Short Communication

Novel linear megaplasmid from *Brevibacterium* sp. isolated from extreme environment

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Brevibacterium sp. Ap13, isolated from flamingo's feces in Laguna Aparejos, a high-altitude lake located at approximately 4,200 m in the northwest of Argentina was previously found to be resistant to multiple antibiotics, and was therefore screened for plasmids that may be implicated in antibiotic resistance. *Brevibacterium* sp. Ap13 was found to contain two plasmids of approximately 87 and 436 kb, designated pAP13 and pAP13c, respectively. Only pAP13 was stably maintained and was extensively characterized by pulsed-field gel electrophoresis to reveal that this plasmid is linear and likely has covalently linked terminal proteins associated with its 5′ ends. This is the first report of a linear plasmid in the genus *Brevibacterium* and may provide a new tool for genetic manipulation of this commercially important genus.

Keywords: Linear plasmid / Terminal protein / *Brevibacterium* / Argentinean high-altitude lakes

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Introduction

Brevibacterium, the sole genus within the family *Brevibacteriaceae*, belongs to a group of loosely related coryneform bacteria within the order Actinomycetales. *Brevibacterium* species are pleomorphic, Gram-positive with a high G+C content (greater than 60%), many of them tolerating high salt concentrations [1]. They are nonmotile and nonspore-forming. So far there are more than 40 different species of *Brevibacterium* described (http://www.dsmz.de/), isolated from various sources, such as poultry, soil and other environmental samples, human skin and dairy products [2, 3]. The genus *Brevibacterium* includes species of environmental, biotechnological, and industrial interest, as well as species of clinical significance [4, 5]. The orange-pigmented *Brevibacterium linens* (the type species) has an important role in the dairy industry as a major component of the microbiota of surface-ripened cheeses, including Limburger, Münster, Roquefort, Brick, and Gruyère [5, 6]; it notably contributes to flavor and color of these cheeses. The production of proteolytic enzymes, carotenoids, and flavor compounds by *B. linens* has been well studied [5, 7, 8].

Descriptions of *Brevibacterium* plasmids exist, however, exclusively of circular ones and with rather limited characterization. Sizes of such plasmids vary between 4.3 and 70 kb [2]. They have been found in strains of *Brevibacterium lactofermentum*, such as pBL70, pBL700 [9], and the cryptic plasmid pBL1 [10], and in *B. linens*, such as pBL100 [9], the cryptic element pBL33 [11] as well as pRBL1 [12], pBLA8 [13], and pLIM [2], for which sudies were performed in some detail. However, up to the present, no linear extrachromosomal DNA elements have been reported for *Brevibacterium*.

Linear plasmids of Gram-positive bacteria were originally described to occur in *Streptomyces rochei* [14]. They have since been found in a number of *Streptomyces* spp., several Mycobacteria and rhodococci, *Clavibacter michiganensis* (a plant pathogen), *Planobispora rosea*, *Arthrobacter nitroguajacolicus* Rü61a, and a *Terrabacter* sp. [15–18].

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These linear replicons belong to a class of genetic elements called invertrons, which are characterized by terminal proteins covalently attached to each DNA 5′ end and terminal inverted repeats [19]. Linear plasmids are known to confer several valuable qualities to its host, such as the production of secondary metabolites [20], heavy metal resistances [21], catabolic traits [16], or hydrogen autotrophy [22].

Characterization of *Brevibacterium* sp. Ap13, a strain previously isolated from Argentinean high-altitude lakes [23], unexpectedly for such environment exhibited multiple resistance to antibiotics. To answer the question if this characteristic is plasmid-encoded, we analyzed *Brevibacterium* sp. Ap13 by means of pulsed-field gel electrophoresis (PFGE).

Material and methods

Strain cultivation was done in Luria-Bertani (LB) medium [24] on a rotary shaker (Innova®44, New Brunswick Scientific GmbH, Nürtingen, Germany) at 160 rpm or on solid LB agar at 30 °C. Agarose gel electrophoresis was carried out as described in Sambrook *et al.* [24]. GeneRuler™ 1 kb DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany) was used as size standard.

