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# BACTERIA ISOLATED FROM A SUGARCANE AGROECOSYSTEM: THEIR POTENTIAL PRODUCTION OF POLYHYDROXYALCANOATES AND RESISTANCE TO ANTIBIOTICS

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## ABSTRACT

In this investigation, a sugarcane agroecosystem at a coastal tableland, in the northeast of Brazil, was screened to obtain bacteria strains able to synthesize poly-β-hydroxyalkanoates (PHA), using sucrose as the main carbon source. The potential to synthesize PHA was tested qualitatively by Sudan Black staining of colonies growing in different carbon sources: sucrose, glucose, fructose, propionate and cellulose. In a typical sugarcane crop management system, the plantation is burned before harvesting and vinasse, a by-product of alcohol production, is used in a fertirrigation system causing, probably, selective pressures on the microbiota of natural environments. Eighty-two bacteria strains, belonging to 16 different genera and 35 different species, were isolated. The data showed that 11 strains (*ca* 13%), nine of which belonging to the genus *Pseudomonas*, presented a strong Sudan Black staining in several carbon sources tested and, simultaneously, showed multiple resistance to antibiotics. Resistance to antibiotics is an advantageous feature for the biotechnological production of PHAs. The total number of isolates with multiple resistance to antibiotics was 73, and 38% of them belong to the genus *Pseudomonas*. Among the isolates, *ca* 86% and 43% grew in the presence of 10-100 U/ml of penicillin and/or 100-300 mg/ml of virginiamycin, respectively. These antibiotics are utilized in the alcohol distillery we investigated. The results suggest that some agroecosystem environments could be considered as habitats where bacteria are submitted to nutritional unbalanced conditions, resulting in strains with potential ability to produce PHAs, and also, to an increase in the microbial diversity.

**Key words:** soil bacteria, poly-β-hydroxyalkanoate, PHA, resistance to antibiotics, sugarcane agroecosystem, vinasse

## INTRODUCTION

Poly-β-hydroxyalkanoic acids (PHA) are biodegradable polymers that accumulate in the citosol of microbial cell, as granules, under unbalanced growth conditions with high C:N ratio in the medium. $C_4$ to $C_{18}$ hydroxyalkanoates can be the monomers of different polymers. The first and more abundant PHA found in the microbial biomass was poly-β-hydroxybutirate (PHB) which under limiting environmental conditions may constitute as much as 90% of the dry cell weight (11, 27). A biodegradable plastic with the registered trade name BIOPOL is already industrially produced by Imperial Chemical

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Industries (ICI), of England, as the copolymer poly-3HB/poly-3HV which is less brittle then PHB and has been used as packaging material. The ICI obtain copolymers from mutant strains of *Alcaligenes euthrophus* H16, using glucose as main carbon source (5). However, glucose is an expensive substrate for producing biodegradable plastic in industrial scale. *Alcaligenes latus* is able to produce PHB from sucrose, a cheaper substrate, but in smaller amount than *A. eutrophus.* Thus, other carbon sources like sodium propionate, fructose, valerate, octanoate, etc have been tested for PHA production, as well as the expression of the structural genes for the key enzymes of PHA synthesis from *Alcaligenes eutrophus* in other organisms, such as *E. coli* and *S. cerevisiae* (2,14,25).

Antibiotic is considered, in ecological terms, a natural product of microorganisms, synthesized as a secondary metabolite, when excess substrate concentration is available. They are produced mainly by actinomycetes and they are widespread, though evidences of their production in the environments are considered to be limited (33), but they are forthcoming (9, 17, 29). Besides the production of antibiotics by the microbiota in natural environments, it is quite impressive nowadays the general use of antibiotics in many activities leaded by man, as animal husbandry, agriculture, hospitals and prophylaxis. This extensive and careless usage of antibiotics has encouraged growth of resistant strains leading to an imbalance in prior relationships between susceptible and resistant bacteria (6, 13). The resistance strategies used by bacteria can be either by mutation or by plasmid acquisition and the seletive pressure exerted by the presence of the antibiotic will induce resistance to antimicrobial agents among bacteria. Efficient transfer of R plasmids between bacteria of diverse origin under simulated natural conditions, even in the absence of antimicrobial agents, demonstrate that R plasmids can spread among bacterial strains of humans, animal, and fish origins that are unrelated either evolutionarily or ecologically (10). Transformation and transduction are, besides conjugation, also important mechanisms of gene transfer in the environment (31). Data confirm that the more frequent antibiotic-resistant strains correspond to the commonest and largest amounts of antibiotics produced and used commercially (16). It was suggested (32) that, in tropical soils, resistant microbial population to antibiotics may be selected from the natural soil microbiota upon contact with these compounds. The resistance to antibiotics would be an advantage for PHA industrial production, once the bacterium that produces this compound could be cultivated free of competitors.

