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# Plasmids Roles in Virulence of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) Races, the Causal Agent of Bacterial Blight of Cotton

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**Plasmids were obtained from strongly virulent *Xcm* strains ( HVS) and moderately virulent strains (6, 7, 10, 11 and 12) from different origin (USA, Nicaragua, Sudan, Burkina Faso, Turkey and Greece). Seventeen plasmid bands could be differentiated. The sizes ranged from 3.9 to 73.4 Mdal. The number of plasmid bands per strain varied from 1-6. On the average, each *Xcm* strain harboured 3 plasmid bands. Only one plasmid band (28.3 Mdal) was common to all *Xcm*-strains with two negative exceptions of the so-called HV strains. However, a correlation between specific plasmid profiles and strain origin was determined in several cases, independent of race designation. Our studies did not prove that the *Xcm* plasmids play a role in virulence or in resistance to antibiotics or heavy metal ions.**

**Key words:** Plasmid, Virulence, Bacterial Blight of cotton, *Xanthomonas* sp.

Bacterial blight of cotton caused by *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) is an economically important disease worldwide, resulting in yield losses of 10-30% of seed cotton (Verma, 1986; Zomorodian and Rudolph, 1993). Factors affecting the yield loss caused by *Xcm* are 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. Yield losses are also variably depending on the infected plant organs. Thus yield losses varied from 1%, when leaves were infected, to as much as 50% after extensive leaf, stem, and boll infection (Verma, 1986; Zomorodian and Rudolph, 1993).

In the United States, 1.0% was the average yield loss of cotton caused by bacterial blight at the period from 1952 to 1984 (El-Zik, 1986). In Sudan, Knight (1949) recorded 46% yield loss in a susceptible upland variety compared to a resistant variety. In Australia, where bacterial blight is present in most cotton growing areas, the average incidence of bacterial blight on bolls in pure seed crops was found to be 30.4% during the 1985 – 1986 seasons (Allen and West, 1987). Allen (1986) and Allen and West (1987) demonstrated that 3.3% of planting seed had been infested with the bacterial blight pathogen. The disease is potentially very destructive in areas where wind driven rain or sprinkler irrigation disseminates the pathogen (Brinkerhoff, 1963 and 1970; Innes, 1983).

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In the frame of several cooperative research projects between the Department of Plant Pathology and Plant Protection of the University of Göttingen and institutions in Nicaragua, Turkey, Sudan, Indonesia and Greece aspects of epidemiology, diagnosis, host parasite-interaction and control were studied (Zachowski and Rudolph, 1988; Zachowski *et al.*, 1989, 1990 a, 1990 b; Ahmed *et al.*, 1997; Kucera, 1998; Huang, 2000; AbdelRehim and Rudolph, 2002). During these studies a broad collection of *Xcm*-strains from different countries and belonging to different races were incorporated into the GSPB (Göttingen Collection of Phytopathogenic Bacteria). This valuable bacterial collection made it possible to study several open questions of diagnosis, race identification and taxonomy by traditional microbiological as well as by genetic methods.

*Xanthomonas campestris* pv. *malvacearum* is a Gram negative bacterium, motile by a single polar flagellum, occurring in short rods, chemoorganotrophic and obligatory aerobic (Bradbury, 1986). The name of the species was changed to *axonopodis* by Vauterin *et al.* (1995). However, since this name is not yet generally accepted by the scientific community (Schaad *et al.*, 2000) the earlier species name *X. campestris* is being used here.

Different symptoms are noted according to the plant part infected. Seedling blight are the disease symptoms when seedlings are infected (Wickens, 1956), while the infection of the stem and the sympodial and monopodial branches leads to black arm (Zomorodian and Rudolph, 1993). Angular leaf spot is the disease term when leaves are infected, where the bacteria enter to the parenchymatous leaf tissue through stomata and multiply causing water soaking appearance resulting from bacterial slime containing extracellular polysaccharides which is hydrophilic and fills the intercellular spaces of the leaf tissue (Rudolph *et al.*, 1989). In some cases, the water soaked lesions may follow the main veins of the leaf blade resulting in the death of the plant's growing point or in the destruction of the terminal bud. Boll blight occurs by infection of the calyx, bracts or receptacle, or through the vascular system from the infection of a fruiting branch (Zomorodian and Rudolph, 1993).

Plasmids are autonomous genetic elements that can replicate independently of the chromosome and can be separated physically from it in the laboratory. Bacterial plasmids are generally circular double stranded DNA molecules that are covalently closed in each strand and are transmitted from one generation to another during cycles of growth and cell division as autonomous self-replicating units. The main exception to this rule is the so-called "linear" plasmid found in *Borrelia* spp. Plasmids have been found in the vast majority of bacterial genera in which they have been sought (Stanisich, 1988), and have also been reported in some lower eukaryotic microorganisms such as yeasts (Broach, 1981). The plasmid DNA content of a cell normally comprises less than 5% of the total DNA.

