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The Haemolytic Activity of *Gluconacetobacter diazotrophicus*

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Abstract: *G. diazotrophicus is a Gram negative and acid acetic bacterium that could be isolated from the roots, stems and leaves of sugarcane, and from the coffee plant, citrus fruits and others. This acetic acid bacterium has demonstrated beneficial effects in the cultures (use as biofertilizer) and it has been recognized for its nitrogen fixation ability, both as plant associated ands free-living bacteria. On the other hand, the acetic acid bacteria are microorganisms found in the environment that are also used in the food industry. However, in recent years reports have been raising concerns related to human bacteremia, endocarditis, and pneumonia, associated with acetic acid bacteria. In this work, we presents some evidence in relation to the possible pathogenic role of G. diazotrophicus due to the hemolytic capacity of this bacterium.*

Keywords: *Gluconacetobacter diazotrophicus, Acetic acid, Bacterium, Haemolytic activity, Sugarcane, Immunocompromised patient.*

1. INTRODUCTION

Gluconacetobacter diazotrophicus is a Gram negative bacterium that was first isolated from sugarcane plant (Cavalcante and Döbereiner, 1988; Döbereiner *et al*., 1993; Gillis *et al*. 1989). It has been found associated in the roots, stems and leaves of sugarcane (Cavalcante and Döbereiner, 1988; Gillis *et al*. 1989; Reis *et al*., 1994). In the late 90's, *G. diazotrophicus* was isolated from the coffee plant and some citrus fruits such as pineapple and others (Cruz *et al*., 2001; Döbereiner *et al*., 1993; ; Eskin *et al.*, 2014; Guedes *et al.*, 2008; Jimenez-Salgado *et al.*, 1997; Madhaiyan *et al*., 2004; Tian *et al.*, 2009). *G. diazotrophicus* has been studied due to its beneficial effects in plants. For example, this bacterium has been proposed as biofertilizer (Cavalcante and Döbereiner, 1988). Additionally, I has been studied for its nitrogen fixation ability both as plant associated and free-living bacteria (Flores-Encarnación *et al.*, 1999; Gillis *et al*., 1989). *G. diazotrophicus* belongs to the acetic acid bacteria and is tolerant to low pH (Flores-Encarnación *et al.*, 1999; Galar and Boiardi, 1995). The acetic acid bacteria are microorganisms found in the environment and widely used in the food industry. Nevertheless, the potential risks for human associated to the use of acetic acid bacteria in food processing have been poorly studied (Alauzet *et al.*, 2010; Bassetti *et al*., 2013; Juretschko *et al*., 2010; Yamada *et al*., 1999). In recent years different authors have reported human cases of bacteremia, peritonitis, lymphadenitis, endocarditis, and post-operative pneumonia, associated with acetic acid bacteria such as *Asaia bogorensis*, *Granulibacter bethesdensis*, *Gluconobacter* spp, *Acetobacter cibinongensis,*and *Acetobacter indonesiensis* (Bittar *et al.*, 2008; Greenberg *et al.*, 2006; Gouby *et al*., 2007; Snyder *et al.*, 2004; Tuuminen *et al.*, 2006). The present study is aimed to look for evidence on the possible pathogenic role of *G. diazotrophicus* when it comes into direct contact with the cells of eukaryotic organisms, especially erythrocyte cells.

2. MATERIAL AND METHODS

2.1. Bacterial Strains

G. diazotrophicus PAL5 was maintained in LGI medium described by Reis *et al.* (1994). *Escherichia coli* CFT073 was grown at 37°C in Luria-Bertani broth.

