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Plasmids in Different Races of *Xanthomonas campestris* pv. *malvacearum* (*Xcm*), Synonym, *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 axonopodis* pv. *malvacearum* (*Xam*) were screened for their plasmid content. One Strain belonging to race 4, and has been attenuated as mutant from race 18 strain and show low virulence than race 18, despite it possesses the same plasmids of race 18 strains. According to plasmid profiles of these strains, 5 groups could be distinguished. First group includes the strain of race and 4 strains of race 18 isolated from Nicaragua, 1986. The other 4 strains of races 18, isolated from Nicaragua, 1986; USA, 1986; Sudan 1991 were distinguished in 4 distinct groups according of their plasmid profile. The attenuated strain of race 4 was more sensitive to antibiotics and heavy metal ions than race 18 strains. No indication that plasmids play a distinct role in virulence of *Xam* strains.

Key words: Plasmid, *Xanthomonas*, Antibiotics, Heavy metals.

The genus *Xanthomonas* consider an economically important group of bacterial pathogens. One of the much important disease is bacterial blight, which causes by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye, synonym, *Xanthomonas axonopodis* pv. *malvacearum* (Vauterin *et al.*, 1995, 2000).

Bacterial blight of cotton is an economically important disease worldwide, resulting in yield losses of 10-30 % 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.

It was reported that *Xam* have virulence wide range. Hunter *et al.,* 1968, has identified 19 *Xam* races using different cotton cultivars (Acala 44, Stoneville 2B-S9, Stoneville 20, Mebane B-1, 1-10B, 20-3, 101-102B, Gregg, Empire B4, and DPxP4). These races were increased to 32 physiological races using 7 cotton cultivars (Verma and Singh, 1974).

These races distribute in different countries, for example race 1 is widespread in Australia, India and USA. Races 2 – 5 were recorded in India and USA; race 6 in Nigeria, Zymbawe and India; race 18 in Australia, USA, Africa, India and Nicaragua (Abdo-Hasan *et al.,* 2008). In Syria,

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Abdo-Hasan, 2002 has identified 6 *Xam* races (1, 2, 8, 21, 32).

Plasmids which are autonomous genetic elements that can replicate independently of the main chromosome, are important and widely occurring constituents of plant pathogenic bacteria (Coplin, 1982). In some cases, the plasmid composition of a particular phytopathogenic bacterium proved to be highly variable, with considerable differences in size range and number of plasmids, as reported by Burr *et al.*, 1988 for *Pseudomonas syringae* pv. *papulans*. Bender and Cooksey 1986 showed that *Pseudomonas syringae* pv. *tomato* harboured 5 size classes of plasmids (29-101 kb), and 10 size classes of plasmids (4.2-35.1 Mdal, 1-4 per strain) were found in *Xanthomonas campestris* pv. *pruni* (Randhawa and Civerolo, 1987).

Other plant pathogenic bacteria are much less variable in their plasmid content, for example *Clavibacter michiganensis* sub sp. *sepedonicus* contains only a single 50.6 kb plasmid. Half of the pathogen strains analysed by Mogen *et al*., 1988 harboured the plasmid, while in the other strains southern hybridization showed the plasmid to be integrated into the chromosome.

In addition to its known roles in antibiotic and heavy metal resistance; xenobiotic compounds degradation; genetic evolution during gene transfer, other possible roles of plasmids were supposed in phytopathogenic bacteria. Virulence, symptoms development, is of these supposed roles of plasmids in phytopathogenic bacteria.

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) strongly virulent strains (race 18), and one strain weakly virulent strains of races 4, which originated as a mutant of a race 18 strain (GSPB 1386) in addition to one strain of *Erwinia stewartii* that was used as a plasmid molecular markers.

Cultivation of bacterial cultures, (Huang, 2000)

The lyophilized samples were suspended in King's B liquid medium for 30 min (King *et al*., 1954), and then some droplets of this suspension were streaked on NGA (Nutrient Glucose Agar) plates and incubated for 3 days at 30 °C.

Antibiotic susceptibility test

The standardized single disk method (Bauter *et al.,* 1966) was used for measuring the antibiotic susceptibility of *Xam* strains.

Heavy metals and MIC values (Ghosh *et al.,* 1997).

The MICs (Minimal Inhibition Concentration) were detected in triplicate by determining bacterial growth on NGA plates containing a different concentrations (0.25 mM, 0.5 mM, 1 mM, 5 mM, 10 mM and 20 mM) of metal ions Zn^{++}, Co^{++}, Ni^{++}, Pb^{++}.

Plasmid extraction

A modified method of Birnboim 1983, was used for plasmid isolation (AbdelRehim, 2005).

Plasmid curing

Xam strain (GSPB1386) was treated with Acrydin orang (100 to 300 µg/ml) and SDS (1.0 to 500 µg/ml). Morphology and other characteristics of treated colonies were noted., colonies were randomly picked and screened for plasmids. DNA concentrations were calculated by determining the OD at 260 nm and 280 nm. Equal concentrations of plasmid DNA were loaded in 0.7 % agarose gel and separated by electrophoresis in TAE buffer at 70 volt for 3 h.