In the northeast of Brazil, the sugarcane is an extensive cash crop plantation spread over many diverse soil ecosystems. One of the most utilized of such ecological systems is the coastal tableland (or 'tabuleiro', as it is named in this part of Brazil). In a typical sugarcane crop management system, every plantation is burned before harvesting, for economical reasons. The vinasse generated as a by-product of alcohol distillery (for every liter of alcohol produced, 11-15 liters of vinasse are discharged to wastewater stabilization ponds), is used in a fertirrigation system, where 300-600 $m^3$ of the wastewater are spread per hectare, before sugarcane crop renovation. Some soil, chemical, physical and ecological implications of this process have been commented elsewhere (15, 28).The distillery here studied, produced 56 million liters of alcohol, from August/1992 to March/1993, which means that at least 616 millions liters of vinasse should be recycled in the sugarcane agroecosystem. In the cultivated area of the Usina Japungu (*ca* 13 thousands hectares), 3 thousands hectares are fertirrigated with wastewater containing vinasse, once a year, before sugarcane crop renewal. In the fermentors of the distillery (with capacity of 350,000-550,000 liters), variable quantities of antibiotics are used for controlling bacteria populations that compete with *Saccharomyces cerevisae* for the sucrose of the sugarcane juice. The crop management and processes above described, we believe, contribute to likely selective pressures on the microbiota of natural environments, with differing consequences on microbial populations involved in biogeocycling of essential nutrients to sugarcane plantation. The poor tablelands soils, whose acid pH (5.0 to 5.5) and low nutrients contents are limiting factors to productivity, may be particularly affected by this crop management system, once many hectares of these ecosystems have been abandoned as unproductive, when yield decreases to less than 40 Mg of sugarcane.$ha^{-1}$.$yr^{-1}$. Physical and chemical soil properties seem not to change over some time of cropping; and vinasse, used for fertirrigation, showed to benefit the microbial biomass, in one year observation (15). However, intensive sugarcane cultivation and vinasse effect on soil properties, in a long-term basis, lack further investigations. The possible relation between environments under stress or strong perturbation and

the biodiversity increase, is another ecological parameter that has been investigated by some authors (4, 30) and is also considered in the present study.

In the present investigation, a screening of bacteria living in environments under anthropic pressure in the sugarcane agroecosystem was performed, searching for **a**) strains with potential to produce PHA using different carbon sources, mainly sucrose; **b**) strains with multiple resistance to antibiotics and **c**) microbial diversity of the isolates in an attempt to use these parameters as bioindicators of anthropic pressure on natural microbiota of diverse environments, in the sugarcane agroecosystem.

## MATERIALS AND METHODS

**Environment selected.** The investigations were performed over two years (1992-94) in the Usina Japungu, an alcohol distillery situated in the municipality of Santa Rita, Paraíba State, Brazil. The local climate type is As' of Köppen classification, hot and humid, and the annual mean rainfall is 1,640 mm. The soil here studied is an oxisol (15). A large and a small stabilization ponds were investigated. The large one receives vinasse from the fermentors and water used for washing the sugarcane before being processed. The small one receives vinasse only. The temperature of the wastewater in the stabilization pond, measured at 10:30, in May 23rd, 1994, was 37°C. The vinasse as an effluent, coming out straight from the fermentors, reaches a temperature between 80 and 90°C, $BOD_5$ 20,000 to 35,000 mg/L, pH 3.6 to 4.0 and total solids 21.8 g/L. Vinasse applied to soil (*ca* 195 $m^3.ha^{-1}$) supplies twice more K than the amount required by sugarcane plantation, therefore, a serious threat to soil salinization (15). The samples collected (**Group A**) and the field experiments carried out in the area we investigated (**Group B**) are organized in distinct types as described. **Group A**: samples collected from soil, from the borders of the wastewater stabilization pond and from the wastewater itself, numbered from I to VII. I) Soil amended with sugarcane bagass (20 cm depth); II) soil amended with sugarcane bagass and other organic residues from the distillery (10 cm depth); III) soil amended with the wastewater (10 cm depth); IV) soil from the borders of the wastewater stabilization pond, in a place with a mixture locally named '*gelose*', which means hydrogel or agar; V) soil from the borders of the stabilization pond, in a place covered temporarily by the wastewater; VI) vinasse collected straight from the fermentors; VII) wastewater from the stabilization pond. **Group B**: field experiments, where small pieces of sugarcane into nylon bags (45 µm mesh size) were buried in several soil environments or submersed in the large and in the small stabilization ponds. These samples were numbered from VIII to XII. VIII) samples from the borders of the large stabilization pond (20 cm depth), in a place covered temporarily by the wastewater; IX) samples from the borders of the large stabilization pond (10 cm depth), in a place covered permanently by the wastewater; X) samples from the soil of sugarcane plantation (10 cm depth), not burned before harvesting; XI) samples from the soil of sugarcane plantation (10 cm depth), burned before harvesting; XII) samples submersed in the small stabilization pond.

**Samples processing and bacteria identification.** Soil samples (2 g) and sugarcane pieces contained in the nylon bags (2 g), were processed as follow (19): they were mixed in 250 ml of water (6,000 r.p.m.) for 2 min. Wastewater and vinasse samples were mixed manually for 2 min. Serial dilutions were performed using buffers with the same pH of the samples collected from the environments (7). Soil pH was determined in water (1:2.5 v/v), according to Allen *et al.* (1). Aliquots from each dilution were transferred to nutrient broth (NB) and incubated at 30°C and 37°C. After 24 h the cultures were streaked in agar nutrient and incubated to form isolated colonies (19). The colonies were characterized morphologically and then replicated for further biochemical tests, for identifying the isolated species (8, 26). Standards strains from the collection of the Departamento de Antibióticos, Universidade Federal de Pernambuco were used for comparison: *Bacillus subtilis* (DAUFPE-16; Waksman, USA); *B. anthracis* (DAUFPE-09; Escola Nacional de Ciências Biológicas, México), *B. cereus* (DAUFPE-11; Departamento de Antibióticos, UFPE), and *Pseudomonas aeruginosa* (DAUFPE-39; Instituto Tecnológico-2633).

