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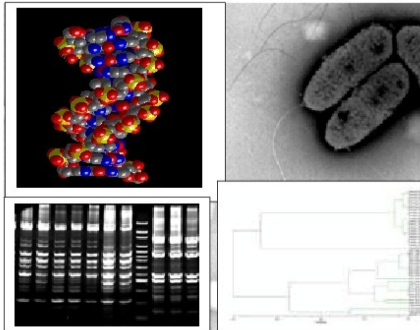

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INRODUCTION

Physiological, chemotaxonomical and genetic characterization of new *Xanthomonas* strains isolated from different host plants.

The present studies were carried out to fullfill the requirement for identifying several bacterial strains which had been isolated from new host plants as xanthomonads. Since these strains originated from new host plants (*Catharanthus* spp., *Lobelia* spp., *Isotoma axillaris*) for which diseases caused by xanthomonads had not been reported earlier, further characterizations were necessary in order to denote correct or new species or pathovar name.

This first step concerning primary characteristics of the genus *Xanthomonas* included microscopic investigation, Gram staining, electron microscopic investigation to detect the motility organs (flagella), some physiological and pathological tests which are related to this genus and finally examination of the unique pigment for xanthomonads, xanthomonadin.

Isolations from plant tissue and soil debris often yield many yellow-pigmented bacteria. It may be difficult to distinguish colonies of *Xanthomonas* spp. visually from colonies of saprophytic bacteria by color, especially with *Xanthomonas* species that do not produce copious amounts of extracellular polysaccharide (EPS). Because the yellow xanthomonadin pigments of *Xanthomonas* are unique to the genus, pigment extraction and chromatographic analysis can be an important tool for bacterial genus identification (Irey and Stall, 1982).

The most important chemotaxonomic significance of xanthomonadin pigments is that they are found in nearly all *Xanthomonas* species but do not occur in any non-xanthomonad (Starr, 1944; Starr and Stephens, 1964; Starr *et al*., 1977). Therefore, unique pigments (xanthomonadin) produced by *Xanthomonas* strains are considered an adequate chemotaxonomic marker for the genus *Xanthomonas*. Moreover, xanthomonads can thereby easily be distinguished from the many other genera of yellow-pigmented bacteria which are isolated from the plants also (Goodfellow *et al.,* 1976).

Originally, each variant of the genus *Xanthomonas* showing a different host range or producing different disease symptoms was classified as a separate species, which can be

described as the "new host-new species" method (Starr, 1981). This led to a complex genus that finally contained more than 100 species. Later on, 140 pathovars have been defined within the genus *Xanthomonas* (Bradbury, 1986; Hayward, 1993).

Several attempts have been made to classify pathovars and strains by using alternative features of the pathogen. Serological test (Benedict *et al.,* 1989; 1990), fatty acid profiling (Stead, 1992; Vauterin *et al.,* 1992), genomic and plasmid DNA analysis (Berthier *et al.,* 1993; Denny *et al.,* 1988; Hartung and Civerolo, 1987; Hildebrand *et al*., 1990; King, 1989; Lazo and Gabriel, 1987; Lazo *et al*., 1987; Leach *et al.,* 1990; Pecknold and Grogan, 1973), and protein analysis (Van Zyl and Steyn, 1990; Vauterin *et al*., 1991; Vauterin *et al*., 1990) have been used to classify pathovars and strains of different species. However, these techniques are often time-consuming, too expensive, or too insensitive for use in routine diagnosis. Therefore, new methods have been developed in recent years to rapidly identify and classify closely related pathogenic bacteria on the basis of genomic fingerprinting approach.

Many different PCR genetic fingerprints are used in identification and characterization of the genetic diversity of phytopathogenic bacteria: AP-PCR (arbitrary primed PCR) (Welsh *et al.,* 1990); RAPDs (randomly amplified polymorphic DNA) (Williams *et al.,* 1990); rep-PCR (repetitive sequence-based PCR) (Versalovic *et al*., 1994) and AFLP (amplified fragment length polymorphism) (Vos *et al*., 1995).

Because of their sufficient conservation, the rRNA genetic locus is used in a universal organization of evolutionary relationships (Cedergen *et al.,* 1988). The utility of the rRNA sequence as a taxonomic tool has been amply demonstrated in bacteria, where 16S RNA sequence analysis have completely redefined phylogenetic relationships (Fox *et al*., 1980; Lane *et al*., 1985; Woese, 1987; Woese and Fox, 1977). In addition to highly conserved areas that have been used to study the relationships among distant taxa, the 16S sequence contains more variable regions that have been useful in the differentiation of genera and species (Goebel *et al.,* 1987).

In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species, 16S, 23S, and 5S genes. These genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the level of genus and species. Within a single genome there are frequently multiple rRNA genetic loci; spacer regions found within

these loci also show a significant degree of variation in length and sequence. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacer (Brosius *et al*., 1981; Loughney *et al.,* 1982). There is a different approach to identify the bacterial genera and species using this technique, the PCR product can be digested with a restriction enzyme, and the resulting fragments can be resolved electrophoretically.

The amplified fragment length polymorphism (AFLP) technique is one of a number of DNA fingerprinting procedures that takes advantage of the polymerase chain reaction (PCR) to amplify a limited set of DNA fragments from a specific DNA sample (Vos *et al.,* 1995). AFLP fingerprints can be used to distinguish even very closely related organisms, including near isogenic lines. Most importantly, AFLPs have been shown to be reproducible and reliable. This is at least partially due to the fact that limited sets of generic primers are used and these are annealed to the target under stringent hybridization conditions.

By using this technique, Janssen *et al.* (1996) demonstrated the superior discriminative power of AFLP towards the differentiation of highly related bacterial strains that belong to the same species or even biovar, highlighting the potential of this novel fingerprinting method in epidemiological and evolutional studies. In 1999, Restrepo *et al.*, used AFLP as a novel PCR-based techniques, to characterize the genetic diversity of Colombian *Xam* (*Xanthomonas axonopodis* pv. *manihotis*) isolates.

In these studies (chapter 2) the following genetic techniques were used to characterize the new Xanthomonas strains: a) 16S-23S Intergenic Transcribed Spacer-PCR (ITS) using a specific primer in comparison with standard *Xanthomonas* strains and digestion of the amplified fragment with *Hae* III restriction enzyme (RFLP); b) 16S rDNA amplification; c) Rep-PCR fingerprinting (ERIC and BOX); d) Amplified Fragment Length Polymorphism (AFLP).

The plasmid pattern of different races of the phytopathogenic bacterium *Xanthomonas campestris* pv. *malvacearum* causing bacterial blight of cotton.

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).

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, different 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.

Plasmids are autonomous genetic elements that can replicate independently of the chromosome and can be separated physically from it in the laboratory. Plasmids are important and widely occurring constituents of plant pathogenic bacteria (Coplin, 1982). 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.

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 only 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.

CHAPTER 1

PATHOLOGICAL AND PHYSIOLOGICAL TESTS INCLUDING CHARACTERIZATION OF XANTHOMONADINS