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Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium

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Abstract

Onion bacterial blight, caused by *Xanthomonas* sp., is a potentially severe disease in several tropical and subtropical areas. Although little research has been undertaken on this pathosystem, seed transmission of the pathogen has been hypothesized. Because of an important bacterial microflora naturally associated with onion seeds, detection of the pathogen is difficult using non-selective agar media. A new semi-selective medium, whose selectivity was obtained by a combination of four antibiotics, was developed. The new NCTM1 medium contained (per liter) yeast extract 7 g, peptone 7 g, glucose 7 g, agar 15 g, neomycin 10 mg, cephalexin 30 mg, trimethoprime 3 mg, pivmecillinam 100 mg and propiconazole 20 mg. Plating efficiencies, using 16 pure cultures of the pathogen, ranged from 79% to 142%, with an average of 110% compared to the basal medium. All onion *Xanthomonas* sp. strains from several countries grew on NCTM1 medium. The pathogen was repeatedly isolated using this medium from seed samples containing approximately 10^6 saprophytic bacteria per gram, as well as from symptomless plant material. *Xanthomonas* sp. was detected only in seeds originating from one infected seed production site. This is the first report of selective isolation of *Xanthomonas* sp. from onion seeds. NCTM1 medium should be a valuable tool to study the ecology and epidemiology of *Xanthomonas* sp. causing onion bacterial blight.

Introduction

A bacterial disease of onion, named onion bacterial blight (OBB), caused by a *Xanthomonas* sp. was first described in Hawaii in 1975 (Alvarez et al., 1978). A bacterial disease inducing similar symptoms was reported in the USA in the 1940s, but the identification of the associated bacterium was uncertain (Alvarez et al., 1978). The pathogen was recently recorded again in Texas (Isakeit et al., 2000) and Colorado (Schwartz and Otto, 2000). OBB has now been reported in other tropical and subtropical areas, including the Barbados (Paulraj and O' Garro, 1993), neighboring islands (O' Garro and Paulraj, 1997), and Réunion Island (Roumagnac et al., 1997). Strains of *Xanthomonas* sp. that are pathogenic to onion and originating from Brazil, Cuba, and Mauritius Island are available in international culture collections, although no reports describe the associated disease in these countries. The taxonomic position of strains of *Xanthomonas* sp. associated with OBB has not been assessed (Dye et al., 1980; Vauterin et al., 1995; Young et al., 1996).

Typical leaf lesions start as lenticular, watersoaked spots which extend and eventually coalesce. Lesions develop into dry, chlorotic spots with tissue collapse and holes. Severe infections induce leaf dieback resulting in a reduction of bulb size. Similar lesions have been observed on onion inflorescences in Réunion Island. Bulb infection has never been reported and probably does not occur. Bacterial strains isolated in the Barbados were pathogenic to French bean, soybean,

winged bean, field pea, moth bean, lima bean, garlic, chives, shallot, and leek (Bowen et al., 1998; O' Garro and Paulraj, 1997). However, the host range remains unclear, as onion strains of *Xanthomonas* sp. from Hawaii were reported to induce typical HR on bean (Alvarez et al., 1978).

The ecology and epidemiology of the causal agent of OBB is poorly understood. Alvarez et al. (1978) hypothesized that OBB development in Molokai was associated with seed transmission by seed lots originating from mainland USA. Plants from these seed lots, when established in a virgin area of Oahu, developed typical OBB lesions, from which *Xanthomonas* sp. was isolated. However, the pathogen was not isolated from onion seeds, because an efficient semi-selective medium was not available.

Semi-selective media have been developed for isolation of several xanthomonads and have proved to be useful for elucidating the ecology and epidemiology of related diseases (Civerolo et al., 1982; Claflin et al., 1987; Davis et al., 1994; Di et al., 1991; Fessehaie et al., 1999; Kritzman, 1991; Mabagala and Saettler, 1992; Mc Guire et al., 1986; Norman and Alvarez, 1994; Rhandawa and Schaad, 1984; Schaad and White, 1974; Sijam et al., 1992). Semi-selective media developed for xanthomonads so far often include kasugamycin as an inhibitor of some components of the bacterial microflora. The world production of this antibiotic has stopped, making it difficult to use these media.

The purpose of this study was to develop a new semi-selective medium allowing the isolation of the OBB *Xanthomonas* from microbiologically contaminated material. This new medium was used to evaluate onion seed contamination by the pathogen in Réunion Island.

Materials and methods

Bacterial strains and media

Strains used in this study are listed in Table1 and include *Xanthomonas* sp. causing OBB in several countries and strains from the bacterial microflora associated with onion seeds and leaves, isolated in Réunion and neighboring islands, but which do not belong to the genus *Xanthomonas*. These strains are referred to as OBM (for onion bacterial microflora) in the text below. All strains in Table1 were identified using the Biolog GN system (Bochner, 1989). Cultures were stored as lyophiles and/or in a $-80\,^{\circ}$C freezer and were checked for purity on YPGA (yeast extract 7 g, peptone 7 g, glucose 7 g, agar 15 g, distilled water 1000 ml, pH 7.2). Cultures used for all tests were grown 24 h at $28\,^{\circ}$C on YPGA medium. Propiconazole (20 μg ml^{-1} – commercially available as Polyflor™ from Novartis Agro) was added to all media to prevent growth of fungi. Pathogenicity was checked on onion, cv. Red Creole, by spray-inoculating plants at the 4–5 leaf stage until run-off with bacterial suspensions prepared in 0.01 M sterile Sigma™ 7–9 buffer (pH 7.2) containing either 10^7 cell ml^{-1} or 10^4 cell ml^{-1} (10 plants per strain and bacterial concentration). Plants sprayed with sterile buffer were used as controls. Plants were incubated at $30 \pm 1\,^{\circ}$C with relative humidity close to saturation. Lesion development was recorded after 4, 6, and 8 days. All the strains classified as *Xanthomonas* sp. in Table 1 were pathogenic on onion.

