Research Article

Characterization of Plasmid pPO1 from the Hyperacidophile Picrophilus oshimae

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Picrophilus oshimae and Picrophilus torridus are free-living, moderately thermophilic and acidophilic organisms from the lineage of Euryarchaeota. With a pH optimum of growth at pH 0.7 and the ability to even withstand molar concentrations of sulphuric acid, these organisms represent the most extreme acidophiles known. So far, nothing is known about plasmid biology in these hyperacidophiles. Also, there are no genetic tools available for this genus. We have mobilized the 7.6 Kbp plasmid from P. oshimae in E. coli by introducing origin-containing transposons and described the plasmid in terms of its nucleotide sequence, copy number in the native host, mode of replication, and transcriptional start sites of the encoded ORFs. Plasmid pPO1 may encode a restriction/modification system in addition to its replication functions. The information gained from the pPO1 plasmid may prove useful in developing a cloning system for this group of extreme acidophiles.

1. Introduction

Picrophilus torridus and Picrophilus oshimae are the most extreme organisms with respect to acidophilic combined with thermophilic lifestyle known to date. These species represent thermoacidophilic archaea, originally isolated from a dry solfataric field in Northern Japan [1]. Together with the genera Thermoplasma and Ferroplasma they form a phylogenetically distinct group of free-living, moderately thermophilic and acidophilic organisms within the Euryarchaeota. The two species of the Picrophilus genus are so far unsurpassed in their ability to grow at pH values around 0, with an optimum at pH 0.7. Also, P. oshimae has been shown to maintain an unusually low intracellular pH of 4.6, in contrast to other acidophilic organisms where this value is usually close to neutral [2]. P. torridus and P. oshimae share similar physiological properties and are morphologically indistinguishable. On the other hand, they differ in their DNA restriction fragment patterns, their 16S rDNA gene sequences, and the presence of extrachromosomal elements. Plasmids of 8.3 kb and 8.8 kb, which showed strong cross-hybridization in southern blot analysis, have been isolated from samples later assigned to P. oshimae but not from samples assigned to P. torridus [3]. Unlike the situation in the Sulfolobales order and especially in the genus Sulfolobus, where a large number of genetic elements have been characterized [4], little is known of extrachromosomal elements in the Thermoplasmatales. To date, the only sequenced and characterized plasmid from this phylogenetic order is pTA1 isolated from Thermoplasma acidophilum [5]. The analysis of plasmid pPO1 from P. oshimae reported here should prove useful in developing genetic tools for this group of organisms.

2. Materials and Methods

2.1. Strains and Plasmids. P. oshimae was obtained from DSMZ (DSM 9789) and was grown in a modified Brock’s medium with a pH of 0.7 at 55°C as described previously [3, 6]. The E. coli strain JM104 (pir⁺) was kindly provided by Professor Ruth Schmitz-Streit (University of Kiel, Germany). pPO1 was isolated from exponentially growing P. oshimae cells using a QIAprep Miniprep Kit (Qiagen), and total DNA used in real time PCR was prepared by the alkaline lysis method [7].
Table 1: Oligonucleotides used in this study.

| Primer         | Sequence (5’–3’)                        | Description                              |
|----------------|------------------------------------------|------------------------------------------|
| RT1.fors/rev   | AATATGGCCTGGAGATAGCG/AGACGACACTTCCGGATACG | Used in quantitative PCR for plasmid copy number determination |
| RT2.fors/rev   | ATCGACGATGCGCTACTCCT/GGGCAAGAGAAGCCATTAAAC |                                          |
| 16S.Pt.fors/rev| TGCCTCTCCAGATACAG/CCGGCATTTGTAATCTCCAG   |                                          |
| RC1.rev1/2     | TCGGATTTAAGCGGCGTCTA/CCTGAAGCTGCTTTATACAT | ORF1 inner/outer 5’/RACE                |
| RC2.rev1/2     | CAAAGGGTGGAGATGTATAGC/TCGAAGGCCAGCATTAGTG | ORF2 inner/outer 5’/RACE                |
| RC3.rev1/2     | TATCTCTGACCTTTATTTCC/TCCAGGGGCTTAAATGAT | ORF3 inner/outer 5’/RACE                |
| RC4.rev1/2     | TTTTCCTGTAATCCCTTACCC/ACATCCTTGGTGATGCCTTTC | ORF4 inner/outer 5’/RACE                |
| RC5.rev1/2     | TCGGAGTAGAATTTACCTGTAG/GGATAAGGATTACCTCTGTTAG | ORF5 inner/outer 5’/RACE                |
| RC6.rev1/2     | GCAATTAGGACAGGTCCGATAAC/ACTCACCACACCTACAC | ORF6 inner/outer 5’/RACE                |

2.2. Transposon Insertion and Sequencing of pPO1. Random insertions of an E. coli replication origin containing transposon in pPO1 were generated with the EZ-Tn5 (R6Kori/KAN-2) Insertion Kit (Epicentre Biotechnologies) as described by the manufacturer. E. coli JM104 was transformed with 1 μL of the transposition reaction and plated on LB medium supplemented with kanamycin (50 μg/mL). A total of 30 plasmids were recovered from the kanamycin-resistant colonies and were sequenced bidirectionally from the ends of the Tn5 transposons. The obtained sequences were assembled with the Staden Package software (http://staden.sourceforge.net/), and the remaining gaps were sequenced by the primer walking method. The complete plasmid sequence was determined on both strands with an average sequence coverage of 3.4.

