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Identification of a highly virulent strain of *Xanthomonas axonopodis* pv. *malvacearum*

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Abstract A highly virulent strain (HVS) of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) was first reported in Africa in 1983, infecting all commercial cultivars of cotton including the immune cv. '101–102B'. The HVS was considered to be a new race of pathovar *malvacearum* (race 20). Here we studied a HVS (GSPB 2388) isolated in Sudan, which causes symptoms that clearly differed from the typical angular water-soaked spots of bacterial bright of cotton. Our investigations showed that extracellular cellulase activity of this HVS was higher than that of the control strain GSPB 1386 (race 18). Additionally, SDS-PAGE indicated that the HVS cell wall contained short LPS molecules with fewer O-chain repeating units, lacking in GSPB 1386. The higher cellulase activity and the distinct lipopolysaccharide of HVS are correlated with the higher virulence and deviating symptom formation. Rep-PCR fingerprinting showed that the HVS was very closely related to other strains of *Xam*.

Keywords Cellulase · Lipopolysaccharide · Rep-PCR

Abbreviations

CMC	carboxyl methyl cellulose
GSPB	Göttinger Sammlung phytopathogener Bakterien
HVS	highly virulent strain
LPS	lipopolysaccharide
RBB	remazol brilliant blue R
Xam	*Xanthomonas axonopodis* pv. *malvacearum*

Introduction

Bacterial blight of cotton (*Gossypium hirsutum*), caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), is an important and potentially destructive bacterial disease worldwide. Cotton yield losses in excess of 20–30% have been reported in the past (Delannoy et al. 2005). According to a test on an array of cotton lines, *Xam* can be divided into 18 races (Hunter et al. 1968). A HVS of *Xam* appeared in Africa in 1988, that could overcome major resistant genes against bacterial blight (*B*2, *B*4, *B*7, *BIn*, *BN*, and *B2B3*) and infected all the commercial cultivars

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including 'Mebane 101–102B' (Chakrabarty et al. 1997; Follin et al. 1988). Although the HVS was reported 20 years ago and has been widely prevalent creating much difficulty in breeding resistant cultivars (Akello and Hillocks 2002), specific pathogenic action is still little known.

In many cases, plant defence responses are initiated by a gene-for-gene interaction. A plant resistance gene (*R*) interacts with the corresponding avirulence gene (*avr*) from the pathogen that recognises the pathogen and induces a rapid hypersensitive reaction (HR) at the site of pathogen invasion. In addition to the *R* gene-mediated highly specific mechanisms, plants acquire the ability to recognize invariant pathogen-associated molecular patterns (PAMPs) that are characteristic of microbial organisms but that are not found in potential hosts (Medzhitov and Janeway 2002; Nurnberger et al. 2004). PAMPs include peptidoglycans, lipoteichoic acid of Gram-positive bacteria, and lipopolysaccharides (LPS) of Gram-negative bacteria. LPS may contribute to the exclusion of plant-derived antimicrobial compounds promoting the ability of a bacterial plant pathogen to infect plants. LPS may suppress rapid defence responses such as the oxidative burst and exerts long-term influences, including transcriptional adjustment to pathogen attack (Tellstrom et al. 2007). LPS can also be recognized by plants to directly trigger some defence-related responses (Dow et al. 2000). Moreover, LPS can induced defence-related genes and activate nitric oxide synthase, which plays a major regulatory in plant defence responses and cell death events associated with pathogen attack (Zeidler et al. 2004).

Many plant-pathogenic bacteria, including those belonging to the genus *Xanthomonas*, secrete extracellular enzymes include depolymerising enzymes, such as cellulases or pectinases (Chapon et al. 2001). These enzymes play an important role in the pathogenesis (Kazemi-Pour et al. 2004). Extracellular enzymes directly or indirectly affect the bacterial population and symptoms in the host, and even determine whether or not the infection will successful.

In this study, we have investigated a HVS isolated in Sudan that infected and caused symptoms in all ten cotton lines used as different from the typical cotton bacterial blight and other HVS reported previously. To further investigate its pathogenesis, LPS and extracellular cellulase were analysed. Additionally, rep-PCR fingerprinting was carried out to determine the genetic relationship between the HVS and other *Xam* strains.

