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Full Length Research Paper

Plasmids in races of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), the causal agent of bacterial blight of cotton

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Nine strains belonging to 2 races of *Xanthomonas campestris* pv*. malvacearum* (*Xam*), synonym, *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) were screened for their plasmid content. One strain belonging to race 4, which has been attenuated as a mutant from race 18 was less virulent than race 18, even though it possessed the same plasmids as race 18 strains. Five groups could be distinguished based on their plasmid profiles. The first group includes the race and 4 strain and 4 isolates of race 18 from Nicaragua that were isolated in 1986. The other 4 isolates of races 18, isolated from Nicaragua in 1986; the USA in 1986; and Sudan in 1991 were separated into 4 distinct groups according to their plasmid profile. The attenuated strain of race 4 was more sensitive to antibiotics and heavy metal ions than the race 18 strains. There was no indication that these plasmids play a distinct role in virulence of these *Xam* strains.

Key words: Plasmid, *Xanthomonas*, antibiotics, heavy metals.

INTRODUCTION

The genus *Xanthomonas* is an economically important group of bacterial pathogens. One of the more important diseases is bacterial blight of cotton, which is caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye, synonym, *Xanthomonas axonopodis* pv. *malvacearum* (Vauterin et al., 2000).

Bacterial blight is an economically important disease worldwide and, result in yield losses of 10 to 30% of seed cotton (Verma, 1986; Zachwski and Rudolph, 1988). The stage at which the plant is infected, the environmental conditions at the time of infection, and the degree of plant resistance to the pathogen are important factors effecting yield losses. *Xam* is reported to have a wide virulence range. Hunter et al. (1968), identified 19 *Xam* races based on virulence to different cotton cultivars (Acala 44, Stoneville 2B-S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4 and DPxP4). The number was increased to 32 physiological races using 7 cotton cultivars (Verma and Singh, 1974).

These races are distributed in different countries, for example race 1 is widespread in Australia, India and the USA. Races 2 to 5 were recorded in India and USA; race 6 in Nigeria, Zymbawe and India; and race 18 in Australia, the USA, Africa, India and Nicaragua (Abdo-

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Table 1. Bacterial strains used in this study.

(GSPB) Nr.	Race	Origin/date of isolation
Xam 1252	18	USA 1986
Xam 1384	18	Nicaragua 1986
Xam 1385	18	Nicaragua 1986
Xam 1386	18	Nicaragua 1986
Xam 1429	18	Nicaragua 1986
Xam 1432	18	Nicaragua 1986
Xam 1435	18	Nikaragua 1986
Xam 3012	18	Sudan 1991
Xam 1430	4	Nicaragua 1986
Erwinia stewartii GSPB 2628		

Xam = Xanthomonas axonopodis pv*. malvacearum*, *Es = Erwinia stewartii*.

Hasan et al., 2008). Five *Xam* races (AbdelRehim, 2005; Abdo-Hasan et al., 2008; Chakrabarty et al., 1995; Mills, 1990; Verma, 1986) has been identified in Syria (Abdo-Hasan, 2002).

Plasmids (autonomous genetic elements that can replicate independently, the main chromosome), are important and widely occurring constituents of plant pathogenic bacteria (Coplin, 1982). The plasmid composition of a particular phytopathogenic bacterium may be highly variable, with considerable differences in size and number of plasmids, reported by Burr et al. (1988) for *Pseudomonas syringae* pv. *papulans*. Bender and Cooksey (1986) reported that *P. syringae* pv. *tomato* could contain 5 different size plasmids (29 to 101 kb), while *X. campestris* pv*. pruni* could have 10 sizes of plasmids (4.2 to 35.1 Mdal with, 1 to 4 per strain) (Randhawa and Civerolo, 1987).

Other plant pathogenic bacteria are much less variable in their plasmid content. For example *Clavibacter michiganensis* subsp. *sepedonicus* contains only a single 50.6 kb plasmid. Half of the pathogen strains analysed by Mogen et al. (1988) harboured the plasmid, while southern hybridization analysis showed the plasmid was integrated into the chromosome of the other strain.

Plasmids are involved in antibiotic and heavy metal resistance; degradation of xenobiotic compounds; genetic evolution during gene transfer, other possible roles affecting virulence, and symptoms development of phytopathogenic bacteria. This study evaluated the plasmid founds in *Xam*.

