Defining Components of the Chromosomal Origin of Replication of the Hyperthermophilic Archaeon *Pyrococcus furiosus* Needed for Construction of a Stable Replicating Shuttle Vector[▽][†]

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We report the construction of a series of replicating shuttle vectors that consist of a low-copy-number cloning vector for *Escherichia coli* and functional components of the origin of replication (*oriC*) of the chromosome of the hyperthermophilic archaeon *Pyrococcus furiosus*. In the process of identifying the minimum replication origin sequence required for autonomous plasmid replication in *P. furiosus*, we discovered that several features of the origin predicted by bioinformatic analysis and *in vitro* binding studies were not essential for stable autonomous plasmid replication. A minimum region required to promote plasmid DNA replication was identified, and plasmids based on this sequence readily transformed *P. furiosus*. The plasmids replicated autonomously and existed in a single copy. In contrast to shuttle vectors based on a plasmid from the closely related hyperthermophile *Pyrococcus abyssi* for use in *P. furiosus*, plasmids based on the *P. furiosus* chromosomal origin were structurally unchanged after transformation and were stable without selection for more than 100 generations.

Pyrococcus species are hyperthermophilic marine archaea that grow anaerobically at temperatures near and above 100°C (19). Their interesting biology, evolutionary history, and potential commercial utility make them an important group to study (9, 47). We recently reported an efficient method for DNA transformation for *Pyrococcus furiosus* (27), which provides the basis for the development of methods for genetic manipulation. A markerless deletion of the orotidine monophosphate (OMP) decarboxylase (*pyrF*) locus was constructed in the *P. furiosus* genome, generating a mutant that is a uracil auxotroph and resistant to 5-fluroorotic acid (5FOA). Complementation by the wild-type *pyrF* allele restored uracil prototrophy and 5FOA sensitivity to this strain. We took advantage of this marker to construct replicating shuttle vectors for use in *P. furiosus*.

Replicating vectors facilitate a variety of genetic manipulations, and vectors capable of shuttling between any host of interest and *Escherichia coli*, perhaps the most convenient biological host for DNA manipulation, are especially useful. Naturally occurring plasmids have been identified in archaeal species (7, 10–12, 16, 22, 51), and some of them have been used to develop shuttle vectors in the haloarchaea (25) and methanogens (49). A shuttle vector between *E. coli* and *Thermococcus kodakarensis*, a close relative of *P. furiosus* (4, 36), was reported, which combined a commercial *E. coli* cloning vector with a naturally occurring plasmid, pTN1, from *Thermococcus nautilus* (44, 46). This plasmid was used successfully to express

a hemagglutinin (HA) epitope-tagged version of RpoL, a subunit of *T. kodakarensis* RNA polymerase, illustrating the utility of such vectors (44). Plasmid pGT5, a naturally occurring plasmid in *Pyrococcus abyssi* (17), was used to create a shuttle vector capable of replicating in both *P. abyssi* and *E. coli* (29), and most recently, a shuttle vector based on pGT5 was used to express a gene encoding His$_6$-tagged subunit D of RNA polymerase in *P. furiosus* (50). Our attempts to construct a stable shuttle vector for *P. furiosus* based on pGT5 were unsuccessful.

Pyrococcus species are predicted to have a single origin of replication with both bacterial and eukaryotic features (28, 37). Other archaea (5, 30, 42) have multiple replication origins, and eukaryotes have hundreds or even thousands of replication origins (41). Eukaryotic replication origins are poorly defined in terms of sequence as well as the number and nature of auxiliary proteins that facilitate their function, and there is increasing evidence that origin maintenance in many eukaryotes is controlled by epigenetic factors whose function is also poorly understood (3, 45). *Saccharomyces cerevisiae* is a notable exception in that it has well-defined origins called autonomously replicating sequence (ARS) elements, generally 100 to 200 bp in length, containing multiple *cis*-acting sequence elements (38) and binding sites for the origin recognition complex (6). This complex is composed of origin recognition complex (Orc) proteins 1 to 6 (6) and a DNA binding protein, Cdc6, first identified in yeast as a gene whose product is involved in cell cycle control (15, 23). Most bacteria have a single origin sequence flanked by polymerases and *dnaA* (35), which encodes a protein that binds DNA within the origin (20) and functions to recruit DnaB and DnaC to form the replication initiation complex. While DnaA is only distantly related to Cdc6, it provides the same function in nucleating the protein complex. Replication origin regions in both bacteria and archaea are AT rich (5, 41).