2.2. Growth Conditions

G. diazotrophicus strain was grown in modified LGI liquid medium containing (g/L): K_2HPO_4, 2 g; KH_2PO_4, 0.6 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.02 g; $Na_2MoO_4.2H_2O$, 0.002 g; $FeCl_3.6H_2O$, 0.06 g, NH_4Cl 0.05 g; sucrose, 50 g. The pH was ajusted to 5.5 with acetic acid (Reis *et al.*, 1994). Culture was grown aerobically at 30°C in a 50 mL-working-volume Erlenmeyer flask stirred at 200 rpm for 72 hours. Growth was determined by measuring the absorbance at 546 nm. The absorbance at 546 nm of culture was ajusted to 0.02 and active inoculum (100 μL) was harvested and washed twice with 20 mM phosfate buffer (pH 7.0). *E. coli* strain was grown in Luria-Bertani broth (LB) containing (g/L): yeast extract, 5 g; tryptone sodium casein, 10 g; sodium chloride, 10 g. The pH was ajusted to 7.0. *E. coli* was grown in a 50 mL-working-volume Erlenmeyer flask steady at 37°C for 24 hours. The absorbance of culture was ajusted to 0.02 and active inoculum (100 μL) was harvested and washed twice with 20 mM phosfate buffer (pH 7.0).

2.3. Haemolytic Activity Assay

The haemolytic activity was determinated measuring the concentration of released hemoglobin from an erythrocyte suspension, which was prepared by placing 100 μL of sheep erythrocytes in 900 μL of 0.9% NaCl (erythrocyte suspension). The hemoglobin concentration was determinated spectrophotometrically using 18 mM benzidine as developer, 5.25 M acetic acid and 0.043% hydrogen peroxide and measuring absorbance at 515 nm according to the methodology described by Pujades (2006).

2.4. Hemolysis Kinetics

Hemolysis kinetics were quantified by measuring the increase of released hemoglobin from the reaction mixtures containing: 800 μL of 0.9% NaCl, 100 μL of erythrocyte suspension and 100 μL of active bacterial inoculum, which was prewashed and resuspended in sterile 0.9% NaCl. Then the mixtures were agitated gently. Assays were performed in triplicate. The mixtures containing *G. diazotrophicus* were incubated at 30°C for 0, 10, 20, 30 y 40 minutes. *E. coli* was used as positive control which was incubated at 37°C under the same conditions described above. At the end of incubation time, an aliquot of 100 μL was taken from the mixture and benzidine was added to quantify the concentration of released hemoglobin. As a negative control was used a mixture of erythrocytes in 0.9% NaCl. As reference study, total hemolysis was produced by adding 100 μL of sheep erythrocytes to 1 mL of sterile distilled water and incubated for 5 minutes at room temperature. The debris were removed by centrifuging at 3,500 rpm for 5 minutes. Then, aliquots of hemolysate were taken: 10, 20, 40, 60 and 100 μL. For each sample, protein and hemoglobin concentrations weres quantified.

2.5. Protein Assay

The protein concentration was determinated according to the methodology described by Lowry *et al*., (1951).

2.6. Growth In Plate

The haemolytic activity of *G. diazotrophicus* was assayed on blood agar plates. They were prepared in the laboratory by adding 5% sheep blood in brain heart infusion agar (Bacto-Difco-BBL). *G. diazotrophicus* was inoculated in cross groove on blood agar plates and it was incubated at 30°C for 72 hours (in humidity chamber to prevent drying of the culture medium).

3. RESULTS

As it is detailed in the Material and methods, the haemolytic activity was determined spectrophotometrically by measuring the concentration of released hemoglobin from an erythrocyte suspension. For this, benzidine was used as developer and absorbance at 515 nm was measured. The mixtures containing *G. diazotrophicus* were incubated at 30°C for 0, 10, 20, 30 y 40 minutes. *E. coli* was used as a positive control, and a mixture of erythrocytes as a negative control. The mixtures containing *E. coli* were incubated at 37°C under the same conditions described above. As can be seen in Fig. 1, cell lysis occurs from the contact of *G. diazotrophicus* with the cell suspension, and it also occurs in the test using *E. coli* as positive control. In the latter case, an increased hemolysis was observed. After 40 minutes the concentration of released hemoglobin in the positive control was quantified around 17 μg, while the concentration of released hemoglobin by *G. diazotrophicus* at the

