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Metabolic and Evolutionary Relationships among *Pyrococcus* Species: Genetic Exchange within a Hydrothermal Vent Environment

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Pyrococcus furiosus and *Pyrococcus woesei* grow optimally at temperatures near 100°C and were isolated from the same shallow marine volcanic vent system. Hybridization of genomic DNA from *P. woesei* to a DNA microarray containing all 2,065 open reading frames (ORFs) annotated in the *P. furiosus* genome, in combination with PCR analysis, indicated that homologs of 105 ORFs present in *P. furiosus* are absent from the uncharacterized genome of *P. woesei*. Pulsed-field electrophoresis indicated that the sizes of the two genomes are comparable, and the results were consistent with the hypothesis that *P. woesei* lacks the 105 ORFs found in *P. furiosus*. The missing ORFs are present in *P. furiosus* mainly in clusters. These clusters include one cluster (Mal I, PF1737 to PF1751) involved in maltose metabolism and another cluster (PF0691 to PF0695) whose products are thought to remove toxic reactive nitrogen species. Accordingly, it was found that *P. woesei*, in contrast to *P. furiosus*, is unable to utilize maltose as a carbon source for growth, and the growth of *P. woesei* on starch was inhibited by addition of a nitric oxide generator. In *P. furiosus* the ORF clusters not present in *P. woesei* are bracketed by or are in the vicinity of insertion sequences or long clusters of tandem repeats (LCTRs). While the role of LCTRs in lateral gene transfer is not known, the Mal I cluster in *P. furiosus* is a composite transposon that undergoes replicative transposition. The same locus in *P. woesei* lacks any evidence of insertion activity, indicating that *P. woesei* is a sister or even the parent of *P. furiosus*. *P. woesei* may have acquired by lateral gene transfer more than 100 ORFs from other organisms living in the same thermophilic environment to produce the type strain of *P. furiosus*.

The shallow marine volcanic vents of Vulcano Island, Italy, have proven to be a rich source of thermophilic archaea. More than a dozen organisms have been isolated from this location (2, 22, 26, 27, 34, 50, 57, 58, 62, 63, 68), including two species of *Pyrococcus*, *Pyrococcus furiosus* (26) and *Pyrococcus woesei* (68). *P. furiosus* was the first of these organisms to be discovered in the Vulcano Island ecosystem and is now one of the best studied of the hyperthermophilic archaea. It grows optimally at temperatures near 100°C and utilizes peptides and carbohydrates as carbon and energy sources, generating organic acids and hydrogen or, if elemental sulfur is present, hydrogen sulfide as end products. The physiology of *P. woesei* appears to be very similar to that of *P. furiosus*. These organisms have the same growth temperature range and use the same carbon sources and terminal electron acceptors (26, 68). The genome of *P. furiosus* has been sequenced. It is 1.9 Mb long and contains more than 2,000 open reading frames (ORFs) (51). Although the genome of *P. woesei* has not been sequenced, 19 protein sequences and two RNA sequences are available in public databases, and all of these sequences exhibit at least 99% identity to their homologs in *P. furiosus* (5, 13–15, 16–19, 21, 35, 39, 41, 52, 64, 70); this includes the 16S rRNA sequences, which are 100% identical (39).

The question arises as to how these two *Pyrococcus* species originated and what the evolutionary relationship between them is. This is an intriguing issue given the fact that these organisms are found in the same geothermal environment.

Indeed, because of the identity of their 16S rRNA sequences, the striking similarities in their physiological properties, and the disruption of a putative Na$^+$/H$^+$ antiporter gene (*napA*, PF0275) by an insertion sequence (IS), it was recently concluded that *P. woesei* should be classified as a subspecies of *P. furiosus* (39). To evaluate the overall genetic differences between the two organisms, we utilized DNA microarrays based on the complete genome of *P. furiosus* (55, 56). The fundamental question to be addressed is, does *P. woesei* contain homologs of all the genes found in *P. furiosus*? Moreover, if the answer is no, what are the consequences of any differences in terms of evolution, physiology, and metabolism? So far, genome-based DNA microarray comparisons have been restricted to mesophilic bacteria, where the goals were to determine the presence or absence of genes associated with pathogenic and nonpathogenic strains (10, 28, 33, 42, 49, 53, 67). We show here that the results of DNA microarray comparisons allow testable predications to be made about physiology and metabolism. In addition, the whole-genome approach also provides an opportunity to gain insight into interactions between members of the Vulcano Island environment. This may provide a means to assess global genetic exchanges that potentially occurred at a time when primitive archaea lived on a hot earth and acquired or disseminated genetic innovation, such as stress resistance or utilization of a carbon source, for survival.