The origin of replication in several archaeal genera was first

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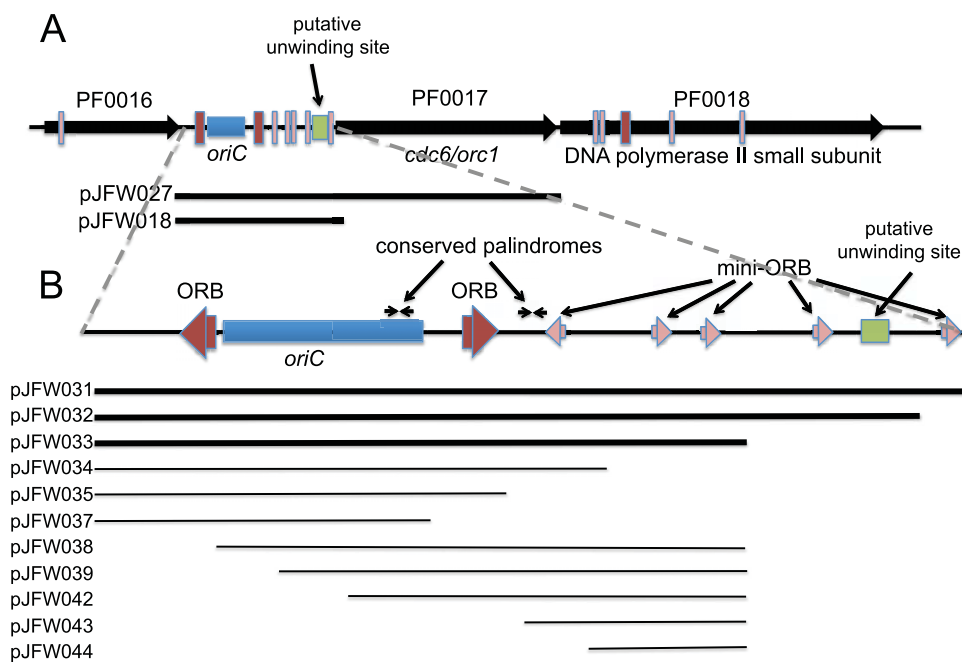
FIG. 1. Region of the *P. furiosus* chromosome predicted to contain the origin of replication, *oriC* (A), with an expanded view of the intergenic space (B). ORBs are indicated in red, mini-ORBS are shown in pink, and the putative unwinding site is shown in green. The chromosomal regions that were cloned into pJFW017 to produce various plasmids are indicated by black lines below the diagrams. Conserved palindromes are marked by inverted black arrows. Inserts that resulted in plasmids capable of autonomous replication in *P. furiosus* are indicated by thick black lines.

predicted by the cumulative GC skew, an early bioinformatic method used to find prokaryotic replication origins (21). In *P. abyssi* the location of the chromosomal replication origin was predicted to be within an ~80-kb region that contained a large intergenic space and genes for several putative replication proteins (28). This intergenic space, neighboring genes, and features are conserved in *P. furiosus* (Fig. 1). While the organization of putative protein binding sites and replicative proteins around the *P. furiosus oriC* is bacterial in nature, the proteins that likely bind the sequences are eukaryotic. In the location where *dnaA* often resides in bacteria, there is a single gene homologous to the *cdc6/orc1* eukaryotic replication proteins (40). Previous studies of the function of the origin sequences of *Halobacterium* sp. strain NRC-1 *in vivo* showed that a 750-bp region containing *oriC* requires the adjacent *orc7* gene in *cis* for autonomous plasmid replication (8). In *Sulfolobus solfataricus*, there are three origins of replication (30, 42), and for each origin of replication, a *cdc6* gene is adjacent but is not required in *cis* for the origin to function in plasmid replication (14). Evidence for the location of the *P. furiosus* origin and for the role of putative replication proteins is based entirely on bioinformatic and *in vitro* analyses. Cells emerging from replication arrest were shown previously to incorporate radiolabeled nucleotides in the DNA sequence at the predicted origin (37). Chromatin immunoprecipitation of the Cdc6/Orc1 protein showed a significant enrichment of the predicted *oriC* DNA region (32), and DNA binding studies showed that the *Sulfolobus* Cdc6/Orc1 protein binds this region of *P. abyssi* as well as *Sulfolobus* DNA *in vitro* (33, 42). The sequence of the sites of protein binding (referred to as origin recognition boxes [ORBs]) suggested that inverted repeat elements flanking the predicted origin, conserved among archaea, were involved in