To examine the plasmid profiles, firstly the plasmid has to be separated from the chromosomal DNA. The earliest plasmid isolation procedures depended on the separation of covalently closed circles of plasmid DNA from chromosomal DNA fragment by ultracentrifugations on cesium chlorid gradients containing high concentrations of ethidium bromide (Currier and Nester, 1976). This procedure is still being used in order to prepare large quantities of pure plasmid DNA.

Later on numerous rapid mini-prep methods for isolation of plasmid DNA on a small scale were developed that can be applied to large numbers of bacterial isolates for typing purposes and epidemiological studies (Towner and Cockayne, 1993). The best yields are obtained with smaller plasmids, partly because of their large copy numbers and because they are less prone to physical damage during the isolation process. But also plasmids > 100 kb in size can be visualized if appropriate care in handling is taken (Towner and Cockayne, 1993).

A rather common role of plasmids is mediation of resistance to antibiotics in naturally occurring bacteria associated with animals, particularly in areas where the antibiotics are applied extensively (Gale *et al.*, 1972). In plant-associated bacteria plasmid-mediated resistance to streptomycin has only rarely been reported (Burr *et al.*, 1988). Several laboratories demonstrated a conjugative transfer of antibiotic resistance between plant pathogenic bacteria and bacteria associated with plants and animals (Chatterjee and

Starr 1973; Cho *et al*., 1975; Gibbins *et al*., 1976; Lacy and Leary 1975; Panopoulos *et al*., 1975)

Thomas *et al.* (1988) demonstrated that a 68-megadalton (Mdal) plasmid which was designed pCPP501 was present in streptomycin-resistance field strains of *Pseudomonas syringae* pv. *papulans* but was not observed in streptomycin-sensitive field strains. The authors found that streptomycin resistance was transferred from streptomycin-resistant donor strains Psp 34 and Psp 36 to streptomycin-sensitive recipient trains after mating on nitrocellulose membrane at frequencies of $4.7X\ 10^{-3}$ and $2.0X\ 10^{-2}$ per recipient cell, respectively. It would appear that the potential spread of pCPP501 or a similar plasmid to other plant pathogenic or human bacteria could pose serious economic and health risks.

Plasmids are important and widely occurring constituents of plant pathogenic bacteria (Coplin, 1982). In a survey of plasmids among phytopathogenic xanthomonads, no plasmids were detected in *Xanthomonas campestris* pv. *citri, Xanthomonas campestris* pv. *pruni* (strain 13052), and *Xanthomonas campestris* pv. *oryzae* strains 507 and 604 (Lin *et al.*, 1979) . Single plasmids with molecular masses of 26 and 43 megadaltons (Mdal) were reported to occur in *Xanthomonas campestris* pv. *pruni* (strain 8 D51) and *Xanthomonas campestris* pv. *vitians* (strain 068790), respectively (Civerolo, 1985).

Five different plasmids were observed in seven isolates of *Xanthomonas campestris* pv. *vesicatoria*; however, none of these plasmids was associated with spontaneous mutation from avirulence to virulence (Dahlbeck *et al.*, 1977). No phenotypic functions have been associated with any of these indigenous plasmids in *Xanthomonas* species (Civerolo, 1985).

Indigenous plasmids have been detected in every strain examined of *Xanthomonas campestris* pv. *malvacearum* isolated from cotton (Lazo and Gabriel, 1987; Chakrabarty, 1992; Chakrabarty *et al*., 1992). According to the results of Lazo and Gabriel (1987) the majority of the plasmid harboring *Xcm* strains contained only one plasmid, but some carried two or more.

However, when Das and Verma (1996) examined the plasmid profile of four races of *Xcm* they found that races 32, 31 and 26 (Indian races system; Verma, 1986) contained five plasmids each, while race 30 (= race 7 in table 2) contained only four plasmids. It was discussed by Chakrabarty *et al.* (1995) that virulence may be affected by certain plasmids. These authors isolated 3 plasmids of 55.0, 31.2 and 7.4 Kb from one strain of *Xcm* belonging to race 32. After incubation of the bacterial culture at 42 $^{o}$C, plasmids were lost and the virulence of the strain was reduced.

In 1998, Sathyanarayana and Verma reported that the highly virulent race 32 strains harbored five plasmids of 60, 40, 10, 5.5 and 2.2 Kb. This race (32) is able to neutralize five B-genes ($B_7$, $B_4$, $B_2$, $B_{IN}$ & $B_N$) (Verma, 1995). The same authors also demonstrated that the moderately virulent strains of race 26 (Indian system) harbored 3 plasmids (60, 40 & 10 Kb) and can neutralize three B-genes ($B_4$, $B_2$ & $B_{IN}$), and the lowly virulent race 5 strains contained one plasmid (10 Kb) and can neutralize only one B gene ($B_{IN}$). Sathyanarayana and Verma (1998) reported that a strain of race 32 (= race 18 of the American system) became avirulent after plasmid curing by exposing to Mitomycin C at 6 µg/ml. When the 10 Kb plasmid (common in the three races) was transferred to avirulent plasmid-cured strains, the virulence for gene $B_{IN}$ was restored (Sathyanarayana and Verma, 1993; Verma, 1995).