Cells were grown in LB medium to an OD_{600} of 2–3. After washing, bacterial cell pellets were used for preparation of cell plugs for PFGE following the procedure of Ravel *et al.* [21]. PFGE was carried out in an electric field apparatus (CHEF DR-III; Bio-Rad, Melville, NY) using 1% (w/v) pulsed-field certified agarose (Bio-Rad, Melville, NY). Electrophoresis was performed at pulse times of 50 to 90 s for 22 h or 1 to 12 s for 15 h, in 0.5× TBE buffer containing 100 μM of thiourea, at 14 °C, and at a voltage of 6 V cm^{-1}. Concatemers of λ DNA and a Low Range Ladder (New England BioLabs, Ipswich, MA) were used as size standards. DNA was stained with SYBR Gold (Molecular Probes, Inc., Eugene, OR). To isolate plasmid DNA, the plasmid DNA bands were excised from pulsed-field (PF) agarose gels and the DNA was obtained by electroelution using dialysis bags (ZelluTrans, V series, MWCO 10,000, Carl Roth GmbH, Karlsruhe, Germany) or the Elutrap System (Whatman Group, Dassel, Germany) followed by ethanol precipitation.

Treatment of isolated pAP13 with λ exonuclease and exonuclease III was performed as described by Meinhardt *et al.* [25] with modifications introduced by Rose and Fetzner [17]. *Sma*I-linearized cloning vector BlueScribe KS Minus (pKS-) (Stratagene, GenBank accession no. L08784.1) was used as reference in reaction controls.

Genotypic characterization of the isolate was performed by PCR using universal primers 27_f and 1492_r [26]. The sequence of the amplified 16S rRNA gene was compared with published 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI) database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the approximate phylogenetic affiliation was determined. Sequence alignment with closely related and representative *Brevibacterium* species and construction of a phylogenetic tree was done with MEGA4.1 (http://www.megasoftware.net) using the neighbor-joining method.

Results and discussion

Brevibacterium sp. Ap13, an orange/white pigmented strain, was previously isolated from flamingo's feces in Laguna Aparejos, a high-altitude lake located at approximately 4,200 m in the northwest of Argentina [23]. Analysis of the 16S rDNA sequence (GenBank accession no. AM711595.2) suggested that the strain belongs to the genus *Brevibacterium*, as highest similarity was found to several *Brevibacterium casei* strains. Fig. 1 shows a neighbor-joining tree indicating the phylogenetic position of the isolate in comparison to related *Brevibacterium* strains. *Arthrobacter nicotianae* DSM 20123^T served as the outgroup. Since *Brevibacterium* sp. Ap13 forms a discernable subclade next to the *B. casei* strains, it might be considered as a related representative of *B. casei* or even a novel species within the genus *Brevibacterium*.

Phenotypic characterization of *Brevibacterium* sp. Ap13 [23] revealed multiple resistance to antibiotics, including colistin, ampicillin, ceftazidime, cefalotin, trimethoprim/sulfamethoxazole, and cefepime, which is highly remarkable since the place of isolation is considered pristine and far away from human influences. Especially resistance to cefepime is surprising as this fourth-generation cephalosporin was developed rather recently [27]. There are few reports about *Brevibacterium* species describing multiple resistances to antibiotics [28–30], however, all these strains were isolated from clinical specimens which is not surprising, since multiresistant microorganisms are quite common in clinical situations.

