymerase chain reaction method. Appl Environ Microbiol 59: 1143–1148

Herbers K, Conrads-Strauch J and Bonas U (1992) Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. Nature 356: 172–174

Ibrahim G and Bayaa B (1989) Fungal, bacterial and nematological problems of citrus, grape and stone fruits in Arab countries. Arab J Plant Prot 7: 190–197

Leite RP, Egel DS and Stall RE (1994) Genetic analysis of *hrp*–related DNA sequences of *Xanthomonas campestris* strains causing diseases of citrus. Appl Environ Microbiol 60: 1078–1086

Malavolta Jr VA, Carvalhom LV, Neto JR, Rossetti V, Nogueira EMC and Palazzo DA (1987) Reaction of different Citrus and relatives to bacterial cancrosis C (*Xanthomonas campestris* pv. *citri* [Hasse] Dye). In: Studart Montenegro HW and Moreira CS (eds) Proc Int Soc Citriculture Vol 1 (pp 363–364). Int Soc Citriculture, Sao Paulo

Namekata T and Balmer E (1977) Comparative studies on pathogenicity among causal agents of the three citrus canker. In: Carpena O (ed.) I Congreso Mundial de Citricultura Vol II (pp 659–662). INIA, Murcia

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonuclease. Proc Natl Acad Sci USA 76: 5269–5273

Palm ME and Civerolo EL (1994) Isolation, pathogenicity and partial host range of *Alternaria limicola*, causal agent of *'Mancha Foliar de los Citricos'* in Mexico. Plant Disease 78: 879–883

Pruvost O, Hartung JS, Civerolo EL, Dubois C and Perrier X (1992) Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus bacterial canker disease. Phytopathology 82: 485–490

Sokal RR and Rohlf FJ (1969) Biometry: the principles and practice of statistics in biological research. W.H. Freeman and co, San Francisco

Stall RE, Miller JW, Marco GM and Canteros BIC (1982) Pathogenicity of the three strains of citrus canker organism on grapefruit. In: C. Lozano and P. Gwin (eds) Proc 5th Int Conf Plant Path Bact (pp 334–340). Centro Internacional de Agricultura Tropical (CIAT), Cali

Stall RE and Civerolo EL (1991) Research relating to the recent outbreak of Citrus canker in Florida. Ann Rev Phytopathol 29: 339–420

Swarup S, De Feyter R, Brlansky RH and Gabriel DW (1991) A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit cankerlike lesions on citrus. Phytopathology 81: 802–809

Swarup S, Yang Y, Kingsley MT and Gabriel DW (1992) A *Xanthomonas citri* pathogenicity gene, *pth*A, pleiotropically encodes gratuitous avirulence on nonhosts. Mol Plant–Microbe Interact 5: 204–213

Vauterin L, Swings J, Kersters K, Gillis M, Mew TW, Schroth MN, Palleroni NJ, Hildebrand DC, Stead DE, Civerolo EL, Hayward AC, Maraite H, Stall RE, Vidaver AK and Bradbury JF (1990) Towards an improved taxonomy of *Xanthomonas*. Int J Syst Bacteriol 40: 312–316

Vauterin L, Yang P, Hoste B, Vancanneyt M, Civerolo EL, Swings J and Kersters K (1991) Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate - polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA - DNA hybridization. Int J Syst Bacteriol 41: 535–542

Vauterin L, Hoste B, Kersters K and Swings J (1995) Reclassification of *Xanthomonas*. Int J Syst Bacteriol 45: 472–489

Vernière C, Devaux M, Pruvost O, Couteau A and Luisetti J (1991) Studies on the biochemical and physiological variations among strains of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus bacterial canker disease. Fruits 46: 162–170

Vernière C, Pruvost O, Civerolo EL, Gambin O, Jacquemoud-Collet JP and Luisetti J (1993) Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. Appl Environ Microbiol 59: 243–249

Vernière C, Pruvost O, Dubois C, Perrier X, Couteau A and Luisetti J (1994) Variations among strains of *Xanthomonas campestris* isolated from citrus distinguished with their sensitivity to antibiotics. In: Lemattre M, Freigoun S, Rudolph K and Swings JG (eds) Proc 8th Int Conf Plant Path Bacteria (pp 247–251). ORSTOM / INRA, Paris

Wu WC, Lee ST, Kuo HF and Wang LY (1993) Use of phages for identifying the citrus canker bacterium *Xanthomonas campestris* pv. *citri* in Taiwan. Plant Pathol 42: 389–395

Yang Y and Gabriel DW (1995) Intragenic recombination of a single plant pathogen gene provides a mechanism for the evolution of new host specificities. J Bacteriol 177: 4963–4968

Young JM, Bradbury JF and Vidaver A (1990) The impact of molecular biological studies on the nomenclature of plant pathogenic bacteria. In: Klement Z (ed.) Proc 7th Int Conf Plant Path Bacteria (pp 659–661). Akademiai Kiado, Budapest

Young JM, Bradbury JF, Gardan L, Gvozdyak RI, Stead DE, Takikawa Y and Vidaver AK (1991) Comment on the reinstatement of *Xanthomonas citri* (ex Hasse 1915) Gabriel et al. 1989 and *X. phaseoli* (ex Smith 1897) Gabriel et al., 1989: indication of the needs for minimal standards for the genus *Xanthomonas*. Int J Syst Bacteriol 41: 172–177

Young JM, Saddler GS, Takikawa Y, De Boer SH, Vauterin L., Gardan L, Gvozdyak RI and Stead DE (1996) Names of plant pathogenic bacteria 1864–1995. Rev Plant Pathol 75: 721–763