



SHORT COMMUNICATION

STABILITY OF PLASMID PROFILES OF HIGHLY VIRULENT RACES OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* DURING STORAGE AND SUBCULTURINGH.P. Narra¹, J. Jayaraman², J.P. Verma³ and R.T.V. Fox¹¹School of Plant Sciences, University of Reading, TOB2 Earley Gate, Reading RG6 6AU, UK²Division of Plant Pathology, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110012, India³Retired Head, Division of Plant Pathology, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110012, India

SUMMARY

Four races of *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) viz. races 23, 27 and 32 (isolated from *Gossypium hirsutum*) and race 23b (from *Gossypium barbadense*) were studied. The plasmid profile of the natural isolates showed four plasmids in races 23 and 23b (ca. 60, 40, 23, 8.2 kb), five in race 27 (ca. 60, 40, 23, 8.2 and 3.7 kb) and six in race 32 (ca. 60, 40, 23, 8.2, 3.7 and 1.6 kb). Continuously sub-cultured laboratory isolates of the *Xcm* races resulted in the loss of all but two plasmids, ca. 60 and 40 kb in size. When the laboratory isolates were passed through cotton (*Gossypium hirsutum*), they regained certain plasmids so that four plasmids were found in race 23 and 23b (ca. 60, 40, 23 and 8.2 kb), five in race 27 (ca. 60, 40, 23, 8.2 and 3.7 kb) and six in race 32 (ca. 60, 40, 23, 8.2, 3.7 and 1.6 kb), which was more or less similar to the original isolates. The isolates recovered from cotton maintained their plasmid profile (except for minor changes in the miniplasmids) after storage for six months at -70°C in 50% glycerol. It is suggested that plasmid profiles among highly virulent races of *Xcm* are unstable during repeated sub-culturing at room temperature, resulting in rapid loss of some plasmids. However, when the cultures were sub-cultured and stored at -70°C the plasmid profile was fairly stable except for the miniplasmids (ca. 3.7 and 1.6 kb).

Key words: bacterial blight, cotton, plasmid, storage, *Xanthomonas campestris* pv. *malvacearum*.

Xanthomonas campestris pv. *malvacearum* (*Xcm*), the causal agent of bacterial blight, causes serious economic losses to cotton, one of the most important fiber crop in the world (Anonymous, 2002). The presence of plasmids in this pathogen is well known (Lazo and Gabriel, 1984). The complete profile of plasmids in *Xcm* has been described, together with their probable role in pathogenicity (virulence) (Sathyanarayana and Verma, 1993); a

high degree of conservation of plasmid DNA sequences within some pathovars has also been reported (Lazo and Gabriel, 1987).

In India, *Xcm* is highly variable and as many as 26 races have been reported. The virulent races overcoming 3-4 major bacterial blight resistant genes (*B*-genes) in the host are most widely distributed in India (Verma, 1986). The virulence of different races (genotypes) of *Xcm* was measured on the basis of the reaction (susceptibility/resistance) of eight differentials of *Gossypium hirsutum* cotton developed by Hunter *et al.* (1968), and numbered according to Verma (1986), taking into consideration the number of genes attacked by the race; e.g. race 32 was considered the most virulent because it could neutralise 4 different bacterial blight resistance genes namely *B*₇, *B*₂, *B*_{In} and *B*_N, whereas the lower numbered races, like races 1-6, were considered least virulent because they could neutralize either only polygenes or one major *B* gene.

The genetic basis for the variability in *Xcm* is not clearly understood. The possibility of plasmid-borne virulence in *Xcm*, on the basis of pattern of race reaction on differentials, and stepwise loss of virulence for single *B*-genes has been predicted (Verma and Singh, 1976). Shaik Abdulla (1993) reported that *Xcm* cultures on YG-CA under mineral oil survived and maintained their race number for several years, but lost virulence to individual *B*-genes, one by one (stepwise), on sub-culturing, and that several isolates became avirulent after 7-8 years. Similar virulence losses were noted on *G. barbadense* cultivars under natural conditions and it was concluded that the extra aggressiveness was lost and the races retained only sufficient virulence for *G. barbadense* cultivars.