**Detection of potential PHA production.** Colonies were grown in minimum unbalanced agar medium (MUA): $NH_2HPO_4.7H_2O$, 6.7 g/L; $KH_2PO_4$, 1.5 g/L; $(NH_4)_2SO_4$, 0.5 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $CaCl_2.2H_2O$, 0.01 g/L; Fe(III)$NH_4$ citrate, 0.06 g/L and trace element solution, 1 ml/L. Trace element solution contained: $H_3BO_3$, 0.3 g/L; $CoCl_2.6H_2O$, 0.2 g/L; $ZnSO_4.7H_2O$, 0.1 g/L; $MnCl_2.4H_2O$,

0.03 g/L; $NaMoO_4.2H_2O$, 0.03 g/L; $NiCl_2.6H_2O$, 0.02 g/L and $CuSO_4.5H_2O$, 0.01 g/L. The carbon source (sucrose, glucose, fructose, sodium propionate or carboxy methyl cellulose) was added at 5.0 g/L (22, modified). Agar was added at 15 g/L. Potential PHA production was detected after five days incubation at 30 or 37°C, by Sudan Black (SB) staining of the colonies. Briefly: a 0.02% SB solution in ethanol 96% was spread over the colonies for 30-60 min, discarded and washed with ethanol 96% (24). Dark blue stained strains were compared to standards strains of *Alcaligenes eutrophus* DSM 545 and *A. latus* 1023 (from IPT-Instituto de Pesquisas Tecnológicas, São Paulo).

**Antibiotics screening procedure.** Plate dilution technique, in nutrient agar (NA), was used for determining the levels of the strains resistance to the following antibiotics and respective concentrations (23): kanamycin sulphate, km, 20 µg/ml (Sigma); tetracyclin, tc, 10 µg/ml (Briston); ampicillin, amp, 50-100-150-200 µg/ml (Wyeth); streptomycin sulphate, sm, 20 µg/ml, (Inlab); nalidixic acid, nal, 20 µg/ml (Sigma). The antibiotics virginiamycin, virg, and penicillin, pen, from SmithKline Beecham, were a gift from Usina Japungu. The maximal concentration used in the alcohol distillery, for all antibiotics, was 20 µg/ml. In this work, the final concentrations for virginiamycin were 100-150-300 µg/ml and for penicillin were 1-10-100 U/ml.

## RESULTS AND DISCUSSION

**Bacteria strains with potential PHA production.** Table 1 shows the bacteria strains (**Group A**) isolated from the environments studied, their ability to grow in different carbon sources and their multiple resistance antibiotic phenotype. From the 40 isolates, 27 strains grew well in sucrose, 32 in glucose, 31 in fructose, 22 in propionate and 7 in cellulose. Samples from sites IV and VII presented the highest number of strains, belonging to 10 and 8 different species respectively. 36% (site IV) and 67% (site VII) of the strains grew well in sucrose, glucose, fructose, and propionate. Positive Sudan Black (SB) staining in the distinct carbon sources is shown in Fig. 1, except for cellulose, because bacteria growing in this carbon source showed very weak staining. Although 85% (34 strains) of the isolates in Group A can be SB positive, a wide range of tint can be observed and only 25% (10 strains) were in the

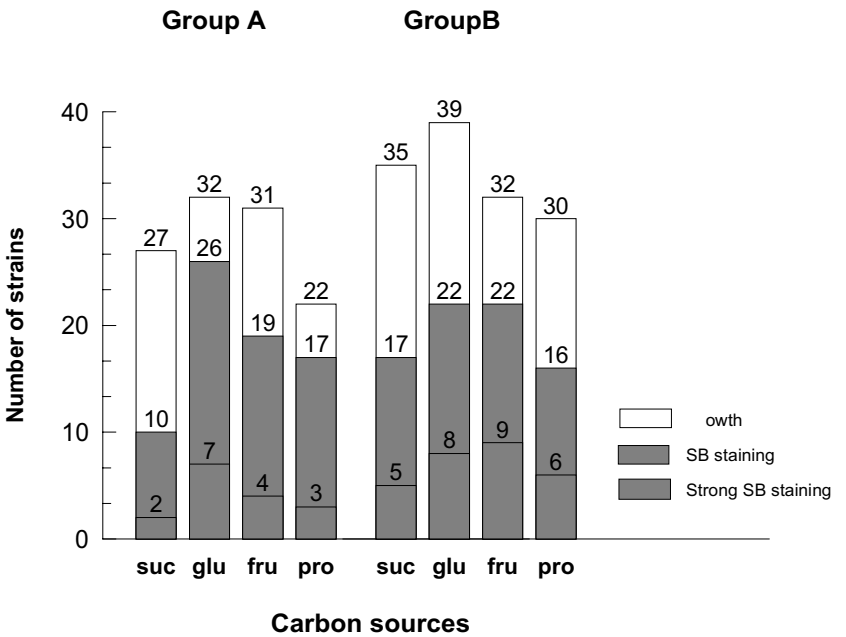


**Figure 1** – Growth and intensity Sudan Black (SB) staining of 82 strains growing in minimum unbalanced agar medium plus sucrose, glucose, fructose or sodium propionate (5.0 g/L), stained with a 0.02% ethanolic SB solution (the numbers on the top of the bars indicate the number of strains).