replication complex formation and binding. The binding of the Cdc6/Orc1 protein to the intergenic space resulted in an unwinding of the DNA, supporting the notion that this was the location of the origin (34). While the intergenic space is AT rich, the DNA sequence is not conserved across *Pyrococcus* species. Further evidence for the position of the origin comes from the fact that DNA at the origin contains a transient replication bubble produced by bidirectional DNA polymerization, and the structure may be isolated by displaying digested total genomic DNA, from actively dividing cells, on two-dimensional gels. Sequences containing a replication bubble were located to a 1-kb fragment that included the intergenic space, possibly overlapping the *cdc6* gene (37). Bioinformatic analyses identified several 13-bp mini-ORB repeats in and around the intergenic space suggested previously to be involved in origin recognition and potential Cdc6/Orc1 binding (26, 33).

Here we present an *in vivo* analysis of sequences at the origin of DNA replication in the *P. furiosus* chromosome and their function in autonomous plasmid replication. DNA fragments containing the putative chromosomal origin as well as predicted protein binding sites and the *cdc6/orc1* gene were cloned into an *E. coli* plasmid vector and tested for the ability to promote autonomous DNA replication in *P. furiosus*. The cloning of the origin sequence without the *cdc6/orc1* gene did not affect its ability to function, suggesting that this protein, while likely required for DNA replication, is not required in *cis*. The location of the origin predicted from *in vitro* analyses was confirmed. Only two of the predicted ORB sequences, however, are required for autonomous plasmid replication. We used this origin sequence in combination with the pSC101 origin from *Salmonella enterica* serovar Panama (13) for rep-
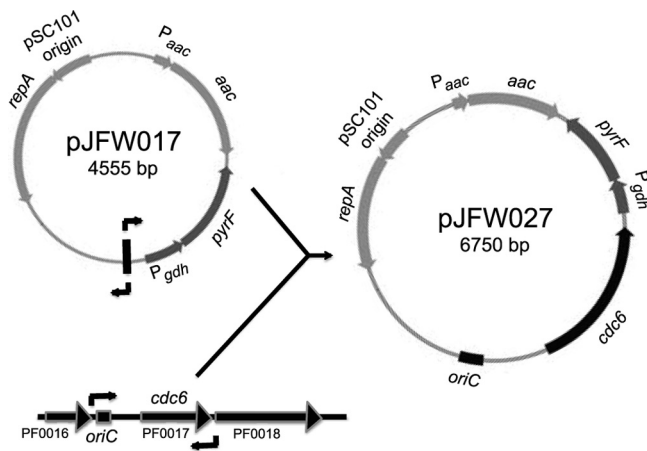
FIG. 2. Construction of pJFW027. A linear DNA fragment containing the entire sequence of pJFW017 was generated by PCR amplification using primers JF266 and JF267 and ligated into the origin fragment indicated in Fig. 1, also generated by PCR amplification using primers JF268 and JF282. Plasmids containing the various origin fragments described in the legend of Fig. 1 were cloned into pJFW017 for testing.

lication in *E. coli* to construct a replicating shuttle vector for *P. furiosus* that is stable in a single copy without selection for more than 100 generations and is structurally unchanged after transformation into *P. furiosus* and back-transformation into *E. coli*.

