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Characterization of Novel Plasmid p1B146 from *Corynebacterium tuberculostearicum*

Wieteska, Łukasz[1]*, Eligia M. Szewczyk[2], and Janusz Szemraj[1]

[1]*Department of Medical Biochemistry, Medical University of Lodz, Mazowiecka 6/8 92-215, Lodz, Poland*
[2]*Department of Pharmaceutical Microbiology, Medical University of Lodz, Pomorska 137 90-235, Lodz, Poland*

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***Corynebacterium tuberculostearicum* B146, a strain derived from healthy human skin, contains a medium copy plasmid, p1B146. This plasmid was cloned and its complete nucleotide sequence determined. As a result, p1B146 was found to be 4,2 kb in size with a 53% G+C content, plus six open reading frames (ORFs) were distinguished. According to a computer-assisted alignment, two of the ORFs exhibited significant similarities to already-known common plasmid proteins, the first being the RepA gene, responsible for plasmid replication *via* a rolling-circle mechanism, and the second being an FtsK-like protein, the function of which remains unclear. The presence and quantity of RNA fragments in the putative ORFs were also evaluated.**

Keywords: *Corynebacterium*, plasmid, rolling-circle mechanism, FtsK-like protein

Corynebacteria are an extensive group of bacteria that are Gram-positive, asporogenous, and pleiomorphic, with a G+C content ranging from 50% to 71% [4]. This group can also be distinguished into the nonpathogenic bacteria such as *C. glutamicum* and *C. callunae*; pathogenic bacteria such as *C. diphteriae*, *C. pseudotuberculostearicum*, and *C. ulcerans*; pathogens for plants such as *C. nebraskense* and *C. michiganense*; and a wide group of opportunistic pathogens such as *C. jejkeium*, *C. urealyticum*, and *C. striatum* [14]. The species *C. tuberculostearicum* belongs to the opportunistic pathogen group and has been isolated from bone marrow, leprosy lesions, and mastitis [9, 18]. In particular, this species has the unique ability to produce tuberculostearic acid, although some recent reports have also found this ability in some other species within *Corynebacterium* spp. [6].

Since the first reports on plasmids derived from corynebacteria in the late seventies and early eighties [10, 12], a number of plasmids from both nonpathogenic and pathogenic species have been isolated and characterized. Differing widely in size, ranging from 1.4 kb to 55 kb, the plasmids from 3 to 8 kb are usually cryptic, whereas the larger size plasmids often encode antibiotic resistance genes [4]. However, the functions of the small cryptic plasmids still remain unknown [20]. No phenotypic profile has been associated with any of the plasmids, and almost all replicate based on a rolling-circle mechanism [20].

Accordingly, this study sequenced a novel plasmid, p1B146, 4.2 kb (GenBank Accession No. HM622074), from *C. tuberculostearicum*, making it the first plasmid to be sequenced from this species. The copy number in the host cells was determined, along with which of the predicted ORFs were transcribed to the RNA. This may help in understanding the role of small cryptic plasmids and be the starting point for developing a new vector for genetic research within the *C. tuberculostearicum* species.

Materials and Methods

Strains and Growth Conditions

Escherichia coli DG-1 chemically competent cells were used for the general cloning purposes and were grown at 37°C in an LB medium [19]. The recombinant *E. coli* strains were grown on an LB medium supplemented with ampicillin (100 μg/ml). *C. tuberculostearicum* strain B146, derived from healthy human skin, was initially collected and partially characterized by the Department of Pharmaceutical Microbiology, Medical University of Lodz. This strain was then grown in a Brain Heart Infusion medium (Difco Laboratories, USA) supplemented with 0.2% Tween 80 (BHIT). The *C. tuberculostearicum* strains CIP 107291 and CIP 107067 (Pasteur Collection Institute) were used as a control for the plasmid distribution.