**Table 1** – Group A strains (types I to VII) isolated from the sugar cane agroecosystem: growth in different carbon sources and resistance to antibiotics

| Types | Strains | Species | Growth in Carbon Sources[1] | | | | | Resistance to antibiotics (μg/ml)[2] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | suc | glu | fru | pro | cel | amp | virg | pen | sm | km | tc | nal |
| I | AS1 | *Enterobacter cloacae* | + | + | + | – | – | 150 | 300 | 100 | – | – | – | – |
| | AS2 | *Enterobacter cloacae* | + | + | + | – | – | 150 | 300 | 100 | – | – | – | – |
| | AS3 | *Enterobacter cloacae* | + | + | + | – | – | 150 | 300 | 100 | – | 20 | – | – |
| | AS4 | *Enterobacter cloacae* | + | + | + | w | w | 150 | 300 | 100 | – | 20 | – | – |
| | AS11 | *Pseudomonas sp.* | – | w | + | w | – | 150 | – | 100 | – | – | 10 | 20 |
| II | AS5 | *Enterobacter cloacae* | + | + | + | – | – | 100 | 300 | 100 | – | – | – | – |
| | AS7 | *Pseudomonas vesicularis* | + | + | + | + | – | 50 | – | 100 | – | – | – | – |
| | AS10 | *Pseudomonas picketii* | – | + | + | + | – | 150 | 150 | 100 | 20 | 20 | 10 | – |
| III | AS12 | *Pseudomonas picketii* | + | + | + | – | – | 150 | – | 100 | 20 | 20 | – | – |
| | AS12A | *Pseudomonas picketii* | – | + | + | + | – | 50 | 300 | 100 | 20 | – | – | – |
| | AS13 | *Bacillus anthracis* | + | + | + | – | – | – | – | – | – | – | – | – |
| | AS13A | *Sporosarcina ureae* | + | + | w | – | – | – | – | – | – | – | – | 20 |
| | AS14 | *Pseudomonas picketii* | w | w | + | + | w | 150 | 300 | 100 | 20 | 20 | 10 | 20 |
| | AS14A | *Bacillus sphaericus* | w | w | + | – | + | – | – | 1 | 20 | 20 | – | 20 |
| IV | AS15 | *Pseudomonas delafieldii* | + | + | + | + | w | 150 | – | 100 | 20 | – | – | 20 |
| | AS15A | *Pseudomonas syringae* | + | + | + | + | – | 150 | 150 | 100 | 20 | 20 | – | – |
| | AS16 | *Serratia marcescens* | + | + | + | + | w | 50 | 300 | 100 | – | 20 | 10 | – |
| | AS16A | *Burkholderia cepacia** | – | + | – | w | – | 150 | – | 100 | 20 | 20 | 10 | – |
| | AS17 | *Citrobacter freundii* | w | w | + | + | w | 50 | 150 | 100 | – | – | – | – |
| | AS17A | *Pseudomonas picketii* | – | + | – | + | – | – | – | – | – | – | – | – |
| | AS17B | *Pseudomonas alcaligenes* | – | w | w | – | – | 150 | 150 | 10 | – | – | 10 | – |
| | AS17C | *Pimelobacter simplex* | + | + | + | w | + | – | – | 10 | – | – | – | 20 |
| | AS18 | *Pseudomonas aeruginosa* | + | + | + | + | – | 150 | 300 | 100 | 20 | 20 | 10 | 20 |
| | AS18A | *Burkholderia cepacia* | + | + | w | + | – | 150 | 300 | 100 | 20 | 20 | 10 | 20 |
| | AS18B | *Pseudomonas pseudoalcaligenes* | – | + | + | + | – | 150 | – | 10 | 20 | – | – | – |
| V | AS76 | *Serratia. marcescens* | + | + | + | + | + | 150 | 150 | 100 | 20 | – | 10 | – |
| | AS76A | *Citrobacter freundii* | w | w | + | + | – | 150 | – | 100 | – | – | – | – |
| | AS77 | *Pseudomonas alcaligenes* | – | + | w | + | – | 150 | 300 | 100 | 20 | 20 | 10 | 20 |
| | AS78 | *Agromyces ramosus* | w | w | + | w | – | 100 | – | 10 | – | – | – | – |
| | AS79 | *Pseudomonas picketii* | + | + | + | + | w | 150 | 300 | 100 | 20 | 20 | 10 | – |
| VI | AS81 | *Bacillus pasteurii* | + | + | + | + | w | – | – | 10 | – | – | – | – |
| VII | AS44 | *Proteus vulgaris* | + | + | + | + | w | 150 | – | 100 | 20 | 20 | – | – |
| | AS44A | *Aeromonas salmonicida* subsp. *salmonicida* | + | + | + | + | + | 200 | 100 | 100 | 20 | 20 | – | – |
| | AS45 | *Aeromonas caviae* | + | + | + | + | + | 150 | – | 100 | – | – | – | – |
| | AS46A | *Pseudomonas succharophila* | + | w | + | + | w | 200 | – | 100 | 20 | – | – | – |
| | AS46B | *Burkholderia cepacia* | + | + | + | + | + | 200 | 100 | 10 | – | – | 10 | – |
| | AS47 | *Pseudomonas pseudoalcaligenes* | + | + | – | w | – | 50 | – | 10 | – | – | – | 20 |
| | AS47A | *Pseudomonas pseudoalcaligenes* | + | + | – | w | – | 50 | – | 100 | – | – | – | – |
| | AS47B | *Aeromonas sobria* | + | + | + | w | + | 200 | – | 100 | – | – | – | – |
| | AS48 | *Kurthia gibsonii* | + | + | – | w | w | – | – | – | – | – | – | 20 |