Materials and methods

Bacterial strains

All bacterial strains used in this study are listed in Table 1. Bacteria were stored as lyophilised stocks and suspended in King's B medium before use. The bacterial suspension were streaked on Nutrient Glucose Agar (NGA) medium (3 g yeast extract, 8 g nutrient broth, 10 g glucose, 15 g agar, 1 l distilled water, pH 7.2) and cultivated at 26°C for 3 days. A single colony was then selected for further characterization.

Test plant cultivation, inoculation, bacterial multiplication analysis *in planta*

Cotton cv. 'Acala 44' (*G. hirsutum*) was grown in a chamber with a relative humidity 85%, temperature at 28/25°C light/dark cycle, and light 16 h 25,000 lux/8 h dark. Bacterial strains were suspended in inoculation buffer (0.01 M $MgSO_4$) to a 10^5 cfu ml^{-1} concentration, and then evenly sprayed on the lower side of the first two not fully-grown young leaves (1/3 size of a mature leaf) following the cotyledons as described (Klement et al. 1990). To determine bacterial multiplication, the uninoculated leaves and top buds were removed after inoculation. Leaf disks were removed with a cork borer once daily and 20 disks were collected for one assessment of bacterial multiplication *in planta*.

Determination of extracellular cellulase activity

Bacteria were pre-cultured on basal medium with 0.75% glucose, 0.5% CMC, and 0.1% yeast extract for 1 day, then transferred to fresh medium (basal medium with 0.5% CMC and 0.1% or 1% yeast extract) and cultivated for 3 days. The bacterial suspension was centrifuged and extracellular cellulase activity in the supernatant was determined by digesting a soluble chromogenic substrate, carboxyl methyl

Table 1 Bacterial strains used in this study

Species and pathovar	Isolate	Race	Location	Host
Xanthomonas axonopodis				
malvacearum	GSPB 2385	11	Greece	Cotton
malvacearum	GSPB 1386	18	Nicaragua	Cotton
malvacearum	GSPB 2388	20 (HVS)	Sudan	Cotton
malvacearum	GSPB 2389	20 (HVS)	Sudan	Cotton
malvacearum	ATCC 49292	20 (HVS)	Burkina Faso	Cotton
malvacearum	ATCC 49293	20 (HVS)	Burkina Faso	Cotton
malvacearum	ATCC 49294	20 (HVS)	Burkina Faso	Cotton
cassavae	GSPB 2437		Rwanda	Cassava
manihotis	GSPB 2511		Nigeria	Cassava
vitians	GSPB 2864		USA	Lettuce
vitians	GSPB 2866		USA	Lettuce
lobeliae	GSPB 2966		Germany	Lobelia
Erwinia caratovora				
carotovora	GSPB 436		DSM 60442	Potato

GSPB Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany, *ATCC* American Type Culture Collection, Virginia, USA, *DSM* Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

cellulose-remazol brilliant blue R (CMC-RBB), kindly offered by Prof. Dr. A.G. Wolf (Universitaet Göttingen, DE). The bacterial supernatant was incubated on CMC-RBB containing-agar, where the cellulase activity was expressed as a clear zone around a well. In another assay the supernatants were incubated with CMC-RBB solution in a Eppendorf tube. The non-degraded substrate was precipitated by addition of HCl, pelleted by centrifugation and discarded. The enzyme activity was measured by photometric extinction at 592 nm in cuvettes. The experiment was carried out as described by Lacy et al. (1990) and Wirth and Wolf (1990). However, the assay system could not differentiate between differences due to changes in cellulase concentration or activity of the enzyme; however, we refer only to cellulase activity in the text.

Extraction and SDS-PAGE of bacterial lipopolysaccharides (LPS)

LPS was prepared as previously described (Senchenkova et al. 2002). The LPS samples were suspended in 0.1 M Tris–HCl (pH 6.8) containing 2% SDS, 10% glycerine and 20 ppm bromophenolblue, then heated for 5 min in a water bath (100°C) and loaded onto 3% stacking gel and 12% separating polyacrylamide gel. After electrophoresis, the gel was silver-stained as described (Tsai and Frash 1982).

Bacterial genomic fingerprinting analysis

Genomic fingerprints were obtained by performing rep-PCR, using the BOX, ERIC and REP-primer sets on Omnigene HBTR3SM7 thermal cycler (Hybaid limited, USA), following the methods described by Louws et al. (1994, 1999). The PCR product was separated on a 5–10% gradient TBE–polyacrylamide gel.