MATERIALS AND METHODS

Array design and DNA preparation. Microarray slides were designed and processed as previously described (55, 56). *P. furiosus* DSM 3638 and *P. woesei* DSMZ 3773 were grown in 1-liter culture bottles using maltose or peptides as the carbon source (65). Cells were harvested at the end of exponential growth, and genomic DNA (gDNA) was isolated by a phenol-chloroform protocol (54).

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Preparation of labeled DNA and hybridization conditions. Labeled DNA was prepared using a Prime-It Fluor kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, except that a dUTP-aminoallyl tag (Sigma, St. Louis, MO) was used. Tagged DNA products and Alexa-labeled DNA were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). Aminoallyl-labeled DNA was coupled with Alexa dyes 488, 546, 594, and 647 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Labeled genomic DNA samples were hybridized to a microarray slide containing PCR products for all 2,065 ORFs in the *P. furiosus* genome (55, 56) using a Genetac hybridization station (Genomic Solutions, Ann Arbor, MI) for 14 h at 65°C. The slides were then washed for 20 s each in 2× sodium chloride-sodium citrate buffer (SSC) containing 0.1% Tween 20, 0.2× SSC containing 0.1% Tween 20, and 0.2× SSC, rinsed in distilled water, and blown dry with compressed air. The fluorescence intensities of the four dyes, which represented one experiment in triplicate with one control, were measured using a Scan Array 5000 slide reader (Perkin-Elmer, Boston, MA) with the appropriate laser and filter settings.

Data analysis. Spots were identified and quantitated using the Gleams software package (Nutec, Houston, TX). The relative fluorescence intensities were averaged from three sets of microarray data generated from slides that contained the *P. furiosus* genome printed in triplicate (a total of nine arrays). The values for spots that gave negative fluorescence intensities with *P. woesei* DNA (78 of the 2,065 spots examined) were converted to an arbitrary value of 200 U, and the values for spots that gave negative fluorescence intensities with *P. furiosus* DNA (16 of 2,065 spots) were converted to the minimum detection limit (2,000 U). Instead of eliminating negative *P. furiosus* numbers, the conversions were used to keep positive *P. woesei* hybridizations for the spots even though the *P. furiosus* control did not. The fluorescence intensities collected from the *P. woesei* data set were divided into the *P. furiosus* data set for fluorescence intensities. The resulting values were then multiplied by −1 and added to 100. Values less than 98 were interpreted to indicate that a *P. furiosus* gene did not have a homolog in the *P. woesei* genome (corresponding to where the *P. woesei* fluorescence intensity was 50% of the *P. furiosus* fluorescence intensity). An ORF analysis was conducted using the InterProScan tool (version 3.3; http://www.ebi.ac.uk/interpro/). ISs were analyzed using ISfinder (http://www-is.biotoul.fr/). Homologs of *P. furiosus* ORFs in the genomes of *Pyrococcus abyssi* (9, 24), *Pyrococcus horikoshii (*31, 40), and *Thermococcus kodakaraensis* were defined as ORFs which encoded proteins that exhibited at least 75% sequence similarity over at least 75% of the protein length when the data were analyzed by BLASTP.

PCR and sequencing. Primers were synthesized and PCR products were sequenced by the University of Georgia Integrated Biotech Laboratories (http://www.ors.uga.edu/ibl/index.html/). PCR analyses were carried out in triplicate with different annealing temperatures and positive controls. The primers used to amplify the Mal I locus were forward primer 5′-AAT ACG CTC ATA GAA TCA AAG-3′ and reverse primer 5′-CCC TAT GAC TGC CTT TGG ATT-3′. All PCR reagents were obtained from Stratagene, and previously described standard molecular biology techniques were used (54).

Growth studies. The two types of maltose used in the growth studies were 95% grade (M2250; Sigma) and 99% grade (M9171; Sigma). Roussin's black salt (RBS) {Na$^+$[Fe$_4$S$_3$(NO)$_7$]$^-$} (8) was provided by Martin Hughes (King's College, London, United Kingdom). The dried powder was suspended in degassed water to a final concentration of approximately 0.9 μM prior to use.

