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DIVERSITY OF *Pectobacterium* STRAINS BY BIOCHEMICAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION

DIVERSIDADE DE ISOLADOS DE Pectobacterium spp. PELA CARACTERIZAÇÃO BIOQUÍMICA, FISIOLÓGICA E MOLECULAR

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ABSTRACT: *Pectobacterium* is a complex taxon of strains with diverse characteristics. It comprises several genera, including *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya*, and *Pantoea*. *Pectobacterium* and *Dickeya* cause diseases in a wide range of plants, including potatoes, where they are causative agents of soft rot in tubers and blackleg in field-grown plants. Characterizing *Pectobacterium* species allows for the analysis of the diversity of pectinolytic bacteria, which may support control strategies for plant bacterial diseases. The aim of this study was to perform biochemical, physiological, and molecular characterizations of *Pectobacterium* spp. from different sites and host plants. The isolated strains were characterized by the glucose fermentation test, Gram staining, catalase activity, oxidase activity, growth at 37 ºC, reducing substances from sucrose, phosphatase activity, indole production, acid production from different sources (sorbitol, melibiose, citrate, and lactose), pathogenicity in potato, and hypersensitivity reactions. Molecular characterization was performed with species-specific primers ECA1f/ECA2r and EXPCCF/EXPCCR, which identify *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* (Pcc), respectively, and with primers 1491f/L1RA/L1RG and Br1f/L1RA/L1RG that differentiate Pcc from *Dickeya chrysanthemi* and from *P. carotovorum* subsp. *brasiliensis*. The strains were identified as belonging to the genus *Pectobacterium*, though they did not fit the biochemical nor the molecular classification standards for subspecies differentiation, indicating significant diversity among the strains.

KEYWORDS: Phytobacteria. Potato blackleg. Polymerase chain reaction. *Solanum tuberosum*.

INTRODUCTION

Potato propagation is potentially associated with the dissemination of pathogens, including bacteria belonging to the genus *Pectobacterium*. These bacteria, the causative agents of blackleg and soft rot diseases (LOPES; QUEZADO-DUVAL, 2001), are capable of producing pectinolytic enzymes that lead to plant death in the field and rot in potato tubers under either field or storage conditions. Controlling the diseases is difficult, since the bacteria present large genetic variability and can survive in a large number of host plants. This makes it difficult to select resistant cultivars (BRISOLLA et al., 2002).

Isolation, detection, identification, and characterization of *Pectobacterium* species can be accomplished by using selective culture medium containing pectate (CUPEELS; KELMAN, 1974), by biochemical, physiological (DE BOER; KELMAN, 2001), molecular (TOTH; AVROVA; HYMAN, 2001), and serological tests (ALLAN; KELMAN, 1977), and by biological baits (TAKATSU; MELO; GARCIA, 1981). Latent infection in tubers can be detected by incubation, lenticella sampling (DE BOER; KELMAN, 1975), and direct seeding of tuber extract dilutions on Bulmer crystal violet pectate medium (PÉROMBELON; KELMAN, 1987). The pathogenicity test can be carried out by inoculating the bacterium in potato tubers or by stem inoculation of potato plantlets (DICKEY; KELMAN, 1988).

In the potato crop, there is the possibility that other subspecies or even yet unidentified species of *Pectobacterium* are involved in blackleg and soft rot diseases (FESSEHAIE; DE BOER; LEVESQUE, 2002). This is a fact that may have important epidemiological implications; it may influence how specific control strategies are chosen to manage the diseases. Therefore, it is of great importance to correctly identify *Pectobacterium* strains.

There is a need for studies involving the identification and characterization of pectobacteria in potato tubers. Therefore, our aim was to perform biochemical, physiological, and molecular characterization of *Pectobacterium* strains isolated

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from potato plants at different sites and compare them to bacteria isolated from other hosts.

MATERIAL AND METHODS

Pectobacterium strains were isolated from different field-growing infected plants at different sites (Table 1). Indirect isolation was done using biological potato baits. Strains were further plated in 523 medium (1% sucrose, 0.8% hydrolyzed acid casein, 0.4% yeast extract, 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, and 1.5% agar) (KADO; HESKETT, 1970) and incubated at 28 °C for 48 h. Purified strains were stored in 40% glycerol and 523 broth (v v^{-1}) at -80 °C.