Results

Race identification and symptom development

In order to determine the race of isolate GSPB 2388 from Sudan, ten standard cotton lines (Hunter et al. 1968) were inoculated with this strain. As negative or positive controls, one strain from Nicaragua (GSPB 1386, race 18) and three HVS strains (ATCC 49292, 49293, and 49294) isolated in Burkina Faso (Follin 1983; Follin et al. 1988) were simultaneously tested. The results show that GSPB 2388, ATCC49292, and 49294 infected all ten lines, indicating that they are HVS, whereas 49293 has lost its virulence and could not infect any cotton line. GSPB 1386 caused symptoms in all cotton lines excepts Mebane 101–102B, identifying it as race 18 (Table 2).

Table 2 Pathogenicity of several *X. a.* pv. *malvacearum* strains on differential cotton cultivars

Cultivars for race assortment	Strains tested				
	GSPB 1386	GSPB 2388	ATCC 49292	ATCC 49293	ATCC 49294
Acala 44	+	+	+	−	+
Stoneville 2B–S9	+	+	+	−	+
Stoneville 20	+	+	+	−	+
Mebane B-1	+	+	+	−	+
Mebane 1–10B	+	+	+	−	+
Mebane 20–3	+	+	+	−	+
Mebane 20–3	+	+	+	−	+
Mebane 101–102B	−	+	+	−	+
Gregg B	+	+	+	−	+
Empire B4	+	+	+	−	+
DP × P4	+	+	+	−	+
Race	18	20 (HVS)	20 (HVS)	Avirulent	20 (HVS)

+ Infected, − no symptom

GSPB 1386 caused typical symptoms of bacterial blight (Fig. 1). One week after inoculation, water-soaked angular leaf spots appeared. The water-soaked spots enlarged and bacterial slime was secreted from the water-soaked spots about 3 weeks after inoculation. The HVS (ATCC49294) from Burkina Faso also caused water-soaked angular spots but the infected area turned necrosis 4 weeks after inoculation. In contrast, the HVS (GSPB 2388) from Sudan did not cause angular water-soaking, but caused chlorotic spots which enlarged and became necrotic 3 weeks after inoculation. Additionally, the chlorotic and nectrotic areas were not obviously restricted by leaf veins and no bacterial slime could be observed.

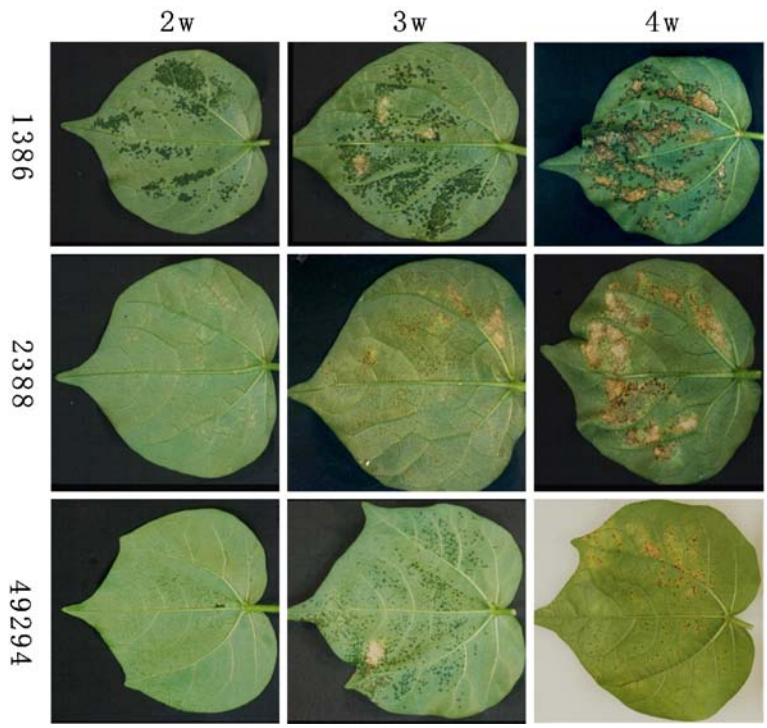

Fig. 1 Comparison of the symptoms caused by race 18 GSPB 1386, and HVS (GSPB 2388, ATCC49294) of *X. axonopodis* pv. *malvacearum* on cotton cv. 'Acala 44'. The inoculated leaves were photographed at 2, 3, or 4 weeks after inoculation

Symptom incited by the HVS from Sudan differs significantly from that of race 18 and HVS from Burkina Faso.