MATERIALS AND METHODS

Strains, media, and growth conditions. *E. coli* strain DH5α was used for plasmid DNA constructions and preparations. Standard techniques for *E. coli* were performed as described previously (43). Apramycin was used for selection at 50 mg/ml. Wild-type strain *P. furiosus* DSM 3638 (19) and the *P. furiosus* COM1 Δ*pyrF* strain (27) were grown anaerobically in a defined medium with cellobiose as the carbon source (27) at 90°C for 16 to 20 h in 100-ml serum bottles containing 50 ml of liquid medium or on medium solidified with Phytagel (1%, wt/vol; Sigma) for 60 h. The *P. furiosus* COM1 Δ*pyrF* strain was used as a host for all DNA transformation experiments. *P. abyssi* strain GE5 (16, 18) was grown in a liquid base salts medium (1) containing 0.5% (wt/vol) casein hydrolysate and 0.2% (wt/vol) elemental sulfur for 40 to 48 h at 90°C under anaerobic conditions. Total genomic DNA was isolated as described previously (27), except that DNA was precipitated with isopropanol and resuspended with 50 μl TE buffer (10 mM Tris, 1 mM EDTA) containing RNase A (100 ng/ml).

Construction of vectors and transformation of *P. furiosus*. To construct pJFW027 and pJFW018, PCR products containing the indicated regions of the chromosome (Fig. 1) were ligated into a linear DNA fragment containing the entire pJFW017 plasmid (Fig. 2), also generated by PCR using primers JF266 and JF267. To generate plasmids pJFW031 to pJFW044, primers with restriction sites added to the 5′ ends were used to allow the digestion and subsequent directional cloning of origin-containing fragments into pJFW017. The 5′ end of each fragment contained a BamHI site, and the 3′ end contained a ClaI site. The PCR amplification of pJFW017 was done by use of primers JF266.2 and JF267.2 with the same restriction sites. Primers used in these constructions are listed in Table 1, and DNA sequences of the primers are shown in Table S1 in the supplemental material. *E. coli* strain DH5α cells were transformed by electroporation in a 2-mm-gap cuvette at 2.5 V. Plasmid DNA was isolated from liquid cultures by using QIAprep Spin Miniprep columns (Qiagen Inc.). For DNA transformations, the *P. furiosus* COM1 Δ*pyrF* strain was grown for 16 to 20 h in defined liquid medium containing 20 μM uracil. Plasmid DNA (100 to 200 ng) was added to 100 μl of culture and plated onto the defined medium without uracil. Prototrophic colonies were inoculated into liquid medium for DNA isolation. The presence of plasmid sequences in *P. furiosus* was confirmed by PCR amplification of the *aac* gene, present only on the plasmid, from *P. furiosus* total genomic DNA by using primers JF263 and JF264 (Table 1).

Assessment of plasmid maintenance, stability, and copy number. To assess plasmid maintenance, *P. furiosus* transformants were serially subcultured every 24 h for 10 days in selective and nonselective liquid media. After each passage, the culture was diluted 100-fold with base salts, and 30 μl of diluted culture was plated onto selective medium to determine the number of prototrophic colonies, i.e., those maintaining the plasmid. The cell density of the liquid culture was determined by direct cell counting using a Petroff-Hausser counting chamber. To assess the structural stability of the plasmid, total genomic DNA isolated from five independent *P. furiosus* transformants containing pJFW027 was used to back-transform *E. coli* for plasmid isolation and restriction digestion analysis. To determine plasmid copy numbers, total genomic DNA was isolated from *P. furiosus* plasmid transformants and digested twice with 10 U of HpaI for 120 min at 37°C. The restriction fragments were separated by electrophoresis in a 1.0% (wt/vol) agarose gel and transferred onto nylon membranes (Roche, Manheim, Germany). Primers GL021 and GL023 (27) were used to amplify the glutamate dehydrogenase (*gdh*) promoter from wild-type *P. furiosus* total genomic DNA to generate a digoxigenin (DIG)-labeled probe by random priming with DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Manheim, Germany). The membrane was incubated at 42°C and washed at 65°C. Band intensities were determined by using a Storm 840 PhosporImager (GE Healthcare) equipped with ImageQuant v.5.4 software (Molecular Dynamics).