***** Formerly, *Pseudomonas cepacia*

[1] suc, sucrose; glu, glucose; fru, fructose; prop, propionate; cel, celulose. Strong growth (+); weak (w) and no growth (–);

[2] amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used.

superior limit of the range (++ or +++). Among the strains growing in sucrose, glucose, fructose, and propionate, *ca* 7%, 22%, 13%, and 14%, respectively, are strong SB stained. These strains were found in the sites II, III, IV and VII, from which, four of them having the highest staining, could be potential PHA producers: two *Pseudomonas picketii*, one *Pseudomonas delafieldii*, and one *Burkholderia* (formerly *Pseudomonas*) *cepacia* (Table 3). It is noteworthy that *P. delafieldii* is strong SB stained when growing either on sucrose, glucose, fructose or propionate. The environments IV and VII are related, directly or not, to vinasse and, therefore, they are likely under stress.

In **Group B** (field experiments using sugarcane pieces), from the 42 strains isolated, 35 grew well in sucrose, 39 in glucose, 32 in fructose, and 30 in propionate (Table 2). The highest number of different species was found in samples from sites XI and XII. Also in Group B, 30 strains (71%) were SB positive in the different carbon sources tested and *ca* 30% (12 strains) was in the superior limit of the range. Among the strains growing in sucrose, glucose, fructose, and propionate, *ca* 14%, 20%, 28%, and 20%, respectively, are strong SB stained (Figura 1). Seven of these strains, from field experiments IX, X, XI and XII, could be potential PHA producers: *Pseudomonas picketii* (3 strains) using propionate as the main C source; *Pseudomonas delafieldii* (3 strains) using sucrose as the main C source and glucose, fructose or propionate; and *Burkholderia cepacia* (1 strain) using glucose and fructose (Table 3). Interestingly, from the 10 strains identified in the field experiment XI (sugarcane plantation burned before harvesting), 5 (50%) were strong SB stained. In the sample from not burned plantation, 25% of the isolated strains were strong SB stained.

In both Groups A and B, eleven strains (5 *P. picketii*, 4 *P. delafieldii*, and 2 *Burkholderia cepacia*) presented the strongest Sudan Black staining intensity (+++) in the indicated C source, and simultaneously, showed multiple resistance to antibiotics (Table 3), which may represent an advantage for biotechnological PHB production. The environments III, IV, VI, VII, IX, XI, and XII could be considered as habitats where bacteria are submitted to unbalanced nutritional growth condition, caused by the introduction of vinasse utilized in the fertirrigation and the use of fire in the sugarcane plantation. These environmental conditions could exert a positive selective pressure for potential PHA producers strains. From the 11 strongest SB staining strains, 8 came from these environments (*ca* 73%). Four *P. delafieldii* strains (AS15, AS60, AS61A, and AS64) were highly SB stained in sucrose, a cheaper C source, and also, multiresistant to antibiotics, which may represent a good potential for biotechnological purpose. Production of PHAs by *P. delafieldii* has not yet been reported in the literature although synthesis of PHAs is a common feature of pseudomonads when grown on hydrocarbons, and expression of PHA synthase gene from *P. aeruginosa* in *E. coli* leads to PHA accumulation in the cells growing in LB medium containing fatty acids (21).

**Bacteria strains antibiotics-resistant.** A total number of 35 strains with multiple resistance to antibiotics was isolated from all the environments investigated in **Group A**. Among these strains, *ca* 46% belong to genus *Pseudomonas* (Table 1). The sets of samples collected from the stabilization pond, IV ('*gelose*') and VII (wastewater), show high species variability (16 different species) with multiple resistance to antibiotics. These environments, as pointed out in the previous item, are related to vinasse and are possibly under stress. But from the vinasse collected straight from the fermentors (temperature between 80 and 90°C), only a strain of *Bacillus pasteurii* was isolated, a typical endospore-forming bacterium. The majority of the bacteria strains were resistant to penicillin (90%) and ampicillin (*ca* 82%) and thirty-three strains were simultaneously amp/pen-resistant. The percentage of isolates resistant to virg and sm was *ca* 47% and 42% respectively. The percentage of isolates resistant to km, tc or nal is lower than 40% in each case. From the 22 soil strains related to the large stabilization pond (III, IV, V), *ca* 45% showed multiple resistance to five or more antibiotics.

