A proposal to rename the hyperthermophile *Pyrococcus woesei* as *Pyrococcus furiosus* subsp. *woesei*

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Summary *Pyrococcus* species are hyperthermophilic members of the order Thermococcales, with optimal growth temperatures approaching 100 °C. All species grow heterotrophically and produce H₂ or, in the presence of elemental sulfur (S⁰), H₂S. *Pyrococcus woesei* and *P. furiosus* were isolated from marine sediments at the same Vulcano Island beach site and share many morphological and physiological characteristics. We report here that the rDNA operons of these strains have identical sequences, including their intergenic spacer regions and part of the 23S rRNA. Both species grow rapidly and produce H₂ in the presence of 0.1% maltose and 10–100 µM sodium tungstate in S⁰-free medium. However, *P. woesei* shows more extensive autolysis than *P. furiosus* in the stationary phase. *Pyrococcus furiosus* and *P. woesei* share three closely related families of insertion sequences (ISs). A Southern blot performed with IS probes showed extensive colinearity between the genomes of *P. woesei* and *P. furiosus*. Cloning and sequencing of ISs that were in different contexts in *P. woesei* and *P. furiosus* revealed that the *napA* gene in *P. woesei* is disrupted by a type III IS element, whereas in *P. furiosus*, this gene is intact. A type I IS element, closely linked to the *napA* gene, was observed in the same context in both *P. furiosus* and *P. woesei* genomes. Our results suggest that the IS elements are implicated in genomic rearrangements and reshuffling in these closely related strains. We propose to rename *P. woesei* a subspecies of *P. furiosus* based on their identical rDNA operon sequences, many common IS elements that are shared genomic markers, and the observation that all *P. woesei* nucleotide sequences deposited in GenBank to date are >99% identical to *P. furiosus* sequences.

Keywords: hyperthermophile, sulfur reduction, transposon.

Introduction

Pyrococcus species are hyperthermophilic members of the order Thermococcales, with optimal growth temperatures near 100 °C. All known *Pyrococcus* species grow optimally and

relatively rapidly at temperatures above 90 °C and produce H₂S from elemental sulfur (S⁰) (Zillig et al. 1987). Two *Pyrococcus* species, *P. furiosus* (Fiala and Stetter 1986) and *P. woesei* (Zillig et al. 1987), were isolated from marine sediments at a Vulcano Island beach site in Italy. They have similar morphological and physiological characteristics: both species are cocci and move by means of a tuft of polar flagella. They are heterotrophic and grow optimally at 95–100 °C, utilizing peptides as major carbon and nitrogen sources. Both species use carbohydrates such as maltose, cellobiose and starch. Growth of *P. furiosus* on β-glucans such as chitin, laminarin, cellobiose and cellulose has been reported (Kengen et al. 1993, Gueguen et al. 1997, Bauer et al. 1999, Blamey et al. 1999, Gao et al. 2003). Neither species requires S⁰ for growth, but S⁰ appears to stimulate growth via detoxification of H₂, the end product of metabolism. Compared with *P. furiosus*, *P. woesei* may grow at higher concentrations of H₂ produced during cultivation (Mukund and Adams 1991). *Pyrococcus furiosus* requires tungsten (10–25 µM Na₂WO₄) for optimal growth in S⁰-free medium (Bryant and Adams 1989). The optimal pH and NaCl concentration for growth of *P. furiosus* may be slightly higher and cover a broader range than for *P. woesei* (pH 6–8 and 15–35 g NaCl l⁻¹ for *P. furiosus*, and pH 6 and 30 g NaCl l⁻¹ for *P. woesei*) (Fiala and Stetter 1986, Zillig et al. 1987), although these differences may also reflect variation among laboratories.

Three *Pyrococcus* genome sequences (*P. furiosus*, *P. horikoshii* and *P. abyssi*) are available in GenBank. In contrast, only 20 sequence accessions from *P. woesei* are available in GenBank, and no 16S rDNA sequence was available. Our preliminary studies revealed that the *P. woesei* genome also contains insertion sequence (IS) elements (GenBank accession nos. AF420277 and AF443788). This study was initiated to determine whether the genomic content and positioning of the IS elements provide a measure of relatedness between *P. woesei* and *P. furiosus*.