PFGE. The pulsed-field gel electrophoresis (PFGE) procedure was adapted from the procedure described by Borges et al. (6). Gel plugs were made by suspending cells of *P. furiosus* or *P. woesei* to a concentration of 5 × 10^9 cells/ml in 1% (wt/vol) agarose. The plugs were incubated with 0.1 M EDTA, 1% (wt/vol) cetyltrimethylammonium bromide, 1% (wt/vol) sodium dodecyl sulfate, 1% (vol/vol) Triton X-200, and proteinase-K (2.0 mg/ml) for 24 h at 42°C. The plugs were washed twice with 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA (TE buffer) for 15 min at 4°C, incubated with 1 mM phenylmethylsulfonyl fluoride in 10 mM TE buffer at 23°C for 2 h, washed with 10 mM TE buffer at 23°C for 2 h, and equilibrated with 1× restriction enzyme buffer (New England Biolabs, Ipswich, MA) for 20 min at 4°C. Using fresh 1× restriction enzyme buffer, each plug was incubated with 25 U of NotI (New England Biolabs) for 16 h at 37°C. The plugs were inserted into a 1% (wt/vol) agarose gel and electrophoresed using the CHEF-DR II system (Bio-Rad, Hercules, CA) for 20 h at 200 V with an alternating field (90°/90 s for 1 h and 90° from 1 to 25 s for 19 h). DNA bands were stained with ethidium bromide.

RESULTS

gDNA from *P. woesei* was hybridized to the DNA microarray containing spots representing the 2,065 ORFs annotated in the *P. furiosus* genome. The fluorescence intensities were compared directly with those obtained using gDNA from *P. furiosus*. The results showed that close homologs of 1,890 (92%) of the 2,065 *P. furiosus* ORFs were present in the *P. woesei* genome. This included all 21 sequences of *P. woesei* genes available in the NCBI database (http://www.ncbi.nlm.nih.gov/) that are known to be (virtually) identical as determined by direct sequence comparisons. The remaining 175 *P. furiosus* ORFs (representing 8% of the *P. furiosus* genome) were not detected in *P. woesei* DNA at a significant level by microarray analysis, implying that close homologs of these genes are not present in the *P. woesei* genome. It is possible that some genes are apparently absent because they are highly divergent. However, this seems unlikely given the almost complete identity of all genes (and proteins) examined so far in the two species. The arrangement of the proposed missing ORFs on the *P. furiosus* genome is shown in Fig. 1. It is readily apparent that one striking feature is that in *P. furiosus* the missing ORFs form clusters or ORF islands. This pattern is not unlike the previously proposed (25) suggestion that these ORFs form islands or gene cassettes because of functional interactions. If *P. woesei* is as closely related as suspected and synteny is conserved, then the regions of missing ORFs are restricted to specific areas of the genome.

The veracity of the microarray results was assessed by direct PCR analysis using primer pairs covering 137 of the 175 ORFs proposed by the microarray results (78% coverage). PCR products of the expected size were obtained for all 137 ORFs analyzed using gDNA from *P. furiosus*, but only 32 ORFs yielded PCR products when *P. woesei* gDNA was used. This analysis therefore confirmed the absence in the *P. woesei* genome of homologs of 105 *P. furiosus* ORFs (or 77% of the ORFs indicated by the DNA microarray analysis). Table 1 lists the *P. furiosus* ORFs missing from the *P. woesei* genome. Of the 105 ORFs, 93 were present in 20 gene clusters consisting of two or more genes, and these clusters are indicated in Table 1 (in which shading indicates potential operons). Analysis by InterPro of the amino acid sequences revealed that 37 of the 105 ORFs (35%) encode proteins with unknown functions that have no known homologs in other organisms. Conversely, 407 of the 444 ORFs that are annotated as hypothetical ORFs in *P. furiosus* appear to be present in the *P. woesei* genome according to the DNA microarray analysis. This suggests that these hypothetical ORFs in *P. furiosus* do indeed encode proteins. The results of a BLASTP analysis of the 105 *P. furiosus* ORFs not present in *P. woesei* and ORFs of three closely related species, *P. abyssi* (9, 24), *P. horikoshii* (31, 40), and *T. kodakaraensis* (29), are shown in Table 1. Only seven of the *P. furiosus* ORFs have homologs in the genomes of all three species, and only 11 ORFs have homologs in two of the three organisms. In *P. furiosus*, these ORFs are scattered in the genome, except for the gene cluster containing PF0764 to PF0770, a homolog of which is found in *P. abyssi* but not in the other two organisms.