MATERIALS AND METHODS

Bacteria

Nine *Xam* bacterial strains were obtained as lyophilized samples from the GSPB bacterial collection (Göttinger Sammlung phytopathologener Bakterien) (Table 1). Eight (8) were highly virulent strains of race 18, and one was a weakly virulent strain (races 4, which originated as a mutant of race 18 strain, GSPB 1386). One strain of *Erwinia stewartii* was used as a plasmid molecular marker.

Cultivation of bacterial cultures

The lyophilized samples were suspended in King's B liquid medium for 30 min before streaking several drops of this suspension on NGA (Nutrient Glucose Agar) plates and incubating for 3 days at 30°C (18a).

Antibiotic susceptibility test

The standardized single disk method was used to measure the antibiotic susceptibility of the *Xam* strains.

Heavy metals and MIC values

The minimal inhibition concentration (MIC) was determined in triplicate by measuring bacterial growth on NGA plates containing a different concentrations (0.25, 0.5, 1, 5, 10 and 20 mM) of metal ions Zn^{++}, Co^{++}, Ni^{++} and Pb^{++}) (Ghosh et al., 1997).

Plasmid extraction and curing

A modified method of Birnboim (1983), was used for plasmid isolation (AbdelRehim, 2005). *Xam* strain (GSPB1386) was treated with acrydine orange (100 to 300 µg/ml) and SDS (1.0 to 500 µg/ml) for curing. Colony morphology and other characteristics were noted. Colonies were randomly picked from NGA plates and screened for plasmids. DNA concentrations were calculated by determining the optical density (OD) at 260 and 280 nm. Equal concentrations of plasmid DNA were loaded in 0.7% agarose gel and separated by electrophoresis in TAE buffer at 70 V for 3 h.

RESULTS

Antibiotic susceptibility test

Sensitivity of the *Xam*-strains to 14 antibiotics is summarized in Table 2. All the strains were resistant to

Table 2. Resistance of *Xam* strains to different antibiotics.

Antibiotics	*Xam* Race 4	*Xam* Race 18
Sulphamethazol + Trimethoprim 23.75 + 1.25 µg (SXT)	-	+
Oxacillin 5 µg (OX)	+	+
Penicillin 10 µg (P)	+	+
Cefalexin 30 µg (CN)	+	+
Clindamycin 10 µg (CC)	+	+
Chloramphenicol 30 µg (C)	-	-
Tetracyclin 30 µg (TE)		
Polymyxin B 300 I.E. (PB)		
Vancomycin 30 µg (VA)		
Tobromycin 10 µg (NN)		
Kanamycin 5 µg (K)	-	ve
Gentamycin 10 µg (Gm)		
Ofloxacin 10 µg (OFX)		
Nalidixic acid 30 µg (NA)		

(+) = Tolerant (growth), (-) = sensitive (no growth).

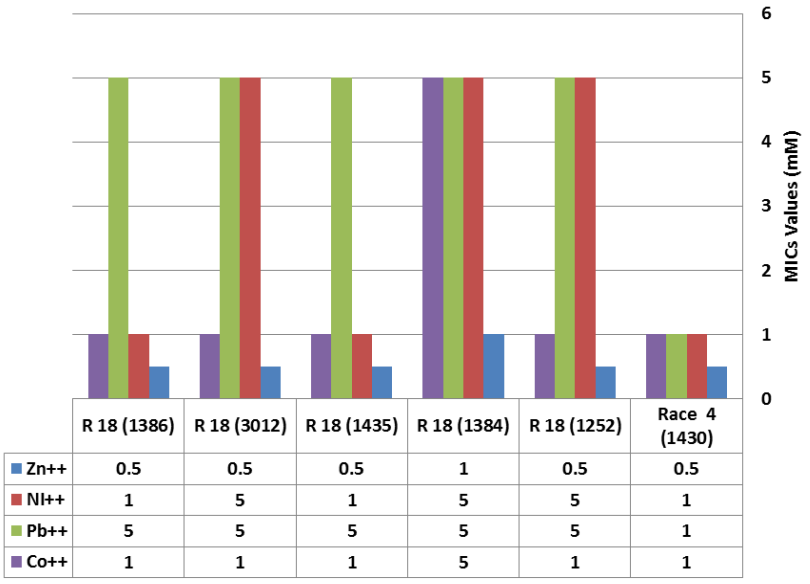

Figure 1. Heavy metals tolerance (MIC value) of different *Xam* strains.

