A Physical Map of the Hyperthermophilic Bacterium *Aquifex pyrophilus* Chromosome

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A genomic map of the hyperthermophilic hydrogen-oxidizing bacterium *Aquifex pyrophilus* was established with *NotI* (GC/GGCCGC), *SpeI* (A/CTAGT), and *XbaI* (T/CTAGA). Linking clones and cross-hybridization of restriction fragments revealed a single circular chromosome of 1.6 Mbp. A single flagellin gene and six rRNA gene units were located on this map by Southern hybridization.

Aquifex pyrophilus is a novel marine hyperthermophilic hydrogen-oxidizing bacterium that grows between 67 and 95°C, with an optimum temperature of 85°C (7). Phylogenetic analyses on the basis of small-subunit rRNA sequence comparisons place *A. pyrophilus* on a line representing the earliest divergence yet encountered in the bacterial evolutionary tree (4). Although physiological, molecular, and phylogenetic data for this species have been accumulated (1, 4, 7), nothing is known about its genome structure or genetic organization because of difficulties in isolating mutants and the lack of an established gene transfer system. However, the antiquity of *A. pyrophilus*, its extreme thermophily, and biotechnological interest in this species were reasons for constructing a chromosomal map as the basis for subsequent genetic studies. In recent years, the combination of rarely cutting restriction enzymes with pulsed-field gel electrophoresis (PFGE) techniques has been crucial for the physical mapping of small genomes (19), and reasonable agreement with the genetic maps has been demonstrated (8, 20). We therefore used contour-clamped homogeneous electric field (CHEF) (6) electrophoresis and field inversion gel electrophoresis of restriction fragments to determine the size of the *A. pyrophilus* genome and hybridization techniques to establish a physical map. Moreover, we determined the locations of a single flagellin gene and six rRNA genes (rDNA units) on this genomic map.

Preparation of intact chromosomal DNA. *A. pyrophilus* DSM 6858 was cultivated in modified SME* medium (7) with 0.2% Na₂S₂O₃ or 0.05% elemental sulfur in an H₂-CO₂-O₂ (79.75:19.75:0.5; 300 kPa) atmosphere at 85°C for 16 h with gentle shaking to a cell density of approximately 10⁸ cells per ml. Cells (from a 400-ml culture) were washed with 50 ml of STE buffer (100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA [pH 8.0]), resuspended in 2.5 ml of STE buffer, and mixed with an equal volume of 1.4% InCert agarose (FMC BioProducts, Rockland, Maine) to achieve final concentrations of 0.7% agarose and 8 × 10⁹ cells per ml. The suspension was pipetted into 15 wells (200 µl each; approximately 1.6 × 10⁹ cells) of gel plug molds (Bio-Rad, Richmond, Calif.) and chilled at 4°C for 20 min. The plugs were treated with 2 volumes of EC-lysis buffer (6 mM Tris-Cl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% Brij 58, 0.2% deoxycholate, 0.5% *N*-lauroylsarcosine) containing 1 mg of lysozyme per ml and 20 µg of DNase-free RNase per ml and incubated at 37°C for 16 h. The

buffer was removed, and 2 volumes of ESP buffer (0.5 M EDTA [pH 8.0], 1% *N*-lauroylsarcosine, 1 mg of proteinase K per ml) were added. The incubation was continued at 50°C for 48 h with gentle shaking and one change of buffer. Gel plugs containing 1 to 2 µg of intact genomic DNA per 100-µl plug were stored in 0.5 M EDTA (pH 8.0) at 4°C.