Materials and methods

Organisms and growth in maltose

Pyrococcus furiosus DSM 3638 and *P. woesei* DSM 3773 were grown under anaerobic conditions at 95 °C in Pf medium containing (per liter): 24 g NaCl, 4 g Na₂SO₄, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.03 g H₃BO₃, 10.8 g MgCl₂·6H₂O, 1.5 g CaCl₂·2H₂O, 0.025 g SrCl₂·6H₂O, 5 g tryptone, 1 g yeast extract, 1 ml resazurin (0.2 g l⁻¹ solution), and 5–10 g sulfur powder; the pH was adjusted to 6.8 (González et al. 1998). Cells were harvested by centrifugation and DNA was extracted as described by Charbonnier and Forterre (1995).

Pyrococcus furiosus and *P. woesei* were grown in maltose in a medium identical to Pf except that S⁰ was omitted and maltose (1%, w/v) and sodium tungstate (10–100 µM) were added. Cell densities were determined (from triplicate cultures) by the acridine orange staining technique. Briefly, samples (0.1–1.0 µl) were mixed with 37% formalin saturated with sodium borate (10–25 µl), 0.01% acridine orange (0.2 ml) and diluted with Milli-Q water to a final volume of 1 ml. The solutions were filtered through 0.22-µm polycarbonate membranes (GE Osmonics, Minnetonka, MN) and the membranes mounted on microscopic slides. Cell counting was performed randomly on at least 50 × 50 µm squares. Cell numbers were determined by the following equation: Cell numbers ml⁻¹ = number of cells per 10 × 10 µm squares × 2300 ml⁻¹/volume in ml.

Phylogenetic analyses of nucleotide sequences

Nucleotide sequences of *P. woesei* were compared by BLAST against genomic sequences of *P. furiosus*, *P. horikoshii* OT3 and *P. abyssi* available at the GeneMate Web site (<http://comb5-156.umbi.umd.edu/genemate/>). All available nucleotide sequences of *P. woesei* were obtained from GenBank. The accession nos. are: X15329, AY443493, AF443788, AF420277, AF043283, U84155, Y09481, AF240464, AF177906, AF239672, X59857, X73527, X60161, X70668, X67205, U56247, U10285, M83988, M83987 and AF461062.

16S rDNA of *P. woesei*

Sequencing of the 16S rRNA-tRNA^{Ala}-23S rRNA gene cluster in *P. woesei* was performed with primers 344F (5'-ACGG GGC GCA GCA GGC GCGA-3'), 345R (5'-CCCT TCC GCC CCG AGC-3'), 346F (5'-AACG GGT CCG ACG GTG-3'), 347R (5'-CCGC GAT CCG AAC TGA-3'), 348F (5'-CCTC TTG CAT ACA TAA CTT CTC-3') and 349R (5'-TTC CCG CAT TGC GGA-3') on both strands. Ambiguous nucleotides obtained from sequencing data were re-sequenced.

Primers and PCR conditions

All primers were designed based on the genomic sequence of *P. furiosus* (<http://www.genome.utah.edu/>). Coordinates 1 and 702 are assigned to adenines of the initiation and termination codons of the putative transposase gene (*tnp*), respectively.

Nucleotide sequences of the three primers specific for *tnp* are: 5'-ATG AAG GCT GAG AGC ATT CTA TAC TC-3', 5'-ATG AAG TCT GAA ACC ATT ATT TAC TGG-3' and 5'-TTA GGA TAA CTG GGG CAT CAC-3'. These primers bind specifically to *tnp* at coordinates 1–26, 1–27 and 682–702, respectively. Primers 4 to 8 were designed to bind specifically to the *napA* locus of the *P. furiosus* genome (binding coordinates 286645–286663, 286694–286716, 286776–286797, 286922–286947 and 285765–285786, respectively). The nucleotide sequences of these primers are: 5'-TCC ATG CTA GTC TTT TTGC-3', 5'-CGA GTG AAC ATC CGA CAA TCT TA-3', 5'-TTA AGG CAA CTT CCA TCC TTGG-3', 5'-AGA ATC ATT AGA ACC AGA AGA GTA GC-3' and 5'-TAG CAA GGG GAT GAT AAC ATGG-3'. An outward and conserved primer sequence (5'-CTT CAA GAG GTG ATA CCC CAG TTA TCC-3') was designed to bind specifically to the 3' end of *tnp* (binding coordinates 672–699).