To gain further insight into the differences between the genomes of *P. furiosus* and *P. woesei* and the proposed absence of 105 ORFs in the latter organism, a PFGE analysis was performed with DNA isolated from both species after digestion using the NotI restriction enzyme. For *P. furiosus* DNA, this enzyme should generate six DNA fragments that are ap-
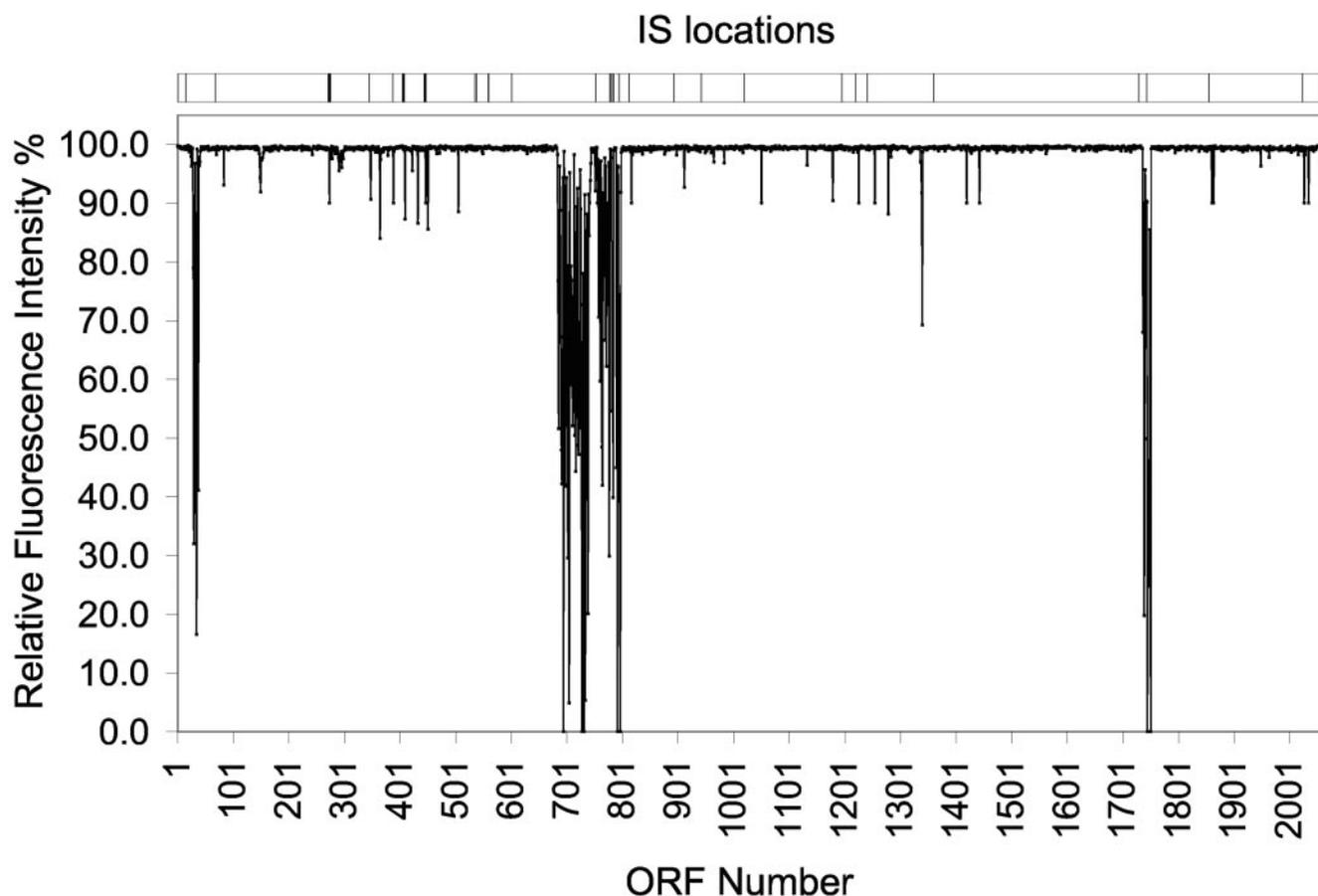
IS locations

FIG. 1. Hybridization of genomic DNA from *P. woesei* to the *P. furiosus* DNA microarray. The data were normalized as described in Materials and Methods. The abscissa indicates *P. furiosus* ORFs (ORFs 1 to 2065). The bar at the top indicates the positions of *P. furiosus* insertion sequences.

proximately 43, 132, 224, 385, 416, and 709 kbp long. Assuming that the two genomes differ only by the 105 ORFs, treatment of *P. woesei* DNA should also yield six fragments, two of which (42 and 132 kbp) are the same as fragments in *P. furiosus*. Each of the other four fragments from *P. woesei* DNA are predicted to be smaller (206, 371, 401, and 667 kbp) than the corresponding fragments from *P. furiosus* DNA. PFGE analysis revealed the expected six bands from *P. furiosus* DNA, and six bands were also seen after digestion of *P. woesei* DNA, all of which corresponded to the *P. furiosus* DNA fragments (data not shown). Five of the bands appeared to be the same in both species since differences of less than 20 kb were not resolved. However, the sixth fragment (667 kb) from *P. woesei* DNA was distinguishable from the fragment from *P. furiosus* DNA (which was predicted to be 41 kb larger). Given that no fragments were obtained from *P. woesei* DNA that were larger than predicted, we concluded that the genome of this organism is approximately the same size as the *P. furiosus* genome and lacks all 105 ORFs (equivalent to 88.8 kbp) predicted by the microarray and PCR analyses.

