ISSN 1022-7954, Russian Journal of Genetics, 2016, Vol. 52, No. 11, pp. 1131–1136. © Pleiades Publishing, Inc., 2016.
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GENETICS
OF MICROORGANISMS

# Nucleotide Sequence and Structural Analysis of Cryptic Plasmid pBL90 from *Brevibacterium lactofermentum*

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Received December 30, 2015

**Abstract**—The nucleotide sequence of cryptic plasmid (designated as pBL90) detected in the cells of *Brevibacterium lactofermentum* DSM 1412 was determined. The length of plasmid DNA is 67826 bp. Comparison of the nucleotide sequence of pBL90 with known plasmid sequences showed no long regions of significant homology. Computer analysis of the plasmid DNA revealed 29 open reading frames (ORFs). The amino acid sequences of 15 ORFs (approximately 25% of plasmid length) have a high (>70%) level of identity to proteins from different plasmids of *Corynebacterium* representatives, including replicative proteins. Unusual in pBL90 is the presence of replicative genes from two different families and types of replication.

*Keywords: Corynebacterium* plasmids, replicative genes, cryptic plasmid pBL90, lysine, transposon, vector

**DOI:** 10.1134/S1022795416100070

## INTRODUCTION

Bacteria of *Corynebacterium* and *Brevibacterium* genera are widely used in industrial biotechnology for manufacture of amino acids, detergents, steroids, and other bioactive compounds [1]. The presence of vectors for cloning is necessary for creation of producers of cell metabolism. Currently, there is widespread use of vectors produced on the basis of plasmid pBL1 that was isolated from the strain of *C. glutamicum* ATCC 13869 [2]. Plasmids identical to pBL1 were detected in many natural strains of *B. lactofermentum* [3]. In spite of the presence of vectors for corynebacteria, the demand for new vectors suitable for already existing ones remains.

In the present work, results of identification and analysis of the new plasmid from *B. lactofermentum* DSM 1412 compatible for plasmid pBL1 are presented.

## MATERIALS AND METHODS

*Strains, medium, and conditions for growth.* The *B. lactofermentum* DSM 1412 strain (received from the German collection of microorganisms, DSM) is widely used in research for construction of amino acid producer strains and is a source of genomic DNA. The mutant variant of this strain *Brevibacterium* sp. 90 is known as a producer of lysine [4]. Cultivation of *Brevibacterium* strains was provided on BH medium (Becton Dickinson) at 30°C during continuous stirring (300 rpm).

*Molecular genetic methods.* Extraction of total DNA from *B. lactofermentum* DSM 1412 and preparation of samples were provided by known molecular biological methods [5, 6]. Determination of the DNA sequence was conducted via pyrosequencing by the GS FLX genomic analyzer with usage of the GS XLR70 Sequencing Kit. The volume of the sequence was 150 Mp, which corresponded to 45-fold overlap of the full genome. Assembly of the sequence of selected reads into long “contigs” that unite them was provided by GS De Novo Assembler (Roche). The search for open reading frames with ability to code proteins was performed by MG-RAST software [7]. Analysis of ORFs was performed with the help of the NCBI software package and database (http://www.ncbi.nlm.nih.gov).

## RESULTS AND DISCUSSION

During analysis of genome structure of the *B. lactofermentum* DSM 1412 strain via full-genome sequencing, we found two ring DNA molecules (plasmids) with size of ~4 kb and ~68 kb. At the same time, these plasmid DNA are absent in *Brevibacterium* sp. 90 strain, producer of lysine [4], that was received from the strain of *B. lactofermentum* DSM 1412 as the result of multiple cycles of mutagenesis. The presence of plasmid DNA in cell-free lysates of the DSM 1412 strain and the absence in its mutant derivative were