Since similar strong indications for the role of plasmids in affecting virulence of phytopathogenic bacteria have not been reported earlier, our investigations aimed to proof this hypothesis by analyzing plasmid profiles of *Xcm*-strains belonging to several races. Most of these strains originated from cooperative research projects between the University of Göttingen and Nicaragua, Turkey, Indonesia or Sudan.

## MATERIALS AND METHODS

### Bacteria

Seventy two *Xcm* bacterial strains were obtained as lyophilized samples from the GSPB bacterial collection (Göttinger Sammlung phytopathologener Bakterien) (Table 1). Six (6) were highly virulent strains (HVS), 6 belonging to races 6, 8 belonged to race 7, 2 rom race 10, 4 from race 11, and one strain belonging to race 12 and originated from sub-culturing of starin 2387 (race 11). *Erwinia stewartii* strain was used as a plasmid molecular markers.

Table 2 shows the reaction of differential upland cotton lines against the eight tested races of pathovar *malvacearum*.

**Cultivation of bacterial cultures**

The lyophilized bacterial cultures were suspended in King,s B (King *et al.*, 1954) liquid medium for 30 min, then some droplets of this suspension were streaked onto NGA plates and incubated for 3 days at 30 °C. A typical colony was selected, transferred on chalk agar slants, incubated for 2 days at 26 °C and preserved at 16 °C for not more than 6 months (Stead, 1990).

**Plasmid extraction**

A modification of Birnboim's method (1983) was used for plasmid isolation. Once plasmid DNA has been precipitated, it must be visualized before it can be analysed. Normally this is achieved by electrophoresis through an agarose gel. Horizontal slab agarose gel with a concentration of 0.7% in TAE (Tris-acetate EDTA) 1 x buffer was used (50 x TAE: 2 M Tris base, 50 mM EDTAx2$H_2O$ and 57.1 ml glacial acetic acid, the pH was adjusted to 8.5) (Towner and Cockayne, 1993).

**Resistance to antibiotics**

The standardized single disk method (Bauer, *et al.*, 1966) was used for measuring the antibiotic resistance of *Xcm* strains.

**Heavy metal resistance and determination of MIC values (Ghosh *et al.*, 1997)**

The MICs (Minimal Inhibition Concentration) were determined in triplicate by growing the bacterial strains on NGA plates containing different concentrations (0.25 mM, 0.5 mM, 1 mM, 5 mM, 10 mM and 20 mM) of studied metal ions (Zn, Co, Ni, Pb).

## RESULTS

**Plasmid profiles**

**HV strains**

The plasmid profiles of the HV strains revealed four groups (fig. 1, table 3). The three strains isolated from Sudan and one strain from Burkina Faso (no. 1829) showed a high degree of similarity. Strain 2923 from Burkina Faso did not reveal any plasmid band.

**Table 1.** Bacterial strains used in this study

| (GSPB) Nr. | Race | Origin and date of isolation | (GSPB) Nr. | Race | Origin and date of isolation |
|---|---|---|---|---|---|
| *Xcm* 1246 | 6 | USA | *Xcm* 1250 | 11 | USA |
| *Xcm* 1583 | 6 | Turkey 1987 | *Xcm* 2385 | 11 | Greece 1994 |
| *Xcm* 1584 | 6 | Turkey 1987 | *Xcm* 2387 | 11 | Greece 1994 |
| *Xcm* 1585 | 6 | Turkey 1987 | *Xcm* 3010 | 11 | Sudan 1994 |
| *Xcm* 1586 | 6 | Turkey 1987 | *Xcm* 2420 | 12 | Greece 1994 |
| *Xcm* 3005 | 6 | Sudan 1994 | *Es* SW 42625 | | Obtained from Prof. K. Geider, Heidelberg 1996 |
| *Xcm* 1247 | 7 | USA | | | |
| *Xcm* 1412 | 7 | Turkey 1986 | | | |
| *Xcm* 1415 | 7 | Turkey 1986 | *Es* SS104 2628 | | Obtained from Prof. K. Geider, Heidelberg 1996 |
| *Xcm* 1418 | 7 | Turkey 1986 | | | |
| *Xcm* 1420 | 7 | Turkey 1986 | | | |
| *Xcm* 1580 | 7 | Turkey 1987 | *Xcm = Xanthomonas campestris* pv. | | |
| *Xcm* 1582 | 7 | Turkey 1987 | *malvacearum, Es = Erwinia stewartii* | | |
| *Xcm* 3004 | 7 | Sudan 1994 | HVS = highly virulent strains | | |
| *Xcm* 1249 | 10 | USA | ATCC= American Type Culture | | |
| *Xcm* 3013 | 10 | Sudan 1994 | Collection | | |
| *Xcm* 1828 | HVS | Burkina Faso | | | |
| *Xcm* 1829 | HVS | Burkina Faso | | | |
| *Xcm* 2388 | HVS | Sudan 1994 | | | |
| *Xcm* 3006 | HVS | Sudan 1994 | | | |
| *Xcm* 3008 | HVS | Sudan 1994 | | | |
| *Xcm* 2923 | HVS | Burkina Faso 1984ATCC 49294 | | | |