Susceptibility to antibiotics

Susceptibility to 63 antibiotics (amikacin, amoxicillin, amoxicillin + clavulanic acid, ampicillin, aztreonam, bacitracin, cefamandole, cefazolin, cefepime, cefixime, cefotaxime, cefotiam cefoxitin, cefpodoxime, cefsulodin, ceftriaxone, cefuroxime, cephalexin, cephalothin, chloramphenicol, ciprofloxacin, colistin, dibekacin, doxycycline, erythromycin, flumequine, fusidic acid, gentamycin, imipren, kanamycin, latamoxef, lincomycin, pivmecillinam, metronidazole, mezlocillin, minocycline, nalidixic acid, neomycin, netilmycin, nitroxoline, ofloxacin, oxolinic acid, oxytetracycline, pefloxacin, phosphomycin, pipemidic acid, piperacillin, polymyxin B, pristinamycin, rifampicin, sisomycin, spectinomycin, spiramycin, streptomycin, sulfamide, teicoplanin, ticarcillin, ticarcillin + clavulanic acid, tobramycin, trimethoprime, trimethoprime + sulfamethoxazole, vancomycin and virginiamycin) was assayed by the disk susceptibility test (Acar and Goldstein, 1991). All strains listed in Table1 were tested. Antibiotic disks were purchased from Sanofi Diagnostics Pasteur (Marne La Coquette, France) and were used as recommended by the manufacturer. Inhibition diameters were recorded after 48 h of incubation at $28\,^{\circ}$C. Antibiotics which induced small inhibition diameters and small variances among *Xanthomonas* sp. strains were selected for further analysis. Minimal inhibitory concentrations (MICs) for selected antibiotics were determined again by the agar dilution susceptibility test (Barry, 1991) for all onion bacterial microflora strains listed in Table 1

Table 1. Bacterial strains used in this study

Strain	Origin	Year of isolation/ obtained from	Genus identification (Biolog GN)[c]	Other numbers
Xanthomonas sp.				
JR512[b]	Barbados		*Xanthomonas*	P360
JR513[b]	Barbados	?/D. Stead	*Xanthomonas*	P361
JR514[b]	Barbados	(CSL, UK)	*Xanthomonas*	P362
JR515	Barbados		*Xanthomonas*	P363
JR516	Barbados		*Xanthomonas*	P372
JQ792	Brazil	1987/J.R. Rodrigues	*Xanthomonas*	IBSBF 594
JQ793	Brazil	Neto (Inst. Biologico, SP Brazil)	*Xanthomonas*	IBSBF 595
JR505[a]	Cuba	?/R. Samson	*Xanthomonas*	A1
JR507[ab]	Cuba	(INRA, France)	*Xanthomonas*	A6
JR525[b]	Cuba		*Xanthomonas*	C1
JQ778	Hawaii	1974/LMG	*Xanthomonas*	LMG 576
JQ779	Hawaii	1975/LMG	*Xanthomonas*	LMG 577
JQ780	Hawaii	1975/LMG	*Xanthomonas*	LMG 578
JQ781	Hawaii	1976/LMG	*Xanthomonas*	LMG 579
JQ782	Hawaii	1980/LMG	*Xanthomonas*	LMG 580
JQ783[b]	Hawaii	1980/LMG	*Xanthomonas*	LMG 943
JQ784	Hawaii	1989/LMG	*Xanthomonas*	LMG 9487
JQ785[b]	Hawaii	1989/LMG	*Xanthomonas*	LMG 9488
JQ786	Hawaii	1989/LMG	*Xanthomonas*	LMG 9489
JQ787	Hawaii	1989/LMG	*Xanthomonas*	LMG 9490
JQ788	Hawaii	1989/LMG	*Xanthomonas*	LMG 9491
JQ789	Hawaii	1989/LMG	*Xanthomonas*	LMG 9492
JQ790	Hawaii	1989/LMG	*Xanthomonas*	LMG 9493
JQ791[b]	Hawaii	1989/LMG	*Xanthomonas*	LMG 9494
JR649[b]	Mauritius	1996/S. Benimahdu (AREU, Mauritius)	*Xanthomonas*	ON1
JR650	Mauritius	1996/S. Benimahdu (AREU, Mauritius)	*Xanthomonas*	ON2
JR651	Mauritius	1996/S. Benimahdu (AREU, Mauritius)	*Xanthomonas*	ON3
JR652-1, JR652-2, JR652-3,	Mauritius	1996/this study	*Xanthomonas*	
JR652-4, JR652-5, JR653[b],	Mauritius	1996/this study	*Xanthomonas*	
JR654-1, JR654-2, JR654-3,	Mauritius	1996/this study	*Xanthomonas*	
JR654-4, JR654-5, JR654-6[b],	Mauritius	1996/this study	*Xanthomonas*	
JR655	Mauritius	1996/this study	*Xanthomonas*	
JS958	Mauritius	1997/S. Saumtally (MSIRI, Mauritius)	*Xanthomonas*	3582
JS959[b]	Mauritius	1997/S. Saumtally (MSIRI, Mauritius)	*Xanthomonas*	3583
JS960	Mauritius	1997/S. Saumtally (MSIRI, Mauritius)	*Xanthomonas*	3584
JS961	Mauritius	1997/S. Saumtally (MSIRI, Mauritius)	*Xanthomonas*	3585
JS962	Mauritius	1997/S. Saumtally (MSIRI, Mauritius)	*Xanthomonas*	3586
JQ632-1, JQ632-2, JQ632-3,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ632-4, JQ708[b], JQ734,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ736-1, JQ736-2, JQ737-1,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ737-2, JQ738-1, JQ739-1,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ739-2[b], JQ740-1, JQ740-2,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ741-1[b], JQ741-2, JQ741-3,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ741-4, JQ741-5, JQ741-6,	Réunion (France)	1995/this study	*Xanthomonas*	