Identification of Strain B146

A 16S rDNA fragment of 1,370 bp was amplified using the primer set 27mf (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T-3'). The amplicon was purified using a QIAquick PCR Purification Kit (Qiagen) and sent

*Corresponding author
Phone: +48 42 678 06 20; Fax: +48 42 678 24 65;
E-mail: l.wieteska@yahoo.com

for sequencing (Genomed - Sequencing Laboratory, Warsaw, Poland). A computer analysis of the 16S rDNA sequence was performed based on NCBI BLAST research and leBIBI (Bio Informatic Bacteria Identification). For additional phenotypic identification, an API Coryne biochemical test was also used.

Plasmid Purification

The plasmid DNA isolation from the *Corynebacterium* was performed using a modified alkaline-SDS lysis protocol [2]. For this purpose, the *C. tuberculostearicum* was grown in a BHI (100 ml) medium for 24 h at 37°C. Cells were subsequently harvested by centrifugation at 4,000 rpm for 20 min at 4°C. The cell pellet was then resuspended in 4 ml of buffer R1 containing 20 mg/ml lysozyme and incubated at 37° for 1.5 h. The experimental steps followed the original protocol. For sequencing, the plasmid was cleaned using a QIAquick purification kit. The plasmid DNA isolation from *E. coli* was then performed using a standard alkaline-SDS lysis protocol [2].

Plasmid Cloning and Sequencing

To determine the complete nucleotide sequence of the isolated plasmid p1B146, an overlapping DNA fragment generated with the restriction endonucleases *Nhe*I and *Hin*dIII was cloned in *E. coli* DG-1 at compatible sites of pUC19. The templates for the DNA sequencing were prepared by alkaline lysis of *E. coli* DG-1 clones carrying the recombinant plasmids. The purified templates were subjected to sequencing using the universal pUC19 primers 21 M13 and M13 REV. The complete double-stranded plasmid sequences were established using a primer-walking strategy on the original plasmid p1B146. Each base pair in the plasmids was sequenced on average 4.6 times. The gene prediction and annotation of the assembled plasmid sequences were performed using GeneMark software [1], the NCBI ORF Finder in conjunction with the NCBI, EMBL BLAST tool, and an Antimicrobial Peptide Database and Analysis System [25]. To search for further structural similarities, an analysis of the translated ORFs was also conducted using the HHpred tool [21].

Plasmid Copy Number Determination

The cells were grown in 300 ml flasks containing 100 ml of a BHIT medium incubated at 37°C on a shaker (100 rpm). From the beginning of the log phase, culture volumes of 2.0 ml were periodically removed from the flask and immediately frozen. Before the RT–PCR analysis, the cells were pelleted and resuspended in a TE buffer to obtain an equal OD_{600} of 0.300 (Cecil Ce 2501 spectrophotometer). The quantitative real–time PCR analysis to determine the plasmid copy number was performed by amplifying a 100 bp sequence from p1B146 using the following primers: 5'-TGA GTG CTG TGA GTG ATG-3', 5'-TCG TCC CTC AAC TTT TCC-3'. The PCR reactions were carried out in a Stratagene 3005 detection system using a Brilliant II SYBR Green QPCR Master Mix kit (Stratagene, USA). Each 25 µl sample contained 12.5 µl of 10× SYBR Green Master Mix, 1 µl of each primer (10 µM), 0.375 µl of ROX reference dye (2 µM/µl), 8.125 µl of PCR-grade water, and 2 µl of the bacteria sample. The reactions were incubated for 15 min at 95°C to activate the DNA polymerase and lyse the cells, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C, concluding with 10 min at 72°C. The threshold fluorescence level was determined automatically by the software. To validate the plasmid copy number, a standard curve was made. The concentration of the purified plasmid was measured using a Picodrop spectrophotometer, after which dilutions were made and standardized according to the cell number. Since the cell components released after the incubation at 95°C for 15 min could have inhibited the PCR reaction, each standard curve sample also contained the same amount of viable cells of another *Corynebacterium* sp. strain that did not include plasmid p1B146.