The power of the comparative DNA microarray approach is that it enables predictions regarding metabolism and physiology. Thus, of the ORFs listed in Table 1, of particular interest are the ORFs encoding proteins having known or predicted functions that are amenable to phenotypic analysis. One such gene cluster is the cluster containing PF1737 to PF1751, which includes the Mal I operon found in *P. furiosus* (1, 55) and in the related genus *Thermococcus* (50, 66). This operon encodes an ABC-type maltose/trehalose transporter (*malEFG* and *malK*, represented by PF1739 to PF1741 and PF1744, respectively), as well as a trehalose-degrading enzyme (PF1742) (1, 32, 36, 37, 44, 66). Interestingly, *Thermococcus litoralis*, which contains the Mal I operon, was also isolated from a shallow marine volcanic vent at Vulcano Island, Italy. In contrast, as shown by the BLASTP results (Table 1), *P. horikoshii*, *P. abyssi*, and *T. kodakaraensis* do not have a complete Mal I gene cluster. These three organisms were isolated from deep sea hydrothermal environments (4, 24, 31), perhaps implying that the availability of the Mal I gene cluster is limited to the vicinity of Vulcano Island. However, the apparent absence of the Mal I operon from *P. woesei* is inconsistent with a report that the organism is able to grow on maltose (68). Indeed, in our hands *P. woesei* exhibited very good growth (densities of $>10^8$ cells/ml) when the standard *P. furiosus* maltose-containing medium was used (65). This inconsistency was resolved by the finding that *P. woesei* did not exhibit significant growth on high-purity maltose (99%) rather than the technical grade usually used (95%, which contain 5% glucose and polysaccharides) (Fig. 2).

TABLE 1. *P. furiosus* ORFs that lack homologs in the *P. woesei* genome based on DNA microarray and PCR analyses[a]