Table 1. Continued

Strain	Origin	Year of isolation/Obtained from	Genus identification (Biolog GN)[c]	Other numbers
JQ741-7, JQ742-1, JQ742-2,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ742-3, JQ742-4, JQ743-1,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ743-2, JQ743-3, JQ744-1,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ744-2, JQ745-1[b], JQ745-2,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ745-3, JQ746-1, JQ746-2,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ746-3, JQ752[b], JQ753[a],	Réunion (France)	1995/this study	*Xanthomonas*	
JQ754, JQ755, JQ756, JQ757,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ758[b], JQ759[ab], JQ760,	Réunion (France)	1995/this study	*Xanthomonas*	
JQ761, JQ762, JQ774[b],	Réunion (France)	1995/this study	*Xanthomonas*	
JR511-1[b], JR511-2, JR511-3,	Réunion (France)	1996/this study	*Xanthomonas*	
JR511-4, JR519-1, JR519-2[b],	Réunion (France)	1996/this study	*Xanthomonas*	
JR519-3, JR519-4, JR520-1[b],	Réunion (France)	1996/this study	*Xanthomonas*	
JR520-2, JR520-3, JR520-4,	Réunion (France)	1996/this study	*Xanthomonas*	
JR523-1[b], JR523-2, JR523-3,	Réunion (France)	1996/this study	*Xanthomonas*	
JR523-4, JR628-1, JR628-2,	Réunion (France)	1996/this study	*Xanthomonas*	
JR628-3, JR628-4, JR636-1[b],	Réunion (France)	1996/this study	*Xanthomonas*	
JR636-2, JR636-3, JR636-4,	Réunion (France)	1996/this study	*Xanthomonas*	
JR636-5[b], JR636-6, JR636-7,	Réunion (France)	1996/this study	*Xanthomonas*	
JR636-8, JR637-1, JR637-2,	Réunion (France)	1996/this study	*Xanthomonas*	
JR637-3, JR637-4[b], JR638-1[b],	Réunion (France)	1996/this study	*Xanthomonas*	
JR638-2, JR638-3, JR638-4,	Réunion (France)	1996/this study	*Xanthomonas*	
JR687-1[b], JR687-2, JR687-3,	Réunion (France)	1996/this study	*Xanthomonas*	
JR687-4	Réunion (France)	1996/this study	*Xanthomonas*	
JS711-1	Réunion (France)	1997/this study	*Xanthomonas*	
Bacterial microflora				
JS923, JS924	Mayotte (France)	1997/this study	*Erwinia*	
JR593	Réunion (France)	1996/this study	*Burkholderia*	
JR594-1	Réunion (France)	1996/this study	*Stenotrophomonas*	
JR594-2	Réunion (France)	1996/this study	*Pseudomonas*	
JR656-3	Réunion (France)	1996/this study	*Klebsiella*	
JR656-5, JR656-6	Réunion (France)	1996/this study	*Pantoea*	
JR656-1, JR656-2, JR656-4	Réunion (France)	1996/this study	Not identified[d]	
JS741-1	Réunion (France)	1997/this study	*Burkholderia*	
JS741-2, JS741-4, JS741-12	Réunion (France)	1997/this study	*Flavimonas*	
JS741-5	Réunion (France)	1997/this study	*Klebsiella*	
JS741-6, JS853	Réunion (France)	1997/this study	*Pantoea*	
JS741-14, JS964	Réunion (France)	1997/this study	*Enterobacter*	
JS741-15 JS741-16,	Réunion (France)	1997/this study	*Pseudomonas*	
JS741-3, JS741-7, JS741-8,	Réunion (France)	1997/this study	Not identified[d]	
JS741-9, JS741-10, JS741-11,	Réunion (France)	1997/this study	Not identified[d]	
JS741-13, JS821, JS822,	Réunion (France)	1997/this study	Not identified[d]	
JS823, JS824, JS825, JS826,	Réunion (France)	1997/this study	Not identified[d]	
JS827, JS828, JS829, JS849,	Réunion (France)	1997/this study	Not identified[d]	
JS850, JS851, JS852, JS854,	Réunion (France)	1997/this study	Not identified[d]	
JS855, JS856, JS857, JS963,	Réunion (France)	1997/this study	Not identified[d]	
JS980, JS981, JS982, JS998	Réunion (France)	1997/this study	Not identified[d]	

[a]Strains isolated from garlic.