Polymerase chain reactions (PCRs) were performed in a 50 µl reaction volume. Thermal cycling conditions were: 95 °C for 30 s, 52–55 °C for 40 s and 72 °C for 60–120 s for 30 cycles, depending on the size of the desired product (approximately 60 s per 1 kb fragment).

Nested PCR reactions were performed on dilute samples (1:600). Primers 4 and 7 were employed in negative control reactions.

Southern blotting and comparison of restriction patterns

Genomic DNAs (5 µg) were digested with the restriction enzyme *Pst*I. Approximately 1 µg of restricted DNA was loaded in each lane and separated in 0.9% agarose gels (11 × 14 cm). Linearized plasmids constructed in this study containing a *tnp* sequence served as size markers. Electrophoresis was performed at 30 V in TAE buffer (pH 8.5) for 14 to 15 h in a cold room. Transfer of DNAs to a nylon membrane (Immobilon-Ny⁺ Transfer Membrane, Millipore, Bedford, MA) was performed by perfusion according to the manufacturer's recommendations. Briefly, DNA in the agarose gel was depurinated in 0.25 N HCl for 15 min. The agarose gel was rinsed briefly with Milli-Q water, denatured in 0.5 N NaOH + 1.5 M NaCl for 30 min and neutralized with 1 M TrisCl (pH 8.0) + 1.5 M NaCl for 30 min. The DNA was transferred onto nylon membrane with 20× SSC for 6 to 8 h. Labeled DNA probes were prepared with 25 µCi of ³²P-α-labeled dATP and 0.1 ng of purified *tnp-I* and *tnp-II* sequences as templates. The probes were boiled for 10 min and immediately added to the pre-warmed hybridization mixture. Hybridization was performed following the standard procedures described by Ausubel et al. (1987). Images were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Band intensity was measured with the NIH Image 1.61 program (http://rhea.la.asu.edu/dm/data_tools/nih_image). Relative intensity was estimated with the equation: Index (per unit area) = (band intensity – mean background intensity)/(intensity of a band containing one IS – mean background intensity).

Nucleotide sequences containing an IS were extracted with GeneMate (<http://comb5-156.umbi.umd.edu/genemate/>).

*Pst*I restriction fragments containing an IS sequence were deduced from the genome sequence of *P. furiosus*.

Lambda library construction and screening for insertion sequences

Approximately 5–6 kb *Pst*I restriction fragments from *P. woesei* genomic DNA were gel purified. The fragments (1 µg) were partially digested with the restriction enzyme *Tsp*509I (0.1 units) at 56 °C for 15 min to create AATT sticky ends. The restriction enzyme was removed with a spin column (QIAquick Gel Extraction kit, QIAGEN, Valencia, CA). Approximately 50 ng of the sticky fragments were ligated to λZIPLOX, *Eco*RI Arms vector (Life Technologies, Gaithersburg, MD) with T4 DNA ligase (Promega, Madison, WI) and packaged (Gigapack II Gold Packaging Extract, Stratagene, La Jolla, CA). Propagation of λ phage and excision of plasmid pZL1 were performed following the methods recommended by the manufacturer (Life Technologies). About 1×10^{-5} positive plaques were obtained. Plaques were excised and screened by standard colony hybridization techniques following the methods recommended for the nylon membrane (Immobilon-Ny⁺ Transfer Membrane, Millipore).

Results and discussion

Growth with maltose and tungsten

Pyrococcus woesei showed similar physiological properties to *P. furiosus*. Cell yields (about 1×10^7 cells ml⁻¹) from overnight cultures in Pf medium were not significantly different between species. Representative growth curves for *P. furiosus*

Figure 1. Growth of *P. furiosus* and *P. woesei* in sulfur-free medium containing 1% maltose and 100 µM sodium tungstate. Triplicate cultures of *P. furiosus* and *P. woesei* were incubated anaerobically at 95 °C. Cells were counted in at least 50 of the 50 × 50 µm squares.

and *P. woesei* in the S⁻-free medium containing maltose and 100 µM Na₂WO₄ are shown in Figure 1. One ml of each culture was inoculated into three identical bottles of 100 ml S⁻-free medium containing 1% maltose and 10–100 µM Na₂WO₄ to obtain cell densities of about $7\text{--}8 \times 10^4$ cells ml⁻¹. The cultures were incubated anaerobically at 95 °C. The results in Figure 1 indicate that both *P. woesei* and *P. furiosus* use maltose and tolerate Na₂WO₄ up to 100 µM.