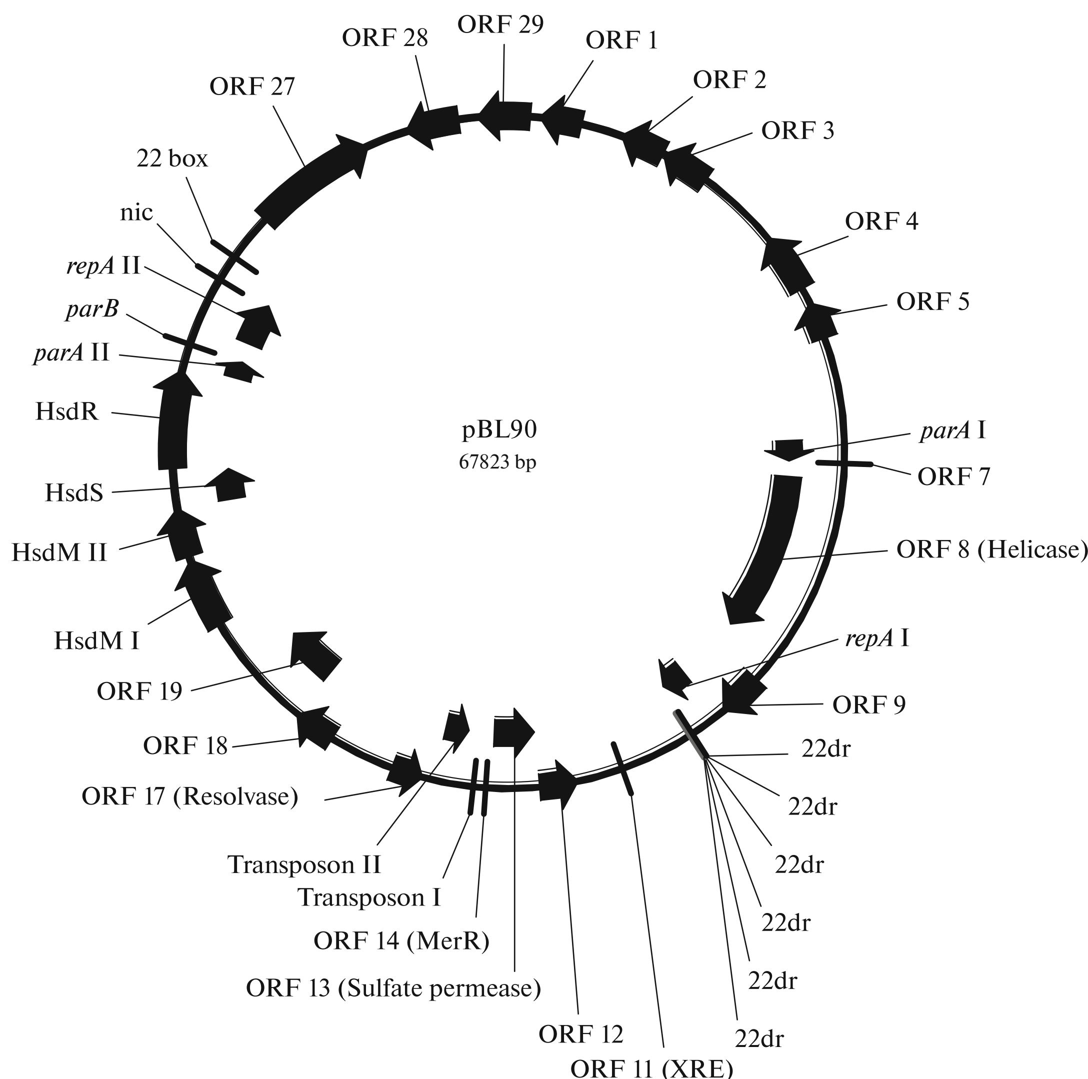


**Fig. 1.** Scheme of plasmid pBL90.

also confirmed by electrophoresis in agarose gel (the data are not presented).

Computer analysis of the nucleotide sequence of small plasmid from *B. lactofermentum* DSM 1412 showed almost full identity to plasmid pBL1 (GenBank no. AF092037) detected in many strains of *Brevibacterium*. On the basis of plasmids of pBL1 family, vectors were created for genetic engineering works with strains of *Brevibacterium* and *Corynebacterium* genera [8, 9].