[b]*Xanthomonas* strains selected for the agar dilution susceptibility test.

[c]All strains identified as *Xanthomonas* by the Microlog 1 software were pathogenic on onion cv. Red Creole. Other strains were not pathogenic on onion, except those identified by the Microlog 1 software as *Burkholderia cepacia* and *Erwinia carotovora.*

[d]The similarity coefficient (Sim) determined by the Microlog software was too low to provide an accurate identification (Sim $\leq$ 0.500).

and for a subset of 29 onion *Xanthomonas* strains (from several countries) which were selected to cover the variations in susceptibility recorded by the disk susceptibility test. Antibiotics were purchased from Sigma™ (Saint Quentin Fallavier, France), except pivmecillinam, which was obtained from Laboratoires Léo (Saint Quentin en Yvelines, France – commercially available as Selexid™). Cephalexin and trimethoprime were dissolved respectively in 1 N sodium hydroxide and 70% ethanol. Other antibiotics were dissolved in 0.01 M sterile Sigma™ 7–9 buffer (pH 7.2). All antibiotics, except pivmecillinam were filter-sterilized prior to addition to agar media cooled down at 42 °C. Tests were performed on YPGA plates in which the concentration of each antibiotic ranged from 0.125 to 128 $\mu g\,ml^{-1}$. Plates were seeded with a Steer's inoculator (Denley, UK). Bacterial inocula were adjusted in 0.01 M sterile Sigma™ 7–9 buffer (pH 7.2) so that each droplet deposited on the agar plates by the inoculator contained approximately 10^4 cells ml^{-1}. Plates were incubated for 48 h at 28 °C prior to scoring. All experiments were replicated once.

Carbon source utilization profiles

The utilization range of carbon and nitrogen sources was determined by the Biolog GN (Hayward CA, USA) technique (Vauterin et al., 1995). Microplates were incubated for 48 h at 28 °C prior to reading (590 nm) with a Biotek EL312e spectrophotometer (Fisher Scientific, Elancourt, France). Identification of strains was performed using the Microlog1 software release 3.50 (Biolog Inc., Hayward CA, USA).

Comparative efficiency of semi-selective media

The selected media (Table 3) were compared to the YPGA basal medium. In a first set of experiments, strains were streaked on each medium. Plates were incubated at 28 °C. Growth was visually scored daily for 5 days. The following global rating system was used: 1 – growth rate identical to the basal medium, 2 – similar amounts of individual colonies formed on the semi-selective medium, but their appearance was delayed approximately by 1 day compared to the basal medium, 3 – confluent growth in the first two quadrants of the plates, but no isolated colonies were formed, 4 – very poor growth, 5 – no growth. The experiment was repeated and mean scores obtained respectively from all *Xanthomonas* and OBM strains for each semi-selective medium were compared pairwise by Mann–Whitney non-parametric *U* test (Statview 5.0, SAS Institute Inc., Cary NC, USA). Inhibition of OBM strains on the four semi-selective media was compared by non-parametric Kruskal and Wallis test (Statview 5.0, SAS Institute Inc., Cary NC, USA). In a second set of experiments, plating efficiency was calculated by plating with a spiral device (Interscience, Saint-Nom-La-Bretèche, France) (Jalenques, 1988) suspensions (10 replicates per strain) adjusted to approximately 5×10^3 cells ml^{-1} prepared from 16 *Xanthomonas* sp. cultures from various origins Plating efficiency (PE) for each strain/semi-selective combination was calculated as

$$\mathrm{PE} = 100 \times \frac{\text{cells ml}^{-1} \text{ on semiselective medium}}{\text{cells ml}^{-1} \text{ on LPGA medium}}.$$

Isolation of Xanthomonas *sp. from onion seeds*

In a first experiment, seed samples collected in Réunion Island from a local 'Chateauvieux' cultivar were assayed. Samples included seeds originating from seed production fields where OBB has or has not been recorded. For each sample, 5 replicates containing 1 g of seed each were soaked in 5 ml of 0.01 M sterile Sigma™ 7–9 buffer (pH 7.2) for 48 h at 4 °C. Seed samples, previously determined to be free of *Xanthomonas* sp., were inoculated with a bacterial suspension prepared from strain JR519-1 (24-h-old culture on YPGA) and containing approximately 5×10^3 cells ml^{-1}, in order to evaluate the ability of *Xanthomonas* sp. to multiply in seed macerates during soaking at 4 °C. Macerates were streaked, after 24 and 48 h, on the semi-selective medium NCTM1 and on YPGA using a spiral device (Interscience, Saint-Nom-La-Bretèche, France) (Jalenques, 1988). Plates were incubated at 28 °C up to 96 h. OBM and *Xanthomonas* sp. colonies were enumerated after 48 and 96 h of incubation, respectively. Growth of OBM on NCTM1 was checked again after 96 h of incubation. Colonies, whose morphology was similar to that of xanthomonads, were further identified by the Biolog GN method (see procedure above) and Koch postulates were verified by the plant inoculation technique described above. Seed lots from which *Xanthomonas* sp. was isolated were further analyzed to determine the percentage of infected seeds. For this purpose, 70 replicates, containing 1 g of seed each, were soaked and analyzed as described above. The contamination rate (p) was determined as: $p =$

