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COMPARATIVE RFLP-ITS ANALYSIS BETWEEN *ENTEROBACTER CLOACAE* STRAINS ISOLATED FROM PLANTS AND CLINICAL ORIGIN

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ABSTRACT

Nineteen strains of *Enterobacter cloacae* including ten isolates from vegetables and eight from clinical origin and one from spring water were analysed by 16S-23S intergenic transcribed spacer (ITS) primers. Type strains of *E. cloacae* (ATCC 13047), *E. dissolvens* (ICMP 1570), and *E. nimipressuralis* (ICMP 1577) were included for comparative purposes. Also, the strains were tested for pathogenicity on onion bulbs. PCR-RFLP results showed that *E. cloacae* strains isolated from plants were homogeneous presenting close similarity among them, whereas the strains from clinical samples were heterogeneous. Pathogenicity tests revealed that the group comprising bacteria from clinical origin also showed capability of were able to induce a collapse of the inner scales of onion bulbs.

KEY WORDS: Pathogenicity, PCR, RFLP analysis.

RESUMO

ANÁLISE COMPARATIVA DE RFLP-ITS ENTRE LINHAGENS DE *ENTEROBACTER CLOACAE* ISOLADAS DE PLANTAS E DE ORIGEM CLÍNICO-HOSPITALAR. Dezenove linhagens de *Enterobacter cloacae* incluindo dez isolados de vegetais, oito isolados clínicos e um de água de fonte foram analisados por meio de PCR-RFLP utilizando primers intergênicos das regiões espaçadoras 16S-23S. Linhagens tipo de *E. cloacae* (ATCC 13047), *E. dissolvens* (ICMP 1570) e *E. nimipressuralis* (ICMP 1577) foram incluídas a título de comparação. A técnica de PCR-RFLP demonstrou que as linhagens vegetais aparecem homogêneas e são claramente distinguidas, enquanto que as de origem clínica mostraram heterogeneidade. Os testes de patogenicidade revelaram que o grupo das linhagens clínicas também induziu colapso interno nas escamas da cebola.

PALAVRAS-CHAVE: Patogenicidade, PCR, análise por RFLP.

INTRODUCTION

Enterobacter cloacae is a bacterium widely distributed in nature, and has been recognized as a nosocomial pathogen, sometimes as a potential or sometimes even as a primary pathogen mainly due to itsability to develop resistance to antibiotics. Several reports of infectious in hospitals have been made, for instance in neonatal intensive care units, surgical wards, burn units, or caused by cross-infection and other types. Articles and revisions on this subject are widely available in the medical literature. Also, often the source of epidemics in hospital environment is associated with contaminated pharmaceutical products. In Brazil this fact was observed by PISANI et al. (1997).

In agriculture *E. cloacae* has been found in many insects such as symbiotic or entomopathogenic and in the surface of vegetables. Several reports have been made with *E. cloacae* strains in the biological control of phytopathogens, for instance *Phytium* spp., *Sclerotinia* sp., *Rhizopus* sp., *Fusarium* spp. and many others.

E. cloacae has emerged in recent years as a phytopathogen of important crops. A reliable evidence of the pathogenicity of *E. cloacae* for plants was noticed by COOTHER & DOWLING (1986) in stored onions. Earlier TANII et al. (1974) had isolated *E. cloacae* from rice seeds in association with *Erwinia, Brevibacterium* and *Pseudomonas* without confirming however involvement in the disease process. Further reports of *E. cloacae* phytopathogenicity were made by WICK etal.

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(1987) concerning mung bean sprouts, by NISHIJIMA et al. (1987) who isolated the bacterium from papaya, by BISHOP & DAVIS (1990) and SCHWARTZ & OTTO (2000) who isolated it. NISHIJIMA et al. (1987) and BISHOP & DAVIS(1990) found in their studies that the type strain of *E. cloacae* from clinical origin were also pathogenic to papaya and onions, respectively. In Brazil, *E. cloacae* causing disease on plants was firstly observed by ROBBS et al. (1995) from samples of diseased melons collected at the northeast areas of the country, showing symptoms of pulp discoloration. Further isolations of the bacterium were made by ROBBS (C.F. Robbs, personal communication) from papaya, mangoes and lettuce.