Whereas in the *B. lactofermentum* DSM 1412 strain we found a huge plasmid (67823 bp) that we marked as pBL90, it does not have homology with known plasmids from *Brevibacterium* and *Corynebacterium*, except regions coding ORFs 11, 13, 16 (Transposon II), 17, and 27 (Fig. 1). The full nucleotide sequence of plasmid pBL90 was deposited in GenBank as no. KU306397. The DNA of plasmid pBL90 was resistant to the AscI, PmeI, SfiI, and SwaI restrictases and had unique sites for FseI, KflI, NotI, and SgrDI. Using MG-RAST, 29 ORFs were found in plasmid pBL90. Analysis of sequences of ORFs via the NCBI software package and database allowed us to detect their homology and predictable functions (table).

An important feature of plasmid pBL90 is the simultaneous presence of two replicative regions in it. In composition of the first region, we found a replicative gene marked as *repA* I, whose product has homology (64%) with replicative protein of plasmid pCRY4 from *C. glutamicum* LP6 [3] relating to the pCRY4 family of low-copied plasmids [10] (Fig. 2).

Minimal replicon pCRY4 has also five direct 22-nucleotide repeats (cgTGCGCAgTAACGGTACcgcC) in the replicative region. It turned out that plasmid pBL90 near the *repA* I gene has at least five copies of sequences of repeats (TGCGCATAAaCGGTcACacatC)

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RepA I 1   M-N----NNNSFESSQGSASDALAERLAQIESGLVDPYTFTSRTLLQATFPHSARAGKEIVLKNGNV 62
C.c.   1   MSNYTPRNQPGHNHGETSAEAELQARLAEIEAGFLDPYTFTSRTLIQATFPHSKREEREVVLANGNV 67
C.d.   1   MTHDSPVPRGGNTRPTAREELEARLEEIEAANQLLDPYAFSSRTLIQTTFPHSSRAGDKLVLKNGAV 67
C.g.   1   MD---DHTLPQPDSGNSDPISDLEARLAEIEAGLGDPLSFTSKTLIQATFPHSAKAGKELVLVNGHT 64

RepA I 63  QVTMYSSKGLPYGVYPRLIMCWLTREAVRRKSLDIDEARIIPLGASLAEFMREIGVDGRS 123
C.c.   68  IVTMYSSKGLPYGVYPRLIMCWLTREAIRRRDLPMNEARVIPLGSSLSQFMREVGIGAAS 128
C.d.   68  TVTMISPNGLPYGVYPRLIMCWLTREALRRRDLPEDEARTIPLGSSLADFMRDVGIMGRS 128
C.g.   65  TVTMYSRHGLPYGSWPRLIMCWLTREALRRQNLPIDEAREIPLNSSLSGFMREVGIGRAT 125

RepA I 124 GGKTGNIANLRKQLTALFSTFISVETIGSSDMEDMPRSFQKIDNSLIADSSTLWWDPKNE 183
C.c.   129 GGKEGNIHRLHHQLTALFSTFITVTTNDTTDVEDMPRSFQRIDNTVVADSSKLWWDPKHP 188
C.d.   129 GGKTGNITSLRKQLRSLFTTMITVEVTNHENRDKRQIVFDQMQNTLIAESSMLWWDTKNP 188
C.g.   126 GGERGTITALKKQMRSLFSTSIGIDIKGDDDL-----KLLDLDESVIAERTEMWWTPRPH 180

RepA I 184 DQLSLMNSSVTLSKVFYQDLIGSAVPLDIAILRAIKRSPMAIDLYCWLTYRLSYHQGFTV 243
C.c.   189 DQLGLQDSSVTLSENFYRDLVSAAVPLNVSMLRQIRRSPLAIDLYCWLTYRLSYHRGVTV 248
C.d.   189 EQLSIQDSSVTLTAGFYRELTCSAVPLDVSILREIRRSPMAIDLYCWMTYRASYQRGITV 248
C.g.   181 DDIDF-EGYIRLSATFYSDLIKSAVPLDTRILRSLKKSPMAIDVYSWLTYRVSYLRYPTV 239