A total amount of 38 strains with multiple resistance to antibiotics was isolated from all the environments investigated in **Group B**. Among these strains, 34% belong to genus *Pseudomonas* (Table 2). Variable numbers of strains with multiple resistance were isolated either from restricted environments of the stabilization pond or from soil of burned and unburned sugarcane plantations. Nine strains showed multiple resistance to 5-7 antibiotics, and 7 of them originated from samples X (not burned) and XI (burned). The majority of the bacteria strains was ampicillin-resistant (*ca* 97%) and penicillin-resistant (*ca* 95%). Thirty-six strains were simultaneously amp/pen-resistant. Forty-two percent

**Table 2** – Group B strains (types VIII to XII) isolated from the sugar cane agroecosystem: growth in different carbon sources and resistance to antibiotics

| Types | Strains | Species | Growth in Carbon Sources[1] | | | | | Resistance to antibiotics (µg/ml)[2] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | suc | glu | fru | pro | cel | amp | virg | pen | sm | km | tc | nal |
| VIII | AS49 | *Klebsiella pneumoniae subsp. pneumoniae* | + | + | + | + | + | 200 | 300 | 100 | – | 20 | – | – |
| | AS50 | *Pseudomonas stutzeri* | + | + | w | w | – | 200 | – | 100 | 20 | 20 | – | – |
| | AS50A | *Pseudomonas delafieldii* | + | + | + | + | – | 150 | 100 | 100 | – | – | – | – |
| | AS51 | *Klebsiella. pneumoniae subsp. pneumoniae* | + | + | + | + | + | 200 | 300 | 100 | – | – | – | – |
| | AS51A | *Kluyvera cryocrescens* | + | + | w | w | – | 200 | – | 100 | – | – | – | – |
| | AS51B | *Pseudomonas pseudoalcaligenes* | w | + | w | w | w | 200 | 150 | 100 | – | – | – | 20 |
| IX | AS68 | *Escherichia hermannii* | + | + | + | + | – | 150 | – | 100 | – | – | – | – |
| | AS68A | *Bacillus cereus* | + | + | + | + | + | 100 | – | 100 | – | – | – | – |
| | AS69 | *Bacillus sphaericus* | + | + | w | + | w | – | – | – | – | – | – | 20 |
| | AS69A | *Pseudomonas stutzeri* | – | w | w | + | – | 50 | 100 | 100 | 20 | 20 | – | – |
| | AS70 | *Bacillus cereus* | + | + | + | + | + | 150 | – | 100 | – | – | – | – |
| | AS70A | *Escherichia hermannii* | + | + | + | + | w | 100 | – | 100 | – | – | – | – |
| | AS70B | *Pseudomonas picketii* | + | + | + | + | + | 100 | 150 | 100 | 20 | 20 | – | – |
| | AS71 | *Escherichia hermannii* | + | + | + | w | – | 150 | – | 100 | – | – | – | – |
| | AS71A | *Bacillus sphaericus* | + | + | + | + | w | – | – | – | – | – | – | 20 |
| X | AS64 | *Pseudomonas delafieldii* | + | + | + | w | – | 150 | 300 | 100 | – | – | 10 | 20 |
| | AS64A | *Serratia marcescens* | + | + | + | + | + | 150 | 150 | 100 | – | – | 10 | – |
| | AS65 | *Pseudomonas delafieldii* | w | + | + | w | – | 150 | – | 100 | – | – | 10 | 20 |
| | AS66A | *Escherichia coli* | + | + | + | + | w | 100 | – | 100 | – | – | – | – |
| | AS67 | *Bacillus cereus* | + | + | + | + | + | 150 | – | 100 | – | – | – | – |
| | AS67A | *Serratia marcescens* | + | + | + | w | + | 150 | 150 | 100 | 20 | – | 10 | – |
| | AS67B | *Pseudomonas picketii* | + | + | – | + | w | 150 | 300 | 100 | 20 | 20 | – | 20 |
| | AS67C | *Bacillus cereus* | + | + | + | + | + | 150 | – | 100 | – | – | – | – |
| XI | AS72 | *Burkholderia cepacia** | + | + | + | + | – | 150 | 300 | 10 | 20 | – | 10 | 20 |
| | AS72A | *Aeromonas salmonicida subsp. smithia* | + | + | + | + | w | 150 | – | – | – | – | – | 20 |
| | AS72B | *Bacillus larvae* | + | + | + | + | + | 150 | – | 10 | | – | – | |
| | AS72C | *Enterobacter(Erwinia) nimipressuralis* | + | + | + | w | + | 150 | – | 100 | – | – | – | – |
| | AS73 | *Burkholderia cepacia* | + | + | + | + | – | 150 | 300 | 100 | 20 | – | 10 | 20 |
| | AS74 | *Bacillus cereus* | + | + | + | + | + | 150 | – | 100 | – | – | – | – |
| | AS74A | *Enterobacter(Erwinia) nimipressuralis* | + | + | + | w | + | 50 | – | 100 | – | – | – | – |
| | AS74B | *Enterobacter cloacae* | + | + | + | w | + | 50 | – | 10 | – | – | – | – |
| | AS75 | *Pseudomonas picketii* | + | + | w | + | – | 150 | 300 | 100 | 20 | 20 | 10 | 20 |
| | AS75A | *Bacillus alcalophilus* | + | + | + | – | + | – | – | – | – | 20 | – | 20 |
| XII | AS60 | *Pseudomonas delafieldii* | + | + | + | + | + | 150 | – | 100 | 20 | – | – | 20 |
| | AS60A | *Pseudomonas syringae* | w | + | – | – | – | 150 | – | 100 | 20 | – | – | 20 |
| | AS61 | *Escherichia hermannii* | w | w | w | + | – | 150 | 100 | 100 | – | – | – | – |
| | AS61A | *Pseudomonas delafieldii* | + | + | + | + | + | 150 | – | 100 | – | – | – | 20 |
| | AS61B | *Arthrobacter amescens* | + | + | + | + | + | – | – | – | – | – | – | – |
| | AS62 | *Klebsiella pneumoniae subsp. pneumoniae* | + | + | + | + | + | 150 | 300 | 100 | – | – | – | – |
| | AS62A | *Bacillus cereus* | + | + | + | + | + | 50 | – | 100 | – | – | – | – |
| | AS62B | *Bacillus coagulans* | w | + | + | + | w | – | – | – | – | – | – | 20 |
| | AS63 | *Pseudomonas aeruginosa* | 0 | w | w | + | 0 | 150 | 300 | 100 | 20 | 20 | 10 | 20 |