In comparing the multiplication kinetic of GSPB 1386 and HVS (GSPB 2388), no significant difference was observed in the period of 0–96 h post-inoculation, but the bacterial concentration of 1386 is evidently higher than that of 2388 in the stationary phase. The maximum bacterial concentrations of the two strains in the inoculated leaves were 2.5×10^8 (1386) and 3.2×10^7 (2388) respectively (Fig. 2).

Extracellular cellulase activity

Extracellular cellulase activity was indicated by the clear zone on agar plates where CMC-RBB was digested by bacterial supernatants (Fig. 3a) and by photometric extinction of digested CMC-RBB in cuvettes (Fig. 3b). In these assays, *Erwinia carotovora* strain GSPB 436 and *Xanthomonas manihotis* strain 2511 served as positive controls, and the culture medium as the negative control. Both assays revealed that HVS GSPB 2388, GSPB 2389 (*Xam* HVS, isolate from Sudan), and ATCC 49294 showed higher cellulase activity than GSPB 1386 (race 18) and GSPB 2385 (*Xam*, race 11). The cellulase activity of HVS from Sudan (GSPB 2388 and 2389) was higher than that from Burkina Faso (ATCC49294) (Fig. 3). Of note, bacterial cellulase activity of all strains excepts GSPB 2511 was affected by the concentration of yeast extract in the medium. The cellulase activity of the bacteria cultivated on medium containing 1% yeast extracts (Fig. 3a, b—A) was higher than that cultivated with 0.1% yeast extract (Fig. 3a, b—B).

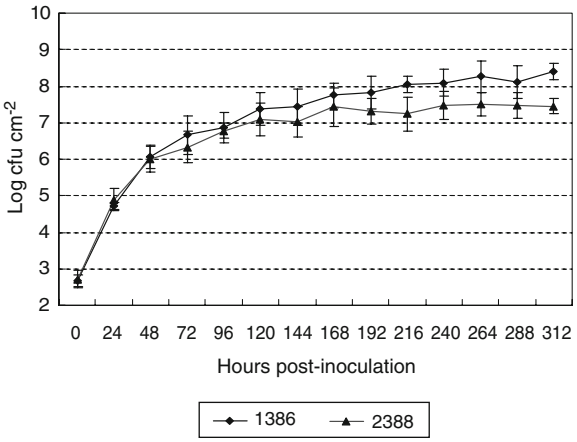

Fig. 2 *In planta* bacterial concentrations of strains GSPB 1386 and GSPB 2388 after inoculation in cv. 'Acala 44'. Values present a mean of three independent plate counts

Characterization of LPS

LPS was isolated and separated by SDS-PAGE and subsequently silver-stained. Nine bands formed with GSPB 1386 LPS and 13 with GSPB 2388 LPS, each band representing a LPS molecule containing lipid A, core region and O-chain repeating units (Fig. 4). The band running on the gel front is the LPS molecule without O-chains. The bands between 56 and 78 kDa of both strains were exactly parallel, reflecting their similar molecular structure. Significant differences could be found in the range of 42.7–56 kDa, where GSPB 2388 had five bands, and GSPB1386 has none, indicating that the HVS from Sudan possesses smaller LPS molecules with fewer O-chain repeating units that were absent in GSPB 1386 (Fig. 4).

Genomic fingerprinting

Primers corresponding to conserved DNA sequences of REP elements, BOXA subunits of BOX elements, and ERIC sequences annealed to genomic DNA and generated unique genomic fingerprints. Rep fingerprint patterns of representative strains of *X. axonopodis* pv. *malvacearum*, *X. axonopodis cassavae*; *X. axonopodis vitians*, *X. axonopodis manihotis*, and *X. axonopodis lobeliae* are shown in Fig. 5. The ERIC-, BOX-, and REP-PCR yielded eight to more than 20 distinct PCR products, ranging in size from approximately 100 bp to over 3 kb. The rep-PCR DNA fingerprints clearly distinguish the different pathovars *X. axonopodis* pv. *vitians* strains (GSPB 2864, lane 6; and GSPB 2866, lane 7), obtained from the same geographical location and isolated in the same year (Table 1). However, the fingerprints of HVS were closely similar to those of *X. axonopodis* pv. *malvacearum* was observed (Fig. 5, lane 1 to 4).

