Research Article

Defluorination of Sodium Fluoroacetate by Bacteria from Soil and Plants in Brazil

Expedito K. A. Camboim,1 Michelle Z. Tadra-Sfeir,2 Emanuel M. de Souza,2 Fabio de O. Pedrosa,2 Paulo P. Andrade,3 Chris S. McSweeney,4 Franklin Riet-Correa,1 and Marcia A. Melo1

1 Unidade Acadêmica de Medicina Veterinária, Universidade Federal de Campina Grande, Avenida Universitária, s/n, Bairro Sta. Cecília, Patos, PB, CEP: 58700-970, Brazil
2 Laboratório de Fixação Biológica de Nitrogênio, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, PR, CEP: 81531-980, Brazil
3 Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, CEP: 50670-901, Brazil
4 CSIRO Livestock Industries, Queensland Bioscience Precinct, Carmody Road, 306, St Lucia, 4067, QLD, Australia

Correspondence should be addressed to Marcia A. Melo, marcia.melo@pq.cnpq.br

Received 1 November 2011; Accepted 11 December 2011

The aim of this work was to isolate and identify bacteria able to degrade sodium fluoroacetate from soil and plant samples collected in areas where the fluoroacetate-containing plants Mascagnia rigida and Palicourea aenofusca are found. The samples were cultivated in mineral medium added with 20 mmol L\(^{-1}\) sodium fluoroacetate. Seven isolates were identified by 16S rRNA gene sequencing as Paenibacillus sp. (ECPB01), Burkholderia sp. (ECPB02), Cupriavidus sp. (ECPB03), Staphylococcus sp. (ECPB04), Ancylobacter sp. (ECPB05), Ralstonia sp. (ECPB06), and Stenotrophomonas sp. (ECPB07). All seven isolates degraded sodium-fluoroacetate-containing in the medium, reaching defluorination rate of fluoride ion of 20 mmol L\(^{-1}\). Six of them are reported for the first time as able to degrade sodium fluoroacetate (SF). In the future, some of these microorganisms can be used to establish in the rumen an engineered bacterial population able to degrade sodium fluoroacetate and protect ruminants from the poisoning by this compound.

1. Introduction

In Brazil, thirteen species of plants causing sudden death associated with physical effort are responsible for nearly 500,000 cattle deaths each year: Palicourea maracgravii, P. aenofofsca, P. juruana, P. grandisflora, Pseudocalymma elegans, Arrabidaea bilabiata, A. japurensis, Mascagnia rigida, M. elegans, M. pubiflora, M. aff. rigida, M. exotropia, and M. sepium [1, 2]. Sodium fluoroacetate was identified as the active principle of P. maracgravii [3] and A. bilabiata [4] and is probably present in other plants of these genera. It disrupts the tricarboxylic acid (TCA) cycle, being first converted to fluorocitrate which in turn inhibits the enzymes aconitase and succinate dehydrogenase resulting in citrate accumulation in tissues and plasma and ultimately causing energy deprivation and death of the animal [5].

The use of sodium fluoroacetate is prohibited in Brazil, occurring exclusively as a natural product in plants. In Australia the compound is used in impregnated baits for the control of rabbit, fox, dingo, and other mammal populations; however, when introduced in the environment it can select fluoroacetate-degrading microorganisms [6].

Microbial degradation of sodium fluoroacetate is catalyzed by a haloacetate halidohydrolase, which is able to cleave the strong carbon-fluorine bond [7]. Twenty-four fluoroacetate-degrading microorganisms were isolated from soil in Central Australia, from seven bacterial genera (Acinetobacter, Arthrobacter, Acreobacterium, Bacillus, Pseudomonas, Weeksella, and Streptomyces) and four genera of fungi (Aspergillus, Fusarium, Cryptococcus, and Penicillium) [8].
The possibility to prevent fluoroacetate poisoning in ruminants by the ruminal inoculation of genetically modified bacteria, containing a gene encoding fluoroacetate dehalogenase has been investigated [9, 10]. A thorough search among samples taken from environment, such as soil, leaf, and digestive tract contents of herbivores that come in contact with fluoroacetate, would be important to ascertain the diversity and prevalence of microorganisms that can degrade these toxins. In the future, genes encoding enzymes from such microorganisms may be used to engineer new bacterial strains able to colonize the rumen and degrade fluoroacetate from toxic plants, thereby protecting the animal from poisoning by this compound.