Table 1. Strains of *Pectobacterium* sp. isolated from different host plants and sites.

Strain	Host	Site	Plant material
UFU A6	Potato	Uberlândia, MG	Tuber
UFU A7	Lettuce	Uberlândia, MG	Leaf
UFU A9	Potato	Santa Juliana, MG	Tuber
UFU A14	Potato	Uberlândia, MG	Tuber
UFU A20	Potato	Santa Juliana, MG	Tuber
UFU A22	Potato	Uberlândia, MG	Tuber
UFU A27	Cassava	Ipiaçu, MG	Root
UFU A33	Lettuce	Uberlândia, MG	Leaf
UFU A37	Tomato	Uberlândia, MG	Fruit
UFU A47	Potato	Santa Juliana, MG	Tuber

Biochemical and physiological characterizations of the strains were carried out by the following tests, which are routinely used to differentiate *Pectobacterium* subspecies (SCHAAD; JONES; CHUN, 2001): glucose fermentation (oxidation/fermentation), Gram staining, catalase activity, oxidase activity, growth at 37 ºC, reducing substances from sucrose, phosphatase activity, indole production, and acid production from sorbitol, melibiose, citrate and lactose (PÉROMBELON; KELMAN, 1987). Potato baits containing the strains were used for hypersensitivity reactions in tobacco and pathogenicity in potato plants.

Genomic DNA from bacterial strains was extracted according to published protocol (SAMBROOK; FRITSCH; MANIATIS, 1989). The PCR reaction used primers ECA1f (5' - GAA CTT CGC ACC GCC GAC CTT CTA - 3') and ECA2r (5' - GCA CAC TTC ATC CAG CGA - 3') (DARRASSE et al., 1994) that amplify a 690-bp fragment particular of *P. atrosepticum*. PCR amplification was performed with an initial denaturation step at 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 65 °C for 45 s, 72° C for 45 s and a final extension of 72 °C for 5 min.

Pectobacterium carotovorum subsp. *carotovorum* (Pcc) specific primers [EXPCCF (5' - CGC CAT CAT AAA AAC AGC - 3') and EXPCCR (5' - GCC GTA ATT GCC TAC CTG CTT AAG - 3')] (EL TASSA; DUARTE, 2004) amplify a fragment of 550 bp. PCR conditions were as follows: 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and 72 °C for 10 min.

The triads of primers 1491f (5' - GAA GTC GTA ACA AGG TA - 3'), L1RA (5' - CAA GGC ATC CAC CGT - 3') and L1RG (5' - CAG GGC ATC CAC CGT - 3') (DUARTE et al., 2004) and of primers Br1f (5' - GCG TGC CGG GTT TAT GAC CT - 3'), L1RA (5' - CAA GGC ATC CAC CGT - 3') and L1RG (5' - CAG GGC ATC CAC CGT - 3') (DE BOER; KELMAN, 2001) allow differentiation of Pcc (fragments of 510 and 550 bp) from *Dickeya chrysantemi* (fragments of 480, 510, and 550 bp) and from *P. carotovorum* subsp. *brasiliensis* (322 bp). The PCR cycle consisted of denaturation at 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 66 °C for 45 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min.

For all primers, the amplification reactions were performed in a final volume of 50 µL, containing 1X Taq buffer (Invitrogen), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 100 ng of each primer, 2.5 U of Taq DNA polymerase (Invitrogen, 5 U μL^{-1}) and 100 ng of DNA template, in the GeneAmp 9700 thermocycler (Applied Biosystems). PCR products were loaded onto a 1.5% agarose gel with 0.20 µg 100 mL^{-1} of ethidium bromide and electrophoresed at 100 V for 40 min. Images were taken using a computerized gel analysis system (Kodak Digital Science 1D).

RESULTS AND DISCUSSION

All bacterial strains showed a round shape, smooth edges, 1 to 3 mm diameter convex colonies, smooth texture, brilliant appearance, opaque optical property, and yellowish cream color. They were Gram-negative, fermentative, catalase positive, and oxidase negative, meeting the phenotypic criteria to be classified as *Pectobacterium* (DE BOER; KELMAN, 2001). Inoculated strains developed soft rot symptoms in potatoes and positive hypersensitivity reactions in tobacco leaves (Table 2).