As antibiotic resistances are often plasmid-encoded traits, we further analyzed *Brevibacterium* sp. Ap13 for the presence of extrachromosomal DNA elements by PFGE, which revealed two plasmid bands, differing in size and intensity (Fig. 2). By comparison with the linear size standard, the smaller and more intensive plasmid, designated pAP13, was estimated to be approxi-

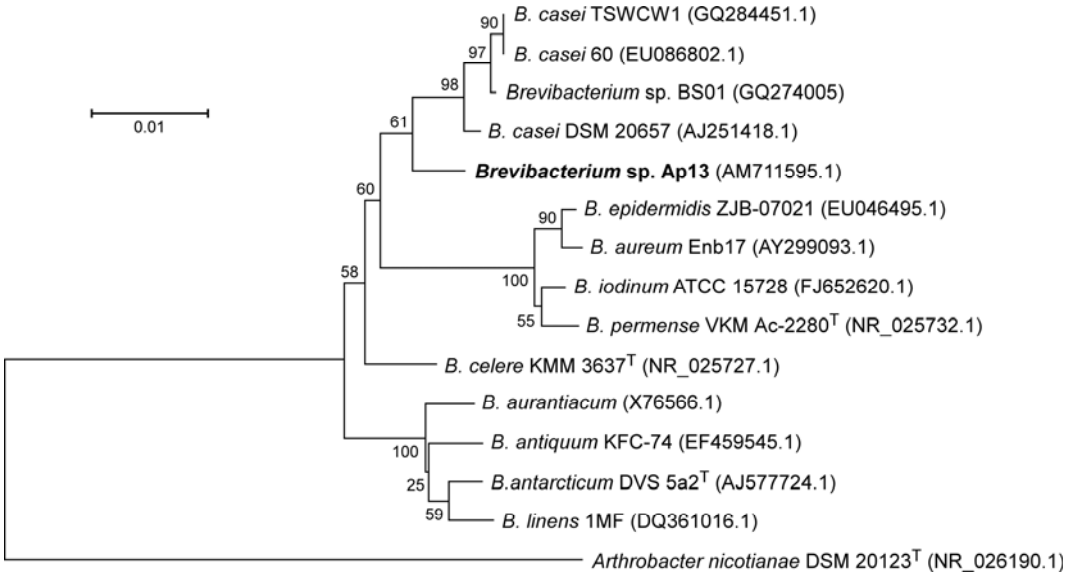

Figure 1. Neighbor-joining tree of aligned sequences of 16S rRNA genes showing relationships between *Brevibacterium* sp. Ap13 and related *Brevibacterium* species. *Arthrobacter nicotianae* DSM 20123^T was used as an outgroup. The bar represents 0.01 substitutions per nucleotide position. Numbers at branch nodes are bootstrap values for 1000 replicates. Accession numbers of the National Center for Biotechnology Information (NCBI) database of each strain are given in brackets.

mately 87 kb in size, whereas the larger one (pAP13c) had an estimated size of approximately 436 kb (Fig. 2A). Using different PFGE running conditions (see Material and methods and figure legends), no changes in the electrophoretic mobility of pAP13 were observed (Fig. 2A and B), indicating that the plasmid is linear rather than circular. However, a considerably altered electrophoretic mobility became evident for pAP13c (Fig. 2A and B), suggesting that this molecule is circular [31]. After successive rounds of cultivation of *Brevibacterium* sp. Ap13, pAP13c was lost, which may indicate its rather low segregational stability under the used growth conditions. Hence, we further concentrated our analyses on pAP13.