Restriction endonuclease digestion of DNA gel plugs. One 200-µl gel plug cut into small pieces (25 µl each) was equilibrated with 500 µl of the recommended restriction endonuclease buffer at 4°C twice for 30 min each time. The buffer was replaced, and 100-µl gel plugs were digested with 20 to 30 U of endonuclease at 37°C for 12 h in a 150-µl reaction volume. The

FIG. 1. CHEF electrophoresis of *A. pyrophilus* DNA digested with *NotI*, *SpeI*, and *XbaI*. (A) Lane 1, *S. cerevisiae* chromosomes; lane 2, *NotI*-digested *A. pyrophilus* DNA. Electrophoresis conditions were as follows: 200 V at 14°C for 15 h with a parabolic pulse of 60 s and for 8 h with a parabolic pulse of 90 s. (B) Lane 1, *XhoI*-digested lambda DNA; lane 2, *HindIII*-digested lambda DNA; lane 3, lambda concatamers (monomers to 20-mers); lane 4, *SpeI*-digested *A. pyrophilus* DNA. Electrophoresis conditions were as follows: 200 V at 15°C for 18 h with a linear ramp from 1 to 20 s. (C) Lane 1, *XhoI*-digested lambda DNA; lane 2, *HindIII*-digested lambda DNA; lane 3, *XbaI*-digested *A. pyrophilus* DNA. Electrophoresis conditions were as follows: 200 V at 15°C for 20 h with a linear ramp from 1 to 17 s. Sizes of the *S. cerevisiae* chromosome standards were 245 (chromosome I), 290 (VI), 370 (III), 460 (IX), 580 (VIII), 630 (V), 700 (XI), 770 (X), 800 (XIV), 850 (II), 945 (XIII), 1,020 (XVI), 1,120 (XV), 1,125 (VII), 1,600 (IV), and 2,200 (XII) kbp. The smallest band in the lambda concatamer ladders is the 48.5-kbp monomer. The buffers were 0.5× TAE buffer for panel A and 0.5× TBE buffer for panels B and C.

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TABLE 1. Restriction fragments of the *A. pyrophilus* chromosome and hybridization data

Restriction enzyme	Fragment	Size (kbp) ^a	Cross-hybridizing probe(s)	Hybridizing gene probe(s) ^b
<i>NotI</i>	N1	800	S1, S2, S3, S6, X1, X3, X4, X8, X9	<i>flaA</i> , <i>rrs</i>
	N2	520	S1, S2, S4, S5, X2, X3, X5, X6, X10, X11	
	N3	290	S2, X2, X7, X9	
	Total	1,610		
<i>SpeI</i>	S1	560	X1, X3, X4, X5, X8	<i>flaA</i>
	S2	530	X2, X7, X9	
	S3	450	X1	<i>rrs</i>
	S4	48	X6, X10, X11	
	S5	35	X5, X6	
	S6	12	X1	<i>rrs</i>
	Total	1,635		
<i>XbaI</i>	X1	620		<i>rrs</i>
	X2	370		
	X3	300		
	X4	180		<i>flaA</i>
	X5	42	N2, S1, S5	
	X6	35		
	X7	28		
	X8	15		
	X9	9		
	X10	8		
	X11	7		
	X12 ^c	0.7		
	Total	1,615		
Mean genome size		1,620		

^a Fragment lengths and the mean genome size were calculated from four (*NotI*) or three (*SpeI* and *XbaI*) independent experiments.

^b Cloned *A. pyrophilus* flagellin (*flaA*) and 16S rRNA (*rrs*) genes were used for probing chromosomal fragments.

^c Detected by the linking clone technique (see Table 2).

restriction buffer was removed after complete digestion of chromosomal DNA, and each gel plug was equilibrated with 1.0 ml of ESP buffer at 4°C for 20 min prior to electrophoresis.