The occurrence of bacterial species from the clinical samples environment on vegetables and *vice-versa* is a recognized fact. Besides *E. cloacae*, other species have been detected causing disease on plants or animals, for instance *Pantoea agglomerans* (MERGAERT et al.,1983; LINDH & URSING, 1986; GAVINI et al.,1989), *Pseudomonas aeruginosa* (LEBEDA et al., 1984), *Agrobacterium* sp. (FRENEY et al., 1985), *Herbaspirillum* "species 3" (BALDANI et al., 1996), *Serratia marcescens* (BERIAM et al., 1993; ROBBS et al., 1998; DORSEY et al., 2000), *Burkholderia cepacia* which is an important phytopathogen (BRADBURY, 1986) and frequently isolated from clinical (VAN LAER et al., 1998) as well as *Burkholderia gladioli* (HOARE & CANT, 1996).

A great number of typing schemes have been used for *E. cloacae*, including biotyping, serotyping, bacteriocin, protein profiles and phage typing. These methods however require an intensive work and lack resolution to detect small changes in the bacterial genome. Analyses based on DNA fingerprints are largely available and they have been employed with advantages in labor, time and precision. The arbitrarily amplified polymorphic DNA (AP-PCR) with randomic primers is able to detect slight genetic differences and has been used in various bacterial species for strains differentiation and epidemiological analysis. Molecular approaches such as the restriction fragment length polymorphism of the 16S-23S rDNA intergenic spacer region (RFLP-ITS) has been widely used to characterize bacterial strains at different taxa level. The ITS region is not under such high selective pressure as the 16S rDNA, another commonly used gene for phylogenetic purposes, and depending on the variation detected it might be a useful tool for discrimination at infraspecific level.

Although the genetic analysis of *E. cloacae* has been carried out by several authors, no studies were performed to investigate the relationship among *E. cloacae* strains coming from clinical and its comparison with isolates from vegetables, which could be useful in studies of nosocomial epidemics. Therefore, an experiment was carried out in order to investigate this relationship by using the RFLP-ITS, a more conserved feature. In addition, the phytopathogenic possibility of *E. cloacae* strains coming from clinical samples was also investigated.

MATERIAL AND METHODS

Bacterial strains and growth conditions - twenty IBSBF Culture Collection deposited strains of *E. cloacae* comprising then strains isolated from plants, eight strains from clinical cases, one strain from water source and the type strain of *E. cloacae* (ATCC 13047), are listed in Table 1. In addition, the type strains of *Enterobacter dissolvens* (ICMP 1570) and *Enterobacter nimipressuralis* (ICMP 1577) were included in this study since they were considered closely related to the type strain of *E. cloacae* (BRENNER et al., 1986; LINDH & URSING, 1991). All strains were grown on nutrient agar (NA) and incubated for 24-48h at 28° C.

DNA extraction – genomic DNA was prepared from a loopful of cells grown for 24 h and washed once in phosphate buffer. The cell pellet was resuspended in 10 ml of TE buffer (40 mM Tris-HCl pH 8.0; 1 mM EDTA) and DNA was extracted according to PITCHER et al. (1989). DNA was resuspended in TE buffer and stored at –20° C.

RFLP of the 16S-23S rDNA intergenic transcribed spacer – the spacer region between the 16S and 23S rDNA was amplified by using known primers for conserved regions: the 16S uni 330 and 23S uni 322 anti described by HONEYCUTT et al. (1995). The reaction was carried out in a final volume of 25 µL and contained 30-50 ng of DNA, 3 mM $MgCl_2$, 100 mM dNTP, 0.5 µM of each primer and 1.5 U of *Taq* polymerase (Cenbiot, Brazil). The cycling conditions were as described in HONEYCUTT et al. (1995). The amplification products were digested with *Sau*3 AI, *Hinf*I or *Dde*I restriction enzymes and separated by electrophoresis in 7% polyacrilamide gel and visualized by silver staining (MORENO et al., 1985).