Virulence was lost progressively but in steps, indicating that for each gene in the host (*i.e.* bacterial blight resistant genes, which may be polygenic or major in nature), there is a corresponding virulence factor in the bacterial pathogen. Some of them, like the virulence factor of *B*₂, were very stable, while for others, like that of *B*_N were unstable, and those of *B*_{In} and *B*₇ showed medium stability (Verma and Singh, 1976). *Vibrio anguillarum*, however, retained viable plasmids during storage and sub-culturing (Pedersen, 1997). Viable strains maintained their plasmid profile throughout the

experiment, except in two cases where the 67 and 200 kb plasmids were lost. Strains stored at -80°C all remained viable and maintained their plasmid profile throughout the study. The presence of five plasmids in race 32, which has the virulence factors for five *B*-genes, has been reported (Sathyanarayana and Verma, 1993). These plasmids were absent in avirulent isolates. The plasmid-cured strains were avirulent and defective in EPS production. The plasmid number was not related to the number of *B* genes attacked.

By contrast, there is no data on the role/effect of storage on the stability of plasmid number or profile in *Xcm*. The present paper reports results on the effect of sub-culturing and storage on the stability of plasmids of *Xcm* races.

Four laboratory isolates of *Xcm* races 23, 27 and 32 (from *Gossypium hirsutum*) and race 23b (from *G. barbadense*), which had been sub-cultured 35 times at 2-week intervals, were used in the study. Inoculations were carried out by the injection-infiltration method. Bacterial inoculum of the laboratory (sub-cultured) isolates was prepared from 18-24 h old colonies in active growth on YGCA (Yeast-Glucose-Chalk-Agar) slants by suspending in distilled water and adjusting OD to 0.1 extinction (approx $5 \cdot 10^6$ cfu ml $^{-1}$) at 620 nm with spectronic-20 colorimeter (Bausch and Lomb, New York, USA) and was referred to as standard inoculum.

Plasmids were obtained from various isolates using the Quiagen[®] (Valencia, CA, USA) columns with some modifications and were run on 0.7% agarose gel. The electrophoresis was carried out for 3-4 h at a constant voltage of 60 volts using Bio-Rad power pack against *lambda*/*Hind* III marker (Genei, Bangalore, India). After the electrophoresis was over, the gel was visualised on UVP-transilluminator and photographed directly by using # 667 polaroid film on a Polaroid MP 4+ Instant Camera System (Vilber Lourmat, Marne-La-Vallée, France). The sizing of the individual plasmids was done using extrapolation by plotting a semilog graph (Narra, 2000).

The plasmid profile of the natural isolates showed four plasmids in races 23 and 23b (ca. 60, 40, 23 and 8.2 kb), five in race 27 (ca. 60, 40, 23, 8.2 and 3.7 kb) and six in race 32 (ca. 60, 40, 23, 8.2, 3.7 and 1.6 kb). The laboratory isolates of *Xcm* races 23, 23b, 27 and 32 were pathogenic but slightly less virulent than their corresponding original isolates. Plasmid profiles of these laboratory isolates showed the presence of only two bands corresponding to ca. 60 and 40 kb (Fig. 1), demonstrating that frequent sub-culturing resulted in loss of both virulence and plasmids. The laboratory isolates were inoculated on host cotton (*G. hirsutum*) and the reisolates showed the presence of 4 plasmids ca. 60, 40, 23 and 8.2 kb in race 23; race 27 showed 5 plasmids including an additional 3.7 kb plasmid, while race 32 had all the plasmids shown in race 23 and 27 and also a unique 1.6 kb