PFGE and Southern transfer. Two 25 µl miniplugs were loaded onto a 1% SeaPlaque GTG agarose gel (FMC BioProducts), and PFGE was performed either in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA [pH 8.3]) at 15°C for the separation of small fragments or in 0.5× TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) at 14°C for large fragments on a Bio-Rad CHEF-DR II system. DNA extracted from exponentially growing cultures of *A. pyrophilus* yielded significantly sharper bands in the electrophoretograms than samples from stationary-phase cultures. *Saccharomyces cerevisiae* chromosomes, lambda DNA concatemers, and lambda DNA digested with either *XhoI* or *HindIII* were used as size standards. Sigma type I agarose (Sigma Co., Munich, Germany) was used for Southern transfer (21). For isotopic labeling of single fragments, DNA bands were cut out after PFGE, and DNA was extracted by using either the phenol-chloroform extraction method (17) or a Magic PCR DNA Purification System (Promega, Madison, Wis.) and random primed with a Random Primed DNA Labeling Kit (Boehringer, Mannheim, Germany) and [α -³²P]dCTP (3,000 Ci/mmol; Amersham Buchler, Braunschweig, Germany). Prior to Southern transfer, gels were exposed to 300-nm UV irradiation for 10 min for DNA fragmentation and then soaked in 0.25 N HCl for 20 min. For reprobing, nylon membranes were stripped by incubation, first in 0.4 M NaOH and then in 0.2 M Tris-HCl (pH 8.9)–0.1× SSPE (1× SSPE is 0.18 M NaCl, 1 mM EDTA, and 10 mM NaH₂PO₄ [pH 7.4])–0.1% sodium dodecyl sulfate at 45°C for 30 min each.

***NotI*, *SpeI*, and *XbaI* digestion patterns and genome size.** In

view of the low (40%) G+C content of *A. pyrophilus* DNA (7), 17 restriction enzymes that recognize GC-rich sites were tested. Of those, only *NotI* (GC/GGCCGC), *SpeI* (A/CTAGT), and *XbaI* (T/CTAGA) yielded convenient numbers of fragments, namely, 3, 6, and 12 bands resolvable by CHEF electrophoresis (Fig. 1). Interestingly, the last two endonucleases share the rare core sequence CTAG (14, 15, 18). Estimated sizes of fragments produced by the three enzymes and their sums are shown in Table 1. The sums consistently yield a total genome size of just over 1.6 Mbp. No band smaller than 7 kbp was detected by CHEF electrophoresis, even at a 50% higher DNA load or with a ramped pulse time of 1 to 5 s for 29 h. The only 0.7-kbp *XbaI* fragment was detected as part of a linking clone (see below). The presence of extrachromosomal DNA in *A. pyrophilus* was excluded by the negative results of (i) conventional 1% agarose gel electrophoresis and (ii) field inversion gel electrophoresis at 7 V/cm with ratio ramping of 1 to 20 s (initial ratio, 2.0; final ratio, 12.0) of undigested *A. pyrophilus* DNA.

Cross-hybridization to order the *NotI* fragments. The three *NotI* fragments of *A. pyrophilus* DNA were conveniently ordered by probing them with the purified and radiolabeled fragments of *SpeI* and *XbaI* digests. DNA fragments for use as probes were recovered from pulsed-field gels by phenol-chloroform extraction. From the hybridization patterns listed in Table 1, it follows that probes S1 and X3 link the *NotI* fragments N1 and N2 and that probe S2 links N1, N2, and N3, with the strongest signal at N3 (not shown). The deduced order of fragments, N1–N3–N2–N1, can be accommodated only by a circular map.

Use of *XbaI* and *SpeI* linking clones to construct a physical map. To resolve the order of *SpeI* and *XbaI* fragments,

FIG. 2. Construction and use of *Xba*I linking clones. (A) *A. pyrophilus* DNA fragments from a complete *Eco*RI digest after intramolecular ligation were linearized with *Xba*I and inserted into the selection vector pUCBM20 to generate a pool of Ap^r *E. coli* JM109 transformants. Individual linking clones were identified by screening for white colonies on Luria-Bertani agar containing IPTG (isopropyl- β -D-thiogalactopyranoside; 50 μ g/ml) and X-Gal (50 μ g/ml) and were further characterized by the *Eco*RI and *Xba*I restriction patterns of

TABLE 2. *Spe*I and *Xba*I restriction fragment contiguities established by Southern blots probed with radiolabeled inserts from *Spe*I- and *Xba*I-generated linking clones