OD_{600} to Cell Number Calibration

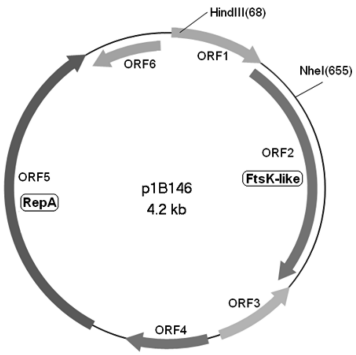
To estimate the correlation between the number of viable cells and the optical density OD_{600}, serial dilutions of the *C. tuberculostearicum* culture were made. Identical volumes of the diluted samples were plated on a BHIT solid medium and incubated for 48 h. Additionally, three independent cultivations were used for the dilution plating experiment, resulting in the following theoretical correlation: $r^2 = 0.97$.

RNA Isolation and cDNA Synthesis

The RNA isolation was performed using a Qiagen RNaeasy Mini kit. The *C. tuberculostearicum* was cultivated to the mid-expotential phase. For each isolation 8×10^7 cells were taken. After adding the RNAprotect reagent and vortexing, the cells were harvested by centrifugation for 10 min at 8,000 rpm. The supernatant was then removed and the pelleted cells were resuspended in 100 µl of a TE buffer containing lysosyme (15 mg/ml, Sigma) and Proteinase K. After 15 min incubation on a shaker incubator, the lysate was used for the subsequent isolation steps according to manufacturer's instructions. The purified RNA was treated for 45 min at 25°C with DNase I (Promega), precipitated, and finally resuspended in RNase-free water. The cDNA synthesis was then performed using an AffinityScript QPCR cDNA Synthesis kit (Stratagene, USA). The reaction mixture contained an RNA sample of around 350 ng.

Plasmid Transcript Identification

The plasmid transcript identification was performed by a Quantitative PCR using a Stratagene 3005 detection system. The procedure was similar to the transcript identification proposed by Park *et al*. [17]. A set of seven pairs of primers specific for six predicted ORFs was used to amplify 99–114 bp sequences (Fig. 1) from the cDNA. The PCR reaction conditions were the same as those used for the plasmid copy number determination. The threshold fluorescence level was determined automatically by the software. A negative control was provided for each primer set using the purified RNA. A



Predicted ORFs			
ORF	Strand	Left end	Right End
1	+	37	435
2	+	425	1516
3	-	1506	1874
4	+	1929	2291
5	+	2442	3848
6	-	3845	4189

Fig. 1. Physical map of plasmid p1B146.
Arrows indicate ORF lengths and direction of transcription. The table on the right shows the predicted ORFs and lengths. The two restriction enzyme sites used for cloning are highlighted.

plasmid transcript identification test was then prepared and triple assayed.

RESULTS AND DISCUSSION

Plasmid Profile and Characterization of *Corynebacterium tuberculostearicum* Strain B146

Corynebacterium sp. strain B146, from the collection of the Department of Pharmaceutical Microbiology, University of Lodz, was isolated from healthy human skin and initially classified as *Corynebacterium* spp. Since the identification of certain corynebacteria can be problematic [6, 13], this study used a sequence comparison of a 16S rDNA fragment and biochemical analysis based on an API Coryne test. The 16S rDNA sequence showed an almost complete similarity (99.93%) to a sequence of *C. tuberculostearicum* strain SK141, with only one different nucleotide in a 1,370-bp-long sequence. Moreover, there was also a great similarity to *C. pseudogenitalium* (99.85%) and *C. accolens* (98.54%). However, the LeBIBI tool classified the strain interchangeably as *C. tuberculostearicum*. Meanwhile, the Api Coryne system generated the numerical code 6100324 (Table 1), which corresponded to *Corynebacterium* CDC group G in the API Coryne profile index, a typical result for *C. tuberculostearicum* [6]. The plasmid profile of the strain also showed the presence of one circular plasmid. To identify the unique restriction enzyme sites, several enzymes were tested (data not shown), among which *Nhe*I and *Hin*dIII were chosen as they only cut p1B146 once. Digested and purified fragments were then used to clone into the *Xba*I and *Hin*dIII sites in a pUC19 cloning vector.