This study aimed to isolate and identify bacteria able to degrade sodium fluoroacetate from soil and plant samples collected in the State of Paraíba, Brazil.

2. Materials and Methods

2.1. Samples Collection. Plant and soil samples were collected in the State of Paraíba, Brazil, in areas where Mascagnia rigida and Palicourea aenofusca were present. Soil samples were collected at the plant base, 1 to 8 cm depth. Leaves and flowers were also collected. All samples were placed in individual 50 mL Falcon type tubes and sent to the laboratory under refrigerated conditions for the immediate cultivation of associated bacteria.

2.2. Bacterial Isolation. Bacterial isolation was performed in 50 mL Falcon type tubes in mineral medium (Brunner) added with vitamins (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium457.pdf) and 20 mmol L⁻¹ sodium fluoroacetate (SF) (Sigma-Fluka) as single-carbon source. This medium will be here designated as Brunner medium. Samples were incubated at 28°C in an orbital shaker. After 48 hours, one mL of the first growth was transferred to test tubes containing nine mL of Brunner medium and incubated under the same conditions described above.

The SF defluorination was measured with an F⁻ selective electrode (Thermo Electron Corporation) in 24-well plates containing 500 µL of culture and 500 µL of Total Ionic Strenght Adjustment Buffer-TISAB (diaminocyclohexane, sodium chloride, and glacial acetic acid, pH 5.5). The fluoride ion released from the microbial degradation of the sodium fluoroacetate was expressed in millimoles (mmol), the defluorination rate of 20 mmol L⁻¹ corresponding to the release of 20 mmol L⁻¹ F⁻.

Samples showing SF defluorination were cultivated in serial dilutions from 10⁻¹ to 10⁻⁹. To obtain pure colonies the highest dilution that presented SF defluorination was plated on Brunner agar (Brunner medium added with agar 1%) and incubated at 28°C for 72 hours. Subsequently, each colony was used to inoculate three test tubes containing 9 mL of Brunner medium, which were monitored for SF defluorination. Pseudomonas fluorescens (strain DSM 8341) was used as positive control for fluoroacetate dehalogenase activity. Nine mL of Brunner medium without bacterial inoculum were incubated under the same conditions to evaluate the sodium fluoroacetate degradation background.

The standard sample (strain DSM 8341) and the bacteria isolated from soil and plants were grown into Brunner medium with increased concentrations of SF (20 mmol L⁻¹, 40 mmol L⁻¹, 60 mmol L⁻¹, and 80 mmol L⁻¹ to 200 mmol L⁻¹) to evaluate the highest defluorination rate. Additionally, to evaluate defluorination in the presence of other carbon sources, strain DSM 8341 and the bacteria isolated from soil and plants were also grown in Brunner medium enriched with yeast extract and glucose, on the following conditions: (1) Brunner medium alone; (2) medium supplemented with yeast extract 0.01% and glucose 2%; (3) medium with yeast extract 0.01%; (4) medium with glucose 2%.

2.3. 16S rRNA Gene Sequence Identification. Bacteria displaying defluorination activity were identified by polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. DNA extraction was performed with Brazorase (LGC Biotechnology) according to the manufacturer’s specifications. 16S rRNA gene was amplified in buffer containing 0.5 µM of 27f and 1492r universal primers [11], 2U of Taq DNA polymerase, 0.2 mM of dNTP and 100 ng of DNA and ultrapure water to a final volume of 20 µL. In the negative control, the DNA volume was substituted by ultrapure water. The amplified products were applied into agarose gel 1% and submitted to electrophoresis. DNA was stained with ethidium bromide and bands visualized with an imaging system (UVP-Bioimaging Systems).