Linking clone	Length (kbp) of insert used as probe	Hybridizing chromosomal fragments
<i>Spe</i> I generated		
pAPSL1	3.6	S2 and S4
pAPSL2	7.8	S1 and S6
pAPSL3	7.3	S1 and S5
pAPSL4	6.9	S2 and S3
pAPSL5	8.3	S4 and S5
pAPSL6	5.4	S3 and S6
<i>Xba</i> I generated		
pAPXL1	4.0	X3 and X5
pAPXL2	3.6	X9 and X7
pAPXL3	6.5	X1 and X9
pAPXL4	1.3	X3 and X4
pAPXL5	6.8	X2 and X7
pAPXL6	7.5	X10 and X11
pAPXL7	3.5	X6 and X10
pAPXL8	3.4	X4 and X8
pAPXL9 ^a	2.7	X1 and X8
pAPXL10	6.6	X5 and X6
pAPXL11	7.0	X11 and X2

^a The insert of pAPXL9 contained an extra 0.7-kbp *Xba*I fragment, X12, located between X1 and X8.

especially the poorly discernible smaller species, a library of linking clones (19) that covers all 6 *Spe*I and 12 *Xba*I sites of the *A. pyrophilus* chromosome was devised. The principle of constructing a pool of *Xba*I linking clones by taking advantage of the frequent cutter *Eco*RI and the single *Xba*I site of the *Escherichia coli* selection vector pUCBM20 (Boehringer) is schematically shown in Fig. 2A. By replacing *Xba*I with *Spe*I, the same scheme was used to construct a pool of *Spe*I linking clones by using the single *Xba*I site of pUCBM20 for insertion (*Xba*I and *Spe*I are compatible because of the common CTAG core of their recognition sequences). From 958 recombinants picked as white colonies from X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates (17), 6 *Spe*I and 11 *Xba*I linking clones were identified by (i) restriction mapping with *Eco*RI and *Xba*I (two sites each) or *Eco*RI and *Spe*I (two and zero sites, respectively) and (ii) Southern hybridization to one unique *Eco*RI fragment and two *Xba*I or *Spe*I fragments from *A. pyrophilus* DNA (Fig. 2B). The linking clones and hybridizing chromosomal fragments are summarized in Table 2. The insert of pAPXL9 is unusual in containing an extra 0.7-kbp *Xba*I fragment, X12, which is retained after partial *Xba*I digestion. Since pAPXL9 links X1 and X8, fragment X12 must

their plasmids. (B) Examples of autoradiographs from CHEF gels probed with the linking clones (Table 2) pAPSL6 (lanes 1 to 3) and pAPXL10 (lanes 4 to 6). Lanes 1 and 4, *A. pyrophilus* DNA digested with *Eco*RI. Conventional agarose (1%) gels were run at constant current of 30 mA in 0.5 $\times$ TBE buffer at room temperature for 12 h. Lanes 2 and 3, *A. pyrophilus* DNA digested with *Spe*I. Lanes 5 and 6, *A. pyrophilus* DNA digested with *Xba*I. CHEF gels were run at 200 V and 14 $^{\circ}$ C in 0.5 $\times$ TAE buffer for 15 h with a parabolic pulse time of 60 s and an additional 8 h with a parabolic pulse time of 90 s for lanes 2 and 5 or at 200 V and 15 $^{\circ}$ C in 0.5 $\times$ TBE buffer for 17 h with a linear ramp from 1 to 20 s for lanes 3 and 6. In lane 5, no hybridization signal was detected, because the pAPXL10 probe hybridized only with two small fragments, X5 and X6, which ran off this gel.

FIG. 3. Physical map of the *A. pyrophilus* chromosome. Restriction fragments and their lengths are listed in Table 1. Map areas assigned to the flagellin (*flaA*) and rRNA (*rm*) genes are indicated by arcs outside the map.

be located between X1 and X8. The combined results indicate the following series of contiguous fragments: S1-S5-S4-S2-S3-S6-S1 and X1-X12-X8-X4-X3-X5-X6-X10-X11-X2-X7-X9-X1. The *SpeI*- and *XbaI*-generated fragments were positioned relative to the *NotI* map (Fig. 3) by cross-hybridization to the *NotI* digest of genomic DNA and to the *SpeI* or *XbaI* digest of the isolated *NotI* fragments.