Strains UFU A6 and UFU A47 (Table 2) did not produce acid from citrate, whereas strain UFU A7 was sorbitol positive. Strains UFU A7 and UFU A20 showed no reduction of substances from sucrose, but the latter showed phosphatase activity. UFU A14 was melibiose-positive for acid production. Based on phenotypic characteristics, it was not possible to differentiate the strains into species nor subspecies level, indicating wide biochemical diversity among them.

Researchers were able to detect biochemical diversity between *Pectobacterium* strains in Chinese cabbage (ALVARADO, 2006), potato tubers, ornamental (KANG; KWON; GO, 2003) and medicinal plants (HU et al., 2008). Alvarado (2006) reported strains of Pcc capable of growing at 37 ºC and producing acid from lactose, but some of them were ineffective at producing acid from sorbitol and had no phosphatase activity. Moreover, 10.2% of the strains evaluated by the author showed a reduction of substances from sucrose and 12.8% were unable to produce acid from melibiose. Opposite results were obtained in our study: the majority of the *Pectobacterium* strains did not reduce substances from sucrose but all of them showed positive reaction to acid production from melibiose.

It can be inferred that there is a great diversity among *Pectobacterium* species, making it difficult to identify them based only on biochemical and physiological parameters. The occurrence of strains with intermediate characteristics impairs correct classification of these bacteria (OLIVEIRA et al., 2003; PALMA, 2006). Phenotypic identification, besides inaccurate, is time-consuming (SAMSON et al., 2001; TOHT; AVROVA; HYMAN, 2001). Seo and Takanami (2002) could not differentiate Pcc from *P. atrosepticum* based on biochemical assays, suggesting that other methodologies should be used for identification at the subspecies level (YAP; BARAK; CHARKOWSKI, 2004).

In the physiological and biochemical characterization of *Pectobacterium* strains isolated from a cold climate region of Brazil, the strains that grew at 37 °C did not necessarily fit the standard characteristics of *P. atrosepticum* (DUARTE et al., 2003). Those were later classified as a new subspecies, named *P. carotovorum* subsp. *brasiliensis* (DUARTE et al., 2004). In a study regarding the occurrence and diversity of pectinolytic bacteria in potato seed tubers, 119 strains were classified as *P. carotovorum* subsp. *brasiliensis* by biochemical tests, 96 as Pcc, and eight did not correspond to any species or subspecies previously described (EL TASSA; DUARTE, 2004). Similar results were found in our study, in which strains recovered from different sites and host plants did not fit into any of the proposed subspecies, suggesting great genetic and phenotypic diversity among the strains imposed by evolutionary factors. Thus, continuous research is needed to characterize and categorize these microorganisms to provide information that can be applied in disease control strategies in agriculturally important crops. Since some strains did not fit within known subspecies characteristics, more tests that link the genetic, biochemical, and physiological heterogeneity of pectinolytic bacteria are necessary.

The primers ECA1f/ECA2r and EXPCCF/EXPCCR did not amplify the genomic DNA from bacterial strains, implying that none of them belonged to the species *P. atrosepticum* or Pcc. Amplicons of 326, 480, and 581 bp were visualized for strains UFU A7, UFU A14, UFU A22, UFU A27, UFU A37, and UFU A47 when primers 1491f/ L1RG/ L1RA were used (Figure 1). Those strains could be classified as *D. chrysanthemi* (480, 510 and 580 bp), except for the 326 bp fragment.

PCR products of 480 and 581 bp for strain UFU A9 (Figure 1) and absence of the 581 bp fragment for strain UFU A20 indicate that they belong to a new subspecies. No DNA amplification was visualized for strain UFU A6.

Primers Br1f/L1RG/L1RA are used to identify *P. carotovorum* subsp. *brasiliensis* by a 322 bp fragment. They generated amplicons only for strain UFU A33 (obtained from lettuce) of 352, 420, and 690 bp sizes (Figure 2). Those fragments cannot be associated to *P. carotovorum* subsp. *brasiliensis*.

Molecular analysis did not confirm the identification of the biochemically characterized strains, reinforcing the hypothesis that they belong to other species / subspecies or that the primers were not able to amplify the expected fragments.