Data analysis – the banding pattern generated by RFLP-ITS was converted in binary matrices assigning 1 for presence and 0 for absence of a designed band. Similarity matrices were constructed by using (S_J/ UPGMA). A dendrogram was constructed with the UPGMA (unweighted pair group method with average) with the program NTSYS-PC (ROHLF, 1989).

Pathogenicity test – pathogenicity tests were performed on mature onion bulbs to verify if strains isolated from clinical and from vegetables could induce disease symptoms. Five bulbs were inoculated through a syringe and hypodermic needle with about 0.5 mL of a bacterial suspension in distilled water of each strain with approximately 10^8 colony forming unity per milliliter (CFU / mL) injected in tissues. Controls were

Table 1 - Code and origin of *Enterobacter cloacae* strains used in this study.

Code IBSBF	Origin / host	Locality / source	Also held as
1140	*Cucumis melo*	Açu, RN	CTAA-35-L4*
1141	" "	" "	CTAA -35-L1
1142	" "	" "	CTAA -35-L2
1143	" "	" "	CTAA -35-L3
1189	" "	" "	CTAA -53-1
1186	*Carica papaya*	Linhares, ES	CTAA -51-1
1188	" "	" "	CTAA -51-2
1184	*Mangifera indicae*	Rio de Janeiro, RJ	CTAA -50-1
1185	" "	" "	CTAA -50-2
1291	*Lactuca sativa*	" "	CTAA -3430
1187	Water source	Uberlândia, MG	CTAA -49-1
1176	Clinical	(CAISM) Campinas, SP	
1177	"	" "	
1178	"	" "	
1231	"	Vitória, ES	
1233	"	" "	
1249	"	IAL 24	
1250	"	IAL 131	
1251	"	IAL 133	
	"	CCT 0456	ATCC 13047

ATCC= American Type Culture Collection, Rockville, Md., USA; CAISM= Unicamp, Campinas, Brazil; CCT= Coleção de Culturas Tropical, Campinas, Brazil; IAL= Instituto Adolfo Lutz, São Paulo, Brazil; IBSBF= Culture Collection of Phytobacteriology Section, Instituto Biológico, Campinas, Brazil.
*Cultures CTAA were provided by Dr. C.F. Robbs.

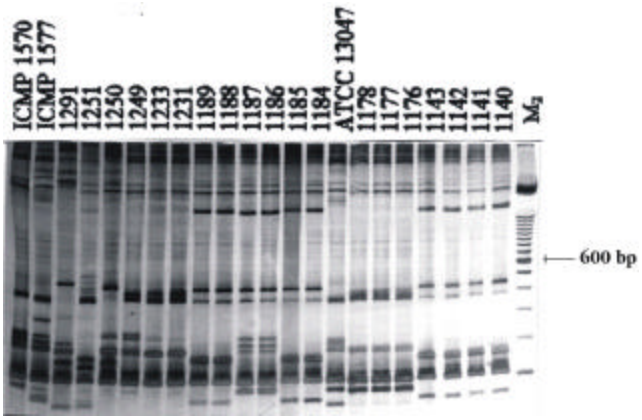

Fig. 1 - RFLP pattern of the 16S-23S ITS rDNA of *Enterobacter cloacae* and type strains of *E. dissolvens* and *E. nimipressuralis* after digestion with *Dde* I (A) and *Sau* 3 AI (B). M_2 100 bp molecular weight marker.