SXT, OX, P, CN and CC except strain GSPB 1430, (race) 4 which was sensitive to SXT. All strains were sensitive to OFX, TE, PB, VA, NN, K, Gm, C and NA antibiotics even at the lowest concentration tested.

Heavy metals tolerance

Different MICs values were observed for the bacterial strains tested (Figure 1), race 4, was the most sensitive to heavy metals, and had MIC values ranging from 0.5 mM (Zn^{++}) to 1 mM (Ni^{++}, Pb^{++}, Co^{++}). Race 18 was more resistant to higher concentrations of these metals, and MIC values of 5 mM were observed in some cases. In contrast, avirulent race 4 strain was less resistant to the heavy metals than virulent strains (Figure 1).

Plasmid profiles

The plasmid profiles of 8 strains belonging to race 18 and one strain of race 4 could be separated into five groups (Figure 2 and Table 3). The four *Xam* strains of race 18 isolated from Nicaragua in 1986 (GSPB, 1386; 1429;

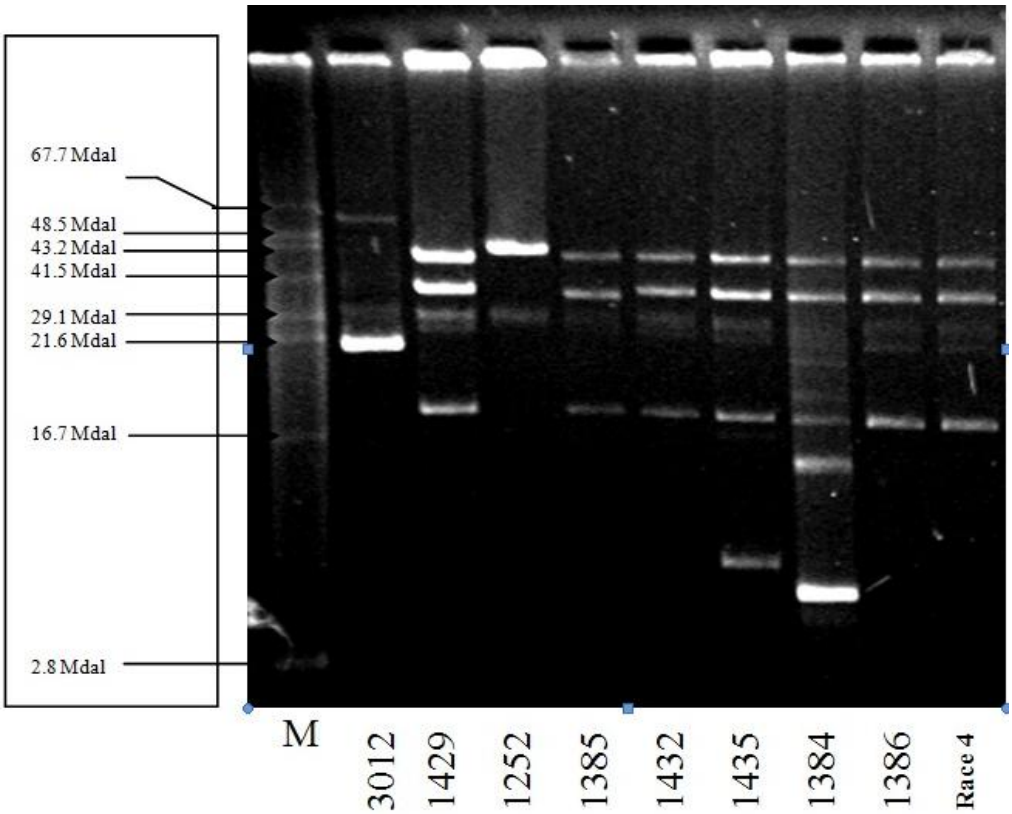

Figure 2. *Xam* plasmid profiles of eight strains of race 18 (GSPB, 3012, 1429, 1252, 1385, 1432, 1435, 1348 and 1386) and one of race 4 (GSPB, 1430). M: Marker, plasmid profile of *Erwinia stewartii,* GSPB 2628.