Table 2. Phenotypic characteristics of *Pectobacterium* strains.

Reference strains/subspecies	Hypersensitivity reaction	Pathogenicity									Acid production from:				Conclusion
	Tobacco	Potato	O/F	Gram	Catalase	Oxidase	Growth at 37 °C	Reducing substances from sucrose	Phosphatase activity	Indole production	Sorbitol	Melibiose	Citrate	Lactose	
Pcc	+	+	F	-	+	-	+	-	-	-	-	+	+	+	*Pectobacterium carotovorum* subsp. *carotovorum*
Pa	+	+	F	-	+	-	-	+	-	-	-	+	+	+	*Pectobacterium atrosepticum*
Dc	+	+	F	-	+	-	+	-	+	+	-	+	+	+	*Dickeya chrysanthemi*
Pcbr	+	+	F	-	+	-	+	+	-	-	-	+	+	+	*Pectobacterium carotovorum* subsp. *brasiliensis*
Pco	+	+	F	-	+	-	+	+	-	-	+	+	+	+	*Pectobacterium carotovorum* subsp. *odoriferum*
Pcb	+	+	F	-	+	-	+	+	-	-	-	-	-	+	*Pectobacterium betavasculorum*
Strains															
UFU A6	+	+	F	-	+	-	+	-	-	-	-	+	-	+	Does not meet biochemical characteristics
UFU A7	+	+	F	-	+	-	+	-	-	-	+	+	+	+	Does not meet biochemical characteristics
UFU A9	+	+	F	-	+	-	+	-	-	+	+	+	-	-	Does not meet biochemical characteristics
UFU A14	+	+	F	-	+	-	+	+	-	-	-	+	-	+	Does not meet biochemical characteristics
UFU A20	+	+	F	-	+	-	+	-	+	-	+	+	+	+	Does not meet biochemical characteristics
UFU A22	+	+	F	-	+	-	+	-	-	-	+	+	-	-	Does not meet biochemical characteristics
UFU A27	+	+	F	-	+	-	+	+	-	-	+	+	-	-	Does not meet biochemical characteristics
UFU A33	+	+	F	-	+	-	+	-	-	-	+	+	-	-	Does not meet biochemical characteristics
UFU A37	+	+	F	-	+	-	+	-	-	-	+	+	-	+	Does not meet biochemical characteristics
UFU A47	+	+	F	-	+	-	+	-	-	-	-	+	-	+	Does not meet biochemical characteristics

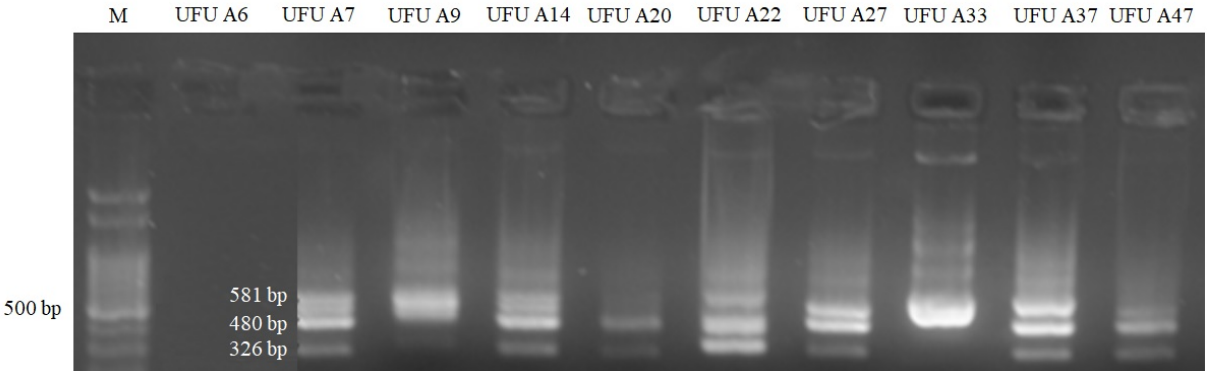

Figure 1. Amplification of DNA fragments from *Pectobacterium* strains UFU A6, UFU A7, UFU A9, UFU A14, UFU A20, UFU A22, UFU A27, UFU A33, UFU A3, and UFU A47 with primers 1491f/L1RA/L1RG. M: molecular marker 1 Kb Plus DNA Ladder (Invitrogen).