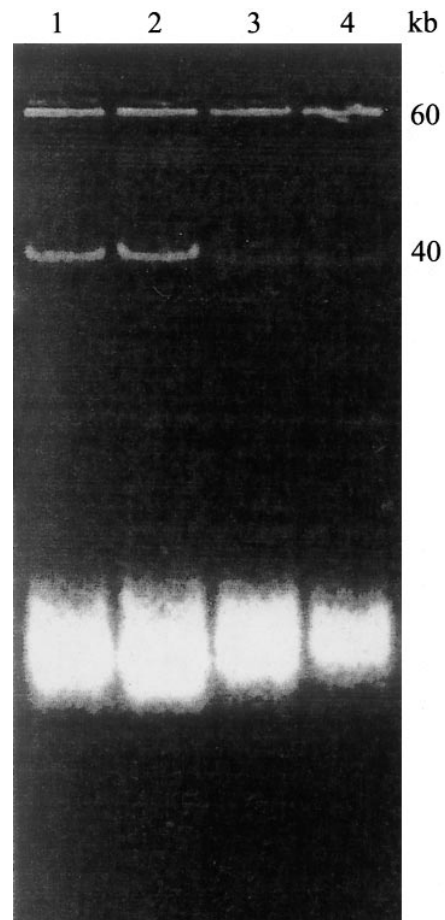


Fig. 1. Plasmid profiles of *Xcm* races: continuously sub-cultured laboratory isolates. 1 = Race 23; 2 = Race 23b; 3 = Race 27; 4 = Race 32.

plasmid (a total of six plasmids) (Fig. 2, Table 1). Reisolates of the laboratory isolate of race 23b (from *G. barbadense*) showed the same plasmids as race 23. When the natural isolates were passed through this host, there was no change in their plasmid profile.

Three reisolates (i.e. the laboratory isolates passed through the host and which had the same plasmid profile as the natural isolates), belonging to races 23, 27 and 32, were stored at -70°C in 50% glycerol. After six months storage all the isolates retained the 60, 40, 23 and 8.2 kb plasmids (Table 1; Fig. 3). However, there were certain minor changes in the miniplasmids, and after six months storage, the 3.7 kb plasmid was missing in the reisolates of race 27; while the 3.7 and 1.6 kb plasmids were missing in the case of race 32 (Table 1; Fig. 3).

Stepwise loss of virulence to individual *B*-genes on repeated sub-culturing of different *Xcm* races on YGCA medium has been shown (Verma and Singh, 1976). However, plasmid profiling of these races, which had lost virulence on repeated sub-culturing, or races from *G. barbadense* with lost aggressiveness was never done.

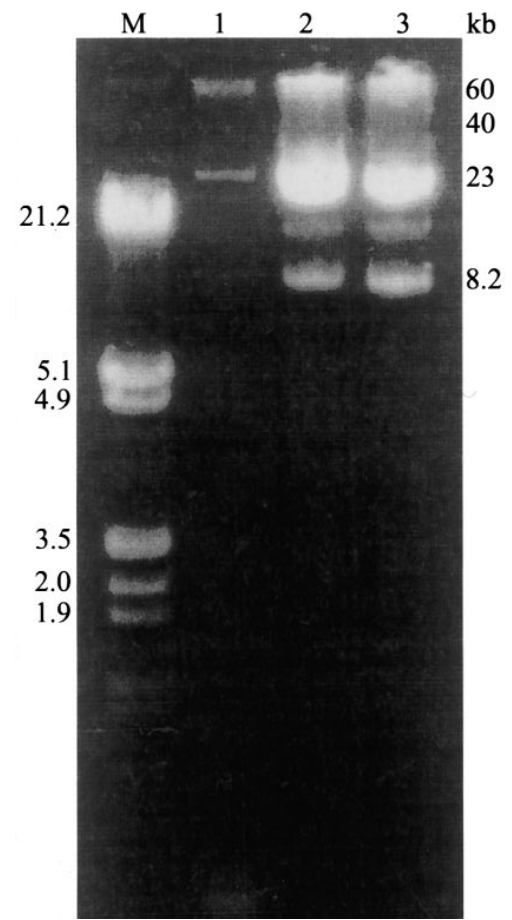
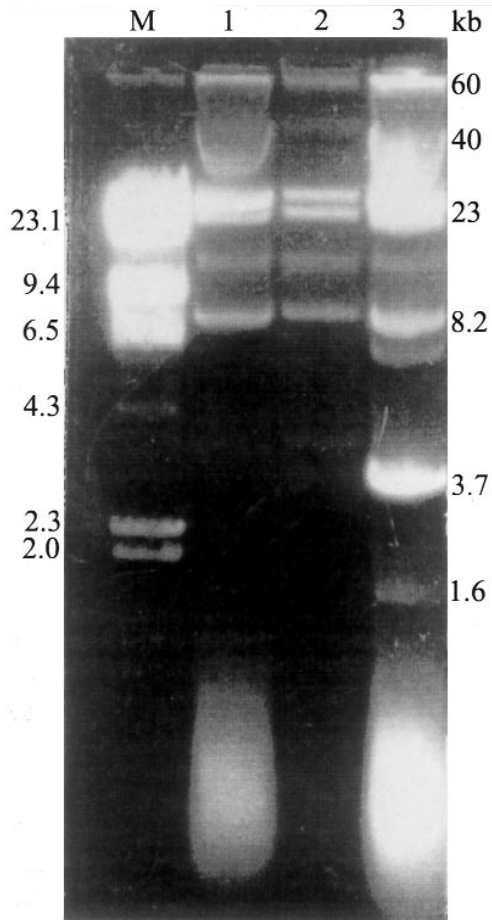