$1 - (Y/N)^{1/n}$ where Y is the number of samples from which the pathogen was not isolated, N is the total number of analyzed samples, and n is the number of seeds per sample (Masmoudi et al., 1994).

In a second experiment, commercial seed samples (Table 5) originating from various countries in the world (Australia, Brazil, France, the Netherlands, South Africa, and USA) and presumed to be free of onion *Xanthomonas* sp. were assayed to determine OBM population sizes in seeds produced under different environmental and technical conditions.

Isolation of Xanthomonas *sp. from symptomless onion leaves*

Symtomless onion leaves were collected in June 1998 from 16 'Chateauvieux' onion commercial production fields (covering major onion production areas in Réunion Island). For each site, 10 randomly collected samples, containing 1 g of leaf tissue each, were homogenized for 2 min in 30 ml of 0.01 M sterile Sigma™ 7–9 buffer (pH 7.2) using a stomacher device (Seward Medical, London, UK). Suspensions were streaked on the semi-selective media NCTM1 and NCTM2 and on YPGA using a spiral device (Interscience, Saint-Nom-La-Bretèche, France) (Jalenques, 1988). Enumeration and identification of colonies was performed as described above.

Results

Susceptibility to antibiotics

Antibiotics for which strains of *Xanthomonas* sp. displayed small inhibition diameters and/or little heterogeneity among strains are listed in Table 2. *Xanthomonas* sp. strains from onion were resistant to cefazolin (98% of the strains), cephalexin (96%), lincomycin (98%), pivmecillinam (100%), metronidazole (100%), and trimethoprime (98%) and showed an intermediate reaction for gentamycin, neomycin, and sisomycin. Among strains of *Xanthomonas* sp. listed in Table 1, JQ752, JQ758, and JQ783 were susceptible to the highest number of antibiotics. MICs for antibiotics listed above were highly variable for OBM strains. Six antibiotics (cefazolin, cephalexin, pivmecillinam, gentamycin, neomycin, and trimethoprime), for which more than 90% of OBM strains had one or more of their MICs lower than those of *Xanthomonas* sp., were further used for the development of semi-selective media.

Ampicillin, amoxicillin and cefuroxime (β-lactam antibiotics) were the antibiotics that showed the most variable inhibition diameters among *Xanthomonas* strains. There was no relationship between the level of susceptibility to these antibiotics and geographic origin or host of isolation.

Table 2. Susceptibility of onion *Xanthomonas* sp. towards selected antibiotics (based on data obtained by the disk susceptibility test)

Antibiotic	Family	Mean inhibition diameter (mm)[a]	Variance	MIC (μg/ml)[b]
Cefazolin	Cephalosporin (1st generation)	6.1	0.7	64–>128
Cephalexin	Cephalosporin (1st generation)	6.2	1.9	64–>128
Pivmecillinam	Amidinopenicillin	6.0	0.0	256–512
Trimethoprime	Diaminopyrimidine	6.1	0.3	8–16
Metronidazole	Imidazole	6.0	0.0	>128
Lincomycin	Lincosamide	6.1	1.0	16–>128
Gentamycin	Aminoglycoside	21.0	3.9	4–16
Neomycin	Aminoglycoside	20.0	3.6	16–32
Sisomycin	Aminoglycoside	21.0	3.3	8–32

[a]Presented values are means of inhibition diameter obtained from all *Xanthomonas* sp. strains listed in Table 1.

[b]Minimal inhibitory concentrations as determined by the agar dilution susceptibility test. Presented values are minimum and maximum MICs obtained from a subset of 29 strains of *Xanthomonas* sp. identified in Table 1.

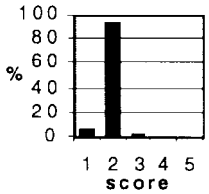
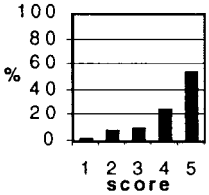
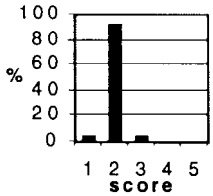
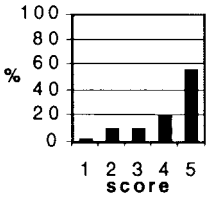
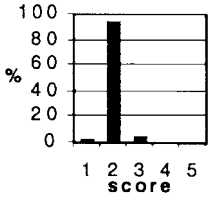
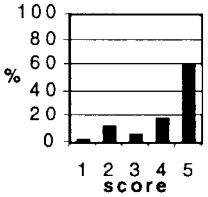
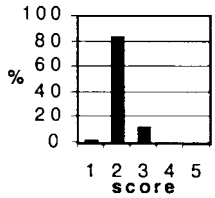
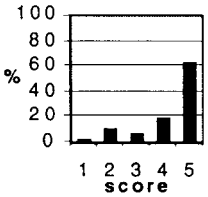
Carbon source utilization profiles