During the first 6 h of incubation, growth of *P. furiosus* and *P. woesei* was not significantly different (cell yields were $1.1 \times 10^6 \pm 3.8 \times 10^5$ and $1.1 \times 10^6 \pm 2.3 \times 10^5$ cells ml⁻¹, respectively), and both cultures entered stationary phase after 10 h of incubation. However, the maximum cell density obtained for *P. woesei* ($3.6 \times 10^6 \pm 6.0 \times 10^5$ cells ml⁻¹ at 10 h) was approximately one log cycle less than for *P. furiosus* ($1.6 \times 10^7 \pm 2.7 \times 10^6$ cells ml⁻¹ at 10 h). By late stationary phase, the cell density of *P. furiosus* had increased slightly (to $3.0 \times 10^7 \pm 6.6 \times$

Figure 2. Micrographs of acridine orange stained cells of *Pyrococcus furiosus* and *P. woesei* growing in sulfur-free medium containing 1% maltose and 100 µM sodium tungstate. The photographs were taken at identical magnification with an oil immersion objective lens. Numbers in parentheses are the volumes of the cultures used to prepare the microscopic slides. (a) *Pyrococcus furiosus* at 19 h. The cell density corresponds to the value shown in Figure 1 (about 3×10^7 cells ml⁻¹). Most cells were single, in pairs or in short chains and rarely in aggregated morphologies. (b) *Pyrococcus woesei* at 19 h. The cell density corresponds to the value for the mid-exponential growth phase shown in Figure 1 (about 1.6×10^6 cells ml⁻¹).

10^6 cells ml^{-1} at 19 h). In contrast, the cell density of *P. woesei* declined about twofold ($1.6 \times 10^6 \pm 4.9 \times 10^5$ cells ml^{-1}) between 10 and 19 h. Figures 2a and 2b show the morphologies of *P. furiosus* (Figure 2a) and *P. woesei* (Figures 2b) at 19 h. Similar cell morphologies and arrangements of *P. woesei* and *P. furiosus* were observed during the exponential phase; most cells were arranged singly or in pairs (picture not shown). Unlike *P. furiosus* (Figure 2a), large aggregates of cells were observed in *P. woesei* (Figure 2b) during the stationary phase, suggesting that *P. woesei* cells in this phase are more sensitive to stress-induced autolysis than cells of *P. furiosus*. Therefore, growth phenotypes of *P. woesei* and *P. furiosus* in the S° -free medium containing maltose and sodium tungstate are not identical.

Identical 16S rDNA sequences

Nucleotide sequences of the 16S rRNA, tRNA^{Ala} and 23S rRNA (partial) cluster from *P. woesei* were determined (GenBank accession no. AY519654) and found to be identical to sequences of the homologous cluster in *P. furiosus* (GenBank accession no. AE010139), including the intergenic spacer regions. The two intergenic spacer regions are sensitive sites for differentiation in the Thermococcales, especially species of *Pyrococcus*, as shown by DiRuggiero et al. (1995).

Analysis of nucleotide sequences

To date, there are 20 GenBank accessions of *P. woesei* sequences available. These nucleotide sequences (28,857 bp) include 22 genes and one three-gene cluster (X59857). BLAST search results for *P. woesei* against the genome sequences of *P. furiosus*, *P. horikoshii* OT3 and *P. abyssi* are summarized in Table 1. The nucleotide sequences of *P. woesei* were highly homologous (99–100% similarities), especially with sequences of *P. furiosus*. They have uniformly lower similarity (approximately 75%) to the homologous sequences of *P. horikoshii* and *P. abyssi*. The genomes of *P. horikoshii* and *P. abyssi* contain no amylase genes homologous to the two *P. furiosus* α -amylase genes (GenBank accession nos. AF240464 and AF177906) and no IS elements, confirming a previous study (DiRuggiero et al. 2000).