RESULTS AND DISCUSSION

The *P. furiosus* chromosomal replication origin functions for stable autonomous plasmid replication. Attempts to construct a stable replicating shuttle vector based on plasmid pGT5 from *P. abyssi* for use in *P. furiosus* were unsuccessful. Plasmids based on pGT5 exist in high copy numbers in *P. abyssi* (29, 50) but show a significantly reduced copy number in *P. furiosus* (50) and cannot be used for the selection of transformants in the closely related *Thermococcus kodakarensis* (44). In an attempt to construct a shuttle vector based on pGT5, the entire plasmid was cloned into pJFW017 that contained a pSC101 origin for replication in *E. coli*, an apramycin resistance gene for selection in *E. coli*, and a wild-type copy of the *P. furiosus* *pyrF* gene for the selection of transformants in the *P. furiosus* COM1 Δ*pyrF* strain. A fragment containing the entire pGT5 plasmid sequence was produced by PCR amplification with primers JF254 and JF270, linearizing the plasmid at a site within pGT5 previously shown not to interfere with replication functions (17, 31), to produce pJFW019 (see Fig. S1 in the supplemental material). This plasmid readily transformed

TABLE 1. Plasmid transformation efficiencies[a]

Plasmid	*oriC* insert position	5′ primer	3′ primer	Transformation efficiency (no. of transformants/μg of plasmid DNA)
pJFW017	None			8.2×10^2
pJFW018	15382–16226	JF268	JF269	9.1×10^5
pJFW027	15382–17576	JF268	JF282	5.8×10^5
pJFW031	15382–16228	JF268.2	JF269.2	6.6×10^5
pJFW032	15382–16187	JF306.2	JF269.2	8.0×10^4
pJFW033	15382–16034	JF305.2	JF269.2	6.5×10^5
pJFW034	15382–15890	JF304.2	JF269.2	5.7×10^2
pJFW035	15382–15786	JF303.2	JF269.2	1.4×10^3
pJFW037	15382–15705	JF301.1	JF269.2	1.4×10^2
pJFW038	15492–16034	JF305.2	JF339	4.7×10^1
pJFW039	15561–16034	JF305.2	JF345	$<4.7 \times 10^1$
pJFW042	15746–16034	JF305.2	JF348	$<4.7 \times 10^1$
pJFW043	15813–16034	JF305.2	JF349	$<4.7 \times 10^1$

[a] Genomic locations are based on the numbering convention of the *Pyrococcus furiosus* (accession number NC_003413.1) genome sequence deposited in GenBank. The detection threshold was 4.7×10^2 transformants per μg of plasmid DNA.

TABLE 2. Maintenance of plasmids in *P. furiosus*[a]

Passage	pJFW018		pJFW027		pJFW019	
	+ura	−ura	+ura	−ura	+ura	−ura
1	187	256	190	194	73	112
2	132	217	97	203	54	268
3	112	232	132	143	11	138
4	146	117	154	165	6	83
5	87	276	100	113	0	77
6	138	197	151	201	0	104
7	144	263	111	122	0	91
8	118	242	87	131	0	94
9	93	213	114	218	0	97
10	107	183	112	169	0	87

No. of cells (header spanning pJFW018, pJFW027, pJFW019)

[a] Transformants containing each plasmid were serially passaged in liquid medium with uracil (+ura) or without uracil (−ura). Following each passage, a diluted culture was plated onto selective medium to determine the number of prototrophic cells remaining.

P. furiosus but was rapidly lost without selection (Table 2) and showed internal deletions after transformation into *P. furiosus* and subsequent back-transformation into *E. coli* (Fig. S2). Other attempts to use pGT5 for the construction of shuttle vectors in *T. kodakarensis* were similarly unsuccessful (44).