ORF[b]	A	B	C	InterPRO annotation	ORF[b]	A	B	C	InterPRO annotation
PF0025	*	*	*	Glutamine amidotransferase II	PF0735				No InterPRO entry
PF0026	*	*	*	DNA polymerase, beta region	PF0737				No InterPRO entry
PF0029				No InterPRO entry	PF0738				S-Adenosylmethionine binding protein
PF0030				No InterPRO entry	PF0739				Bacterial regulator, AsnC/Lrp
PF0031				PLP enzyme, beta subunit	PF0740			*	Copper-transporting ATPase
PF0032				No InterPRO entry	PF0742			*	Ferritin and Dps
PF0034		*		No InterPRO entry	PF0743				Coenzyme A binding domain
PF0035		*		ABC transporter	PF0744			*	ABC transporter
PF0037				ARM repeat fold	PF0758	*	*		HEPN, nucleotide-binding
PF0038	*	*		Beta-lactamase-like	PF0760	*		*	Transposase, IS*605* OrfB
PF0041	*	*	*	Elongator protein 3/MiaB/NifB	PF0762	*	*		No InterPRO entry
PF0151	*			Glycoside hydrolase, family 5	PF0763				No InterPRO entry
PF0152		*	*	Protein of unknown function	PF0764	*	*	*	Archaeal ATPase
PF0154	*	*	*	Glutamine amidotransferase II	PF0765		*		6-Phosphogluconate DH, C terminal
PF0365				Protein of unknown function	PF0766		*		Oxidoreductase, N terminal
PF0366	*	*	*	No InterPRO entry	PF0767		*		Deg T aminotransferase
PF0685				Protein of unknown function	PF0768		*		Transferase hexapeptide repeat
PF0687				Transcription factor TFIIB	PF0769		*		Glycosyl transferase, family 2
PF0689				Prismane-like	PF0770		*		UTP-glucose-1-P$_i$ transferase
PF0691		*		Lambda repressor-like	PF0772	*			DNA polymerase, beta-like
PF0692		*		Prismane	PF0773				No InterPRO entry
PF0693				SirA-like	PF0775				No InterPRO entry
PF0694				Flavodoxin/NO synthase	PF0776		*		No InterPRO entry
PF0695		*		Protein of unknown function	PF0777				No InterPRO entry
PF0697				Sugar transporter superfamily	PF0781		*	*	Nucleotide binding protein, PINc
PF0698				No InterPRO entry	PF0783		*		No InterPRO entry
PF0701	*	*		No InterPRO entry	PF0790				No InterPRO entry
PF0702	*	*		Peptidase M24	PF0791				Glycosyl transferase, group 1
PF0703				No InterPRO entry	PF0792				No InterPRO entry
PF0704				Protein of unknown function	PF0794				UDP-*N*-Ac gluc.-2-epimerase[c]
PF0705				Cytochrome *c* biogenesis	PF0795				Glycosyl transferase, group 1
PF0706				Protein of unknown function	PF0796				Transferase hexapeptide repeat
PF0707				4-Oxalocrotonate tautomerase	PF0797				No InterPRO entry
PF0708				Extrusion protein MatE	PF0798				Glycosyl transferase, family 2
PF0712				No InterPRO entry	PF1337	*	*		TENA/THI-4 protein
PF0713		*		Winged helix DNA binding	PF1339				Protein of unknown function
PF0715				Nitroreductase	PF1340				Protein of unknown function
PF0716				6-Phosphogluconate dehydrogenase	PF1737				No InterPRO entry
PF0717				S-Adenosylmethionine binding motif	PF1738				Ribokinase
PF0718				No InterPRO entry	PF1339				Protein of unknown function
PF0719				No InterPRO entry	PF1740				Membrane transport component
PF0720				No InterPRO entry	PF1741				Membrane transport component
PF0721				NADPH: flavin mononucleotide reductase	PF1742	*			Glycosyl transferase, group 1
PF0722				Alkyl hydroperoxide reductase	PF1743	*			Protein of unknown function
PF0723				Iron permease FTR1	PF1744	*	*	*	ABC transporter
PF0725				Coenzyme A binding domain	PF1745	*	*		L-Fucose isomerase
PF0727				No InterPRO entry	PF1746				Glycoside transferase
PF0729				4Fe-4S ferredoxin	PF1747				Protein of unknown function
PF0730			*	No InterPRO entry	PF1748		*		Membrane transport component
PF0731				Ferritin-like	PF1749		*		Membrane transport component
PF0732				ABC transporter	PF1750	*	*	*	ABC transporter
PF0733				ABC transporter	PF1751	*	*		Thiamine ABC transporter
PF0734				No InterPRO entry					

[a] Shading indicates potential ORF clusters (in which adjacent ORFs can be on the positive or negative strand). An asterisk indicates the presence of a homolog. See text for details.

[b] For each ORF, the presence of a homolog in *P. horikoshii* (A), *P. abyssi* (B), or *T. kodakaraensis* (C) is shown.

[c] UDP-*N*-acetylglucosamine-4-epimerase.

Hence, in agreement with the DNA microarray analysis, maltose does not support growth of *P. woesei*.

A second gene cluster of interest in *P. furiosus*, which was absent in *P. woesei*, is the cluster containing PF0691 to PF0695. This cluster contains an ORF (PF0694) which encodes a protein that exhibits between 32 and 65% sequence similarity to the flavoprotein nitric oxide reductase (NOR) from the anaerobic bacteria *Moorella thermoacetica* (59), *Desulfovibrio vulgaris* (60),

and *Desulfovibrio gigas* (59, 61). The protein encoded by PF0694 has the conserved residues required to coordinate the binuclear nonheme iron site found in NOR (11, 20, 30). Analysis of the genome sequences available for 23 archaea revealed that only 6 of them have a homolog of the gene encoding the bacterial NOR. These organisms include *Archaeoglobus fulgidus* and the methanogens *Methanobacterium thermoautotrophicum*, *Methanococcus janaschii*, *Methanosarcina acetivorans*, and *Methanosarcina mazei*.
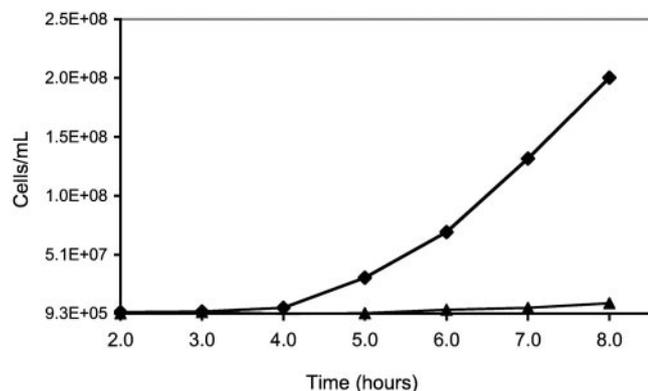
FIG. 2. Growth of *Pyrococcus* species on 99% pure maltose. Symbols: ◆, *P. furiosus*; ▲, *P. woesei*.