Southern blotting and comparison of restriction patterns

The *P. woesei* genome contains IS elements (GenBank accession nos. AF420277 and AF443788) homologous to the IS elements in the *P. furiosus* genome. In *P. furiosus*, the IS elements are grouped into three types according to their nucleotide sequences: type I (11 copies), type II (eight copies) and type III (four copies). BLAST searches of entire IS elements against the complete *P. furiosus* genome revealed an additional four truncated copies of the ISs (approximately 300–700 bp),

Table 1. BLAST results of nucleotide sequences from *P. woesei* against homologous sequences in the genomes of *P. furiosus*, *P. horikoshii* OT3 and *P. abyssi*. Abbreviation: nf = not found.

Accession no. <i>P. woesei</i>	Description	Similarity (%) to homologous sequences of		
		<i>P. furiosus</i>	<i>P. horikoshii</i>	<i>P. abyssi</i>
AY443493	dUTPase	100	85	75
X15329	5S rRNA	97	96	96
AF461062 ^a	<i>napA</i> (Na^+/H^+ antiporter) (disrupted by an IS-III)	99 ^b	69	69
AF461062 ^a	IS element type III	99	nf	nf
AF420277 ^a	Transposase type II	99	nf	nf
AF443788 ^a	Transposase type I	100	nf	nf
AF461062 ^a	IS element type III	99	nf	nf
AF420277 ^a	Transposase type II	99	nf	nf
AF443788 ^a	Transposase type I	100	nf	nf
AF043283	β -Galactosidase	99	66	74
U84155	DNA-dependent DNA polymerase	100	73–74	74
Y09481	Triosephosphate isomerase	100	78	77
AF240464	α -Amylase	99	nf	nf
AF177906	α -Amylase (amyA)	100	nf	nf
AF239672	Pyrolysin	99	nf	nf
X59857	Ef-1a, ribosomal S10 and tRNA-Ser cluster	99	73–83	70–79
X73527	3-Phosphoglycerate kinase	99	71	73–77
X60161	Glutamine synthetase	99	73	75
X70668	Transcription factor IIB	100	79	79
X67205	Translational elongation factor 2	99	83	70–82
U56247	Cell division protein (<i>FtsZ</i>)	99	76	77
U10285	TATA-binding protein	100	78	79
M83988	Glyceraldehyde 3-phosphate dehydrogenase	100	80	75
M83987	Putative ribosomal protein	99	76	76

^a Sequences from this study.

^b Insertion sequence element disrupting *napA* in *P. woesei* was removed before BLAST analysis.

which are classified as type D in this study. The IS elements in all classes contained no *Pst*I restriction sites. To simplify subsequent discussions, IS numbers were assigned and the coordinates of the IS elements in the genome of *P. furiosus* are listed in Table 2. The sizes of fragments containing an IS element between two *Pst*I restriction sites were predicted from the genome sequence of *P. furiosus*. The predicted *Pst*I restriction fragment sizes are summarized in Table 2.

Southern blot analysis of *Pst*I restriction patterns and DNA hybridization with probes specific to *tnp* sequences (Figure 3) revealed that the *P. woesei* genome (lane 1) contains multiple copies of the IS elements shown in Table 2. In the *P. furiosus* genome (lane 2), band numbers 1–7 and a cluster of large fragments (bracket 8) were mapped according to their relative intensities and predicted *Pst*I restriction fragment sizes. Generally, the mapping correlated well with band intensities of bands 1–6. Bands denoted no. 8 contain large unresolved fragments (> 10 kb) and may contain a mixture of divergent IS elements. The *Pst*I restriction fragments predicted for *P. furiosus* did not include an 8 kb fragment containing an IS element, because the 8 kb fragment (band 7) observed for *P. furiosus* may be a divergent IS element and this area was not accounted for in the comparison. The restriction pattern for *P. woesei* (lane 1) showed the same *Pst*I fragments as *P. furiosus* (bands 1 and 3–6 of *P. furiosus*), except for the band corresponding to band 2 in *P. furiosus* (lane 2). These bands contain 10 IS

elements, including two elements of type D in band 3 of *P. furiosus* and one element in band 5, implying that the genomes of *P. furiosus* and *P. woesei* are colinear in dispersed regions throughout the genome.