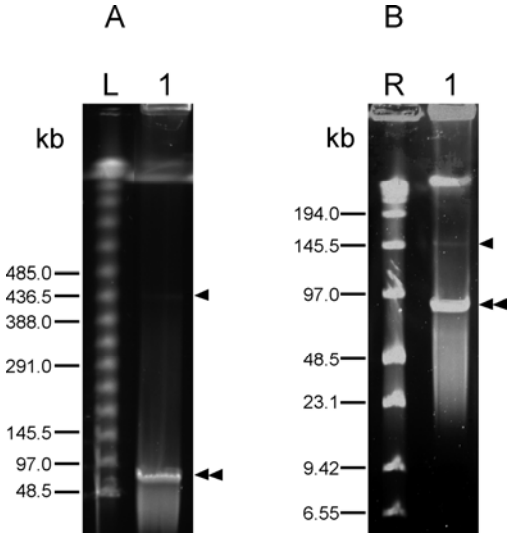

Figure 2. PFGE of total DNA of *Brevibacterium* sp. Ap13 at different running conditions. The plug-embedded and lysed cells were electrophoresed at 6 V cm^{-1} and 14 °C. Pulse times were 50 to 90 s for a 22 h run (A) and 1 to 12 s for a 15 h run (B), respectively. Simple black arrowheads indicate circular plasmid pAP13c and double black arrowheads linear plasmid pAP13. (A) is compiled from lanes of the same gel. L, λ DNA concatemers; R, Low Range Ladder; 1, total DNA of *Brevibacterium* sp. Ap13.

Linear replicons known from streptomycetes and other actinomycetes possess terminal proteins covalently linked to their DNA 5′ ends [18 and references therein]. As a consequence, such genetic elements are protected from cleavage by 5′-3′ exonucleases, but are accessible to degradation by 3′-5′exonucleases. To affirm our assumption, pAP13 being a linear plasmid, we isolated plasmid DNA from a preparative PF gel and subjected it to exonuclease treatment; functionality of the used exonucleases was demonstrated by degradation of *Sma*I-linearized pKS-, serving as the control (Fig. 3, lanes 1, 2, and 3). As shown in Fig. 3 (lane 6), pAP13 was entirely degraded by *Escherichia coli* exonuclease III (a 3′-5′exonuclease). However, no degradation was observed for λ exonuclease (Fig. 3, lane 5), which hydrolyzes DNA in 5′-3′direction. This result clearly demonstrates the 5′ ends of pAP13 being protected. As such, the DNA was primarily treated with proteinase K during plug preparation for PFGE. Nevertheless, plasmid DNA is not amenable to degradation by λ exonuclease, as the covalent linkage of the terminal protein to the plasmid 5′ end cannot be cleaved by proteinase K

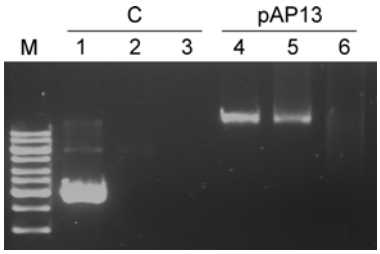

Figure 3. Sensitivity of pAP13 to exonuclease treatment. Cells (proteinase-K-treated) of *Brevibacterium* sp. Ap13 were subjected to preparative PFGE and plasmid DNA was isolated by electroelution. pAP13 was treated with λ exonuclease and exonuclease III, respectively, and analyzed on a 1.5% agarose gel. M, GeneRuler™ 1 kb DNA ladder; C, control DNA (*Sma*I-linearized plasmid pKS-). Lanes 1 and 4, plasmids untreated; lanes 2 and 5, plasmids treated with λ exonuclease; lanes 3 and 6, plasmids treated with exonuclease III.

[31]. Terminal proteins of *Streptomyces* linear plasmids play an essential role in their replication [32, 33, and references therein]. Thus, the proteins covalently bound to the 5′ ends of pAP13 are likely to be instrumental in replication as well.

Concluding remarks

We have detected a linear megaplasmid, pAP13, in a *Brevibacterium* sp., which to our knowledge is not only the first report of a plasmid greater than 70 kb found in *Brevibacterium*, but also the first description of a linear replicon for this genus. Due to the presence of terminal proteins linked to the DNA 5′ ends, pAP13 seems to be similarly structural organized as already known actinomycetes linear replicons. In light of the important role of *Brevibacterium* in the dairy industry, pAP13 might be useful for the construction of vectors for biotechnological purposes. Whether other qualities, such as the observed multiple antibiotic resistances and further genetic traits, that allow survival in pristine and extreme environments, are provided by pAP13, is currently under investigation, as well as sequencing of the plasmid DNA.

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