Table 3. Plasmids in the eight *Xam* strains of race 18 and 4.

Plasmid group	Plasmid bands sizes / Strain no. origin and date of isolation		(54.6 Mdal)	(45.2 Mdal)	(42.4 Mdal)	(35.1 Mdal)	(28.3 Mdal)	(25.6 Mdal)	(16.5 Mdal)	(11.7 Mdal)	(5.3 Mdal)	(3.9 Mdal)
1	1252 USA 1986	Race 18		+			+					
2	1384 Nicaragua. 1986				+	+	+		+	+	+	
3	1435 Nicaragua 1986				+	+	+		+			+
4	3012 Sudan 1991		+				+	+				
5	1386 Nicaragua 1986				+	+	+		+			
	1429 Nicaragua 1986				+	+	+		+			
	1432 Nicaragua 1986				+	+	+		+			
	1385 Nicaragua 1986				+	+	+		+			
	1430 Nicaragua 1986	R 4			+	+	+		+			

1432; 1385) had identical plasmid profiles with the race 4 Strain (GSPB, 1430) and were placed in plasmid group 5. The other two race 18 strains from Nicaragua (GSPB, 1384 and 1435) had different plasmid that were different plasmid pattern than the other race 18 strains placed in Groups 2 and 3, respectively. Strains from the USA (GSPB, 1252) and Sudan (GSPB, 3012) are group 4, respectively, since their plasmid profiles differed considerably from each other as well as from the Nicaragua strains.

Plasmid curing

No plasmids loss was detected from about 300 bacterial colonies of *Xam* strain GSPB 1386, race 18 were detected after screening of about 300 bacterial colonies treated with 300 µg/ml of Acriydine orange and 400 µg/ml of SDS.

DISCUSSION

Bacterial genes, occurring on either chromosomal or plasmid DNA, are involved in a wide range of phenotypic characteristics. The majority of genes involved in infection and development of disease by Xanthomonads are located on the main chromosome, even though, the vast majority of plasmids occurring in all genera of phytopathogenic bacteria are cryptic (Mills, 1990). In recent years, plasmids have been shown to encode a wide range of functions that are important in bacterial-plant interactions (including pathogenicity, virulence factors, production of toxins) and they are also important in determining certain non-pathogenic features (Sigee, 1993).

The ability of a particular pathogen to cause disease depends on two types of genes: pathogenicity genes and virulence genes. Pathogenicity genes are a fundamental requirement for disease to be induced, while virulence genes determine the type of disease and its severity.

Two sets of genes of leaf spot causing bacteria determine the host pathogen interaction. The *hrp* genes are required for "basic pathogenicity": that is the ability to grow as a pathogen inside a plant, but the *hrp*-genes may also cause an hyper-sensitivity (HR) like necrosis in nonhost plants. The second gene set comprises the so-called avirulence (*avr*) genes whose products interact directly or indirectly with plant resistance gene to provoke a defense reaction. The defense reaction is typically a local necrosis that limits pathogen invasion and disease. Race-specific resistance is often genetically specified by dominant single loci in the host that correspond to specific dominant *avr* genes in the pathogen.

Gabriel et al. (1986) concluded that *Xam* carries at least nine identifiable *avr* genes, all of which appeared to be chromosomally determined. Later, De Feyter and Gabriel (1991), suggested the possibility that at least one of the *avr* genes (of the six *avr* genes clustered on a 90-kb plasmid in *Xam*) may be important for conditioning virulence of the pathogen on a susceptible host.

The so-called avirulence genes located in the chromosome or a plasmid have two effects: on a resistant host plant with a matching resistance gene, they will cause a hypersensitive defence reaction, on a susceptible host plant without a matching resistance gene, the *avr*-genes may enhance disease development. Therefore, the question arose whether specific plasmids may be harboured by defined *Xam*-races, as suggested by Sathyanarayana and Verma (1998).