Table 1. Phenotypic pattern of *C. tuberculostearicum* strain B146 (API Coryne kit, bioMérieux).

N	Reactions	Result
Enzymatic activity		
1	Pyrazinamidase	+
2	Pyrrolidonyl arylamidase	+
3	Alkaline phosphatase	+
4	β-Glucuronidase	–
5	β-Galactosidase	–
6	α-Glucosidase	–
7	*N*-Acetyl-β-glucosaminidase	–
8	β-Glucosidase	–
9	Urease	–
10	Gelatinase	–
11	Catalase	+
12	Nitrate reduction	–
Fermentation of sugars		
1	Glucose	+
2	Ribose	+
3	Xylose	–
4	Mannitol	–
5	Maltose	+
6	Lactose	–
7	Sucrose	–
8	Glycogen	–

Plasmid Sequence Bioinformatic Analysis

The whole plasmid p1B146 nucleotide sequence was analyzed (GenBank Accession No. HM622074) and found to be 4,215 bp in size with a G+C content of 53.13%, which falls within the DNA G+C content range of *Corynebacterium* spp. However, the sequence did not exhibit any significant similarity to other already known corynebacterial plasmid sequences. The reference strains of *C. tuberculostearicum*, CIP 107291 and CIP 107067, do not contain this plasmid. An ORF analysis conducted using GeneMark.hmm 2.0 and ORF Finder revealed at least 6 possible genes in both strands (Fig. 1), yet the functions of only two ORFs could be predicted, as none of the others displayed any significant similarity to ORFs in publicly available databases. All the potential ORFs were assessed using the criteria that an ORF should consist of at least 75 codons and start with AUG, GUG, or UUG. To investigate antimicriobal peptides, the possible length of an ORF was decreased to 10.

Replication Region

One of the ORFs, starting at 2,442 and ending at 3,843, encoded a 480 aa putative replication protein (RepA). A sequence analysis showed the highest similarity (identity 36%, positives 52%) to the RepA protein of *C. glutamicum* plasmid pAG3. Other significant matches were assigned to the putative RepA gene of *C. callunae* plasmid pCC1, RepA protein from *C. glutamicum* ssp. *lactofermentum* plasmid pBl1, and RepA protein of *Propionibacterium freudenreichii* plasmid pLME108, with a 35%, 30%, and 31% identity, respectively. An alignment of the sequence to the RepA protein from pAG3 can be seen in Fig. 2. According to these data, plasmid p1B146 would appear to replicate based on a rolling-circle mechanism [15]. In addition, four of the five known conserved motifs in the plasmid RepA protein family were found (Fig. 3), as in other plasmids, for instance, pAG3, pCC1, and pBL1 that lack the Motif IV sequence (Cx_5CPxC). This may suggest, as proposed earlier [24], that these plasmids belong to a distinct subgroup of plasmids within the pIJ101/pJV1 family [11]. The putative origin region of p1B146 was located approximately 400 bp upstream of the *repA* gene (Fig. 4).

FtsK-Like Protein

The predicted ORF2 showed a significant similarity to the FtsK family protein found in *Mobiluncus mulieris* (NCBI Reference Sequence: ZP_03994922.1), FtsK family protein of *Bifidobacterium breve* plasmid pB21a (GenBank Accession

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p1B146: 35   EHARQGARREEDAASARDARLGNKSIKSQQQVEA--PDLQR-RLWALHKTMWKITDYQRL 91
pAG3  : 10   EHATS-APNQATPATHTTGRLGTTDTTKHHKVKALSPALFRAKLWDLQRTMWKVTEDKTL 68

p1B146: 92   RGCHRYIAGGAGAASLRWHEPGRASWASLCTSSSVWASPLSAAAIGKTRAVEVSTALDNW 151
pAG3  : 69   AGCGRWVAGGSAVASLVWQAQGRARWGGLQNSHSVWGSPVASSVICSRRANEVDAAIQAW 128