**Table 2.** Reaction differential upland cotton lines against 7 races of *Xcm* (Hunter *et al*., 1968; Bird, 1985 and Verma, 1986)

| R. C. | 4 | 6 | 7 | 10 | 11 | 12 | 18 | HVS |
|---|---|---|---|---|---|---|---|---|
| 1 | + | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + | + |
| 3 | - | - | - | + | - | + | + | + |
| 4 | - | + | + | + | - | - | + | + |
| 5 | - | + | + | + | - | - | + | + |
| 6 | + | - | + | + | - | - | + | + |
| 7 | - | - | - | - | - | - | - | + |
| 8 | | + | + | + | + | + | + | + |
| 9 | | - | - | - | - | - | + | + |
| 10 | | - | - | - | - | - | + | + |

R = Race no.
C = Cotton differential lines (Line 1= Acala 44, 2 = Stoneville 2B-S9, 3 = Stoneville 20, 4 = Mebane B-1, 5 = 1-10B, 6 = 20-3, 7 = 101-102B, 8 = Gregg, 9 = Empire B4, and 10 = DPxP4)
+ = Susceptible
- = Resistant (No symptoms)

**Table 3.** Plasmids in strains of race HVS

| Group | Plasmid bands (size) and no. Strain no.Place and date of isolation | 5 (51.3 Mdal) | 8 (42.4 Mdal) | 9 (35.1 Mdal) | 10 (28.3 Mdal) |
|---|---|---|---|---|---|
| 1 | 1828 Burkina Faso | + | + | + | |
| 2 | 1829 Burkina Faso | | + | | + |
| 3 | 2388 Sudan 1994 | | | | + |
| | 3006 Sudan 1994 | | | | (+) |
| | 3008 Sudan 1994 | | | | + |
| 4 | 2923 Burkina Faso | | | | |

* Brackets indicate very weak bands.

**Table 4.** Plasmids in strains of race 10

| Group | Plasmid bands (size) and no. Strain no.Place and date of isolation | 5 (51.3 Mdal) | 10 (28.3 Mdal) |
|---|---|---|---|
| 1 | 1249 USA | (+) | |
| 2 | 3013 Sudan 1994 | | (+) |

**Table 5.** Plasmids in races 11, and 12

| Group | Plasmid bands (size) and no.Strain no. Place and date of isolation | 1 (73.4 Mdal) | 2 (66.5 Mdal) | 5 (51.3 Mdal) | 9 (35.1 Mdal) | 10 (28.3 Mdal) | 11 (25.6 Mdal) | 12 (20.0 Mdal) |
|---|---|---|---|---|---|---|---|---|
| 1 | 1250 USA | Race 11 | (+) | | | | + | |
| 2 | 2385 Greece 1994 | | | | + | | (+) | |
| 3 | 3010 Sudan 1994 | | | | | | (+) | + |
| 4 | 2387 Greece 1994 | | | + | | + | + | + |
| | 2420 Greece 1994 | R 12 | | (+) | | + | + | + |

**Race 10**

The plasmid profiles of the strains of race 10 revealed two groups (fig. 2, table 4), however, only one very weak plasmid band appeared in each group.

**Races 11 and 12**

The plasmid profiles of the strains from races 11 and 12 revealed four groups (fig. 3, table 5). The strain of race 12 (GSPB 2420) came with the strain GSPB 2387 of race 11 in the same group, may be because both strains were isolated in Greece. The strains from Sudan (3010) and USA (1250) were characterized by additional bands, although very weak in the USA strain (indicated by an arrow on the figure). Interestingly, band no. 10 appeared in all the strains of races 11 and 12.

**Race 7**

The plasmid profiles of the strains of race 7 revealed five groups (fig. 4, table 6). Group 5 contained most of these strains (GSPB 1418, 1420, 1580, and 1582) which were isolated from Turkey in 1986 and 1987. Also two other strains from Turkey and one from Sudan showed a banding profile very similar to group 5, with band no. 6 missing, or the additional bands no. 13 and 16. On the other hand, the banding profile of strain 1247 from USA differed considerably from all the other strains with two rather weak bands (nos. 1 and 10). Band no. 10 was very weak in all strains with the exception of strain 3004 from Sudan.