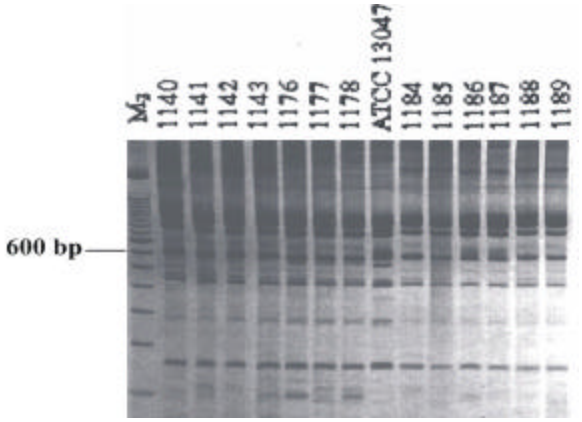

Fig. 2 - RFLP patterns of the 16S-23S ITS region of *Enterobacter cloacae* after digestion with *Sau* 3A I. M_2, 100 bp DNA Molecular Weight Marker.

inoculated with water. The inoculated onion bulbs were maintained at 36°C in a humid chamber. Pathogenicity symptoms were recorded after a week according to the following scale: 0 = tissues remain undamaged and without discoloration; 1 = slight discolored tissues 1 cm around the inoculation point and tissues undamaged; 2 = inner scales discolored or flaccid; 3 = inner scales flaccid or discolored from top to the basis; 4 = flaccid and discolored tissues of half or entire bulb.

RESULTS AND DISCUSSION

The amplification of 16S-23S ITS yielded a unique fragment of 1,000 base pairs approximately. Digestion of the PCR products with the restriction enzymes *Dde*I, *Hinf*I and *Sau*3 AI revealed similarity among the majority of *E. cloacae* strains isolated from plants except for 1186 and 1291 strains. Slight differences were observed in the patterns obtained with *Dde*I or

*Sau*3 AI. The digestion with *Hinf*I generated most of the discriminative bands which could allow the clear differentiation between clinical and phytopathogenic strains (Fig. 1). The *Sau*3 AI digestions yielded a very uniform pattern for all *E. cloacae* strains and only *E. cloacae* ATCC 13047 showed one differential band (Fig. 2).

The data obtained from PCR-RFLP for the 16S-23S ITS region were combined for cluster analysis. Using UPGMA cluster analysis, the similarities among strains from plants ranging up to 95% and different groups were identified as shown in the dendrogram (Fig. 3). The clinical strains showed about 80% similarity to each other except for strain 1251, which is more related to plant strains. One interesting point is the fact that the type strain ATCC 13047 remained as a single branch with lower than 50% similarity to other *E. cloacae* strains investigated. Such a weak relationship of the type strain to other *E. cloacae* strains was also described by LINDH & URSING (1986). The type strains of *E. dissolvens* and *E. nimipressuralis* were closer to each other with approximately 90% similarity but with values lower than 50% in comparison with the type strain of *E. cloacae* ATCC 13047. Although the remarkable differences found in the relatively few strains of *E. cloacae* studied herein, it is evident that *E. dissolvens* and *E. nimipressuralis* showed a pattern that diverged significantly and provided evidence that these species are not related to *E. cloacae*. The strain 1291 was situated in a single branch with approximately 45% similarity with the other *E. cloacae* strains, suggesting that it could belong to another *Enterobacter* species.

The genetic diversity detected in *E. cloacae* is not a novelty. It has been described by several authors in epidemiological studies of nosocomial outbreaks, for instance GARAIZAR et al., (1991), HAERTL & BANDLOW (1993), HARTSTEIN et al. (1995) and DARINI et al. (1999), and a single patient may be infected with different strains (POILANE et al, 1993).

The *E. cloacae* strains examined from clinical origin clearly induced variable levels of symptoms on onion bulbs as did the strains isolated from vegetables, except for strain 1231, which showed no tissue alteration. Reisolations made from scales showing tissues discoloration symptoms yielded bacteria similar to those inoculated and the strains could be divided into four groups according to their capability of destroying the onion tissues, causing a brown discoloration and a decay of the inner scales. This feature is illustrated in Figure 4.

The first group (level 4) comprised strains 1140, 1143 and 1185; the second group (level 3) comprised strains 1184, 1187, 1188 and 1250; level 2 were 1142, 1176, 1233, 1291 and ATCC 13047; level 1 comprised strains 1141, 1177, 1178, 1186, 1249 and 1251. The type strains of *E. dissolvens* and *E. nimipressuralis* showed a similar degree of pathogenicity in the level 2.