An index of one was assigned to Band 2 (ca. 3.5 kb), which represents fragments containing a single IS element. Band 1 (index = 1.9) contains two IS elements (2.1 and 2.2 kb). Band 3 (index = 1.5) contains two type D elements (4.5 and 4.6 kb). Band 4 (index = 2.6) contains two IS elements (ca. 5.2 kb). Band 5 (index = 3.2) contains three IS and one deletion mutant element (ca. 5.8–6.1 kb). Band 6 (index = 3.0) contains two IS elements (ca. 6.8 kb). No predicted fragment size matched band 7 (ca. 8 kb). Bands denoted no. 8 were assumed to contain IS elements in fragments that are greater than 10 kb (see also Table 2).

Mapping the *napA* locus in *P. woesei*

Labeled probes specific to *tnp* sequences were used to locate flanking DNAs at the ends of the IS elements in a λ library containing genomic DNA of *P. woesei*. Twenty positive plaques were isolated and the inserts in pZL1 were sequenced.

Table 2. Element numbers (IS no.), coordinates and predicted *Pst*I restriction fragment sizes (kb). Sizes of fragments containing a single IS element sequence were calculated from the two nearest *Pst*I restriction sites flanking an IS element sequence. Band nos. are numbers assigned to the bands observed in lane 2 of Figure 3.

Figure 3. Restriction patterns from a Southern blot probed with 32 P-labeled *tnp* DNAs. The DNA from *P. woesei* (lane 1) and *P. furiosus* (lane 2) were completely digested with *Pst*I restriction enzyme. Size markers in kilobases (kb) are shown on the left of the Figure. Band numbers were assigned only to the bands from *P. furiosus* and are shown on the right of the figure. Intensities of bands and indices were determined.

Band no.	IS no.	Size (kb)	Type	Coordinates
1	14	2.1	I	797130
1	20	2.2	III	977655
2	3	3.6	I	752270
3	24	4.6	D	250231
3	26	4.5	D	437640
4	13	5.2	I	463555
4	17	5.2	I	1142040
5	1	5.8	I	284983
5	2	5.8	I	397627
5	22	6.1	II	414414
5	27	5.8	D	1443764
6	19	6.7	III	870640
6	11	6.8	I	72986
7	—	~8	—	— ¹
8	4	15.8	I	767495
8	5	15.8	III	771322
8	6	18.1	II	579456
8	7	22.7	II	909866
8	8	12.9	II	1161577
8	9	12.6	II	1612359
8	10	16.8	II	1629433
8	12	11.7	I	361521
8	15	10.8	I	1720267
8	16	10.7	I	1877741
8	18	11.8	III	552546
8	21	25.9	II	12566
8	23	12.4	II	1284310
8	25	10.4	D	253075

¹ No IS element assigned to band.

An alignment of the 20 clones carries a 78-nucleotide-long sequence at the right end of the IS (corresponding to nucleotide numbers 632–779 of a type III IS) joining with a 22-nucleotide-long sequence aligned perfectly to coordinates 286776–286797 in the *P. furiosus* genome. In *P. furiosus*, coordinates 286776–286797 are part of a putative Na⁺/H⁺ antiporter (*napA*) gene (coordinates 285783–286922) that has 69% identity to a putative *napA*-3 (PAB1247) in *P. abyssi* and a hypothetical Na⁺/H⁺ antiporter open reading frame (PH0302) in *P. horikoshii* OT3. The Na⁺/H⁺ antiporter is a membrane protein that exchanges Na⁺ and H⁺ and plays an important role in osmoregulation (Hellmer et al. 2002, Waditee et al. 2002).

To map the *napA* locus in *P. woesei*, sets of primers (Primers 4 to 9) were employed to walk upstream and downstream of the IS sequences mentioned above. Cloning and sequencing of the *napA* locus in *P. woesei* confirmed the presence of an type III IS disrupting the relative coordinates 2828675 and 2828676 and revealed a direct repeat sequence (5'-CCAGGAT-3') flanking the type III IS element. The presence of direct repeats flanking the IS implies that the type III IS element was inserted into the *napA* of *P. woesei* by transposition (Mahillon and Chandler 1998).

The nucleotide sequence of the disrupted *napA* locus (total 1969 bp) was submitted to GenBank (accession no. AF461062). Apart from the type III IS insert and the direct repeat sequences, the nucleotide sequence is 99% similar to the homologous sequence (coordinates 285783–286922) in *P. furiosus* (Table 1). The nucleotide sequence of the type III IS element is 99.5% similar to the type III IS element 20 in the *P. furiosus* genome.