FIG. 4. Products of the Mal I region of *P. furiosus* and *P. woesei* as determined by PCR analysis. The products were analyzed on an agarose gel (0.5%, wt/vol) and were stained with ethidium bromide. The λ/HindIII-φX174/HaeIII DNA ladder was obtained from Stratagene.

As indicated in Table 1, a close homolog of PF0694, which is annotated by InterPro as NO synthase, is not present in *P. abyssi*, *P. horikoshii*, or *T. kodakaraensis*.

In light of the presence of a putative NOR system in *P. furiosus* and the apparent absence of this system in *P. woesei*, the question arose as to whether there are any differences between the two organisms in their responses to reactive nitrogen species (RNS). However, sensitivity of archaea to RNS has not been reported. To investigate the responses of the two *Pyrococcus* species, we used the NO generator known as RBS. This iron-sulfur-nitrosyl compound delivers seven molar equivalents of NO and is a potent antimicrobial agent (7, 8, 38, 47, 48). If *P. woesei* does not contain a homolog of PF0694, the organism should be more susceptible to this NO generator than *P. furiosus*. As shown in Fig. 3, growth studies using RBS showed that *P. furiosus* was not significantly affected by addition of 0.9 μM RBS, while under the same conditions the *P. woesei* cultures were not viable. These results strongly suggest that the cluster containing PF0691 to PF0695, particularly PF0694, plays a key role in detoxifying RNS (3).

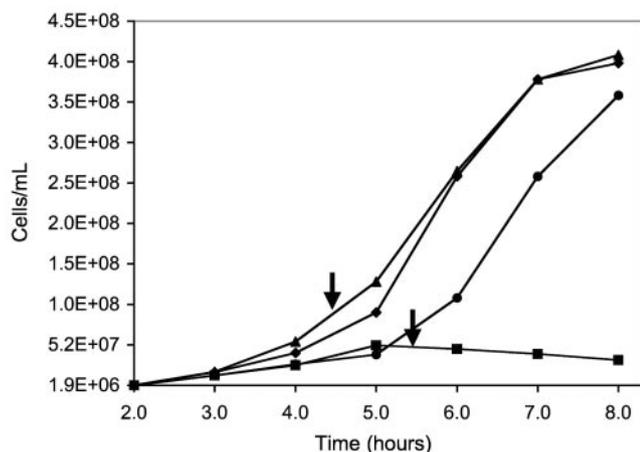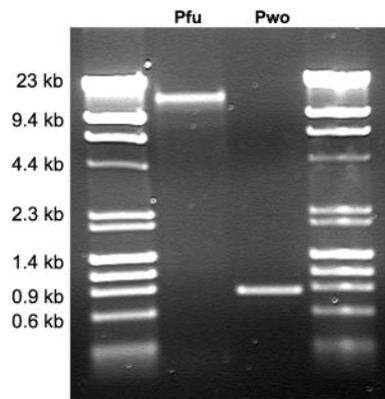It is therefore clear that *P. woesei* and *P. furiosus* share a close genetic origin. Interestingly, analysis of the *P. furiosus* genome revealed that the ORF islands not detected in *P. woesei* are either bracketed by or are close to ISs. This is illustrated in Fig. 1. A notable exception is the putative gene cluster (PF0691 to PF0695) that encodes NOR (Table 1). However, this cluster has an IS on one side (PF0756), and on the other side, adjacent to PF0688, there is a ~3.5-kb stretch of a long cluster of tandem repeats (LCTR). The tandem repeat that composes the LCTR is 29 nucleotides long. An LCTR is also located next to the cluster containing PF0025 to PF0032, which is another ORF cluster that is absent in *P. woesei* (Table 1). LCTRs are noncoding repeat sequences in tandem that are believed to behave like mobile elements and that have been proposed to participate in gene transfer (69). The positions of LCTRs next to some of the ORF clusters that are not present in *P. woesei* strongly support this proposition.