**Race 6**

The plasmid profiles of the strains of race 6 revealed four groups (fig 5, table 7). The plasmid pattern appeared to be more specific for the place and date of isolation than for the race. Thus, 3 of 4 Turkish strains showed an identical plasmid pattern, and the 4$^{th}$ Turkish strains differed by non-appearance of 1 plasmid band. On the other hand, strain 1246 from the USA contained one plasmid band which was not observed in any other strains of race 6, while 3 plasmid bands, present in most of the Turkish strains, were absent. Only one weak plasmid band (no. 10) appeared in all strains of race 6.

**Resistance against antibiotics**

All the strains were resistant to SXT (Sulphamethazol+Trimethoprim 23.75+1.25 µg), OX (Oxcacillin 5 µg), P (Penicillin 10 µg), CN (Cefalexin

**Table 6.** Plasmids in race 7

| Group | Plasmid bands (size) and no.Strain no. Place and date of isolation | 1 (73.4 Mdal) | 2 (66.5 Mdal) | 5 (51.3 Mdal) | 6 (51.3 Mdal) | 10 (28.3 Mdal) | 13 (16.5 Mdal) | 16 (4.8 Mdal) |
|---|---|---|---|---|---|---|---|---|
| 1 | 1247 USA | (+) | | | | (+) | | |
| 2 | 1412 Turkey 1986 | | + | + | | (+) | | |
| 3 | 3004 Sudan 1994 | | + | + | | + | | |
| 4 | 1415 Turkey 1986 | | + | + | + | (+) | (+) | + |
| 5 | 1418 Turkey 1986 | | + | + | + | (+) | | |
| | 1420 Turkey 1986 | | + | + | + | (+) | | |
| | 1580 Turkey 1987 | | + | + | + | (+) | | |
| | 1582 Turkey 1987 | | + | + | + | (+) | | |

**Table 7.** Plasmids in race 6

| Group | Plasmid bands (size) and no.Strain no. Place and date of isolation | 1 (73.4 Mdal) | 2 (66.5 Mdal) | 5 (51.3 Mdal) | 6 (46.3 Mdal) | 10 (28.3 Mdal) |
|---|---|---|---|---|---|---|
| 1 | 1246 USA | + | | | | (+)* |
| 2 | 1584 Turkey 1987 | | (+) | + | | (+) |
| 3 | 1586 Turkey 1987 | | + | + | + | (+) |
| | 1585 Turkey 1987 | | + | + | + | (+) |
| | 1583 Turkey 1987 | | + | + | + | (+) |
| 4 | 3005 Sudan 1994 | | | + | | (+) |

30 µg) and CC (Clindamycin 10 µg). All strains were sensitive to each of OFX (Ofloxacin 10 µg), TE (Tetracyclin 30 µg), PB (Polymyxin B 300 I.U), VA (Vancomycin 30 µg), NN (Tobramycin 10 µg), K (Kanamycin 5 µg), Gm (Gentamycin 10 µg), C (Chloramphenicol 30 µg) and NA (Nalidixic acid 30 µg). The only exception to the general pattern was the finding that all the HV strains showed a moderate sensitivity to oxacillin, 5 µg (OX).

**Table 8.** Minimal inhibition concentration values of different heavy metal ions towards *Xcm* strains belonging to different races

| Race | GSPB | MIC (mM) | | | |
|---|---|---|---|---|---|
| | no. | Zn++ | Ni++ | Pb++ | Co++ |
| 6 | 1246 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 1584 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 3005 | 1.0 | 5.0 | 5.0 | 0.5 |
| | 1586 | 1.0 | 5.0 | 5.0 | 0.5 |
| | 1585 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 1583 | 1.0 | 5.0 | 5.0 | 1.0 |
| 7 | 1247 | 0.5 | 5.0 | 5.0 | 0.25 |
| | 1412 | 0.5 | 5.0 | 5.0 | 0.5 |
| | 3004 | 0.5 | 5.0 | 5.0 | 0.5 |
| | 1415 | 0.5 | 5.0 | 5.0 | 1.0 |
| | 1418 | 0.5 | 5.0 | 5.0 | 0.5 |
| | 1420 | 0.5 | 5.0 | 5.0 | 0.5 |
| | 1580 | 0.5 | 5.0 | 5.0 | 0.5 |
| | 1582 | 0.5 | 5.0 | 5.0 | 1.0 |
| 10 | 1249 | 0.5 | 5.0 | 5.0 | 5.0 |
| | 3013 | 0.5 | 5.0 | 5.0 | 0.5 |
| 11 | 1250 | 0.5 | 5.0 | 5.0 | 1.0 |
| | 2385 | 0.5 | 5.0 | 5.0 | 1.0 |
| | 3010 | 0.5 | 5.0 | 5.0 | 1.0 |
| | 2387 | 0.5 | 5.0 | 5.0 | 1.0 |
| 12 | 2420 | 20.0 | 5.0 | 5.0 | 1.0 |
| HVS | 1828 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 1829 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 2388 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 3008 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 3006 | 1.0 | 5.0 | 5.0 | 1.0 |
| | 2923 | 1.0 | 5.0 | 5.0 | 1.0 |