RESULTS

Antibiotic Susceptibility test

The sensitivity of tested *Xam*-strains towards 14 antibiotics is summarized in table 2. All of strains were resistant to SXT, OX, P, CN and CC except strain GSPB 1430, race 4 was sensitive to SXT. All strains were sensitive to the antibiotics OFX, TE, PB, VA, NN, K, Gm, C and NA.

Heavy metals tolerance

Different MICs values were determined for the bacterial strains tested. As summarized in Fig. 1, the most sensitive race was race 4, which showed a MIC value ranged from 0.5 mM (Zn^{++}) to 1 mM (Ni^{++}, Pb^{++}, Co^{++}). Strains of race 18 were much resistant to higher concentrations of these metals, since MIC values reaches 5 mM in some cases as shown in Fig. 1.

Plasmid profiles

Plasmid profiles of 8 strains belonging to race 18 and one strain of race 4 revealed five groups

(fig. 2, table 3). Four *Xam* strains of race 18 and isolated from Nicaragua in 1986 [GSPB, 1386; 1429; 1432; 1385] were identical in their plasmid profile to race 4 strain [GSPB; 1430] (group 5). One the other hand, the other two race -18 strains from Nicaragua [GSPB, 1384 (group 2); 1435 (group 3)] differ in their plasmid profile and show different plasmid pattern than the other race 18 strains as well. Strains from USA and Sudan [GSPB, 1252 (group 1); GSPB, 3012 (group 4)] differed considerably from each other as well as from the Nicaragua strains.

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*

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

Xam Races Antibiotics		Race 4	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)	-	ve
Vancomycin 30 µg	(VA)	-	
Tobromycin 10 µg	(NN)	-	
Kanamycin 5 µg	(K)	-	
Gentamycin 10 µg	(Gm)	-	
Ofloxacin 10 µg	(OFX)	-	
Nalidixic acid 30 µg	(NA)	-	

(+) = Tolerant, (-) = Sensitive

Table 3: Plasmids in 8 strains of race 18 and one strain of race 4

Group	Plasmid bands (size) 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			+			+			
2	1384 Nicaragua. 1986	Race 18			+	+	+		+	+
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			+	+	+		+	

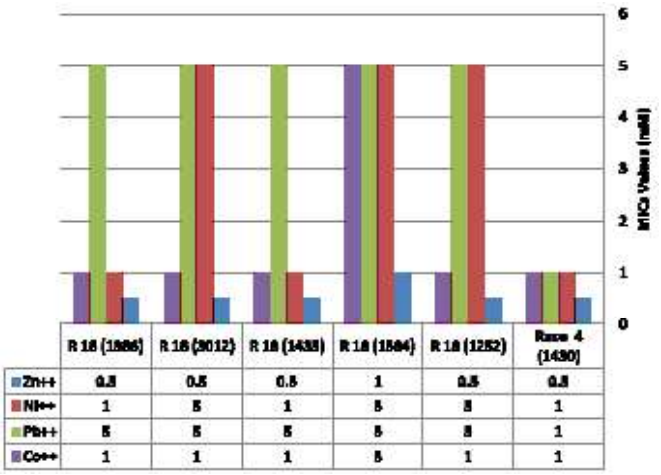
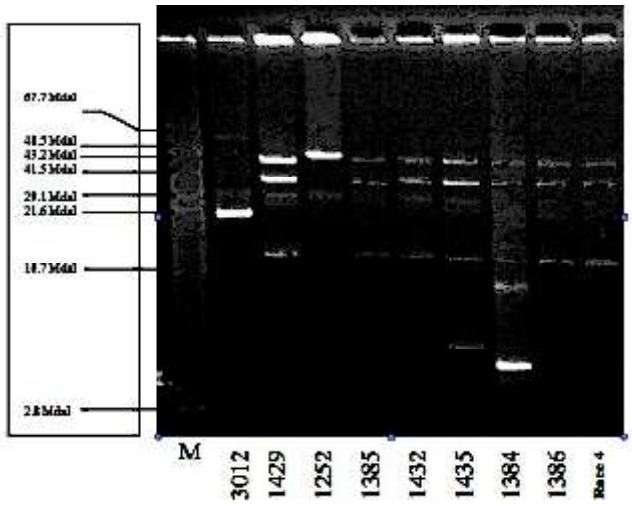


Fig. 1. Heavy metals tolerance of different *Xam* strains belonging to race 18 and race 4



M: Marker, plasmid profile of *Erwinia stewartii,* GSPB 2628.

Fig 2: Plasmid profiles of 8 *Xam* strains of race 18 (GSPB, 3012, 1429, 1252,1385, 1432, 1435,1348, 1386) and one of race 4 (GSPB, 1430)

Plasmid curing

No plasmids loss in selected colonies of *Xam* strain GSPB 1386, race 18 were detected after screening of about 300 bacterial colonies which were randomly picked after plating the serial diluted bacterial cultures treated with 300 µ g/ml of Acrydin orange and 400 µg/ml of SDS.