ISs are mobile elements that can transpose within the genome or into extrachromosomal elements (12, 46). The Mal I gene cluster found in *P. furiosus* (but not in *P. woesei*) is particularly noteworthy as it is packaged as a composite transposon. In other words, the Mal I is bracketed by two identical ISs, and the whole composite transposon (including ISs) is flanked by matching direct repeats (DRs), an indication of insertion as a complete composite transposon. In fact, the sequences of the Mal I gene cluster are virtually identical in *P. furiosus* and *T. litoralis*, and it has been proposed that a lateral gene transfer event was responsible (23, 37). To investigate whether the nature of IS and DR elements around the Mal I gene cluster could provide insight into the phylogenetic relationship between *P. furiosus* and *P. woesei*, the sequence of the relevant region in *P. woesei* was determined. PCR primers were designed to anneal outside the vicinity of the composite Mal I transposon in *P. furiosus* to determine what was present in the corresponding region in *P. woesei*. The same experiment with *P. furiosus* produced a PCR product that was approximately 17 kb long (the Mal I composite transposon is 17,854 bases long), while *P. woesei* produced a fragment that was about 780 bp long (Fig. 4). The sequence of the PCR product from *P. woesei* (accession number DQ202294) revealed that synteny of surrounding ORFs was conserved in *P. woesei* and *P. furiosus*. An

FIG. 3. Growth of *P. woesei* and *P. furiosus* in the presence of the NO generator RBS. The arrows indicate times at which RBS (0.9 μM) was added. Symbols: ▲, *P. furiosus* with RBS; ◆, *P. furiosus* without RBS; ■, *P. woesei* with RBS; ●, *P. woesei* without RBS.
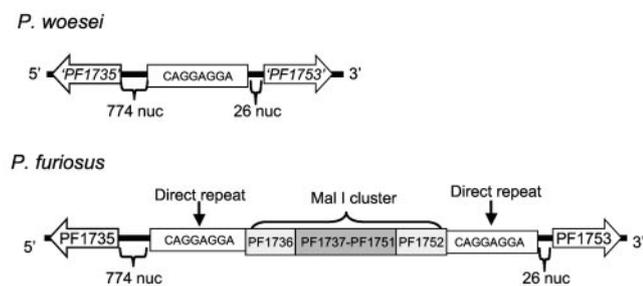
FIG. 5. Sequences of the PCR products of the Mal I regions of *P. furiosus* and *P. woesei*. See text for details. The sequenced ORFs of the unsequenced *P. woesei* genome are labeled '*PF1735*' and '*PF1753*.' The nucleotide sequences of these two ORFs in *P. woesei* are identical to the nucleotide sequences of the corresponding *P. furiosus* ORFs.

eight-nucleotide sequence, CAGGAGGA, was found in the *P. woesei* locus where the Mal I cluster is located in *P. furiosus*. This sequence is not spurious, as the DRs that bracket the *P. furious* Mal I composite transposon have the same sequence (Fig. 5).

DISCUSSION

Insertion sequences are suspected of playing key roles in the shaping of the *P. furiosus* genome that led to evolutionary divergence from *P. abyssi* and *P. horikoshii* (43, 45, 69). A total of 28 transposases are annotated in the *P. furiosus* genome, and our analysis of them showed that they comprise four groups. These groups were given the formal names groups IS*Pfu*1 (8 isoforms, IS*6* family), IS*Pfu*2 (11 isoforms, IS*6* family), IS*Pfu*3 (5 isoforms, IS*982* family), and IS*Pfu*5 (4 isoforms, IS*6* family) after analysis by ISFinder (http://www.is.biotoul.fr/). The ISs that bracket the Mal I gene cluster are isoforms belonging to group IS*Pfu*1. This group is a member of the IS*6* family, which transpose via replicative transposition. This is accomplished by formation of cointegrates between the donor and target sites that resolve, leaving a copy of the IS in the target and the donor site (46). Once a member of the IS*6* family inserts into a locus, the copy remains at the new location, potentially replicating at other target sites in time. However, the PF1735/PF1753 locus of *P. woesei* does not contain an IS or a composite transposon, nor is there any indication that the locus has been involved in replicative transposition. Assuming that the archaeal ISs follow the observed IS*6* replicative progression, we concluded that *P. woesei* is a sister and possibly the parent of *P. furiosus*. A *P. furiosus* strain would therefore be the type strain and presumably acquired ORFs (missing from *P. woesei*) from external sources at temperatures near 100°C in the same hydrothermal environment.

ISs likely play a pivotal role in shaping the genomes of the thermophilic community found at Vulcano Island and at similar locations on Earth. The use of DNA microarrays enables the first step to be taken toward understanding genomic phenomena such as the dissemination of gene cassettes within a community. This focuses attention on gene clusters found in archaeal communities rather than on individual genes or an individual organism. However, as demonstrated here, additional molecular and phenotypic characterizations are necessary to confirm the implications of array results.

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