2.3. Determination of pPO1 Copy Number. The copy number of pPO1 was determined with quantitative PCR (qPCR) by analyzing the ratio of plasmid to genomic DNA in P. oshimae cells grown to the beginning of the stationary phase. Two plasmid (RT1 and RT2) and one genomic (16S rRNA gene) loci were chosen for qPCR, and the determined amplification efficiencies (E) were $E_{RT1} = 1.58$ ($R^2 = 0.998$), $E_{RT2} = 1.95$ ($R^2 = 0.997$), and $E_{16S} = 1.62$ ($R^2 = 0.993$). The real-time PCR measurements were carried out in triplicate with two independent preparations of total P. oshimae DNA which permitted the estimation of the intra- ($C_{intra}$) and interassay ($C_{inter}$) coefficients of variation, $C_{intra} = 1.32\%$ and $C_{inter} = 2.02\%$. These assays were performed using SYBR green (qPCR MasterMix Plus for SYBR green I with fluorescence, Eurogentec) on an iCycler (Bio-Rad); the primers used are listed in Table 1.

2.4. Sequence and Transcriptional Start Sites Analyses. ORF prediction was performed on the EasyGene prediction server (http://www.cbs.dtu.dk/services/EasyGene/); the nucleotide and protein sequences and conserved domains were searched with the NCBI database using the programs BLAST and CDART, respectively. Repeats were identified with the Tandem Repeats Finder program [8]. In order to determine the transcription start sites of the pPO1 ORFs, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was used [9], using the protocol of the manufacturer (FirstChoice RLM-RACE kit; Ambion). Two independent replications of the 5’–RLM-RACE procedure were carried out, including a control without tobacco acid pyrophosphatase treatment. The cDNA obtained was subjected to nested PCR with SuperTaq Plus DNA polymerase (Ambion) using the primers listed in Table 1; the obtained PCR products were column purified (QIAquick PCR purification; QIAGEN) and cloned using a StrataClone PCR cloning kit (Stratagene). A total of 10 colonies per ORF were analyzed, and the plasmid inserts were sequenced in both directions with an ABI 3700 sequencer (Applied Biosystems).

3. Results and Discussion

3.1. Structural Features and Open Reading Frames in pPO1. Complete sequencing of the plasmid pPO1 from P. oshimae resulted in a circular molecule of 7646 bp (GenBank accession number JN032732) and a G + C content of 30.5%. This is considerably lower than the G + C content value of 36% reported for the genomes of P. oshimae (HPLC data, [3]) as well as for P. torridus (genome sequencing data, [10]). Two oppositely situated intergenic regions in pPO1, IG1 and IG2, were found to deviate significantly from the average G + C content (Figure 1). Interestingly, no Tn5 transposon insertions were found in these regions among the 30 plasmids used for sequencing. A possible explanation for the observed bias could be the formation of stable secondary DNA structures rather than the local G + C content which can prevent Tn5 transposition [11]. The intergenic region IG1 was also found to be rich in repeated sequences. A total of four tandem repeats were detected, two of which were localized in IG1 (Table 2).

The nucleotide sequence displayed no regions of homology to other archaeal plasmids or to the genome of P. torridus. A total of 6 ORFs longer than 100 amino acids could be predicted from the nucleotide sequence. ORFs 1 to 4 contained domains which could be assigned to known COG and/or PFAM families (Table 4). ORF 3 appeared to encode a protein of 42.4 kDa which showed the sequence characteristics of an archaeal Orc1/Cdc6 cell division control protein and displayed a high level of amino acid sequence similarity (38% identity) to the Orc1/Cdc6 homologue found on the pTA1 plasmid from T. acidophilum.
Table 2: Tandem repeats in pPO1.

| Tandem repeat | Consensus sequence | Coordinates | Period size | Copy number |
|---------------|--------------------|-------------|-------------|-------------|
| TR1           | TTAATTAAAAAAAATAAAAATAT | 283–328     | 24          | 2.0         |
| TR2           | ATAAATATATATA       | 384–431     | 11          | 3.8         |
| TR3           | AAGTAATGTAGTAAGAGTGT | 6382–6431   | 21          | 2.3         |
| TR4           | AAAAGTAAAAGGTAAGTATAGTGTGTTAAAACCTC | 6452–6530 | 42          | 1.9         |

Table 3: Transcriptional start sites (TSS) of pPO1. ORFs identified by 5' RLM-RACE. The start codons of the ORFs are in bold, the TSS are preceded by an asterisk.