Chakrabarty et al. (1995) assumed that virulence genes in *Xam* were plasmid borne. The authors isolated 3 plasmids of 55.0, 31.2 and 7.4 Kb from one strain of *Xam* belonging to race 18 (American system). After curing them of these plasmids by incubation of the bacterial culture at 42°C, virulence of the strain was significantly reduced. Sathyanarayana and Verma (1998) reported that the highly virulent race 32 (Indian system) harboured five plasmids (60, 40, 10, 5.5 and 2.2 Kb) and the moderately virulent race 26 harboured three plasmids, whereas the less virulent race 5 had only one plasmid. They also reported that a strain of race 32 became avirulent after plasmid loss by curing with Mitomycin C (6 µg/ml) to suggest strongly the role of individual plasmids in neutralizing respective B-genes (Verma, 1995). When the 10 Kb plasmid (common in the three races) was transferred to the avirulent plasmid-cured strain, virulence for gene B_{IN} was restored.

On the average, five plasmid bands were detected per strain in our studies, ranging from two bands in strain GSPB 1252, race 18 from the USA to six plasmid bands in strain GSPB 1384, race 18 from Nicaragua. In contrast, Lazo and Gabriel (1987) found that the majority of the *Xam* strains harbouring plasmid contained only one plasmid, and only a few carried two or more. The reason for this discrepancy is unknown.

Sathyanarayana and Verma (1993, 1998) hypothesized that plasmids play a role in virulence of *Xam*. Chakrabarty et al. (1995) reported that three plasmids of an Indian race 18 strain of *Xam* played a decisive role in virulence. More precise results may be obtained by curing *Xam* strains of plasmids and experiments on transformation with specific plasmids. Thus, Ulaganathan and Mahadevan (1988) heat cured *X. campestris pv. vignicola* of a 95 Mdal plasmid and demonstrated that it did not play any role in virulence but seemed to influence colony morphology.

Hema et al. (2010) assumed that some natural plasmids present in *Xam* races were restored when they were incubated with cotton leaf extract. These plasmids disappeared from the same laboratory subcultured strain. They speculated that this was an adaptation strategy for *Xam* to increase the copy number of genes involved in pathogen aggressiveness which are otherwise present as a single copy in the bacterial chromosome

In contrast in our studies, the plasmid profile of weakly virulent race 4 and highly virulent strains of race 18 were similar; yet, race 18 can infect 9 cotton differentials, whereas races 4 can only infect 2 cotton differentials. Strain GSPB1430 of race 4 (Figure 2 and Table 3) was isolated from cotton in Nicaragua in 1986 and originally classified as a strain of race 18 (originally strain GSPB1386), but was reclassified as race 4 since its virulence had decreased during several transfers on nutrient media (Kucera, 1998). However, this strain was still possessing the same plasmid profile as its mother

strain (1386, race 18).

In our study, several attempts were made to cure race 18, strain 1386 of plasmid(s), but no plasmid(s) free mutants were obtained. Since strain 1430 originated as a mutant of strain 1386, race 18, the virulence of this strain (named race 4) is decreased even though it has the same plasmid profile as some race 18 strains. Thus, our data does not support the hypothesis that plasmid determined virulence. This is consistent with Dixon and Lamb (1990) conclusion that the decisive differences between the *Xam*-races are not located in the plasmids.

Several workers reported that plasmids can affect the resistance of phytopathogenic bacteria to antibiotics. Davies (1986) and Gale et al. (1972) demonstrated that resistance to streptomycin was encoded by plasmid-born genes. Minsavage et al. (1990) concluded that the streptomycin resistance locus in *X. axonopodis* pv. *vesicatoria* was found on 68 kb plasmid.

Stall et al. (1986) demonstrated that resistance of *X. campestris* pv. *vesicatoria* to copper was associated with a conjugative plasmid.

In contrast, our results did not reveal a correlation between a distinct plasmid profile and either antibiotic or heavy metal resistance. The curing experiments with GSPB 1386 were not successful due to the high stability of plasmid in this strain. Based on this data, we concluded that these plasmids do not play a decisive role in the resistance to heavy metals since race 4 lost its resistance to the antibiotic SXT and the heavy metals, Ni^{++} and Pb^{++} even though it possessed the same plasmids as race 18 strain 1386 which was much more resistant to the antibiotics and heavy metals (Figure 1 and Table 2). These results support the hypothesis of Mills (1990), that a majority of the plasmids occurring in all genera of phytopathogenic bacteria are cryptic.

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