The sequencing reaction was performed with BigDye kit according to manufacturer’s recommendations (Applied Biosystems) and the product sequenced in the Genetic Analyzer 3500 XL sequencer (Applied Biosystems).

2.4. Sequence Analysis and Phylogram. 16S rRNA gene sequences were assembled with the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php). DNA sequences were analyzed by Basic Local Alignment Search Tool (BLAST) available on the website of the National Center for Biotechnology Information (NCBI—http://www.ncbi.nlm.nih.gov/BLAST). Species identification was based on maximum score, identity, and coverage values. The Greengenes database and workbench were used to corroborate species identification (http://greengenes.lbl.gov/). The phylogram tree was generated with MEGA 5 software using the default parameters (http://www.megasoftware.net/mega.php) [12, 13].

3. Results

Following the analysis of all 16S rRNA genes, seven isolates were identified as Paenibacillus sp. (ECPB01), Burkholderia sp. (ECBP02), Capriavidus sp. (ECBP03), Staphylococcus sp. (ECBP04), Ancylobacter sp. (ECBP05), Ralstonia sp. (ECBP06), and Stenotrophomonas sp. (ECBP07) (Table 1). Capriavidus sp. and Ralstonia sp. were isolated from both soil and plants samples, whereas Paenibacillus sp., Burkholderia sp. and Ancylobacter sp. were found from soil samples and, Staphylococcus sp. and Stenotrophomonas sp. were isolated from plant samples.
Table 1: Results of BLAST for the 16S rRNA sequences obtained from bacteria isolated from soil and plants samples.

| Isolate code no. | Most similar species* | Isolated from | 16S rRNA sequence length | Coverage (%) | Max score | E-value | Identity (%) |
|------------------|-----------------------|---------------|---------------------------|--------------|-----------|---------|--------------|
| ECPB01           | Paenibacillus sp.     | Soil          | 1425                      | 99           | 2619      | 0.0     | 99           |
| ECPB02           | Burkholderia sp.      | Soil          | 1398                      | 99           | 2553      | 0.0     | 99           |
| ECPB03           | Cupriavidus sp.       | Soil and plant| 1398                      | 99           | 2540      | 0.0     | 99           |
| ECPB04           | Staphylococcus sp.    | Plant         | 1402                      | 100          | 2569      | 0.0     | 99           |
| ECPB05           | Ancylobacter sp.      | Soil          | 1368                      | 99           | 2435      | 0.0     | 99           |
| ECPB06           | Ralstonia sp.         | Soil and plant| 1407                      | 99           | 2551      | 0.0     | 99           |
| ECPB07           | Stenotrophomonas sp.  | Plant         | 1417                      | 99           | 2606      | 0.0     | 99           |

*Genera were identified based on maximum score, identity, and coverage.

In order to further support the attribution of genera based on BLAST maximum scores, identity and coverage values, a phylogram tree was built from the sequences previously trimmed to have the same length and indicated that five isolates were phylogenetically closely related (Figure 1): Ancylobacter sp., three Burkholderiaceae (Cupriavidus sp., Ralstonia sp., Burkholderia sp.) and Stenotrophomonas sp. Two other species, Paenibacillus sp. and Staphylococcus sp. formed a heterogeneous group.

All bacterial isolates displayed SF degradation activity, reaching a 20 mmol L\(^{-1}\) level of released fluoride ion 32 hours after incubation in Brunner medium containing 20 mmol L\(^{-1}\) of SF (Figure 2). The same result was observed with the Pseudomonas fluorescens control strain (DSM 8341). There was no release of fluoride ions when the Brunner medium was incubated in the absence of bacteria.