Fig. 2. Plasmid profiles of *Xcm* races: reisolates (i.e. laboratory isolates after passage through host cotton). M = Lambda/*Hind* III marker; 1 = Race 23; 2 = Race 27; 3 = Race 32.

Fig. 3. Plasmid profiles of *Xcm* races: reisolates stored at -70°C for six months. M = Lambda/*Hind* III/*Eco* RI marker; 1 = Race 23; 2 = Race 27; 3 = Race 32.

Table 1. Plasmid profiles of reisolates of laboratory isolates of *Xcm* before and after storage at -70°C.

Race	Plasmid profile (in kb)		
	Natural isolates/races	Reisolates ^a	
		Before storage at -70°C	After storage at -70°C
23	60	60	60
	40	40	40
	23	23	23
	8.2	8.2	8.2
27	60	60	60
	40	40	40
	23	23	23
	8.2	8.2	8.2
32	3.7	3.7	-
	60	60	60
	40	40	40
	23	23	23
	8.2	8.2	8.2
	3.7	3.7	-
	1.6	1.6	-

^a Laboratory isolates after passage through host possessed two plasmids of 60 and 40 kb.

The present studies now show that continuously sub-cultured laboratory isolates of *Xcm* races lost plasmids and retained only two large plasmids (ca. 60 and 40 kb). When these laboratory isolates were passed through host cotton (*G. hirsutum*) they regained certain plasmids and the plasmid profile was more or less similar to the original isolates. The sizes of the largest plasmids have now been determined to be 55.0 (for the 60 kb), 31.2 (for the 40 kb) and 7.4 (for the 10 kb) (Canteros *et al.*, 1995). The reisolates maintained their plasmid profiles (except for minor changes in the miniplasmids) after storage for six months at -70°C in 50% glycerol. It is suggested that plasmid profile among highly virulent races of *Xcm* is unstable during repeated sub-culturing at room temperature resulting in rapid loss of plasmids, but when the cultures were sub-cultured and stored at -70°C the plasmid profile was fairly stable except for the miniplasmids (3.7 and 1.6 kb).

Two large plasmids of approximately 60 and 40 kb were uniformly present in most of the races and were difficult to eliminate (Das and Verma, 1996). The miniplasmids were removed easily just through a few subcultures on YGCA. It was, therefore, concluded that, as the isolates containing 60 and 40 kb plasmids (with loss of miniplasmids) retained their general virulence, these plasmids are more important for the pathogenicity of *Xcm*. We now have preliminary data showing that when laboratory isolates were exposed to certain preparations such as intercellular fluid or cotton plant leaf extracts, they regained plasmids in a manner similar to passage through the host plant (Prasad *et al.*, 2001). The constancy of the plasmid profile of the natural isolates/races from various geographical sources and their inducible nature (through nutrient amendment) is an indication that bacterial chromosomes play a role in plasmid production. The possibility of plasmids being incorporated into the bacterial genome forming high frequency recombinants cannot be ruled out. Transfer of mitochondrial DNA to the nucleus has been reported in yeast (Ricchetti *et al.*, 1999), and chloroplast DNA in tobacco has been found to incorporate within nuclear DNA at different genomic sites (Maliga, 2003). The copy number of the plasmids may have been reduced to an undetectable level in the laboratory isolates. However, more research must be done before genophore-mediated plasmid production can be confirmed. Some preliminary observations also indicated a virulence increase in *Xcm* through host passage (Duttamajumder and Verma, 1994) and a gain in virulence through host passage was also reported by Brinkerhoff (1963) and Schnathorst (1970).

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