DISCUSSION

Bacterial genes, occurring on either chromosomal or plasmid DNA, are involved in the determination of a wide range of phenotypic characteristics. The majority of genes involved in the induction and development of disease by xanthomonads are located on the main chromosome. On the other hand, the vast majority of the plasmids occurring in all genera of phytopathogenic bacteria are cryptic (Mills, 1990). However, in recent years it has been shown that plasmids encode a wide range of functions that are important in bacterial-plant interactions (including pathogenicity; virulence factors; production of toxins) and that they are also

important in the determination of certain non-pathogenic features (Sigee, 1993).

The ability of a particular pathogen to cause disease depends on two sorts 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 determinate the host pathogen interaction: 1) *hrp* genes which are required for "basic pathogenicity": the ability to grow as a pathogen inside a plant, but the *hrp*-genes may also cause an HR-like necrosis in nonhost plants. 2) The second gene set comprises the so-called avirulence (*avr*) genes the products of which interact directly or indirectly with plant resistance gene products to provoke a defense reaction. The defense reaction is typically a local necrosis which 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.

In 1986, Gabriel *et al.,* concluded that *Xam* carries at least nine identifiable avirulence genes, all of which appeared to be chromosomally determined. Later on De Feyter and Gabriel 1991, suggested the possibility that at least one of the *avr* (avirulence) genes from the six *avr* genes which are 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 was 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 of these plasmids by incubation of the bacterial culture in 42 °C, plasmids were lost and the virulence of the strain was significantly reduced. Sathyanarayana and Verma,1998 reported that the highly virulent race 32 (Indian system) harboured five plasmids of 60, 40, 10, 5.5 and 2.2 Kb, the moderately virulent race 26 harboured 3 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 (6µg/ml), suggesting 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, 5 plasmid bands were detected per strain in our studies, ranging from minimum 2 bands as in strain GSPB 1252, race 18 from USA which possess 2 plasmid bands, to 6 plasmid bands as found in strain GSPB 1384, race 18 from Nicaragua. In contrast, Lazo and Gabriel,1987 found that the majority of the plasmid harbouring *Xam* strains contained only one plasmid, but only few carried two or more. The reason for this discrepancy is unknown.

According to the hypothesis of Sathyanarayana and Verma 1993; 1998, plasmids play a role in virulence of *Xam* strains. Chakrabarty *et al.*, 1995 reported that 3 plasmids of an Indian race 18 strain of *Xam* played a decisive role in virulence. More precise results can only be obtained by curing *Xam* strains from plasmids and experiments on transformation with specific plasmids. Thus, Ulaganathan and Mahadevan, 1988 demonstrated by heat curing that a 95 Mdal plasmid of *Xanthomonas campestris pv. vignicola* did not play any role in the virulence but seemed to influence colony morphology.

In 2010, Hema *et al.,* assumed that some natural plasmids present in *Xam* races were restored in the tested isolates when incubated with cotton leaves extract, after disappearing of these plasmids in the same laboratory subcultured strain. They speculate this as an adaptation strategy for *Xam* to increase copy number of genes involved in pathogen aggressiveness which are otherwise present as single copy in bacterial chromosome However, in our studies, despite, the similarity between plasmid profile of race 4 and strain of race 18, race 18 is highly virulent because it can infect 9 cotton differentials, whereas races 4 can only infect 2 cotton differentials respectively. The strain GSPB1430 of race 4 (fig. 2, table 3) was isolated

from Nicaragua in 1986 as a strain belonging to race 18 (originally strain GSPB1386), since its virulence had decreased during several transfers on nutrient media the strain was reclassified as race 4 (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 done to cure race 18, strain 1386 from plasmd(s), but no plasmid(s) free mutants were got. Since the strain 1430 has originated as a mutant of strain 1386, race 18, the virulence of this strain which was named as race 4 is decreased despite it has the same plasmid profile of race 18 strain. Thus, our data do not support the hypothesis supporting the positive role of plasmid in virulence.

Dixon and Lamb, 1990 have also concluded 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 against antibiotics. Thus, Davies 1986 and Gale *et al.* 1972 demonstrated that the resistance to streptomycin encoded by plasmid-born genes. In 1990, Minsavage *et al.,* concluded that the streptomycin resistance locus in *Xanthomonas axonopodis* pv. *vesicatoria* was found to be on a plasmid of 68 kb.

Concerning heavy metal resistance, Stall *et al.*, 1986 demonstrated that the resistance to copper was associated with a conjugative plasmid in *Xanthomonas campestris* pv. *vesicatoria*.

In contrast, our results did not reveal a correlation between a distinct plasmid profile and each of antibiotic and heavy metal resistance because the curing experiments were not successful due to the high stability of the plasmid in the tested strain (GSPB 1386). But we tend to say that these plasmids do not play a decisive role in the resistance to heavy metals depending on the observation of the resistance of race 4 strain, which loses the resistance towards the antibiotic SXT and the heavy metals, Ni^{++} ; Pb^{++} despite possessing the same plasmids of race 18 strain 1386 which was much resistant to antibiotics and heavy metal tested (fig. 1, table 2). These results supported the hypothesis of Mills 1990, that majority of the plasmids occurring in all genera of phytopathogenic bacteria are cryptic.

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