When samples were grown in different concentrations of SF the maximum rate of defluorination was 140 mmol L\(^{-1}\) F\(^{-}\) in 140 mmol L\(^{-1}\) of concentration. At higher SF concentrations (160 mmol L\(^{-1}\), 180 mmol L\(^{-1}\) and 200 mmol L\(^{-1}\)) the levels of degradation were still 140 mmol L\(^{-1}\). When the isolates were cultured into Brunner medium added with others carbon sources (yeast extract 0.01% and glucose 2%), an intense bacterial growth was observed after 24 hours, but SF degradation achieved only 20 mmol L\(^{-1}\) F\(^{-}\) between 48 to 64 hours with 20 mmol L\(^{-1}\) SF initial concentration.
4. Discussion

_Ancylobacter_ sp., the Burkholderiaceae (Cupriavidus sp., Ralstonia sp. and Burkholderia sp.) and Stenotrophomonas sp. belong to the Alpha, Beta and Gamma-proteobacteria classes, respectively. The two other species, Paenibacillus sp. and Staphylococcus sp. are from Paenibacillaceae and Staphylococcaceae families, respectively, forming a heterogeneous group; the genus assignment based on BLAST values was coherent with the phylogram, which is in agreement with the phylogeny of these bacteria. The existence of a similar haloacid dehalogenase activity among distantly related microorganisms isolated over a restricted area points to a common and effective selective pressure acting in a broad set of soil microorganisms.

Annotated genomic sequences are available at the NCBI genome database for the species _Burkholderia_ sp., _Cupriavidus taiwanensis_, _Staphylococcus saprophyticus_, _Paenibacillus_ sp., _Ralstonia_ sp., and _Stenotrophomonas maltophilia_. The _Ancylobacter dichloromethanicus_ genome has not yet been sequenced, but there is 16S rRNA sequence for this bacterium, _Ancylobacter dichloromethanicus_, belonging to any of the six species described here: _Moraxella_ sp., _Acinetobacter_ sp., _Arthrobacter_ sp., _Bacillus_ sp., _Pseudomonas_ sp., _Weeksella_ sp. _Staphylococcus_ sp., and _Ancylobacter_ sp. the dehalogenases were annotated as haloacid dehalogenase hydrolase domain-containing protein [15, 16], haloacid dehalogenase-like hydrolase [17, 18], 2-haloalkanoic acid dehalogenase [17, 18], haloacetate dehalogenase [18, 19], and dichloromethane dehalogenase [20]. However, when the seven isolated were grown in the presence of SF the substrate was defluorinated. This finding may be explained by the unspecificity of the dehalogenases, which can use as substrates other compounds structurally similar to SF, which is known as cross-adaptation [21]. Liu and colleagues [22] verified that fluoroacetate dehalogenase degrades other halo- genated compounds such as chloroacetate, bromoacetate, iodoacetate, and dichloroacetate. Similar results were obtained by Donnelly and Murphy [23] that found fluoroacetate dehalogenase activity catalyzing chloroacetate, bromoacetate, and ethyl fluoroacetate. Also sodium fluoroacetate can be defluorinated by L-2 haloacid dehalogenase [24]. Another possibility is the lateral transfer of fluoroacetate dehalogenase genes.

Although a couple of other environmental bacteria such as _Moraxella_ sp. [25], _Acinetobacter_ sp., _Arthrobacter_ sp., _Bacillus_ sp., _Pseudomonas_ sp., _Weeksella_ sp., and _Streptomyces_ sp. [8] also present fluoroacetate dehalogenase activity, there are no previous reports on such an activity from microorganisms belonging to any of the six species described here: _Cupriavidus_ sp., _Staphylococcus_ sp., _Paenibacillus_ sp., _Ralstonia_ sp., _Stenotrophomonas_ sp., and _Ancylobacter_ sp., since genes for haloacid dehalogenases were annotated in the genomes of the first five species. It is therefore reasonable to assume that all seven isolates have the fluoroacetate genes in their chromosomes and not in plasmids, which further support the existence of a strong, durable and common environmental selective pressure over these organisms.