RepA I 244 VTWNQLQAQFGAGYPNTARGRVDFKRKLKGSLERVVDAWPEAAAVPTDHGIMLKPGNPSV 303
C.c.   249 VAWEQLRAQFGAGYPDTTRGRRNWKIKVTAALRKVMDAWPEASVSVVDNGLMLKPGAPSV 308
C.d.   249 VTWDQLQRQFGASYQGTARGRRDFKRRLLEALEKVVAAWPEAAVDVTENGLMLRPGAPSV 308
C.g.   240 IKWDQIQGQLGAGYPDTSQGMRNFRKKFLIALNKVIDVWPTDSISIVKNGILLTPGSPSV 299

RepA I 304 PKEI-QQQLR-R-EENNDNP--F 320
C.c.   309 AKNQ-QRELKKC-DTTEDNP--F 326
C.d.   309 PRRVHQEIAK-RYAT--DAENRF 327
C.g.   300 PRRA-QDEFQKRFSIG-DDPL-F 318
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**Fig. 2.** Comparison of amino acid sequence of peptide coded by *repA* I of plasmid pBL90 with replicative proteins of several plasmids of corynebacteria. Presented are amino acid sequences of replicative proteins from plasmids of the following corynebacteria: C.c.—*Corynebacterium callunae*, GenBank no. WP_015453127.1; C.d.—*C. doosanense*, GenBank no. WP_018023049.1; C.g.—*C. glutamicum*, GenBank no. WP_032490505.1. Amino acid residues identical in proteins are marked in black color; similar amino acid residues are marked in gray color.

(Fig. 1). Thus, the first replicative region pBL90 has similar structure with the replicative region of the plasmid of the pCRY4 family with replication according to the θ-mechanism.

In the composition of the second region, we found a replicative gene marked as *repA* II, the product of which has homology (82%) with the replicative protein of plasmid pGA2 from *Corynebacterium lilium* [11] related to the pCG1 family (Fig. 3) [3]. In the replicative region, all plasmids of this family have the conservative sequence (from one to several copies) CrTAAGCArwAhACGGTTCCCC, necessary for autonomous replication [3]. The similar sequence (CaTAAGCAatAgaACGGTgCCCC) was also found in the second replicative region of plasmid pBL90. Moreover, in compound of the second replicative region, we detected a sequence that has homology with the consensus sequence of the replication start point [12].

Near the *repA* II gene of plasmid pBL90, we revealed genes (marked as *parA* I and *parB*) controlling allocation of plasmid DNA to daughter cells; the products of these genes show a high level of homology (89–95%) with Par proteins of plasmids of corynebacteria (table). Thus, the second replicative region of pBL90 has a high level of similarity to the structure of the replicative region of plasmids of the pCG1 family, of which replication by the "roll ring" mechanism is typical.

One more feature of plasmid pBL90 is the presence of genes the products of which show high level of homology (90%) with enzymes of cell metabolism (Yga2A pyruvate dehydrogenase, ORF 7, sulfate permease, and ORF 13), which is not typical of plasmids from corynebacteria.

Moreover, in the composition of plasmid pBL90, we found ORFs coding proteins with a high level of homology (60–90%) with proteins of conjugative transfer.

On a section of the plasmid (45021–53509 bp) (table), a group of genes were located that have homology with genes of the restriction-modification system of the first type (SRM I), which are usually located on the chromosome. SRM I is formed by five subunits that function as a single whole and constitute a complex of two HsdM, two HsdR, and one HsdS. On the plasmid, there are all three types of CPM I subunits (Fig. 1), and at the same time, the HsdM II and HsdS reading frames are fused. The presence of two subunits HsdM I and HsdM II at the same time is unusual. Genes coding subunits of DNA methyltransferases do not have important homology at the DNA level and have only weak (~25%) homology at the protein level in the middle part of the amino acid sequence of enzymes. Consequently, the simultaneous presence of two HsdM genes cannot be explained by