**Resistance to heavy metal ions**

The degree of resistance (or tolerance) to heavy metal ions is expressed by the Minimal Inhibition Concentration (MIC)—values as shown in table 8. Most of the strains of each race showed the same degree of resistance against the heavy metal ions zink, nickel and lead. Only against cobalt, different sensitivies within the races 6, 7, 10 were recorded. An extra ordinarily high resistance to zinc ions was recorded for strain 2420 of race 12 (20 mM). It appeared impossible to correlate higher degree of heavy metal resistance with appearance of specific plasmid bands.

## DISCUSSION

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 and virulence factors, and production of toxins) and that they are also important in the determination of certain non-pathogenic features (Sigee, 1993).

The interactions of phytopathogenic bacteria with potential plant hosts can be divided

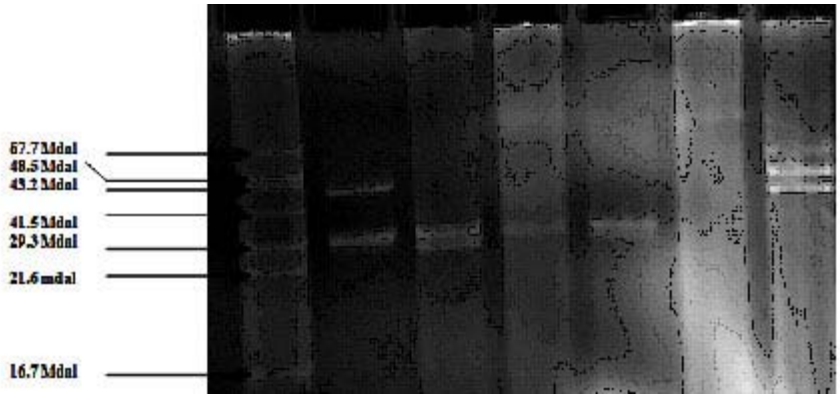


**Fig 1.** Plasmid profiles of 6 *Xcm* (HVS). M: Marker, plasmid profile of *Erwinia stewartii*, GSPB 2628

into two general categories; (1) the compatible interaction which is defined by the ability of a virulent bacterium to incite disease on a susceptible plant host; and (2) the incompatible interaction which results in little or no disease. The latter can occur when an avirulent bacterium induces a defense response in a resistant host plant (avirulence genes). The compatible interaction will typically be expressed by spreading water-soaked or necrotic lesions due to bacterial growth in the infected tissue.

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. These genes control such processes as production

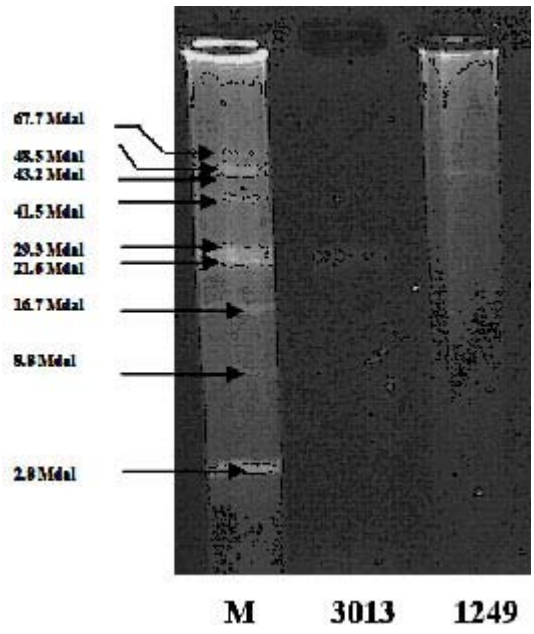


**Fig. 2.** Plasmid profiles of 2 *Xcm* strains of race 10. M: Marker, plasmid profile of *Erwinia stewartii*, GSPB 2628

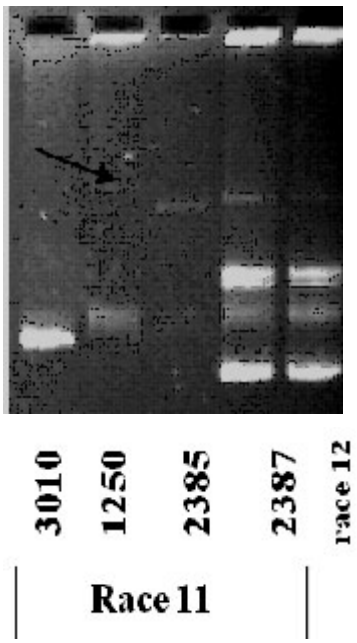


**Fig. 3.** Plasmid profiles of 4 *Xcm* strains of race 11 and one strain of race 12. M: Marker, plasmid profile of *Erwinia stewartii.* (GSPB 2628)