Discussion

Although HVS of cotton bacterial blight has been reported for some time (Follin 1983; Follin et al. 1988), the basis of its pathogenicity is still relatively unknown. Chakrabarty et al. (1997) described a HVS isolate that caused water-soaked spots and from it

cloned an avirulent (*avr*) gene *pthN*. However, no data indicated that the *pthN* was related to its high virulence (Chakrabarty et al. 1997). A strain (GSPB 2388) isolated from Sudan infected all ten cotton lines used for differentiating races of *X. axonopodis* pv. *malvacearum* including immune cv. 'Mebane 101–102B', and was therefore regarded as a HVS of *X. axonopodis* pv. *malvacearum* (Table 2). In order to further characterize the pathogenicity of this highly virulent isolate, a comparable virulent strain (GSPB 1386, race 18) was chosen as the control, which could infect all the nine cotton lines for differentiating races except 'Mebane 101–102B'. Cultivar 'Acala 44' containing no known resistant gene against *X. axonopodis* pv. *malvacearum* was chosen as a susceptible host for both strains. As shown in Fig. 1, GSPB 1386 caused the typical symptoms of cotton bacterial blight, angular water-soaked leaf spots with exudation, whereas HVS GSPB 2388 caused chlorosis and later necrosis, which were not obviously restricted by leaf veins. Additionally, no exudation of bacterial slime occured. HVS from Burkina Faso (ATCC 49294) also caused water-soaked spots, which subsequently turned necrotic. Symptoms caused by HVS from Sudan (GSPB 2388) were distinct from those of other *Xam* strains and hitherto reported HVS (Follin et al. 1988; Chakrabarty et al. 1997).

The bacterial concentration of GSPB 1386 in the stationary phase is higher than that of GSPB 2388 (Fig. 2), which may be explained by the *in planta* environment. GSPB 2388 occurred in unfavourable conditions in leaves that were chlorotic and necrotic, preventing the bacteria from fully filling the intercellular space and limiting cell division.

Extracellular enzymes are important virulence factors. In a *Xam* strain with reduced virulence, cellulase activity was also reduced, while protease activity remained unchanged (Verma and Singh 1975). The HVS (GSPB 2388 and 2389) had a distinctly higher cellulase activity than other *Xam* strains. Cellulase