same time point was 12.5 µg. As shown in Fig. 1, the haemolytic effect observed by both bacteria was constant until 30 minutes and then they produced a strong hemolysis. Also as shown in Fig. 1, hemolysis was not observed in the absence of bacteria (negative control). On the other hand, the haemolytic activity of *G. diazotrophicus* also was assayed on blood agar plates. The plates were incubated at 30°C for 72 hours according to the conditions described in Material and Methods. The results are shown in Fig. 2. *G. diazotrophicus* produced an alpha hemolysis across the plate containing blood agar (Fig. 2B). Fig. 2A shows a blood agar plate that was not inoculated. As indicated in Material and Methods, the blood agar plates were incubated in a humidity chamber in order to avoid dehydration of agars. The results obtained in this study confirmed the ability from *G. diazotrophicus* to lyse the erythrocytes *in vitro*.

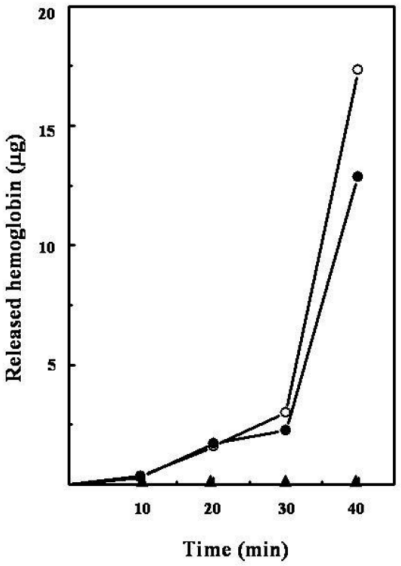

Fig1. *Measurement of haemolytic activity using benzidine, Haemolytic activity from G. dizotrophicus (●); haemolytic activity from E. coli (positive control) (○); negative control (▲)*

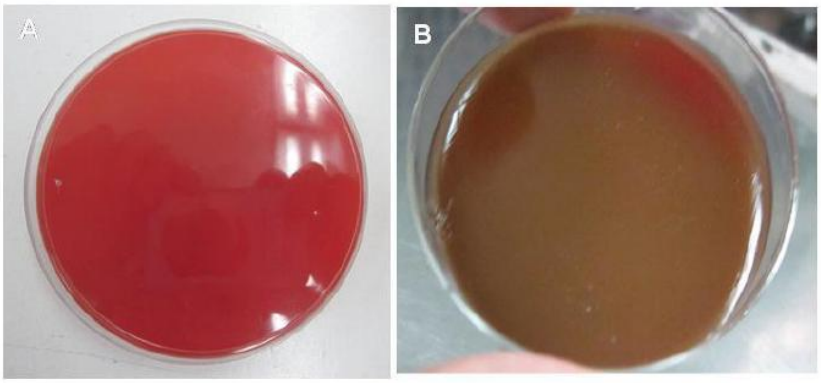

Fig2. *The haemolytic activity of G. diazotrophicus on blood agar plates. A. Blood agar plate not inoculated; B. The alfa hemolysis produced by G. diazotrophicus.*

4. DISCUSSION

Acetic acid bacteria are Gram-negative or Gram-variable, non-spore forming, aerobic, ellipsoidal to rod-shaped cells (Greenberg *et al.*, 2006; Jiménez-Salgado *et al*., 1997; Raspor and Goranovic, 2008). The optimum pH for the growth of acetic acid bacteria is pH 5.0 to 6.5, while they can grow at lower pH values between pH 3.0 and pH 4.0 (Flores-Encarnación *et al.*, 2004; Sengun and Karabiyikli, 2011). Acetic acid bacteria are well known for the ability to oxidize sugars and alcohols resulting an accumulation of organic acids in the environment. They are involved in some important industrial process, for example: the production of L-sorbose from D-sorbitol; D-gluconic acid, 5-keto- and 2-keto-D-gluconate from D-glucose; and dihydroxyacetone from glycerol (Flores-Encarnación *et al.*, 2004; Gupta *et al*., 2001; Raspor and Goranovic, 200). The application of acetic acid bacteria are many, both in the food industry and agriculture. However, in recent years it has been reported different human cases of bacteremia, peritonitis, endocarditis, post-operative pneumonia lymphadenitis, associated with acetic acid bacteria. In such cases, *Asaia bogorensis*, *Granulibacter*