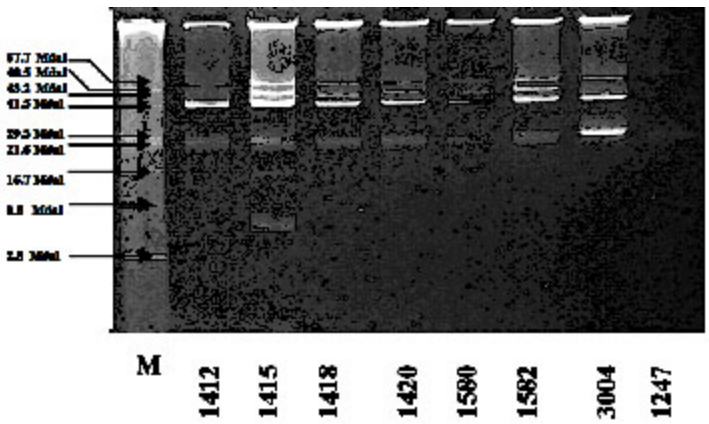


**Fig. 4.** Plasmid profiles of 8 *Xcm* strains of race 7. M: Marker, plasmid profile of *Erwinia stewartii*, GSPB 2628

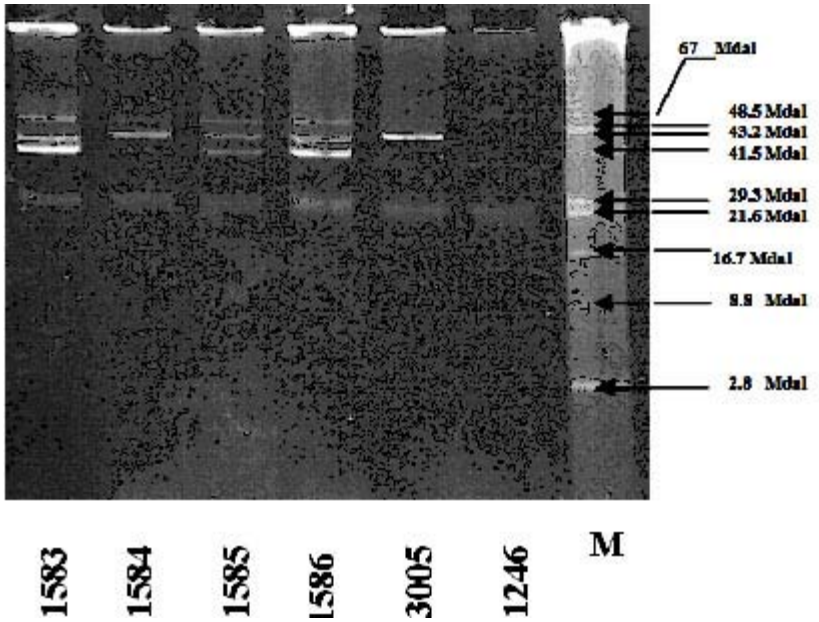


**Fig. 5.** Plasmid profiles of 6 *Xcm* strains of race 6. M: Marker, plasmid profile of *Erwinia stewartii*, GSPB 2628

of toxins, hormones, extracellular polysaccharides and plant cell wall-degrading enzymes.

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. The lack of either member of the gene pair usually results in a compatible (disease) interaction, when the bacterium contains pathogenicity genes (e. g. *hrp*-genes) and virulence genes (see above).

Genetic analysis of very few *Xanthomonas*- caused diseases indicated that a 2 gene for gene2 interaction occurs in certain race/ cultivar combinations (e.g. *X. c*. pvs. *vesicatoria* or *malvacearum*). In this case it is assumed that a resistance gene in a particular host cultivar confers resistance against physiological races that express the matching avirulence genes, so that the HR results (Rudolph, 1993). Elicitors, elicitor receptors, signal interactions, and signal transduction mechanisms have been discussed by Dixon and Lamb (1990) as well as the channel defense model (Gabriel *et al.*, 1986). A relatively simple model of race-specific defense responses in the host plant *Arabidopsis* against different *X.c*.pv. *campestris* strains has been developed (Daniels *et al.*, 1991).

In 1986, Gabriel *et al.*, concluded that *Xcm* 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 *Xcm* may be important for conditioning virulence of the pathogen on a susceptible host.

In conclusion, 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 *Xcm*-races, as was suggested by Sathyanarayana and Verma

origin correlated with the plasmid profile in many cases, but not the race designation.