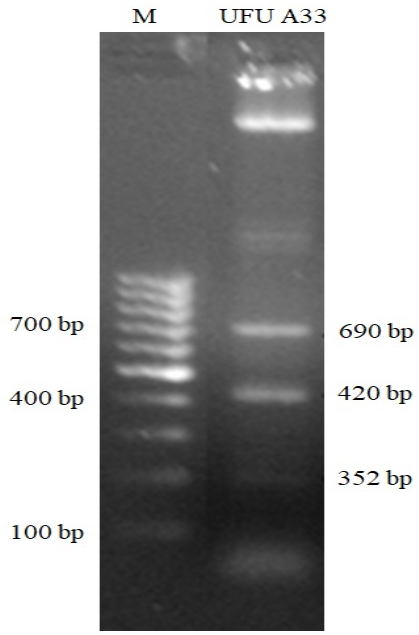

Figure 2. Genomic DNA amplification of *Pectobacterium* strain UFU A33 with primers Br1f/L1RA/L1RG. M: molecular marker 1 Kb Plus DNA Ladder (Invitrogen).

Hu et al. (2008), in the characterization of Pcc from a medicinal herb (*Pinelli ternata*), found two strains apparently classified as Pcc and *P. carotovorum* subsp. *odoriferum*, sharing 97-99% similarity. After conducting more precise studies, they concluded that the strains were Pcc. The authors reported that the differences between both strains and other Pcc species in Europe and North America are related to the geographic distribution and diversity of host plants. Several studies show that pectobacteria are genetically distinct, forming heterogeneous groups even within subspecies (DARRASSE et al., 1994).

Knowledge of diversity is an important prerequisite for the identification of phytobacteria, as well as for taxonomic classifications, to support epidemiological studies and the development of strategies for plant diseases control, especially regarding selection of resistant varieties in breeding programs. This is particularly important when different closely related species and subspecies cause diseases in the same host, as is the case of pectobacteria in potatoes (EL TASSA; DUARTE, 2006).

CONCLUSIONS

The strains evaluated in our study were characterized as belonging to the genus *Pectobacterium*. No further classification was accomplished using biochemical and molecular techniques, demonstrating their great diversity.

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RESUMO: *Pectobacterium* é um táxon complexo de isolados bacterianos com características diversas. Compreende vários gêneros como *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* e *Pantoea*. *Pectobacterium* e *Dickeya* causam doenças em ampla variedade de plantas, incluindo a batateira, na qual são os agentes etiológicos da podridão mole dos tubérculos e da canela-preta de plantas cultivadas em campo. A caracterização de espécies de *Pectobacterium* permite a análise da diversidade de bactérias pectolíticas, podendo auxiliar estratégias de controle de doenças bacterianas em plantas. O objetivo deste trabalho foi caracterizar bioquímica, fisiológica e molecularmente isolados de *Pectobacterium* sp. provenientes de diferentes locais e hospedeiros. Os isolados foram caracterizados pelos testes de fermentação de glicose, Gram, catalase, oxidase, crescimento à 37 ºC, redução de substâncias a partir de sacarose, atividade da fosfatase, produção de indol, produção de ácido a partir de sorbitol, melibiose, citrato e lactose, patogenicidade em batata e reação de hipersensibilidade. Para a caracterização molecular, foram utilizados os pares de *primers* ECA1f/ECA2r e EXPCCF/EXPCCR [específicos para *P. atrosepticum* e *P. carotovorum* subsp. *carotovorum* (Pcc), respectivamente] e as tríades de *primers* 1491f/L1RA/L1RG e Br1f/L1RA/L1RG, para diferenciar Pcc de *Dickeya chrysanthemi* e de *P. carotovorum* subsp. *brasiliensis*. Os isolados foram identificados como pertencentes ao gênero *Pectobacterium,* no entanto, não se enquadraram na classificação bioquímica e tampouco molecular para diferenciação das subespécies, demonstrando a grande diversidade dos mesmos.

PALAVRAS-CHAVE: Fitobactéria. Canela-preta da batata. Reação em cadeia da polimerase. *Solanum tuberosum*.

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