To test whether the predicted *P. furiosus* chromosomal origin of replication could promote stable autonomous plasmid replication, a fragment of the chromosome containing the predicted origin sequence and the gene encoding Cdc6/Orc1 (Fig. 1) was cloned into an *E. coli* plasmid, pJFW017 (Fig. 2), to make pJFW027. We used the transformation efficiency as an assay for plasmid replication (8). As shown in Table 1, transformants of pJFW027 were observed at a frequency of 5.8×10^5 transformants per µg of plasmid DNA. No transformants were observed in the absence of added plasmid DNA, and while some transformants were obtained in experiments with pJFW017, which does not contain an origin sequence (8.2×10^2 transformants per µg of plasmid DNA), this is most likely due to integration by homologous recombination between the *gdh* promoter region (283 bp), driving the transcription of the *pyrF* gene on the plasmid, and the *gdh* locus in the chromosome. In fact, we have observed the integration of nonreplicating plasmid DNA by homologous recombination at the same frequency (27). The transformation frequency of pJFW027 was a thousandfold greater than that of pJFW017, indicating that the plasmid was replicating autonomously. PCR amplification of the apramycin resistance gene, contained only on the plasmids, was used to confirm the presence of plasmid DNA in the transformants. A 950-bp product containing this sequence was obtained from transformant total genomic DNA but not from the wild-type or the *P. furiosus* COM1 Δ*pyrF* strain (see Fig. S3 in the supplemental material).

Attempts to isolate a significant quantity of pJFW027 plasmid DNA from *P. furiosus* were unsuccessful. This is perhaps not surprising, since quantities of plasmids based on the chromosomal origin would be expected to be low or 1 copy per chromosome. In lieu of direct plasmid isolation, we chose to rescue the plasmid by back-transformation to *E. coli*. That transformants contained a replicating copy of the plasmid was shown by using total genomic DNA isolated from 5 independent plasmid transformants to back-transform *E. coli* strain DH5α selecting for apramycin resistance. Back-transformants were obtained at frequencies of 10^4 transformants per µg of DNA, an underestimate of the plasmid transformation, since this frequency is based on the number of transformants per microgram of total genomic DNA, and only covalently closed circular plasmid DNA is capable of transforming *E. coli* strain DH5α at this frequency (24). Plasmid DNA isolated from these back-transformants was indistinguishable from the pJFW027 plasmid DNA used to transform *P. furiosus* by using restriction digestion analysis, indicating that there were no gross rearrangements during transformation and replication in *P. furiosus* or subsequent back-transformation to *E. coli*. When the P_{gdh} fragment, specific to plasmid pJFW027, was used as a probe for the Southern hybridization of total genomic DNA from the *P. furiosus* transformants with DNA digested with either EcoRV (data not shown) or HpaI (Fig. 3), which have a single cleavage site within the plasmid, a single band was detected, showing that the plasmid DNA was not integrated into the chromosome and existed as an autonomously replicating molecule.

To examine plasmid maintenance, transformants of pJFW027 and pJFW018 were serially subcultured in liquid medium with or without selection (i.e., in the absence or presence of uracil) for more than 100 generations and then plated onto minimal medium without uracil to assay plasmid maintenance. No loss of plasmids with *oriC* was detected even without selection (Table 2). In addition, the restriction pattern of plasmid DNA isolated from *E. coli* after transformation into *P. furiosus* and subsequent transformation back into *E. coli* remained unchanged, indicating that no rearrangements of the plasmid DNA occurred (Fig. 4 [data for 1 of 10 transformants tested are shown]).

The *cdc6/orc1* open reading frame is not required in *cis* for replication origin function. In bacteria, *oriC* is often, but not always, adjacent to *dnaA*. In *E. coli*, *oriC* is between *gidA* and *mioC* (which encodes another replication protein), approximately 43 kb from *dnaA*, and can function for the autonomous
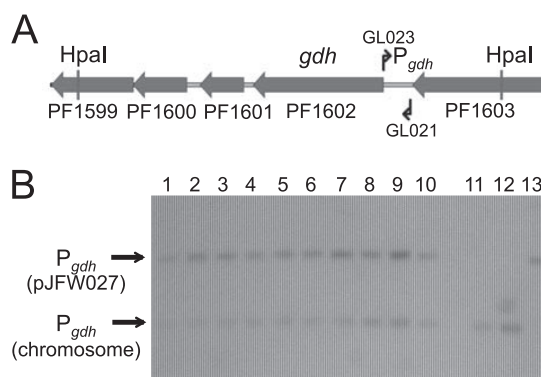