It was observed that the plasmid profiles remained stable, even when the race designation of certain *Xcm*-strains changed within time. Thus, strain 2420 of race 12 developed from strain 2387 of race 11 (Mavridis, personal communication) which was isolated from Greece in 1994 (fig. 3, table 5) and showed still the same plasmid profile of the original strain 2387 although its virulence had increased. The underlying mechanism of the virulence change is not exactly clear, e.g. if some genes were switched on and if they were located on the main chromosome or on plasmids present in these two strains.

Also this observation does not support the hypothesis that genes encoded on plasmids are responsible for race specificity.

It has to be concluded from these studies that the decisive differences between the *Xcm*-races are not located in the plasmids, but probably in the so-called avirulence genes which have been elucidated in several cases (Dixon and Lamb, 1990).

Our results, thus, showed that the dissimilarity between strains of the same race but from different origin was 100%. There was only one exception where two strains of the same race (7) but from different origin (1412 from Turkey and 3004 from Sudan) came together in one group (15). However, these strains did not possess identical plasmid profiles because band no. 10 seemed to be different in both strains (very intensive in strain 3004).

The HV strains comprise a heterogeneous group of strains causing variable symptoms (Huang, 2000). Development of disease symptoms depends on relatively high air humidity (Kucera, 1998). Therefore, these strains have only be isolated in African countries where sprinkler irrigation is being used in cotton culture. The HV strains have in common that they can infect all cotton differentials including the genotype Mebane 101-102B which is resistant against race 18. Therefore, the HV strains have been designated as race 20 by Folin *et al*. (1988). This designation could be confirmed by our genetic analyses described in chapter 2. As reported by Huang (2000), the HV strains from Sudan did not cause water-soaked leaf spots. Instead necrotic leaf spots appeared one week after inoculation which enlarged within the following 4 weeks until leaf drop. In contrast, the HV strains from Burkina Faso caused water-soaked leaf spots within 2 weeks which transformed into necrotic spots 2 weeks later.

The heterogeneity of the HV strains is also reflected by different plasmid profiles. The three strains (1828, 1829 and 2923) which originated from Burkina Faso all possessed different plasmid profiles, whereas the strains isolated from Sudan in 1994 (GSPB 2388, 3006 and 3008) were similar in their plasmid profiles. Interestingly, the HV strains were never grouped together with strains from other races, with the exception of group 5 containing one strain of race 10. This may indicate that plasmids are involved in symptom expression, since the HV strains induce mainly necrosis and are dependent on high air humidity more than the other races of pv. *malvacearum.*

As is discussed above, we could not find any indications that *Xcm*-plasmids are involved in race specificity. Never a correlation between race designation and a unique plasmid profile was observed. Therefore, our studies did not shed light on the possible role of plasmids in pathogenicity and virulence of *Xcm* races. Similarly, Dittapongpith and Richie (1993) demonstrated that no functions could be correlated with the presence of the miniplasmids detected in *Xanthomonas*. Also, Wilk *et al.* (1989) reported that in spite of different plasmids patterns, the strains of *Vibrio salmonicida* were very similar with respect to biological reactions.

More precise results can only be obtained by curing *Xcm* 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. On the other hand, Chakrabarty *et al.* (1995) reported that 3 plasmids of an Indian race 18 strain of *Xcm* played a decisive role in virulence.

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 develops in one of two general ways at the cellular level - either by alteration of the binding affinity of ribosomal

protein to the antibiotic by chromosomal mutations, or by modification or destruction of the antibiotic by periplasmic enzymes commonly encoded by plasmid-born genes in plant pathogenic prokaryotes. In 1990, Minsavage *et al.,* concluded that the streptomycin resistance locus in *Xanthomonas campestris* pv. *vesicatoria* was found to be on a plasmid of 68 kb.

Thomas *et al.* (1988) demonstrated that a 68 (Mdal) plasmid was present in streptomycin-resistant strains of *Pseudomonas syringae* pv. *papulans* but not in streptomycin-sensitive strains. The streptomycin resistance genes appeared to be associated with a conjugative plasmid. Resistance to streptomycin was also found to be associated with a conjugative plasmid in the enterobacteriaceae (Watanabe *et al.,* 1964) and in *Pseudomonas syringae* pv. *tomato* (Bender and Cooksey, 1986).

In our experiments, one HV strain (GSPB 2923) which did not possess any plasmids, showed the same degree of antibiotic resistance than other strains belonging to the same or different races. Before drawing definite conclusions, it would be necessary to perform more experiments with slightly different antibiotic concentrations in the range between bacterial sensitivity and resistance. In another model, Dharmalingam *et al.* (2003) concluded that the presence of plasmids in clinical isolates of *Helicobacter pylori* did not have any correlation with their antibiotic resistance pattern.

Stall *et al.* (1986) demonstrated that the resistance to copper was associated with a conjugative plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Our results did not reveal a correlation between a distinct plasmid profile 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, because one HV strain (GSPB 2923) which does not harbour any plasmid bands also had the same degree of heavy metal resistance as the other *Xcm* strains belonging to the same race (HVS).

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