In all isolates defluorination occurred up to 32 hours of cultivation, with the release of 20 mmol L−1 fluoride ion in the presence of 20 mmol L−1 sodium fluoroacetate. These results were similar to those reported by Davis and colleagues [26] with _Burkholderia_ sp., which also degraded SF in 32 hours, releasing 20 mmol L−1 F− with 20 mmol L−1 of SF. Growing the strains in different concentrations of SF indicated that the highest degradation rate was 140 mmol L−1 F− with 140 mmol L−1 of substrate. For higher fluoride concentrations the defluorination rate did not increase, which may be due to exhaustion of other nutrients.

When the bacteria were cultivated in Brunner medium added with yeast extract and glucose, the dehalogenase activity was delayed, probably due to the availability of the others energy sources and to the difficult cleavage of the strong carbon-fluorine bond in fluoroacetate [7]. In conclusion, seven fluoroacetate degrading bacteria were isolated from both soil and plants, six of which were not previously reported as able to degrade SF. The possibility to use some of these bacteria to establish in the rumen an engineered bacterial population able to degrade fluoroacetate and protect ruminants from the poisoning by this compound should be explored in the future.

Acknowledgment

This work was supported by the INCT for the control of plant poisonings: Grant no. 573534/2008-0.

References

[1] C. H. Tokarnia, P. V. Paixoto, and J. Dobereiner, “Toxic plants affecting heart function of cattle in Brazil,” _Pesquisa Veterinária Brasileira_, vol. 10, pp. 1–10, 1990.

[2] S.V. Schons, T. L. Mello, F. Riet-Correa, and A. L. Schild, “Poisoning by amorimia (Mascagnia) sepium in sheep in Northern Brazil,” _Toxicol_, vol. 57, pp. 781–786, 2011.

[3] M. M. Oliveira, “Chromatographic isolation of monofluoroacetic acid from Palicourea marginata St. Hila,” _Experientia_, vol. 19, no. 11, pp. 586–587, 1963.

[4] H. C. Krebs, W. Kemmerling, and G. Habermehl, “Qualitative and quantitative determination of fluoroacetic acid in _Arrabidea bilabiata_ and _Palicourea marginata_ by 19F-NMR spectroscopy,” _Toxicol_, vol. 32, no. 8, pp. 909–913, 1994.

[5] P. A. Mayes, “O ciclo do ácido cítrico: o catabolismo da acetil-coA,” in _Harper: Bioquimica_, K. R. Murray et al., Ed., pp. 182–189, Athenaeu, São Paulo, Brazil, 9th edition, 2002.

[6] L. E. Twigg and D. R. King, “The impact of fluoroacetate-bearing vegetation on native Australian fauna: a review,” _Oikos_, vol. 61, no. 3, pp. 412–430, 1991.

[7] S. Fetzner and F. Lingens, “Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications,” _Microbiological Reviews_, vol. 58, no. 4, pp. 641–685, 1994.

[8] L. E. Twigg and L. V. Socha, “Defluorination of sodium monofluoroacetate by soil microorganisms from central Australia,” _Soil Biology and Biochemistry_, vol. 33, no. 2, pp. 227–234, 2001.

[9] K. Gregg, C. L. Cooper, D. J. Schafer et al., “Detoxification of the plant toxin fluoroacetate by a genetically modified rumen bacterium,” _Nature_, vol. 12, no. 13, pp. 1361–1365, 1994.

[10] K. Gregg, B. Hamdorf, K. Henderson, J. Kopecky, and C. Wong, “Genetically modified ruminal bacteria protect sheep..."
from fluoroacetate poisoning,” *Applied and Environmental Microbiology*, vol. 64, no. 9, pp. 3496–3498, 1998.