Assignment of *flaA* and *rm* loci to the chromosomal map. A cloned flagellin gene (*flaA*) of *A. pyrophilus* (2a) was located by Southern hybridization in the overlap region of N1 and X4 (kbp 612 to 792). Digestion of genomic DNA with *I-CeuI* endonuclease, which recognizes a 19-bp sequence in the 23S rRNA gene (12, 13), as well as by Southern hybridization with *A. pyrophilus* 16S ribosomal DNA (*rrs*), resulted in six separable bands. If the only genomic *I-CeuI* recognition sites are contained in the 23S rRNA genes (12, 13), this result indicates the presence of at least six rRNA units in the *A. pyrophilus* genome. These units are organized as 16S-23S-5S rDNA, as demonstrated by PCR amplification of the spacers joining 16S-23S rDNA and 23S-5S rDNA. All rDNA units were mapped in one fifth of the circular chromosome, between kbp 288 and 560 (Fig. 3), by *SpeI/CeuI* double digestion and Southern blot analyses. Whether these rRNA genes are clustered or located separately could not be determined with the low resolution of these methods.

Thermophiles and the evolution of genome structure. The hyperthermophilic bacterium *A. pyrophilus* has a circular chromosome with an apparent circumference of 1.6 Mbp. Of the thermophile genomes whose sizes are known, this is the smallest, being slightly smaller than the 1.7-Mbp genomes of *Thermotoga maritima* (9) and of *Streptococcus thermophilus* (10, 11), the 1.74-Mbp genome of *Thermus thermophilus* (3), and the 1.89-Mbp genome of the archaeon *Thermococcus celer*

(16). By contrast, the genomes of such well-studied mesophiles as *E. coli* K-12 (4.7 Mbp [20]), *Streptomyces coelicolor* (8 Mbp [8]), and *Myxococcus xanthus* (9 Mbp [5]) are considerably larger, suggesting that these species have three- to sixfold-higher coding capacities. However, this gain in genetic material is not accompanied by a similar increase in morphological and metabolic complexity, yet typically, greater versatility and a potential for adaptation to changing environments can be observed in these "modern" bacteria. Therefore, the small genomes of bacteria and archaea living at extreme temperatures may define the lower limit for their genetic material with a capacity for 1,500 to 1,700 genes. Small genome size may be a consequence both of the need for fast and faithful replication at elevated temperatures and of phylogenetic antiquity, implying that small chromosomes are an archetypal feature. The larger genomes of the more recent mesophilic species (presumably generated by gene duplication, gene rearrangements, and horizontal exchange of genetic material [8, 10, 22]) have endowed their cells with a greater potential for evolutionary change, as illustrated by cryptic genes, amplifiable DNA, transposable elements, and efficient systems for gene transfer, which are not found in the slowly evolving, ancient thermophiles. By inference, their chromosomes are densely packed with genes, most of which are required for essential functions. Hence, in a population of thermophiles, genetic polymorphism should be extremely rare. It will be interesting to determine whether such bacteria use mobile transposable elements as a primitive means for evolutionary change. In fact, IS1000, an insertion sequence from *T. thermophilus* has been described; however, no transposition activity has been observed (2). Such puzzles are now becoming amenable to experimental testing, as new technologies facilitate the physical and genetic mapping

of the chromosomes of fascinating organisms such as the hyperthermophile *A. pyrophilus*.