All *Xanthomonas* strains used dextrin, glycogen, tween 40, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactulose, maltose, D-mannose, D-psicose, sucrose, D-trehalose, turanose, monomethyl succinate, cis-aconitic acid, α-keto glutaric acid succinic acid, bromosuccinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-glutamic acid, glycyl-L-glutamic acid, and L-serine. Because most OBM strains can also utilize these carbon sources, none of them could be used to favor growth of *Xanthomonas* sp. from samples invaded by saprophytic bacteria. Several of the above mentioned carbon sources allowed efficient growth of *Xanthomonas* sp. in complex media. D-glucose was selected for the semi-selective media.

Comparative efficiency of semi-selective media

Based on results described above, YPGA was used as the basal medium, because it allowed single colonies of *Xanthomonas* sp. to grow within 2 days. Four antibiotic combinations were compared for their ability to inhibit OBM (Table 3). The typical mucoid appearance of *Xanthomonas* sp. colonies was unchanged on

Table 3. Antibiotic combinations added to YPGA basal medium and their effect on the growth of *Xanthomonas* sp. and onion bacterial microflora

Designation	Selected antibiotics	Growth of *Xanthomonas* sp.[a]	Growth of onion microflora strains[a]
NCTM1	Neomycin 10 mg/l Cephalexin 30 mg/l Trimethoprime 3 mg/l Pivmecillinam 100 mg/l		
NCTM2	Neomycin 10 mg/l Cefazolin 30 mg/l Trimethoprime 3 mg/l Pivmecillinam 100 mg/l		
GCTM1	Gentamycin 2.5 mg/l Cephalexin 30 mg/l Trimethoprime 3 mg/l Pivmecillinam 100 mg/l		
GCTM2	Gentamycin 2.5 mg/l Cefazolin 30 mg/l Trimethoprime 3 mg/l Pivmecillinam 100 mg/l		

[a]Bars indicate the percentage of *Xanthomonas* sp. and OBM strains growing on the four semi-selective media. The scoring system is detailed in the Materials and methods and is summarized below : 1 – growth rate identical to the basal medium, 2 – similar amounts of individual colonies formed on the semi-selective medium, but their appearance was delayed approximately by 1 day compared to the basal medium, 3 – confluent growth in the first two quadrants of the plates, but no isolated colonies were formed, 4 – very poor growth, 5 – no growth.

Table 4. Enumerated populations and plating efficiencies of 16 strains of *Xanthomonas* sp. from different countries on YPGA medium and NCTM1 and NCTM2 semi-selective media

Strain	Origin	Cells ml^{-1}			Plating efficiency (%)	
		YPGA	NCTM1	NCTM2	NCTM1	NCTM2
JR515	Barbados	1943 ± 614	2203 ± 226	2039 ± 250	113	105
JR516	Barbados	1799 ± 303	1419 ± 351	1596 ± 203	79	89
JQ792	Brazil	2498 ± 599	2587 ± 425	2449 ± 403	104	98
JR505	Cuba	3205 ± 810	4539 ± 809	3959 ± 566	142	124
JR507	Cuba	6189 ± 459	7744 ± 458	7843 ± 462	125	127
JQ783	Hawaii	4077 ± 743	3289 ± 469	3870 ± 301	81	95
JR649	Mauritius	4791 ± 350	4732 ± 347	4600 ± 190	99	96
JR650	Mauritius	5217 ± 334	6169 ± 327	5230 ± 231	118	100
JS958	Mauritius	3020 ± 410	2811 ± 784	2829 ± 572	93	94
JR523-1	Réunion	4579 ± 621	5575 ± 350	5402 ± 289	122	118
JQ756	Réunion	2006 ± 836	2679 ± 870	2598 ± 959	134	129
JR519-1	Réunion	5256 ± 853	6673 ± 397	6289 ± 415	127	120
JR636-4	Réunion	2486 ± 276	2360 ± 367	2530 ± 284	95	102
JR636-5	Réunion	4360 ± 792	4555 ± 941	4768 ± 684	104	109
JR511-1	Réunion	5274 ± 342	5746 ± 544	5482 ± 424	109	104
JR520-3	Réunion	4565 ± 820	5404 ± 472	5179 ± 365	118	113

semi-selective media. Colonies of *Xanthomonas* sp. were 2–3 mm in diameter on the 4 semi-selective media after 72 h of growth at 28 °C. The growth of strain JQ781 (*Xanthomonas* sp. from onion in Hawaii) was weak on GCTM1 and GCTM2 media (score 4), but better on NCTM1 (score 2) and NCTM2 (score 3) media. The MIC for gentamycin of strain JQ781 was $4\,\mu g\,ml^{-1}$. A synergistic action of antibiotics likely explains the failure of strain JQ781 to grow on GCTM1 and GCTM2 media, making them unreliable for isolation of *Xanthomonas* sp. Growth of OBM strains was always significantly reduced ($p < 0.0001$) compared to that of *Xanthomonas*, whatever the semi-selective media. Furthermore, no differences in inhibition of OBM strains were found in both experiments ($p = 0.91$ and 0.92) on the four semi-selective media.