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RepA II 1   MY---SIARTTSPARLLRRECFHDTPPAYVPAATHDHAPSLWSDTQRYTEENAYQQARDDWFD 60
C.g.    1   MN---SIARTTSQTRLLTRECFHDTPPAYVPAIAHDHAPSLWSDTQRYTEEHAYQQARDDWFD 60
C.e.    1   MSCINSIARTRAHAPQLVRECHHDTLPTYVPVTLGDHAPSLWSDTQRYTDEDTYQRDRDSWFD 63
YS      1   M-----------------RECHHDTLPTYVPVTLGDHAPSLWSDTQRYTDEDTYQRDRDSWFD 46

RepA II 61  RNMVSHQSRDLNDLGWCHHDAEIFLNHIGREALHCAKDRAGIVKAWSKKKDKNGHRKPLV 120
C.g.    61  RNMVSHQPRDLNDLGWCHHDAEIFLNHIGREALHCAKDRAGIVKAWSKKKDKNGHRKPLV 120
C.e.    64  THIVTHQSRELVDTGWCHHDAEILLSHLGRDGLHCAKDRAGITKAWSKTKDQHGHRTPLI 123
YS      47  THIVTHQSRELVDTGWCHHDAEILLSHLGRDGLHCAKDRAGITKAWSKTKDQHGHRTPLI 106

RepA II 121 WPRERVHLAEYIHLTNPTYASVIVIDIDHVGAPGGLLSDLDLFVSDQVKKLSRLRLGPNW 180
C.g.    121 WPRERVHLAEYIHLTNPTYAAVIVIDIDHVGAPGGLLSDLDLFVSDQVKKLSRLRLGPNW 180
C.e.    124 WPRERVHLAEYIHFTNPTYAAVIVIDIDHVGAPGGFLSGLDGFVADKVEKLAHLRLGPNW 183
YS      107 WPRERVHLAEYIHFTNPTYAAVIVIDIDHVGAPGGFLSGLDGFVADKVEKLAHLRLGPNW 166

RepA II 181 IGINPQSGKSQMIWYIDPVYRDEGEKSKPWSLLEALHMELQDIFEADKHFSHGWSRNPIY 240
C.g.    181 IGINPQSGKTQMIWYIDPVYRGNSEISKPWSLLEALHTELQDIFEADKNFSHGWSRNPIY 240
C.e.    184 IGINPESGKSQMIWYIDPVYRTPGEVSKPWSLLEALHTELQAFFEADKNFSHGWSRNPIY 243
YS      167 IGINPESGKSQMIWYIDPVYRTPGEVSKPWSLLEALHTELQAFFEADKNFSHGWSRNPIY 226

RepA II 241 DGDSLEAYRWYAQHHEVFHMRLLSTGLWMLKGETVATLEDKGVADRRQNQRFSSGRELIL 300
C.g.    241 DGDNLDAYRWYAQHHEVFHMRLLSAGLWMLKGDTVATLEDKGVADRRNTQRFSSGRDLIL 300
C.e.    244 SGDNLGAYRWYAQHHEVFHMRLLSTGLWMLKGDTVATMEDKGVADRRHTQRFSSGRELIN 303
YS      227 SGDNLGAYRWYAQHHEVFHMRLLSTGLWMLKGDTVATMEDKGVADRRHTQRFSSGRELIN 286

RepA II 301 AARENTERFRQAQQAREILAGLEDDDLAEAFEASDPDIIDGIRVVWQTPGRAQRDVTAFN 360
C.g.    301 AAKANTERFHQAQQARAVLAGLEDDDLAKAFEASDPDVIDGIRVVWQTPGRAQRDVTAFN 360
C.e.    304 AARANTERARQAMQAREVLAGLEDDDLAKAFEASDPDVIDGVRVVWQSPGRAQRDVTAFQ 363
YS      287 AARANTERARQAMQAREVLAGLEDDDLAKAFEASDPDVIDGVRVVWQSPGRAQRDVTAFQ 346