Identification of type I IS as a genetic marker

The *P. furiosus* genome contains a type I IS element located at coordinates 284983–285764 (Table 2) upstream of the *napA* locus (coordinates 285783–286922). This raises the question whether the type I IS is also present in *P. woesei*. A PCR-based strategy with Primers 9 and 6 was employed (see also relative binding coordinates in Figures 4a and 4b) to amplify DNAs of *P. woesei* and *P. furiosus*. As anticipated, a fragment of about 1079 bp was obtained from the positive control. From *P. woesei*, two fragments (60 and 1858 bp) should have been amplified with Primers 9 and 6; however, only the short fragment was produced because of the blocking of PCR reactions by the distal distant primer pair, Primers 9 and 6 (see map in Figure 4b). Nested PCR amplifications on a diluted (1:600) primary reaction from *P. woesei* using a set of primer pairs (Primers 4 and 7 as negative control, Primers 4 and 3, Primers 9 and 5, and Primers 9 and 6) that bind internally or externally, or both, to the large fragment resulted in a trace amount of PCR fragment containing type I IS (results not shown). These results suggest that a trace amount of the large fragment was produced in the primary amplification reaction with Primers 9 and 6 from the genomic DNA of *P. woesei* and imply that the genome of *P. woesei* contains the type I IS element in the same context as identified upstream of the *napA* locus in the *P. furiosus* genome. Sequencing of the PCR products con-

firmed that the large fragments were identical. Maps comparing the *napA* loci of *P. furiosus* and *P. woesei* are shown in Figures 4a and 4b.

In conclusion, the physiological properties of *P. woesei* are similar to those of *P. furiosus*. Both species grow rapidly in the presence of 0.1% maltose and 10–100 μ M Na₂WO₄ in S⁻-free medium. However, the cell density of *P. woesei* cultures decreased faster than that of *P. furiosus* in stationary phase, because of more extensive autolysis. This demonstrates a phenotypic difference between the two isolates that is probably related to differences in membrane integrity and osmotic function. We speculate that this could be due, in part, to the defective *napA* gene in *P. woesei*. The Na⁺/H⁺ antiporter encoded by *napA* is a membrane protein that exchanges Na⁺ and protons and often plays an important role in osmoregulation (Hellmer et al. 2002, Waditee et al. 2002).

The 16S rRNA, tRNA^{Ala} and 23S rRNA (partial) gene cluster from *P. woesei* (GenBank accession no. AY519654) was sequenced in this study for the first time. The results revealed complete identity with the *P. furiosus* operon (GenBank accession no. AE010139). Comparisons of the 20 *P. woesei* sequences publicly available (Table 1) confirmed identity or high similarity to homologous nucleotide sequences of *P. furiosus*. Restriction patterns of fragments containing IS element (Figure 3) markers support colinearity between the genomes of both species. The presence of two IS elements in the Na⁺/H⁺ antiporter (*napA*) gene in *P. woesei*, including one in the same context as the single type I IS in *P. furiosus*, not only supports our contention that the ISs continue to be active,

Figure 4. Maps of insertion sequence (IS) elements in the *napA* loci of (a) *P. furiosus* and (b) *P. woesei*. Parallel lines represent double-stranded DNAs. Boxes with thick arrows represent IS elements. Thick arrows represent genes. Arrowheads on the thin lines represent forward (pointing right) and reverse primers (pointing left) (see binding coordinates in Materials and methods). Coordinates in the *P. furiosus* genome are shown by the vertical dotted lines. Direct repeat sequences are boxed. The *napA* gene in *P. woesei* contains two IS elements, including the type I IS in the same context as in the genome of *P. furiosus*.

but provides evidence that they contribute to the divergence of the two strains. It has been proposed that the divergence of the *P. furiosus* genome from the genomes of *P. abyssi* and *P. horikoshii* is mediated largely by IS movement or recombination between ISs in *P. furiosus* (Maeder et al. 1999, Lecompte et al. 2001, Zivanovic et al. 2002). We suggest that *P. woesei* and *P. furiosus* are closely related, certainly in comparison with the many *E. coli* strains that continue to be recognized as species, and the new designation *P. furiosus* subsp. *woesei* is proposed.

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