Recovery of *Xanthomonas* sp. on NCTM1 and NCTM2 semi-selective media was compared to recovery on YPGA basal medium (Table 4). Plating efficiency for the 16 strains of *Xanthomonas* sp. ranged from 79% to 142% on NCTM1 (with an average of 110%) and from 89% to 129% on NCTM2 (with an average of 108%).

Isolation of Xanthomonas *sp. from onion seeds*

The growth of 80–100% of OBM in commercial seed samples was inhibited on NCTM1 medium (Table 5). *Xanthomonas* sp. was repeatedly isolated from seed samples originating from one seed production site in Réunion Island where severe OBB had been recorded. Colonies of *Xanthomonas* sp. were isolated after 24 and 48 h of soaking. However, higher populations (by 1 log unit) were recovered after 48 h of soaking. Increase in populations was not caused by the multiplication of *Xanthomonas* sp. in seed macerates during soaking. All of the 12 subcultures, obtained among colonies suspected to be *Xanthomonas* sp. (based on their colony morphology on NCTM1 medium), were identified as *Xanthomonas* by Biolog GN identification system and were pathogenic on onion cv. Red Creole after spray-inoculation. Pure cultures of *Xanthomonas* were re-isolated from induced lesions. Populations of *Xanthomonas* sp. in contaminated samples ranged from 5×10^2 to 2×10^6 cells g^{-1} of seeds. The contamination rate of seeds originating from the contaminated seed production field was approximately 0.4‰. *Xanthomonas* sp. could be detected on NCTM1 medium from seed samples in which seed associated OBM populations on YPGA medium ranged from 8×10^5 to 2×10^6 cells g^{-1}.

Isolation of Xanthomonas *sp. from symptomless onion leaves*

Colonies with a morphology similar to that of *Xanthomonas* sp. were recovered on semi-selective media from 1 or 2 out of the 10 leaf samples in 4 out of the 16 sites assayed, but not on YPGA, as high OBM populations grew on this medium. Populations of *Xanthomonas* sp. in positive leaf samples ranged from 3×10^2 to 1×10^3 cells g^{-1}. All of the nine subcultures,

Table 5. Examples of inhibition of onion bacterial microflora in commercial seed samples using NCTM1 medium

Onion cultivar	Origin	Bacterial microflora[a]		% inhibition[b]
		YPGA	NCTM1	
Chateauvieux	SRPS, Réunion	4.95–6.32	2.82–5.03	80–99
Dessex	Hygrotech, South Africa	2×10^4–1×10^5	ND[c]–4×10^2	98–100
Liberty	Bejo Zaden, The Netherlands	2×10^3–4×10^4	ND–3×10^3	87–100
White Hawk	Bejo Zaden, The Netherlands	5×10^5–1×10^6	1×10^4–1×10^5	91–99
Domingo	Bejo Zaden, The Netherlands	5×10^5–1×10^6	ND–1×10^5	84–100
Rouge Espagnol	Technisem, France	1×10^3–1×10^6	ND–6×10^3	99–100
Princesse	Technisem, France	9×10^5–1×10^6	2×10^3–1×10^5	89–100
Goldor	Technisem, France	5×10^1–8×10^4	ND–6×10^3	92–100
Texas Grano	Technisem, France	2×10^5–8×10^5	7×10^2–2×10^4	89–100
Alix	Novartis, The Netherlands	6×10^2–5×10^5	ND	100
Z512	Yates, Australia	3×10^5–9×10^5	ND–2×10^3	100
Gladalan	Yates, Australia	5×10^5–1×10^6	7×10^3–1×10^5	85–99
Sweet	Rio Colorado, USA	5×10^1–1×10^5	ND–2×10^2	100
Mister Max	Rio Colorado, USA	2×10^2–3×10^5	ND	100
AG558	Agroceres, Brazil	7×10^2–4×10^3	ND	100

[a]Minimum and maximun populations recovered per gram of seed.
[b]Minimum and maximun percentages of inhibition of bacterial microflora (*Xanthomonas* sp. not included) on NCTM1 medium versus YPGA basal medium.
[c]Not detected (detection threshold 2×10^1 cells g^{-1}).

obtained among colonies suspected to be *Xanthomonas* sp. (based on their colony morphology), were identified as *Xanthomonas* by Biolog GN identification system and were pathogenic on onion cv. Red Creole after spray-inoculation. Pure cultures of *Xanthomonas* were re-isolated from induced lesions.

Discussion

Several bacterial diseases caused by xanthomonads are seed-borne (Aggour et al., 1989). Contaminated seed is often an epidemiologically important source of primary inoculum for many bacterial diseases of food crops around the world. As a consequence, seed sanitation is a major component of disease control strategies (Saettler, 1989). Seed transmission of *Xanthomonas* sp. causing OBB was hypothesized by Alvarez et al. (1978). The disease was observed in virgin areas of Molokai (Hawaii), making seed the most likely source of inoculum, but isolation of the pathogen on agar media was unsuccessful.