bethesdensis, Gluconobacter spp, *Acetobacter cibinongensis, Acetobacter indonesiensis* have been isolated (Abdel-Haq *et al.,* 2009; Alauzet *et al*., 2010; Bittar *et al.*, 2008; Greenberg *et al.*, 2006; Greenberg *et al.*, 2007; Gouby *et al*., 2007; Juretschko *et al*., 2010; Snyder *et al.*, 2004; Tuuminen *et al.,* 2006). It has been reported that bacteremia caused by *Asaia bogorensis* in a young patient was favored by a history of intravenous-drug abuse (Goudy *et al*., 2007; Tuuminen *et al*., 2006).

Recreational drug use was a factor that contributed to the development of these diseases (Tuuiminen *et al*., 2006). In 2013, an endocartidis case also associated with acetic acid bacteria and drug use was reported, which denotes that these bacteria are microorganisms that can potentially infect humans (Bassetti *et al.*, 2013). So, it was reported that a multiresistant strain of *Gluconobacter* spp. was associated with endocardial lesions in a 25 year old female intravenous drug abuser. These evidences highlight that acetic acid bacteria should be considered as newly emerging opportunistic human pathogens(Alauzet *et al*., 2010; Bassetti *et al*., 2013; Bittar *et al.*, 2008; Greenberg *et al.*, 2007). The importance of these studies are that acetic acid bacteria are microorganisms commonly found in the environment and used in the food industry (Raspor and Goranovic, 2008; Sengun and Karabiyikli, 2011). In addition to endocarditis, other cases of disease in humans caused by acetic acid bacteria have been reported. For example: peritonitis associated with *Asaia bogorensis* in a patient with a peritoneal dialysis catheter; chronic granulomatous disease associated with *Granulibacter bethesdensis*; bacteremia associated with *Gluconobacter* spp. without specific site localization in a non immunocompromised patient with a history of intravenous drug abuse (Abdel-Haq *et al*., 2009; Alauzet *et al.*, 2010; Bittar *et al.*, 2008; Greenberg *et al*., 2006; Juretschko *et al.*, 2010; Snyder *et al*., 2004;).

On the other hand, *G. diazotrophicus* is an acetic acid bacterium that have been found in sugarcane plant, coffee plant, some citrus fruits such as pineapple (Cavalcante and Döbereiner, 1988; Döbereiner *et al*., 1993; Gillis *et al*. 1989; Reis *et al*., 1994; Jimenez-Salgado *et al.*, 1997; Madhaiyan *et al*., 2004). *G. diazotrophicus* has been studied because it has demonstrated beneficial effects in the cultures tested and it has been proposed for use as biofertilizer. This is an aerobic nitrogen-fixing bacterium (Cavalcante and Döbereiner, 1988; Flores-Encarnación *et al.,* 1999) whose usefulness is associated with the agricultural, in addition to producing auxins such as indole acetic acid (Ríos and Dibut, 2007). However, little or nothing is known about the possible pathogenic role that *G. diazotrophicus* could have. This is the first report in literature presenting evidence of the haemolytic activity of *G. diazotrophicus*. The results of this study indicated that *G. dizotrophicus* lysed an erythrocyte suspension, results obtained both in liquid medium and in blood agar culture plates. This implies that this bacterium has a potential pathogenicity factor responsible for the lysis of erythrocytes. At this point we do not know the nature of it, we have only seen the effect. However, *G. diazotrophicus* could exhibit similar behavior to other acetic acid bacteria that have been previously isolated from different human pathological cases, especially in individuals whose immune response is compromised.