p1B146: 152  FK-----------------QEKQHSVE----------FLTLTLAHDKEQSLQEVWDTLA- 183
pAG3  : 129  ANGAGLTPAHYRGVSTSPSDRKQRGVSVNPVVERGISLMTLTLRHNSKQSLTEVWDAIAG 188

p1B146: 184  YGRGVVAGASWRGGKRYEGDRARFGVKHWIKSVEATHGAHGWHVHLHVLLLLDKELTQDQ 243
pAG3  : 189  CWQAVTNTAAWRGGARTAGDKSRYGIAHWYRAIEVTHGKNGWHVHLHVVLFHDRVLSVDE 248

p1B146: 244  RGALESNIYSRWSAAAQRRGFKAPSRAHGVK-LEKARKDKNGHDLGTYLAKGSIASVAET 302
pAG3  : 249  RDSLADRVFDRWAAKAVRLGMRAPSRDRGIDVVHVAASSDDAKSIGGYTCKGMLSGIA-- 306

p1B146: 303  LSREMTAGQSSKEGRAESRTPFQILDSIRKKWDTSLKNPDVQLWRAWEKDSMGRRQIAWS 362
pAG3  : 307  --AETTTGQVTKEAKGDNRTPFQILGDLGKQY----TKRDHALWLEWEKGSKGRRQTGWS 360

p1B146: 363  KGAKDALGVSAVSDEEAEQQGEESHTTVEVARVEYEEWNRAREDTGEKLRDDLATRGDVV 422
PAG3  : 361  QGTKDVLGINELSDDQIDDSLGDNDQSEVVAMVGRGAWGAIASDTEK--------RAVIL 412

p1B146: 423  EYVAQAKTPGEAHKRAATILRALQV 447
pAG3  : 413  DAVAATDSAEGARKAARDVLKLFGV 437
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Fig. 2. Alignment of putative replication protein of p1B146 and pAG3.
Conserved residues are highlighted in black, conservative changes in grey.

No. DQ497626), and FtsK homolog in *Bifidobacterium breve* plasmid pCIBb1 (GenBank Accession No. AF085719) with an identity of 31%, 30%, and 30%, respectively, and positive of 50%, 46%, and 46%, respectively. There were also many insignificant matches to FtsK-like proteins, FtsK/SpoIIIE, and transfer proteins from various bacterial species. Using an NCBI Conserved Domain Search and EMBL-EBI InterProScan Sequence Search, an N-loop NTPase domain (FtsK/SpoIIIE, pfam01580), signal peptide domain, and two transmembrane regions were also identified. The FtsK/SpoIIIE domain is present in ATP- or GTP-dependent DNA translocases in *E. coli*, and is normally involved in chromosome segregation during cell division and *Bacilius subtilis* sporulation [5]. An HHpred analysis also showed a great structural homology to other FtsK-like proteins, suggesting that this gene could be responsible for plasmid segregation and stability maintenance [16]. Meanwhile, SpoIIIE proteins have been implicated in intercellular chromosomal DNA transfer [8], which in the current case was supported by the presence of a signal peptide domain that has not been detected in many FtsK-like proteins derived from different bacterial chromosomes (data not shown).

As a result of the BLAST search at NCBI and UniProt Knowledgebase research, ORF1, ORF3, ORF4, and ORF6 did not show a significant homology to any proteins in the databases and thus could not be related to any specific function. A search for conserved domains did reveal a putative signal peptide domain in ORF 6, yet no homology protein was identified. Notwithstanding, RNA sequences were detected within the range of each examined ORF (Table 2). The repeatable differences in the C_T values of the RNA fragments showed that the most active part was within the range of ORF 5, identified as the RepA protein gene. Meanwhile, the RNA from the range of ORF 1 and ORF 6 had the smallest C_T values. Since some corynebacteria