FIG. 3. Determination of copy number for pJFW027 in *P. furiosus*. (A) Diagram of the chromosomal region, including the *gdh* open reading frame. HpaI sites are indicated, as are the locations of primers used to generate the *gdh* hybridization probe. (B) Southern blot of pJFW027 transformants. Lanes 1 to 10, DNA isolated from transformants and digested with HpaI; lanes 11 and 12, DNAs from *P. furiosus* wild-type and COM1 Δ*pyrF* strains, respectively; lane 13, pJFW027 plasmid DNA purified from *E. coli*.
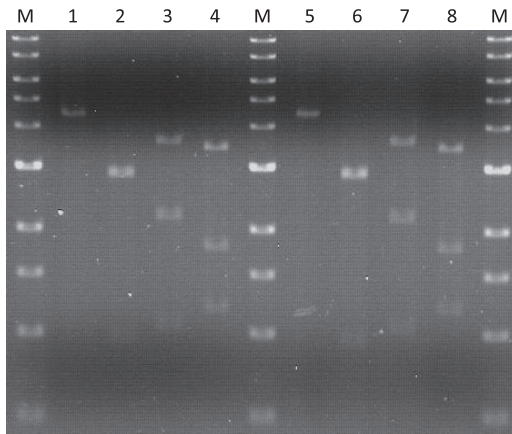
FIG. 4. Restriction analysis of plasmid DNA before and after transformation of *P. furiosus* and back-transformation to *E. coli*. Lanes M, 1-kb DNA ladder; lanes 1 to 4, pJFW018 plasmid DNA isolated from *E. coli* DH5α (lane 1) and digested with AccI (lane 2), AvaI (lane 3), and HindIII (lane 4); lanes 5 to 8, plasmid DNA isolated from *E. coli* DH5α back-transformed from *P. furiosus* transformants (lane 5) and digested with AccI (lane 6), AvaI (lane 7), and HindIII (lane 8).

replication of plasmids without *cis*-acting replicating components (39, 48). In the chromosome of *Halobacterium* NRC-1, *oriC* requires the adjacent *orc7* gene in *cis* for autonomous plasmid replication (8). In *Sulfolobus solfataricus*, there are three origins of replication, and for each origin of replication, a *cdc6* gene is adjacent but is not required in *cis* for the origin to function in autonomous plasmid replication (14). In the sequenced *Pyrococcus* species *P. furiosus*, *P. abyssi*, and *P. horikoshii* as well as the closely related *T. kodakarensis*, there is a single *oriC* adjacent to a *cdc6*/*orc1* homologue, but nothing is known about the requirement of this protein for *oriC* function. To test whether *cdc6*/*orc1* was required in *cis* for autonomous plasmid replication in *P. furiosus*, a fragment containing only *oriC* was cloned into parent plasmid pJFW017 to generate pJFW18 (Fig. 1). As shown in Table 1, plasmids containing the fragment with only the *oriC* sequence without the *cdc6*/*orc1* gene transformed *P. furiosus* as efficiently and were maintained as stably as plasmid pJFW27 carrying the *cdc6*/*orc1* gene, suggesting that the *cdc6*/*orc1* gene is not required in *cis* for stable autonomous plasmid replication.

Only two of the predicted ORB sequences and part of the predicted chromosomal origin sequence are required for plasmid replication. The origin region was predicted previously to contain several ORB and mini-ORB sequences (33), suggested to be binding sites for the replication initiation protein Cdc6/Orc1, which is presumed to facilitate the nucleation of the replication complex. Our analysis using the BLASTN 2.2.24+ algorithm (2) identified three ORB repeats and several mini-ORB repeats by the self-alignment of the sequence of the genomic region containing *oriC* and neighboring genes. These results are similar but not identical to those described previously by Matsunaga et al. (33), in that we found a clustering of mini-ORB repeats in and around *oriC*, but the exact number and position of these mini-ORB repeats were different. In addition, we identified two conserved palindromic sequences (Table 3) conserved in all sequenced *Pyrococcus* species. One of them contains compensating changes within the sequence