5. CONCLUSION

Acetic acid bacteria are microorganisms found in the environment and used for industrial and agricultural purposes. However, in recent years it has become clear that such bacteria are associated with different in several countries, especially in immunocompromised patients. Therefore, acetic acid bacteria are considered as new opportunistic human pathogens. In this study, evidence was obtained that *G. diazotrophicus* had the ability to produce hemolysis, which is important because there are no reports in the literature indicanting that it is a pathogenic potentially bacteria. It will be important to determine other pathogenicity factors present in the bacteria that could cause damage in humans who have contact with it.

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REFERENCES

[1] Abdel-Haq N, Savaçsan S, Davis M, Asmar SI, Painter T and Salimnia H (2009). *Asaia lannaensis* bloodstream infection in a child with cancer and bone marrow transplantation. J. Med. Microbiol. 58**:**974-976.

[2] Alauzet C, Teyssier C, Jumas-Bilak E, Gouby A, Chiron R and Rabaud C (2010). *Gluconobacter* as well as *Asaia* species, newly emerging opportunistic human pathogens among acetic acid bacteria. J. Clin. Microbiol. 48:3935-3942.

[3] Bassetti M, Pecori D, Sartor A, Londero A, Villa G, Cadeo B, Brillo F, Bongiorno D, Campanile F and Stefani S (2013). First report of endocarditis by *Gluconobacter* spp. in a patient with a history of intravenous-drug abuse. J. Infect. 66: 285-287.

[4] Bittar F, Reynaud-Gaubert M, Thomas P, Boniface S, Raoult D and Rolain JM (2008). *Acetobacter indonesiensis* pneumonia after lung transplant. Emerg. Infect. Dis. 14:997-998.

[5] Cavalcante VA and Döbereiner J (1988). A new acid-tolerant nitrogenfixing bacterium associated with sugarcane. Plant Soil. 108:23-31.

[6] Cruz ES, Souza EMDE, Weber OB, Baldani IVO, Do J and Bioquı D (2001). 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from nanana (*Musa* spp.) and pineapple (*Ananas comosus*). Appl. Envirom. Microbiol. 67:2375-2379.

[7] Döbereiner J, Reis VM, Paula MA and Olivares FL (1993). Endophytes diazotrophs in sugar cane, cereals and tuber plants. Curr. Plant Sci. Biotechnol. Agric. 17:671-676.

[8] Eskin N, Vessey K. and Tian L (2014). Research progress and perspectives of nitrogen fixing bacterium, *Gluconacetobacter diazotrophicus*, in monocot plants. Intern. J. Agron. 2014:1-13.

[9] Flores-Encarnación M, Contreras-Zentella M, Soto-Urzua L, Aguilar-Gutiérrez GR., Baca BE and Escamilla-Marván JE (1999). The respiratory system and diazotrophic activity of *Acetobacter diazotrophicus* Pal 5. J. Bacteriol. 181:6987-6995.

[10] Flores-Encarnación M, Sánchez-Cuevas M, Ortiz-Gutiérrez F (2004). Las PQQ-deshidrogenasas. Un novedoso ejemplo de quinoproteínas bacterianas. Rev. Lat. Microbiol. 46:47-59.

[11] Galar ML and Boiardi JL (1995). Evidence for a membrane-bound pyrroloquinoline quinone-linked glucose dehydrogenase in *Acetobacter diazotrophicus*. Appl. Microbiol. Biotechnol. 43:713–716.

[12] Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt RM, Stephan MP, Teixeira KRS, Döbereiner J and De Ley J (1989). *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int. J. Syst. Bacteriol. 39:361-364.

[13] Gouby A, Teyssier C, Vecina F, Marchandin H, Granolleras C, Zorgniotti I and Jumas-Bilak E (2007). *Acetobacter cibinongensis* bacteremia in human. Emerg. Infect. Dis. 13:784-785.