RepA II 361 HALKTAARLNRAGKKMTDDAIIDAYRAAYEVAHSVGADDRSREEPPMRDLRSLARRVRGY 420
C.g.    361 HALKTAWRLNRAGKKMTDDAIIDAYRAAYEVAHSVGADDRSREEPPMRDLRSLARRVRGY 420
C.e.    364 HALKTAGRLNRAGKKMTDDAIIDAYREAYEVAHSVGADDRPREEPPMRDLRSLARRVRGY 423
YS      347 HALKTAGRLNRAGKKMTDDAIIDAYREAYEVAHSVGADDRPREEPPMRDLRSLARRVRGY 406

RepA II 421 VSSNKRVDCAAPKETPNDSRMSPQERKALATLGRKGAIVSNSRRWASPSSPAAQASMTAL 480
C.g.    421 VSSNKRVERAVPKEAPSNKRMSPQERKALATLGRRGGKKA-SERWKDPNSDYVQAELKKL 479
C.e.    424 VASNKRVEHAVVKENPTDTRMRPQERKALATLGRRGGKKA-AERWKDPESDYAKNQLETL 482
YS      407 VASNKRVEHAVVKENPTDTRMRPQERKALATLGRRGGKKA-AERWKDPESDYAKNQLETL 465

RepA II 481 AEINQRRKAQSRIGKRTVANVVDEYQIDFNRLPTLQELVSATGLSQSTVQRHLKKTGVALPRGRRSSQKRLH 552
C.g.    480 EAANRRRAVQGAGTRGRVLSIYSQTIVDRGSSPSARQIAEELGVTKRTVNMHLK----AL---REAGM--LN 542
C.e.    483 KKTQRKKKIQGQTTRARIQAFIGEQYINLGRVPTRKEIATEVGCTSRTVTTHLA----AL---RNAGLLPGE 547
YS      466 KKTQRKKKIQGQTTRARIQAFIGEQYINLGRVPTRKEIATEVGCTSRTVTTHLA----AL---RNAGLLPGE 530
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**Fig. 3.** Comparison of amino acid sequence of peptide coded by *repA* II of plasmid pBL90 with replicative proteins of several plasmids of corynebacteria. Shown are amino acid sequences of replicative proteins from plasmids of the following corynebacteria: C.g.—*Corynebacterium glutamicum*, GenBank no. NP_776231.1 (PM 12948627); C.e.—*C. efficiens*, GenBank no. WP_011069221.1; YS—*C. efficiens* YS-314, GenBank no. EEW50820.1. Amino acid residues identical in proteins are marked in black color; similar amino acid residues are marked in gray color.

simple duplication with further collection of mutations; it is possibly related to participation in formation of a cluster of mobile elements.

The presence of a high number of ORFs in plasmid pBL90 can also be explained by activity of mobile elements present in the plasmid. In the composition of the plasmid, there are two transposases with a high level of homology (90–95%) with transposases earlier found in *Corynebacterium* strains (table). One of them has also a high similarity (95%) at the level of the nucleotide sequence.

Thus, plasmid pBL90 with large size (67823 bp) found in the strain of *B. lactofermentum* DSM 1412 has a complicated structure. There are two replicative regions in it; each has similarity to plasmids related to different families of corynebacterial replicones and different mechanisms of replication [3]. In addition, in the plasmid, we detected ORFs the products of which are elements of the system of conjugative transfer, system of restriction–modification, and system of metabolic pathways of cells. This complicated structure of plasmid pBL90 can be the result of activity of mobile elements providing association of two replicons and transfer of different cell genes to the plasmid. The simultaneous presence of two plasmids (pBL90 and pBL1-like plasmid) in the *B. lactofermentum* DSM 1412 strain confirms their belonging to different groups of compatibility. Considering the fact that plasmid pBL1 is the basis for creation of vectors, identification of a plasmid of another group of compatibility