[11] W. G. Weisburg, S. M. Barns, D. A. Pelletier, and D. J. Lane, “16S ribosomal DNA amplification for phylogenetic study,” *Journal of Bacteriology*, vol. 173, no. 2, pp. 697–703, 1991.

[12] J. Felsenstein, “Confidence limits on phylogenies: an approach using the bootstrap,” *Evolution*, vol. 39, pp. 783–791, 1985.

[13] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, “MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods,” *Molecular Biology and Evolution*, vol. 28, no. 10, pp. 2731–2739, 2011.

[14] T. Kurihara, T. Yamauchi, S. Ichiyama, H. Takahata, and N. Esaki, “Purification, characterization, and gene cloning of a novel fluoroacetate dehalogenase from *Burkholderia* sp. FA1,” *Journal of Molecular Catalysis B*, vol. 23, pp. 347–355, 2003.

[15] S. Lucas, A. Copeland, A. Lapidus et al., “*Paenibacillus* sp. Y412MC10, complete genome,” 2011, http://www.ncbi.nlm.nih.gov/.

[16] S. Lucas, A. Copeland, A. Lapidus et al., “*Stenotrophomonas maltophilia* R551-3, complete genome,” 2011, http://www.ncbi.nlm.nih.gov/.

[17] M. Kuroda, A. Yamashita, H. Hirakawa et al., “Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 37, pp. 13272–13277, 2005.

[18] C. Amadou, G. Pascal, S. Mangenet et al., “Genome sequence of the beta-rhizobium *Capriavidus taiwanensis* and comparative genomics of rhizobia,” *Genome Research*, vol. 18, pp. 1472–1483, 2008.

[19] D. Ward, A. Earl, M. Feldgarden et al., “The genome sequence of *Ralstonia* sp. strain 5_7_47FAA,” 2011, http://www.ncbi.nlm.nih.gov/.

[20] J. E. Firsova, N. V. Doronina, E. Lang, C. Spröer, S. Vuilleumier, and Y. A. Trotsenko, “Ancylobacter dichloromethanicus sp. nov.—a new aerobic facultatively methylotrophic bacterium utilizing dichloromethane,” *Systematic and Applied Microbiology*, vol. 32, no. 4, pp. 227–232, 2009.

[21] I. S. Melo and J. L. Azevedo, “Como isolar microrganismos degradadores de moléculas xenobióticas,” in *Microbiologia Ambiental*, I. S. Melo and J. L. Azevedo, Eds., pp. 167–183, EMBRAPA-CNPMA, Jaguariuna, Brazil, 1997.

[22] J. Q. Liu, T. Kurihara, S. Ichiyama et al., “Reaction mechanism of fluoroacetate dehalogenase from *Moraxella* sp. B,” *Journal of Biological Chemistry*, vol. 273, no. 47, pp. 30897–30902, 1998.

[23] C. Donnelly and C.D. Murphy, “Purification and properties of fluoroacetate dehalogenase from *Pseudomonas fluorescens* DSM 8341,” *Biotechnology Letters*, vol. 31, pp. 245–250, 2009.

[24] W. Y. Chan, M. Wong, J. Guthrie et al., “Sequence- and activity-based screening of microbial genomes for novel dehalogenases,” *Microbial Biotechnology*, vol. 3, no. 1, pp. 107–120, 2010.

[25] H. Kawasaki, K. Tsuda, I. Matsushita, and K. Tonomura, “Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella* sp. strain B,” *Journal of General Microbiology*, vol. 138, no. 7, pp. 1317–1323, 1992.

[26] C. K. Davis, S. E. Denman, L. I. Sly, and C. S. Mcsweeney, “Development of a colorimetric colony-screening assay for detection of defluorination by micro-organisms,” *Letters in Applied Microbiology*, vol. 53, no. 4, pp. 417–423, 2011.