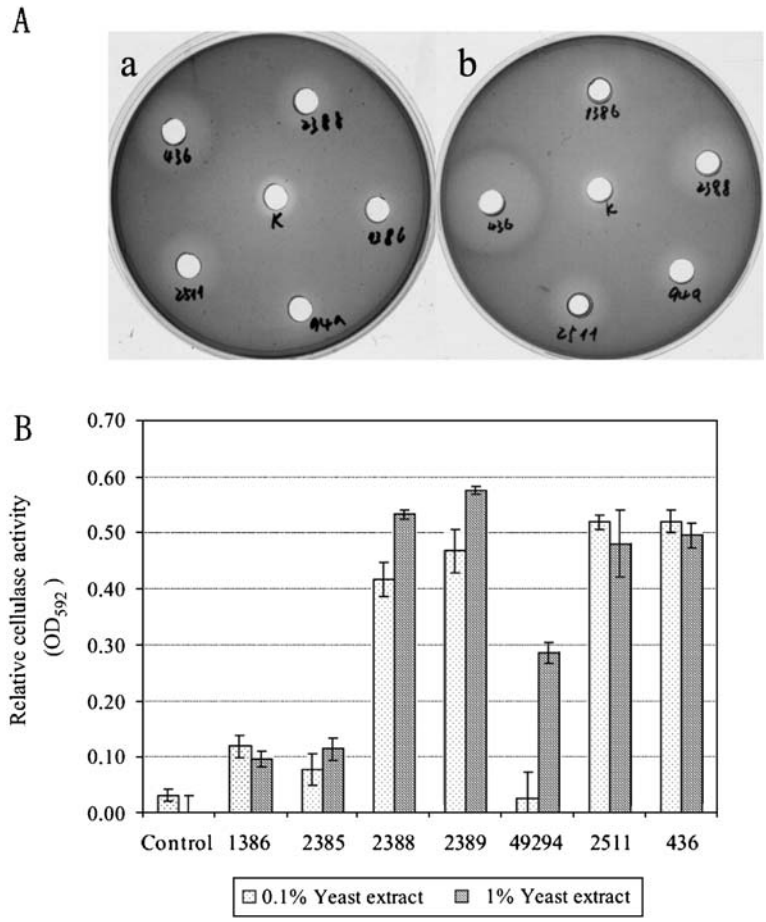

Fig. 3 Determination of cellulase activity. **a** On agar medium containing chromogenic substrate with supernatants from strain GSPB 1386 (*Xam*, *1386*), GSPB 2388 (*Xam* HV, *2388*), GSPB 2511 (*X. axonopodis manihotis*, *2511*), GSPB 436 (*E. carotovara carotovora*, *436*), ATCC 49294 (*Xam* HVS, *94a*), and control (*k*), cultured in medium containing 0.1% yeast extract (*A*), or 1% yeast extract (*B*); **b** by photometric extinction measured for supernatants of strain GSPB 1386 (*Xam*), GSPB 2388 (*Xam* HVS), GSPB 2389 (*Xam* HVS), GSPB 2511 (*X. axonopodis manihotis*), GSPB 436 (*E. carotovara carotovora*), ATCC 49294 (*Xam* HVS), and control, incubated with soluble substrate CMC-RBB clarified and read at OD_{592}. Values present a mean of three independent experiments

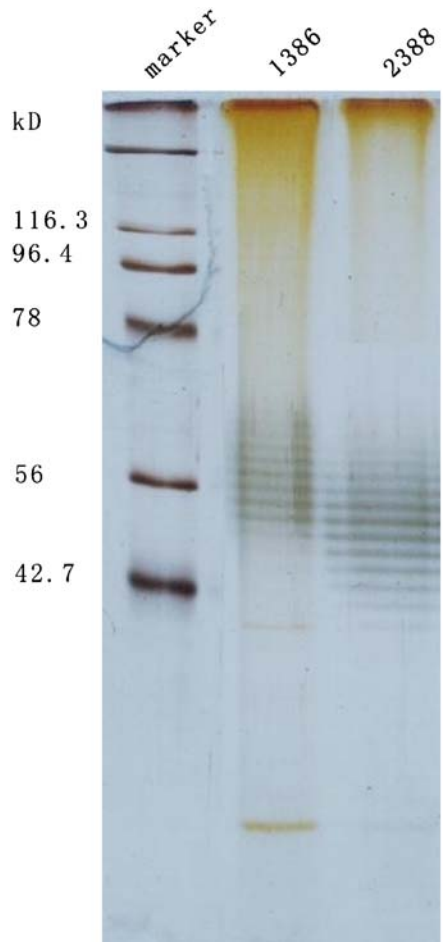

Fig. 4 SDS-polyacrylamide gel electrophoresis of lipopolysaccharide GSPB 1386 and GSPB 2388 and silver-stained after electrophoresis

activity of HVS (ATCC49294) was lower than that of HVS from Sudan, but significantly higher than that of GSPB 1386 and 2385 (Fig. 3). The possibility could not be ruled out that cellulase plays a key role for HVS in breaking the resistance of 'Mebane 101–102B'. Furthermore, the leaf chlorosis and necrosis caused by HVS were not restricted by leaf vein and did not develop into angular spots, indicating that the higher cellulase activity may enable HVS to penetrate the vein restriction. Additionally, the cellulase synthesis of HVS seems to highly dependent on the supply of organic substrates, because yeast extracts added to medium affected cellulase activity (Fig. 3). Similar results have also been reported in *E. carotovara carotovara* (Chatterjee et al. 1995).