	Motif I	Motif II	Motif III	Motif V
	tutltxxx	xpHuHuuux	uxxYuxKxxx	wxeyexaxxgrraxxwsrglr
p1B146	F**LTLT**LA**H**	**H**V**HLHVLL**L	**L**GT**Y**LA**K**GSI	**W**RAW**E**KDSM**GRR**QIA**WS**KGAK
pAG3	L**LTLT**LP**H**	**H**V**HLHVVL**F	**I**GG**Y**TC**K**GML	**W**LEW**E**KGSK**GRR**QTG**WS**QGTK
pCC1	L**MTLT**LR**H**	**HPHFHVLL**F	**M**GA**Y**TA**K**GIA	**W**R**EYE**ATTR**G**VKQTS**WS**TGAK
pBL1	M**F**VG**T**VR**H**	**H**L**H**RN**MLL**F	**M**AT**Y**LA**K**GMS	**W**R**EYE**VGSKNL**R**S-S**WSR**GAK
pLME108	M**VT**M**T**MR**H**	**H**V**HLHVLV**F	**L**AR**Y**LS**K**AQF	**W**F**E**W**E**KGSR**GRR**QIG**WS**AG**LR**

Fig. 3. Alignment of conserved motifs of Rep proteins from most similar plasmids (NCBI BLAST).
The Rep protein of plasmid p1B146 is compared with the following plasmids: pAG3 from *C. glutamicum* (formerly *C. melassecola*) (Takeda *et al*., 1990; GenBank Accession No. AY172684), pCC1 from *C. callunae* (Venkova-Canova *et al*., 2004; GenBank Accession No. AJ308231), pBL1 from *C. glutamicum* ssp. *lactofermentum* (Fernandez-Gonzalez *et al*., 1994; GenBank Accession Number No. AF092037), and pLME108 from *Propionibacterium freudenreichii* (Dasen *et al*., GenBank Accession No. AJ006662). The most conserved amino acid residues are shown in boldface letters.

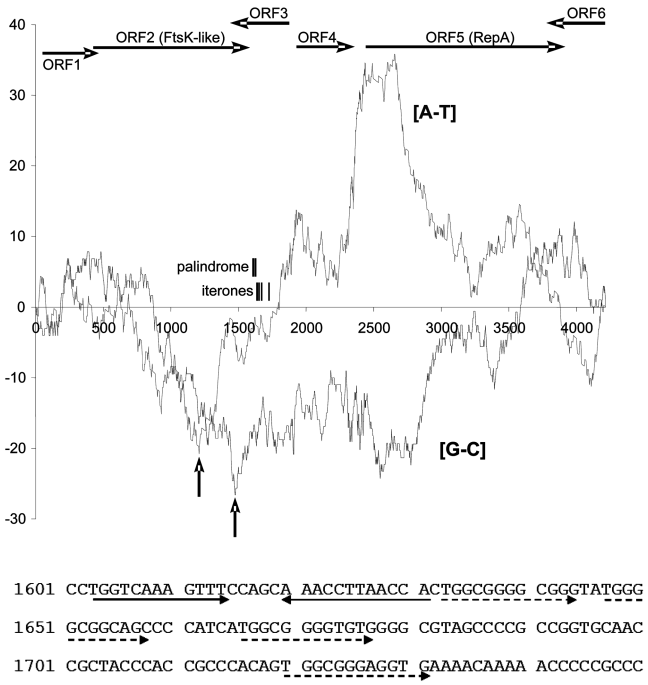

Fig. 4. Putative origin of replication of p1B146.
The chart shows differences in [G-C] and [A-T] content (cumulative deviation from mean subtraction value). Arrows point to the global minimum associated with the replication initiation area. In the same region, one imperfect palindromic sequence (12 bp) and four consecutive 12 bp direct repeats (iterons) were independently spotted.

are known to display antimicrobial/bacteriostatical activity, an alignment was also performed using the Antimicrobial Peptide Database and Analysis System, along with an additional search for shorter ORFs, yet this yielded no significantly positive results. It should be mentioned that all known corynebacterial bacteriocin genes are localized in plasmids. However, according to Tauch *et al*. [22], the chromosome of *C. jeikeium*, which is closely related to *C. tuberculostearicum,* includes some insertion sequences that were previously found in corynebacterial plasmids.