* Formely *Pseudomonas cepacia*

[1] suc, sucrose; glu, glucose; fru, fructose; prop, propionate; cel, celulose. Strong growth (+); weak (w) and no growth (–);

[2] amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used.

**Table 3** – Bacteria strains isolated from the sugarcane agroecosystem, with their respective potential for producing polyhydroxyalcanoates (Sudan Black stained) in the carbon sources tested, and their resistance to antibiotics.

| Strains Group A* | Source | Species | Carbon Source | | | | Antibiotics |
|---|---|---|---|---|---|---|---|
| | | | suc | glu | fru | pro | |
| AS10 | II | *P. picketii* | – | +++ | – | ++ | Amp/Tc/Sm/Km/Nal/Virg/Pen |
| AS12 | III | *P. picketii* | – | ++ | ++ | – | Amp/Sm/Km/Pen |
| AS12A | III | *P. picketii* | – | ++ | – | +++ | Amp/Sm/Virg/Pen |
| AS15 | IV | *P. delafieldii* | +++ | +++ | +++ | +++ | Amp/Sm/Nal/Pen |
| AS15A | IV | *P. syringae* | – | – | ++ | – | Amp/Sm/Km/Virg/Pen |
| AS16A | IV | *B. cepacia** | – | ++ | – | – | Amp/Tc/Sm/Km/Virg/Pen |
| AS17C | IV | *P. simplex* | ++ | – | – | – | Nal/Pen |
| AS18A | IV | *B. cepacia** | – | +++ | – | – | Amp/Tc/Sm/Km/Nal/Pen |
| AS44A | VII | *A. salmonicida* | – | ++ | – | – | Amp/Sm/Km/Virg/Pen |
| AS46A | VII | *P. saccharophila* | – | – | ++ | – | Amp/Sm/Pen |
| Group B* | | | Suc | Glu | Fru | Pro | |
| AS50A | VIII | *P. delafieldii* | – | ++ | ++ | ++ | Amp/Nal/Virg/Pen |
| AS60 | XII | *P. delafieldii* | +++ | +++ | +++ | +++ | Amp/Sm/Nal/Pen |
| AS61A | XII | *P. delafieldii* | +++ | ++ | ++ | +++ | Amp/Nal/Pen |
| AS64 | X | *P. delafieldii* | +++ | +++ | +++ | – | Amp/Tc/Sm/Nal/Pen |
| AS65 | X | *P. delafieldii* | – | ++ | ++ | – | Amp/Tc/Nal/Virg/Pen |
| AS67B | X | *P. picketii* | – | – | – | +++ | Amp/Sm/Km/Nal/Virg/Pen |
| AS70B | IX | *P. picketii* | – | ++ | – | +++ | Amp/Sm/Km/Virg/Pen |
| AS72 | XI | *B. cepacia** | ++ | +++ | +++ | – | Amp/Tc/Sm/Nal/Virg/Pen |
| AS73 | XI | *B. cepacia* | – | – | ++ | – | Amp/Tc/Sm/Nal/Virg/Pen |
| AS74A | XI | *E. (Erwinia) nimipressuralis* | – | – | ++ | – | Amp/Pen |
| AS74B | XI | *E. cloacae* | – | – | ++ | – | Amp/Pen |
| AS75 | XI | *P. picketii* | ++ | ++ | – | +++ | Amp/Tc/Sm/Km/Nal/Virg/Pen |

++/+++/ = Sudan Black staining, in the carbon sources tested: sucrose, Suc; glucose, Glu; fructose, Fru; sodium propionate, Pro.
*Formerly *Pseudomonas cepacia*.
Amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used

of the isolates were resistant to virg and/or nal. The percentage of isolates resistant to sm, km or tc is lower than 30% in each case.

Among the 73 isolates with multiple resistance to antibiotics we emphasize the multiple resistance to seven antibiotics of *P. picketii* (AS14), *P. aeruginosa* (AS18), *P. alcaligenes* (AS77) and *B. cepacia* (AS18A) in Group A and *P. picketii* (AS75) and *P. aeruginosa* (AS63) in Group B. More than 95% of the isolates are resistant to amp or pen in the concentration range tested and about 48% are resistant to virg. The unknown proportions and specificity of antibiotics used in the fermentors of the alcohol distillery (sometimes they use a mixture called by them of 'cocktail'), do not allow in the present study to establish any plausible relation between the use of antibiotics in the distillery and the high numbers of antibiotics-resistant strains we isolated. Despite the personal information from technicians of the distillery chemical laboratory on greater use of penicillin, the possibility of some sort of selective pressure of this antibiotic on the microbiota environments here studied, deserves a deeper investigation; otherwise, several strains with multiple resistance to antibiotics, were also isolated from the soil under unburned sugarcane plantation (Table 2, site X, a place in a similar condition to a natural vegetation), and from all the other sites from groups A and B. Although there is a general agreement that the pool of resistance genes in the environment is amplified by the use of antimicrobial agents (12, see *Science*, 264: 359-393, 1994), the mechanisms of gene transfer between bacteria of different origins can spread the resistance genes

efficiently in the microbial world, even in the absence of antimicrobial agents (10).