As described above, LPS is a key factor of pathogenesis. Earlier studies revealed that structures of the O-repeating units of GSPB 1386 and 2388 were indistinguishable (Senchenkova et al. 2002). This study revealed that five visible bands could be detected in the range of 42.7–56 kDa in the LPS preparation of HVS, which were absent in GSPB 1386, suggesting that HVS GSPB 2388 possesses some smaller LPS with fewer repeating units of O-side polysaccharide that are lacking in strain GSPB

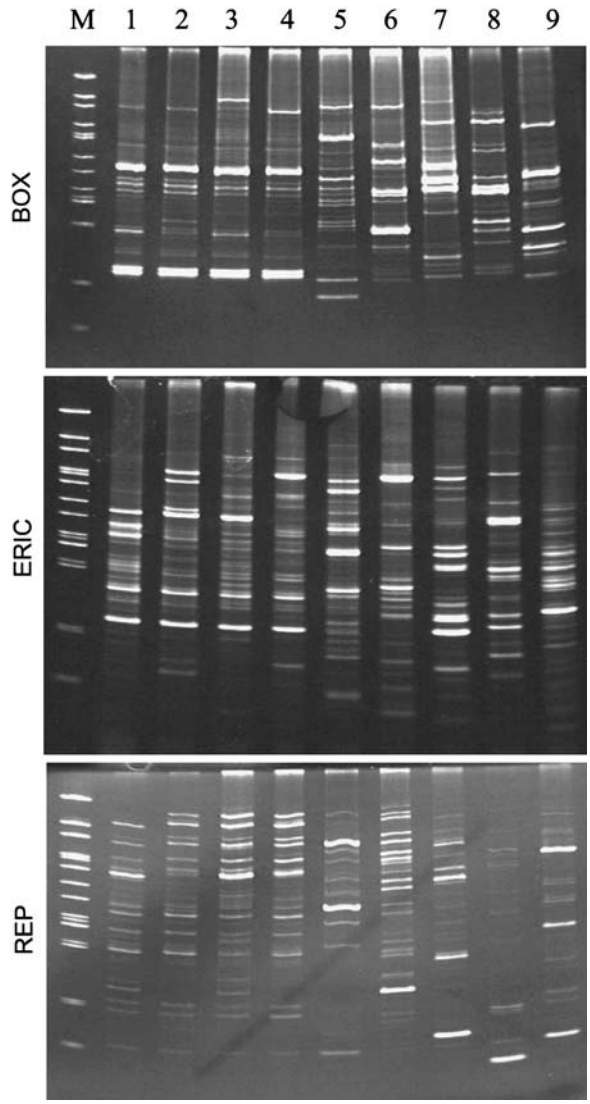

Fig. 5 ERIC-, BOX-, and REP-PCR fingerprinting patterns of genomic DNA of *X. axonopodis* isolates GSPB 1386 (lane *1*), GSPB 2385 (lane *2*), GSPB 2388 (lane *3*), ATCC49294 (lane *4*); *X. axonopodis cassavae* isolate GSPB 2437 (lane *5*); *X. axonopodis vitians* isolates GSPB 2864 (lane *6*), GSPB 2866 (lane *7*); *X. axonopodis manihotis* isolate GSPB 2511 (lane *8*); *X. axonopodis lobeliae* isolate GSPB 2966 (lane *9*). The left lane *M* shows the DNA molecular size ladder

1386 (Fig. 4). Newman and colleagues (Dow et al. 2000; Newman et al. 1997) have extensively studied the biological significance of bacterial LPS by using a series of LPS using a series of LPS derivates isolated from defined mutants, concluded that plant cells can recognize different structures within bacterial LPS to trigger alterations in a plant's response to avirulent pathogens. Although the biological significance of the smaller LPS with fewer O-repeating units of HVS is still not clear, it may be correlated with pathogenesis and higher virulence.

The pathovar taxon used for plant pathogens refers to a strain or set of strains with the same or similar characteristics, differentiated at the infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts, while race distinguishes strains that give different responses on different cultivars. Where there are clear differences in symptomatology and disease biology on the same host, separate pathovars are warranted (Swings and Hayward 1990). Under this definition, designation of HVS from Sudan as a new pathovar of *Xanthomonas axonodopois* may be warranted. In order to determine whether this classification was reasonable, a rep-PCR fingerprinting analysis was carried out. However, genomic fingerprints showed a very close relationship between HVS and other strains of *X. axonopodis* pv. *malvacearum* and evident distinction from other pathovars, suggesting that it probably is not a separate pathovar.

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