List of ORFs found in plasmid pBL90

| ORF | | Supposed protein | | Homology | |
|---|---|---|---|---|---|
| name | limits, bp | size, aa | function | GenBank no. | % of identity by amino acids |
| ORF 1 | 2281–893 | 462 | Conjugative transfer | WP_042392468.1 | 70 |
| ORF 2 | 5078–3594 | 494 | Function is not clear | WP_005326123.1 | 61 |
| ORF 3 | 6686–5082 | 534 | Function is not clear | WP_015453148.1 | 54 |
| ORF 4 | 11393–9423 | 656 | Cutinase | WP_051613677.1 | 41 |
| ORF 5 | 13133–11898 | 411 | Component of pyruvate dehydrogenase complex | | 90 |
| ORF 6 (*parA* I) | 16433–17215 | 261 | ATPase parA | WP_032406388.1 | 63 |
| ORF 7 | 17239–17679 | 146 | Function is not clear | WP_011091137.1 | 90 |
| ORF 8 | 17805–23999 | 2064 | Helicase | WP_030176357.1 | 40 |
| ORF 9 | 24873–26363 | 496 | Function is not clear | WP_018297448.1 | 33 |
| ORF 10 (*repA* I) | 26590–27552 | 320 | Replication of plasmid | WP_015453127.1 | 64 |
| ORF 11 | 30127–30372 | 81 | Hypothesized transcription regulator (answer to influence of xenobiotics) | WP_011091138.1 | 99 |
| ORF 12 | 32856–31594 | 420 | Glycosyltransferase | WP_050036060.1 | 45 |
| ORF 13 | 34412–32853 | 519 | Sulfate permease | WP_018297444.1 | 90 |
| ORF 14 | 34589–34993 | 135 | Hypothesized transcription regulator (answer to stress influence) | AJE68854.1 | 70 |
| ORF 15 (Transposon I) | 35344–35002 | ~114 | Transposase | WP_034666750.1 | 92 |
| ORF 16 (Transposon II) | 36279–35383 | 299 | Transposase | WP_019194348.1 | 97 |
| ORF 17 | 37693–36662 | 343 | Resolvases | WP_053546271.1 | 97 |
| ORF 18 | 39824–41212 | 462 | Function is not clear | CPV54868.1 | 62 |
| ORF 19 | 41205–43274 | 689 | Function is not clear | WP_032384845.1 | 68 |
| ORF 20 (HsdM I) | 45021–47312 | 763 | System of restriction–modification type I, HsdM-subunit | WP_006063140.1 | 36 |
| ORF 21 (HsdM II) | 47426–49036 | 536 | System of restriction–modification type I, HsdM-subunit | WP_011075868.1 | 83 |
| ORF 22 (HsdS) | 49033–50217 | 394 | System of restriction–modification type I, HsdSsubunit | ACL39088.1 | 50 |
| ORF 23 (HsdR) | 50318–53509 | 1063 | System of restriction–modification type I, HsdR-subunit | BAC19139.1 | 76 |
| ORF 24 *parA* II) | 53705–54379 | 224 | Allocation of plasmid DNA to daughter cells | WP_011091133.1 | 89 |
| ORF 25 (*parB*) | 54369–54668 | 99 | Allocation of plasmid DNA to daughter cells | WP_011091134.1 | 95 |
| ORF 26 (*repA* II) | 55111–56769 | 552 | Replication of plasmid | NP_776231.1 | 82 |
| ORF 27 | 59073–63161 | 1362 | Conjugative transfer | WP_053546272.1 | 97 |
| ORF 28 | 66212–64464 | 582 | Conjugative transfer | WP_015453156.1 | 58 |
| ORF 29 | 603–66807 | 540 | Endopeptidase of M23 family | WP_031264785.1 | 62 |

allows us to create a new batch of compatibility vectors for genetic construction in cells of corynebacteria.

## ACKNOWLEDGMENTS

This work was performed under financial support of the Ministry of Education and Science of the Russian Federation (unique project identifier RFMEFI62614X0003) with usage of UNU—National Bioresource Center Russian National Collection of Industrial Microorganisms.

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*Translated by V. Kharcheva*