Considering the clinical strains, the pathogenicity evaluation showed that most of the strains were classified in the intermediate levels except for strain 1250 which presented a particular strong pathogenicity reaction. The results indicated that clinical strains were able to induce symptoms in the plant species tested.

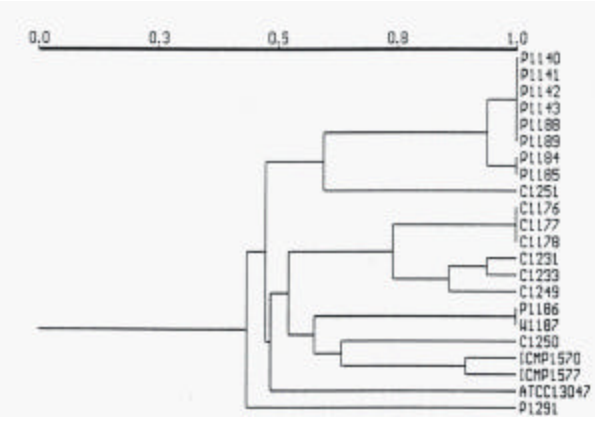

Fig. 3 - Dendrogram (s_j / UPGMA) derived from RFLP of the 16S-23S ITS region digested with Dde I, Hinf I and Sau 3A I restriction enzymes of *Enterobacter cloacae* and type strains of *E. dissolvens* and *E. nimipressuralis*. The letter before the number of the strains designates the origin: C (clinical), P (plants) and W (water).

Fig. 4 - Pathogenicity levels of *Enterobacter cloacae* on onion bulbs after artificial inoculation. A, strain 1251 (level 1); B, strain 1184 (level2); C, strain 1250 (level 3); D, strain 1140 (level 4).

When bacterial strains isolated from plants were evaluated for pathogenicity interesting differences were observed and no correlation was found in the host origin and pathogenicity to onion. Also, the majority of these strains showed a marked phytopathogenicity at levels 3 and 4. The mode of infection of *E. cloacae* strains to onion bulbs remain undetermined, however it seems possible that enzymes production by the bacterial cells may affect the cell wall of the plant host by increasing the permeability of cell membranes as it occurs, for instance, in the soft-rot *Erwinia* (BARRAS et al., 1994).

Several epidemiological studies revealed that *E. cloacae* of an opportunistic pathogen and the reservoir for strains involved in nosocomial infections is the gastrointestinal tract of patients as well as contaminated water or parenteral nutrition and cross-contamination. The observations presented in this paper indicated that clinical strains of *E. cloacae* although differentiable from the plant strains by RFLP-ITS, were able to induce some kind of "symptom" and to multiply on onion tissue.

Indeed, *E. cloacae* has been isolated from post-harvest vegetables (BARTOLONI et al., 1989), from vegetables and cooked meat samples in restaurants (SORIANO et al., 2001), and from manufactured yoghurts (CANGANELLA et al., 1999), and it is known that they persist in insects for at least three days (ARMSTRONG et al.,1989). These facts may contribute to the survival and dispersal of the bacterium by contaminating foods or materials and act as a "reservoir" in the hospital environment. In this manner, the phytopathogenicity capability of *E.cloacae* could be regarded as another presumable source of inoculum. Questions that arise are related to the host range (plants) of the pathogen, their survival on spoiled vegetables and in insects and the establishment of the minimal bacterium population required for a infection process. Also, the knowledge of the adhesive properties of the strains such as exopolyssacharide production and fimbriae (LIVRELLI et al., 1996) could provide more information useful aiming at the control of its populational expansion and prophylaxis measures.

Another point to be investigated is the patterns of antibiotic resistance in strains coming from food, plants and clinical samples which could provide (or not) a evidence that contaminated vegetables can indeed introduce the bacteria in the hospital environment.

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