In the present study, the efficiency of four semi-selective media was compared. Antibiotics included in these media were selected based on their ability to inhibit more than 90% of a collection of onion bacterial microflora strains at concentrations that do not inhibit *Xanthomonas* sp. Our attempt to identify carbon sources which would have a negative effect on the growth of some components of onion bacterial microflora was not successful. Because all strains of *Xanthomonas* sp. readily utilized D-glucose, this substrate was chosen as the primary carbon source. More generally, it was decided to use YPGA as the basal medium because excellent plating efficiencies were recorded for xanthomonads with this medium. Additionally, the aspect of *Xanthomonas* sp. colonies on YPGA and on the newly developed semi-selective media makes them easily distinguishable from other yellow-pigmented bacteria. Thus, the use of starch as primary carbon source, which allows an easy identification of starch-hydrolyzing *Xanthomonas* colonies (Dhanvantari and Brown, 1993; Fukui et al., 1994) but tends to delay their appearance, was unnecessary.

Although all four semi-selective media were efficient to selectively isolate *Xanthomonas* sp. (Table 3), media GCTM1 and GCTM2, that both contain gentamycin, inhibited the growth of strain JQ781 of *Xanthomonas* sp. NCTM2 medium, but not NCTM1 medium, delayed the growth of three strains of *Xanthomonas* sp. As there was no significant difference in the ability of the four media to inhibit the onion bacterial microflora, NCTM1 medium, which contains four different antibiotics (cephalexin, pivmecillinam, neomycin and trimethoprime) and a fungicide (propiconazole), is recommended. Cephalexin is a narrow spectrum cephalosporin (β-lactam antibiotic), which inhibits some Gram negative bacteria (Bryan and

Godfrey, 1991). Several xanthomonads are resistant to narrow-spectrum cephalosporins (Weng et al., 1999). In *Xanthomonas campestris* pv. *campestris*, this resistance is associated with the presence of a gene coding for a β-lactamase which shows homology with amber class A/Bush group 2 β-lactamases (Weng et al., 1999). Furthermore, the commercial synthesis of cephalexin is currently done using bacteria belonging to the genus *Xanthomonas* (Rhee et al., 1980). Pivmecillinam is a β-lactam antibiotic, that alone has an activity against many Enterobacteriaceae and usually provides a synergistic effect against many Gram negative bacteria when associated with an other β-lactam antibiotic (Eliopoulos and Moellering, 1991). Neomycin belongs to the aminoglycoside family, a family that has a broad spectrum activity which is improved by β-lactam antibiotics (the latter increase cell permeability to aminoglycosides) (Eliopoulos and Moellering, 1991). Trimethoprime, an antibiotic belonging to the diaminopyrimidine family, inhibits Gram positive bacteria (Neu, 1991), and has a synergistic effect when combined with aminoglycosides against several genera in Enterobacteriaceae (Eliopoulos and Moellering, 1991). Propiconazole was found to be more efficient than the conventionally used cycloheximide for inhibiting the growth of fungi (data not shown). The combination of antibiotics included in NCTM1 medium inhibited the growth of 80–100% of saprophytic bacteria in seed samples from various regions in the world but did not drastically affect the growth of onion *Xanthomonas* sp., which was only delayed by one day for most strains.

Protocols for extraction and isolation of xanthomonads from seeds often include a pre-washing step, which primarily reduces surface microflora (Roth, 1989). In preliminary experiments (data not shown), a pre-washing step did not significantly reduce the saprophytic microflora which can develop on NCTM1 medium. Soaking of onion seed was performed in sterile buffer, as sterile distilled water was reported to sometimes adversely influence the viability of xanthomonads. Soaking of seeds for 24 and 48 h at 4 °C was compared (data not shown). Although soaking for 24 h allowed the detection of *Xanthomonas* sp. from naturally contaminated seed, soaking for 48 h increased the recovery of the target organism and did not significantly increase saprophytic bacterial populations recovered on NCTM1 medium. It is not known if the better recovery of *Xanthomonas* sp. after soaking for 48 h is due to an internal location of the bacteria in seeds.

Xanthomonas sp. using NCTM1 medium was repeatedly isolated from local 'Chateauvieux' onion seed samples originating from a diseased seed production site, but not from healthy fields. A relationship between severity of foliar lesion development and seed contamination might exist, as it has been shown for the *X. translucens* pv. *translucens*/wheat pathosystem (Tubajika et al., 1998). Successful isolations of the pathogen were performed in samples that contained approximately 10^6 saprophytic bacteria per gram of seeds (as indicated by the non-selective YPGA medium). Higher populations of saprophytic bacteria associated with onion seeds (whatever their origin) were uncommon, making NCTM1 a suitable medium for isolation of the pathogen in highly polluted samples. NCTM1 medium should be useful for the detection of *Xanthomonas* sp. from seed in other countries where OBB occurs. The epidemiological significance of seed-borne inoculum is presently under investigation in Réunion Island.

Low populations (approximately 10^2–10^3 cells g^{-1}) of *Xanthomonas* sp. were selectively isolated from symptomless onion leaves in several fields in Réunion Island. The biological significance of asymptomatic populations of onion *Xanthomonas* sp. is not known. Nevertheless, this new medium is likely to be a valuable tool to study the ecology and epidemiology of *Xanthomonas* sp. associated with onion.

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