Plasmid Copy Number (PCN)

The PCN of p1B146 was determined using the QPCR method described previously [3]. The real-time QPCR amplification and analysis were performed using a Stratagene 3005 detection system (for the detailed protocol, refer to Materials and Methods). Absolute quantification determines the exact copy concentration by relating the C_T value to a standard curve. The amplification specificity was detected using a melting curve analysis and showed a sharp peak for the PCR product at 84.3°C, indicating the absence of any nonspecific products within the analyzed temperature range.

Table 2. Differences between plasmid p1B146 RNA concentrations and predicted ORFs (ΔCt).

Concentration of RNA for predicted ORFs						
ORF	ORF 1	ORF 2	ORF 3	ORF 4	ORF 5	ORF 6
ΔC_T	8.76	10.76	10.24	12.45	11.31	9.23

Values after subtracting controls. Seq5 and Seq6 have close ΔC_T values, belonging to the same ORF 5, which was identified as a putative RepA protein. The highest ΔC_T was detected for a sequence within the range of ORF 4, yet it was not associated with any functional homolog in a BLAST analysis.

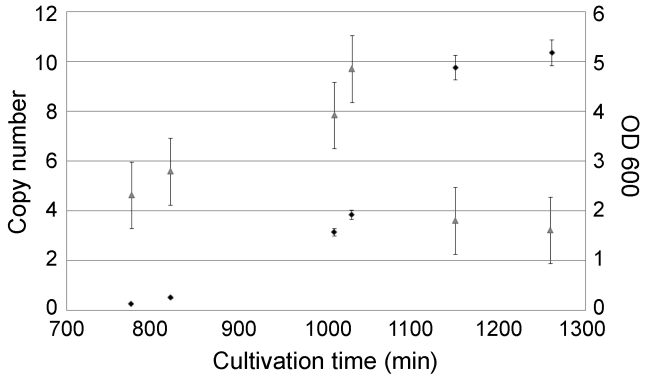

Fig. 5. Time-course evolution of plasmid copy number in *C. tuberculostearicum* (marked as grey triangles) and corresponding growth curve (marked as black diamonds).

A variation in the plasmid copy number during cell growth was also revealed (Fig. 5), where the PCN increased during exponential cell growth, from 4.6 plasmids/cell at the beginning of the cultivation (OD_{600} = 0.120) to 9.7 plasmids/cell (OD_{600} =1.92) in the middle. Then, at the end of the cultivation in the stationary phase, the PCN was 3.2 plasmids/cell (Fig. 5). This plasmid p1B146 copy variation during cell growth was also similar to results previously obtained for pFM20 and pFM46 [3]. The average PCN for p1B146 classified it as a low copy number plasmid. However, if the results from the middle of the exponential growth phase are used to determine the average PCN, instead of the cells harvested throughout this phase, plasmid p1B146 should be classified as a medium copy number plasmid, corresponding to other plasmids from this group [15].

In conclusion, this study described a small 4.2 kb, medium copy cryptic plasmid derived for the first time from *C. tuberculostearicum*. Computer analysis and identified patterns of the RepA protein allow the claim that this plasmid replicates based on a rolling-circle mechanism. None of the ORFs predicted by GeneMark or additionally calculated small ORFs included the properties of an antimicrobial peptide. A quantitative RT–PCR facilitated the detection of an RNA product within the range of each predicted ORF, which implied the presence of unknown genes. One such product from a predicted ORF (from 425 to 1,516) was identified as an FtsK-like protein. A conserved domain search of this ORF revealed two additional

transmembrane domains and a signal peptide at the beginning. The exact function of this FtsK-like protein remains unclear. It is worth mentioning that many corynebacterial and bifidobacterial plasmids contain similar Ftsk-like genes [20]. Although the role of small cryptic plasmids in *Corynebacterium* sp. remains unknown, some of them, owing to their small size, have been successfully used to develop shuttle vectors [20, 23, 24]. Thus, plasmid p1B146 could also be used as a framework for shuttle vector construction for further genetic research on *C. tuberculostearicum*.

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