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REFERENCES

1. Acca, M., M. Bocchetta, E. Ceccarelli, R. Creti, K. O. Stetter, and P. Cammarano. 1994. Updating mass and composition of archaeal and bacterial ribosomes. Archaeal-like features of ribosomes from the deep-branching bacterium *Aquifex pyrophilus*. *Syst. Appl. Microbiol.* **16**:629–637.
2. Ashby, M. K., and P. L. Bergquist. 1990. Cloning and sequence of IS1000, a putative insertion sequence from *Thermus thermophilus* HB8. *Plasmid* **24**:1–11.
- 2a. Behammer, W., Z. Shao, W. Mages, R. Rachel, K. O. Stetter, and R. Schmitt. Submitted for publication.
3. Borges, K. M., and P. L. Bergquist. 1993. Genomic restriction map of the extremely thermophilic bacterium *Thermus thermophilus* HB8. *J. Bacteriol.* **175**:103–110.
4. Burggraf, S., G. J. Olsen, K. O. Stetter, and C. R. Woese. 1992. A phylogenetic analysis of *Aquifex pyrophilus*. *Syst. Appl. Microbiol.* **15**:352–356.
5. Chen, H., I. M. Keseler, and L. J. Shimkets. 1990. Genome size of *Myxococcus xanthus* determined by pulsed-field gel electrophoresis. *J. Bacteriol.* **172**:4206–4213.
6. Chu, G., D. Vollrath, and R. W. Davis. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* **234**:1582–1585.
7. Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter. 1992. *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Syst. Appl. Microbiol.* **15**:349–351.
8. Kieser, H. M., T. Kieser, and D. A. Hopwood. 1992. A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome. *J. Bacteriol.* **174**:5496–5507.
9. Kim, C. W., P. Markiewicz, J. J. Lee, C. F. Schierle, and J. H. Miller. 1993. Studies of the hyperthermophile *Thermotoga maritima* by random sequencing of cDNA and genomic libraries. *J. Mol. Biol.* **231**:960–981.
10. Leblond, P., P. Demuyter, J. M. Simonet, and B. Decaris. 1991. Genetic instability and associated genome plasticity in *Streptomyces ambofaciens*: pulsed-field gel electrophoresis evidence for large DNA alterations in a limited genomic region. *J. Bacteriol.* **173**:4229–4233.
11. Le Bourgevis, P., M. Mata, and P. Ritzenthaler. 1989. Genome comparison of *Lactococcus* strains by pulsed-field gel electrophoresis. *FEMS Microbiol. Lett.* **59**:65–69.
12. Liu, S., A. Hessel, and K. E. Sanderson. 1993. Genomic mapping with I-CeuI, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc. Natl. Acad. Sci. USA* **90**:6874–6878.
13. Marshall, P., and C. Lemieux. 1992. The I-CeuI endonuclease recognizes a sequence of 19 base pairs and preferentially cleaves the coding strand of the *Chlamydomonas moewusii* chloroplast large subunit rRNA gene. *Nucleic Acids Res.* **20**:6401–6407.
14. McClelland, M., and A. S. Bhagwat. 1992. Biased DNA repair. *Nature (London)* **355**:595–596.
15. McClelland, M., R. Jone, and M. Nelson. 1987. Restriction endonucleases for pulsed field mapping of bacterial genomes. *Nucleic Acids Res.* **15**:5985–6005.
16. Noll, K. M. 1989. Chromosome map of the thermophilic archaeobacterium *Thermococcus celer*. *J. Bacteriol.* **171**:6720–6725.
17. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. Schmid, K., M. Thomm, A. Laminet, F. G. Laue, C. Kessler, K. O. Stetter, and R. Schmitt. 1984. Three new restriction endonucleases MaeI, MaeII and MaeIII from *Methanococcus aeolicus*. *Nucleic Acids Res.* **12**:2619–2628.
19. Smith, C. L., and G. Condemine. 1990. New approaches for physical mapping of small genomes. *J. Bacteriol.* **172**:1167–1172.
20. Smith, C. L., J. G. Econome, A. Schutt, S. Kico, and C. R. Cantor. 1987. A physical map of the *Escherichia coli* K12 genome. *Science* **236**:1448–1453.
21. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
22. Weinstock, G. M. 1994. Bacterial genomes: mapping and stability. *ASM News* **60**:73–78.