**Microbial diversity and environmental bioindicators.** Eighty-two bacteria strains were isolated from the sugarcane agroecosystem. In microbial diversity terms, 16 different genera and 35 different species of bacteria were isolated from the two groups of samples. Group A samples presented 12 different genera and 24 different species and Group B presented 10 genera and 21 species. The microbial diversity showed to be greater in samples related to the wastewater from the stabilization ponds (IV, 10 species; VII, 8 species; XII, 8 species) and the soil sample from the sugarcane plantation burned before harvesting (XI, 8 species). Odum's (18) classical observation that communities with low energy cost for maintaining the entropy (high respiration: biomass ratio), divert their energy supply into diversity, may be happening to the microbiota of the environments here investigated. A high respiration: biomass ratio observed in unproductive soil irrigated with vinasse, in the tableland soil of the Usina Japungu (15) gives support to this theory.

Discussion about biodiversity and environmental stress is controversial. Atlas (1984) pointed out diversity changes in response to environmental stress, showing both tendencies: (a) increase in diversity by selective toxicity, eliminating dominant organism; (b) diversity decreases by elimination of many species due to toxicity or increase in particular populations. Torsvik *et al.* (30) observed diversity decrease in perturbed soil due to agriculture, as compared to undisturbed environments. A molecular analysis of microbial diversity in Amazonia soils, based on PCR amplification of small-subunit rRNA, showed microbial population shifts related to deforestation in the Amazonian forest, with predominance of *Bacillus* and high G+C gram-positive-like sequences in pasture and predominance of *Clostridium* and unclassified bacteria in the forest (4). *Bacillus* seems to be a 'natural indicator' of inhospitable environmental conditions and their endospore forming characteristic certainly explains their occurrence in these situations.

Among the isolates in Group A, sporulating bacteria are present only in site III, soil amended with vinasse. That is the case for the highly virulent animal pathogen *B. anthracis*, with high tolerance range to temperature (-5 to 75 °C), to pH acidity (from 2 to 8) and salt (up to 25% NaCl) (20). It is noteworthy to remind that vinasse causes salinization of soils. In Group B, sporulating bacteria were found in all samples, except for VIII. Nevertheless, the highest percentage of sporulating strains was found in the borders of the large stabilization pond, in a place pemanently covered by wastewater (site IX). Bacteria strains with special ability to degrade recalcitrant compound, like *Burkholderia cepacia* (16), were found in the environments under greater selective pressure (sites IV, VII and XI). These altered conditions, of anthropic origin, may have contributed to an increase in the bacteria diversity and may also explain the greater occurrence of *Bacillus*, an endosporing bacterium. Atlas (3) conclusions are still certainly valid, as he said that though diversity measurements are a reflection of the dynamic status of an ecosystem, they do not show a cause and effect relationship between a particular level of stress and a particular species composition of the community.

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## RESUMO

### Bactérias isoladas de um agrossistema de cana-de-açúcar: produção potencial de polihidroxialcanoatos e resistência a antibióticos

Neste trabalho, um agrosistema de cana de açúcar em tabuleiro litorâneo do nordeste do Brasil, foi rastreado para obtenção de bactérias capazes de sintetizarem polihidroxialcanoatos (PHA) usando sacarose como principal fonte de carbono. O potencial para sintetizar PHA foi testado

qualitativamente por coloração, com Sudan Black, de colônias crescendo em diferentes fontes de carbono: sacarose, glicose, frutose, propionato e celulose. Num sistema de manejo típico do cultivo da cana-de-açúcar, a plantação é queimada antes de cada colheita e a vinhaça, subproduto da produção de álcool, é utilizada num sistema de fertirrigação causando, provavelmente, pressões seletivas sobre a microbiota dos ambientes naturais. Oitenta e duas linhagens de bactérias, pertencentes a 16 diferentes gêneros e 35 diferentes espécies foram isoladas. Os dados mostraram que 11 linhagens (13%), 9 das quais pertencentes ao gênero *Pseudomonas*, apresentaram intensa coloração por Sudan Black em algumas das fontes de carbono testadas e mostraram, simultaneamente, múltipla resistência a antibióticos. Resistência a antibióticos, é uma característica vantajosa à produção biotecnológica de PHAs. O número total de isolados com múltipla resistência a antibióticos foi 73, dos quais, 38% pertencentes ao gênero *Pseudomonas*. Entre os isolados, 86% e 43% cresceram na presença de 10-100 U/ml de penicilina e 100-300 µg/ml de virginiamicina. Estes antibióticos são utilizados na Usina em estudo. Os resultados sugerem que alguns ambientes do agrossistema podem ser considerados como habitats onde as bactérias estão submetidas a condições nutricionais desbalanceadas, contibuindo para estabelecimento de linhagens com habilidade potencial de produzir PHAs e, também, para o aumento da diversidade microbiana.

**Palavras-chave:** poli-β-hidroxialcanoato, PHA, resistência de bactérias a antibióticos, agrossistema da cana-de-açúcar, vinhaça.

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