| ORF-1 | ORF-2 |
|-------|-------|
| ...ATCAATTATTCTCTGTTTTACCTTTTACTTTT*TACTATTTTTATT TACTCATTATAATAATTAGGTATTAGTATATATGTA GACGCCCTGTAAATCCGAGTTTATGCAAGACTAAATTACGAC TAC... |
| ...TAAAGGGCATGTGAAA*GAACCTAATACAAACAAAGGCGTAC AATGAAATTTCATTTTTAACCTAATCAGACCTAAATATTTAGAGAAGAGGTACGATTACAGGCCTTGAATCCGAGTTTTAATTGCAAGAC TAC... |
| ORF-5 | ORF-6 |
| ...TATGACAAAGCATCAGAATTAGGTGAGGGGCAAGG ATGATTTCATTACAATCTTTTGGGAAAGGTCTAATGCAAGACAACAGTTAATGAAAAATGAGTTTTAATATAATGAGTGATTA... |
| ...CATTAAGGGTTGCGAAAGGATGAAA*GATACAGAG GAATATAAGTGGAATGAAATGAATGAAGTAGATAAGGGTTATACCTAATTACACTCACTTATACACTTATATTAGTGATATATACGAC... |

[5]. Although Rep proteins are most often implicated as replicator initiators of plasmids, no homolog of a known Rep protein could be identified in pPO1. We could not detect single-stranded DNA intermediates in cell extracts of exponentially growing P. oshimae, neither by nondenaturing southern hybridization nor by S1 nuclease-based protection assay [12] (data not shown). Therefore, most probably pPO1 replicates via a theta mechanism, and ORF 3 is likely to be a replication initiator. The localization of ORF3 and the intergenic region IG1 further supports this prediction as IG1 carries the characteristics of an archaeal replication origin site (oriC). Archaeal oriC sites are typically AT-rich, repeat-containing intergenic regions and are most often located upstream of genes coding for Orc1/Cdc6 homologs [13]. Similar overall architecture of the Orc1/Cdc6 ORF and the oriC site can be found in the pTA1 plasmid from the related T. acidophilum [5]. Clearly, further experiments are needed to unequivocally determine the mode and origin of replication for both pPO1 and pTA1.

Additionally, pPO1 seemed to encode genes for a restriction/modification system (R/M system, ORFs 5 and 2) and for a recombinase (ORF 4). Notably, the ORFs of the putative R/M system were found to have homologs only in the bacterial lineage, the ones with the highest level of amino acid sequence similarity belonging to the group of high GC Gram positive Bacteria.

3.2. Plasmid Copy Number Determination. pPO1 copy number was determined by measuring the abundance of two plasmid DNA loci relative to a genomic DNA locus using quantitative real-time PCR. The results obtained for the two plasmid loci were in good agreement with each other, that is, 11.8 ± 3.6 copies per genome equivalent for RT1 and 15.4 copies ± 2.7 for RT2. The DNA samples used for these measurements were from cultures grown to the beginning of the stationary phase.

Figure 1: Schematic map of the pPO1 plasmid. Arrows designate ORFs, repeats are marked as triangles, and deviation from the average GC-content is plotted on the inner circle.
3.3. RACE Analysis of Transcriptional Start Sites. The transcrip-
tional start sites (TSS) of four of the pPO1 ORFs could be
mapped by the RLM-RACE procedure (Table 3). No PCR
products were obtained for ORFs 3 and 4, most probably
indicating low levels of transcription of these ORFs under
the experimental growth conditions. Despite the colinear
organisation of ORFs 1, 6, and 4 and in addition the overlap
of ORFs 3 and 2, no polycistronic RNA could be detected by
5’ RLM-RACE.

3.4. Potential of pPO1 for Vector Development. To date no
cloning system or transformation method is available for
the hyperacidophilic organisms of the Picrophilus genus.
Plasmid pPO1 could now serve as the starting point for
development of a recombinant cloning vector. Judging from
the pPO1 sequence, the size of the plasmid regions needed for
autonomous replication to be included in the construction of
a shuttle vector can presumably be reduced considerably, for
example, by deletion of the R/M system genes. A host/vector
system for the genetic modification of Picrophilus strains
will help to study in more detail the basis of extreme ther-
moacidophilic adaptation in these unique archaeal microor-
ganisms which are capable of life around pH 0.

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Table 4: Annotation of the predicted ORFs of pPO1 and assignment to existing COGs.

| ORF | COG family (description) | Annotation |
|-----|--------------------------|------------|
| 1   | COG0433, (FtzK, DNA segregation ATPase FtsK) | FtzK-related protein |
| 2   | COG0863, (DNA modification methylase) | Putative N6–N4 Mtase |
| 3   | COG1474, (Cdc6-related protein, AAA superfamily ATPase) | Cdc6 |
| 4   | COG1961, (PinR, site-specific recombinases) | DNA invertase/resolvase family protein |
| 5   | No | Type II restriction enzyme |
| 6   | No | Beta-subunit of acetyl-CoA carboxylase |