[14] Greenberg DE, Ding L, Zelazny AM, Stock F, Wong A, Anderson VL, Miller G, Kleiner DE, Tenorio AR, Brinster L, Dorward, DW, Murray PR and Holland SM (2006). A novel bacterium associated with lymphadenitis in a patient with chronic granulomatous disease. PloS Pathog. 2:260-267.

[15] Greenberg DE, Porcella SF, Zelazny AM, Virtaneva K, Sturdevant DE, Kupko JJ and Holland SM (2007). Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis.* J. Bacteriol. 189:8727–8736.

[16] Guedes HV, dos Santos ST, Perin L, Teixeira KRS, Reis VM and Baldani JI (2008). Polyphasic characterization of *Gluconacetobacter diazotrophicus*. Braz. J. Microbiol. 39:718–723.

[17] Gupta A, Singh VK, Qazi GN and Kumar A (2001). *Gluconobacter oxydans*: its biotechnological applications. J. Mol. Microbiol. Biotechnol. 3:445-456.

[18] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

[19] Jimenez-Salgado T, Fuentes-Ramirez LE, Tapia-Hernandez A, Mascarua-Esparza MA, Martinez-Romero E, Caballero-Mellado J (1997). *Coffea arabica L.*, a new host plant for *Acetobacter diazotrophicus*, and isolation of other nitrogen-fixing acetobacteria. J. Appl. Environ. Microbiol. 63:3676-3683.

[20] Juretschko S, Beavers-May TK and Stovall SH (2010). Nosocomial infection with *Asaia lannensis* in two paediatric patients with idiopathic dilated cardiomyopathy. J. Med. Microbiol. 59:848–852.

[21] Madhaiyan M, Saravanan VS, Jovi DB, Lee H, Thenmozhi R, Hari K and Sa T (2004). Occurrence of *Gluconacetobacter diazotrophicus* in tropical and subtropical plants of Western Ghats, India. Microbiol. Res. 159:233-243.

[22] Pujades A. La hemoglobina y sus derivados. Características generales y métodos de determinación cuantitativa. En. Vives Corrons, J.L. y Aguilar Bascompte, J.L. (Eds). Manual de técnicas de laboratorio en Hematología. 3ª Edición. Madrid, España. Elsevier. 2006. pp: 149-172.

[23] Raspor P and Goranovic D (2008). Biotechnological applications of acetic acid bacteria. Crit. Rev. Biotechnol. 28:101-124.

[24] Reis VM, Olivares F and Döbereiner J (1994). Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J. Microbiol. Biotechnol. 10:401-405.

[25] Ríos Y and Dibut B (2007). *Gluconacetobacter diazotrophicus*: Un microorganismo promisorio en la elaboración de biopreparados. Cultivos Tropicales. 28: 19-24.

[26] Sengun IY and Karabiyikli S (2011). Importance of acetic acid bacteria in food industry. Food Control. 22:647-656.

[27] Snyder RW, Ruhe J, Kobrin S, Wasserstein A, Doline C, Nachamkin I and Lipschutz J (2004). *Asaia bogorensis* peritonitis identified by 16S ribosomal RNA sequence analysis in a patient receiving peritoneal dialysis. Am. J. Kidney Dis. 44:15-17.

[28] Tian G, Pauls P, Dong Z, Reid L and Tian ML (2009). Colonization of the nitrogen-fixing bacterium *Gluconacetobacter diazotrophicus* in a large number of Canadian corn plants. *Canadian Journal of Plant Science*. 89: 1009-1016.

[29] Tuuminen T, Heinäsmäki T and Kerttula T (2006). First report of bacteremia by *Asaia bogorensis*, in a patient with a history of intravenous-drug abuse. J. Clin. Microbiol. 44:3048-3050.

[30] Yamada Y, Hosono R, Lisdyanti P, Widyastuti Y, Saono S, Uchimura T (1999). Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. J.Gen. Appl. Microbiol. 45:23-28.