that retain the perfect palindromic structure, suggesting that these are not random sequences within this highly repetitive region of DNA and may potentially be binding sites for other replication proteins or have a structural role in replication. These palindromes are not present in the *oriC* region of the closely related *Thermococcus* species, however, suggesting that if they have a function, it may be specific to *Pyrococcus*. To test whether these sequences were required for autonomous plasmid replication, plasmids containing various portions of the region around the predicted origin were constructed and tested for the ability to replicate. The smallest insert able to promote autonomous plasmid replication was the 653-bp fragment cloned into pJFW033. As shown in Table 1, only two of the three ORB sequences, and only a part of the sequence predicted to contain the origin, were required for plasmid replication. The predicted unwinding site, for example, is apparently not required for autonomous plasmid replication.

Replicating shuttle vectors based on the chromosomal origin exist in single copies. To determine the approximate copy number of the *oriC*-based plasmids, a PCR product generated from the P_{gdh} promoter was used as a probe in Southern hybridization experiments with total genomic DNA from *P. furiosus* wild-type cells and pJFW027 transformants. Since P_{gdh} is present in one copy both on pJFW027 and in the *P. furiosus* chromosome, a densitometry analysis of the amount of DIG-labeled probe hybridized to each one allowed an estimation of the number of plasmid copies per chromosome (Fig. 3). The relative intensities of the plasmid-derived hybridization signal to the chromosomally derived hybridization signal of P_{gdh} for the EcoRV and HpaI digests ranged from 1.4 to 1.8 for 10 transformants tested, indicating that the *oriC*-based plasmids exist in a single copy per chromosome.

Conclusions. The functional analysis of the replication origin of the *P. furiosus* chromosome reported here showed that only two of the three ORB sequences, those flanking an AT-rich sequence most conserved in arrangement and sequence among the *Archaea* (42), and no more than three of the mini-ORB sites are required for autonomous plasmid replication. In particular, the DNA-unwinding site, predicted by P1 endonuclease assays (34), is not required for autonomous plasmid

TABLE 3. Conserved palindromic sequences within the
Pyrococcus oriC region[a]

Species (GenBank accession no.)	Sequence	Genomic position
Palindrome 1		
P. furiosus (NC_003413.1)	ATATTTAAATAT	15641–15674
P. abyssi (NC_000868.1)	TATTTAAATA	123223–123232
P. horikoshii (NC_000961.1)	TATTTAAATA	111307–111316
Palindrome 2		
P. furiosus (NC_003413.1)	ATTAgaTTAAtcTAAT	15809–15824
P. abyssi (NC_000868.1)	ATTAagTTAAccTAAT	123072–123087
P. horikoshii (NC_000961.1)	ATTAagTTAActTAAT	111155–111170

[a] Base differences are indicated by lowercase type; underlining indicates a base that deviates from the palindrome consensus. Genomic locations are based on the numbering of the genome sequences deposited in GenBank.

replication, nor are any of the predicted ORB or mini-ORB sequences within the DNA polymerase small-subunit open reading frame. We emphasize that we have not ruled out the possibility that these sequences are important for chromosomal replication and that they may serve to promote additional Cdc6/Orc1 binding for chromosomal replication initiation. The open reading frame encoding the Cdc6/Orc1 protein present adjacent to the predicted origin sequence is not required in *cis* for autonomous plasmid replication. Vectors based on *P. furiosus oriC* were stably maintained for more than 100 generations without selection and showed no evidence of rearrangement after replication and transformation between *E. coli* and *P. furiosus*. The smallest *oriC* fragment identified in this study capable of conferring autonomous replication was 653 bp in length, and vectors based on the origin exist in a single copy per chromosome in the cell. Two conserved short palindromes were identified within the origin region that are conserved among *Pyrococcus* species but not in the closely related species *Thermococcus kodakarensis*, suggesting that if they have a function, it may be specific to *Pyrococcus* species. We anticipate that these vectors will have utility for homologous and heterologous gene expression, as well as providing a tool for the study of natural competence, and *in vivo* studies of replication and recombination in *P. furiosus*.

ACKNOWLEDGMENTS

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