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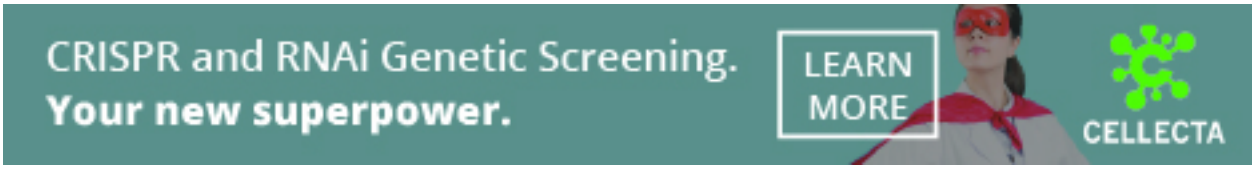

RESEARCH

The Marine Bacterium *Pseudoalteromonas haloplanktis* Has a Complex Genome Structure Composed of Two Separate Genetic Units

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The genome size of *Pseudoalteromonas haloplanktis*, a ubiquitous and easily cultured marine bacterium, was measured as a step toward estimating the genome complexity of marine bacterioplankton. To determine total genome size, we digested *P. haloplanktis* DNA with the restriction endonucleases *Not*I and *Sfi*I, separated the fragments using pulsed-field gel electrophoresis (PFGE), and summed the sizes of the fragments. The *P. haloplanktis* genome was 3512 ± 112 kb by *Not*I digestion and 3468 ± 54.1 kb by *Sfi*I digestion. *P. haloplanktis* is also shown to have a complex genome structure, composed of two large replicons of ~2700 and 800 kb. Three pieces of evidence support this conclusion: (1) Two separate bands are always seen in PFGE of undigested *P. haloplanktis* DNA; (2) restriction digests of the larger band are missing a band of ~650 kb compared with restriction digests of total genomic DNA; and (3) a 16S rDNA probe hybridized to the larger replicon but not to the smaller. To our knowledge, *P. haloplanktis* is the first marine bacterium shown to have a complex genome structure.

The structure and size of bacterial genomes are becoming increasingly important to microbial ecologists. This interest in bacterial genomics was stimuated in part by the cloning of 16S rRNA genes from nucleic acids extracted from environmental samples, which is now used routinely as a method for assessing microbial diversity (Ammann et al. 1995). These studies resulted in the recognition that uncultured species are important components of many natural ecosystems. The first cloning and sequencing of large genomic DNA fragments from an uncultured species were reported recently (Stein et al. 1996). Although the information obtained by random sequencing of this fragment failed to identify the physiology of the organism, in principle, genomic DNA sequencing can be used to understand the physiology of an uncultured microbe. Such attempts to link physiologically relevant genes with phylogenetically informative genes, such as 16S rRNAs, depend on assumptions about the complexity of bacterial populations, the size distributions of bacterial genomes, and the organization of bacterial chromosomes in natural systems. Similar considerations apply to genetic measurements of microbial diversity; Farrelly and colleagues (1995) recently emphasized that the sizes and copy numbers of genomes can greatly affect the frequencies of 16S rDNAs in natural populations.

The possibility that bacterial genomes might be reconstructed from contiguous, large DNA fragments cloned from natural populations relies on the assumption that bacterial genomes are single circular chromosomes. However, with the discovery of two separate circular chromosomes in *Rhodobacter sphaeroides* 2.4.1 (Suwanto and Kaplan 1989, 1992), the concept of the single circular chromosome that contains the entire genome of the prokaryote has been revised. A number of prokaryotes have complex genome organizations, including multiple chromosomes (Suwanto and Kaplan 1989, 1992; Zuerner 1991; Allardet-Servent et al. 1993; Michaux et al. 1993; Zuerner et al. 1993; Cheng and Lessie 1994) and large megaplasmids (Mergeay et al. 1985; Friedrich et al. 1986; Bancroft et al. 1989; Sobral et al. 1991; Lopez-Garcia et al. 1994; Muro-Pastor et al. 1994). The extent of such complexity has been largely unexplored, and, in particular, the genome structure of marine bacteria has, until now, been uncharacterized.

Marine bacterioplankton have served as a

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testbed for the development of methods for studying complex mixtures of nucleic acids derived from environmental samples (Giovannoni et al. 1990; Stein et al. 1995). Significantly, in this regard, it has been suggested that marine bacterioplankton have unusually small genomes (Robertson and Button 1989; Button et al. 1993). One approach to addressing this issue is the measurement of the genome sizes of model cultured marine bacterioplankton. There are a few species of bacterioplankton that have been cultured and are also commonly found in rDNA libraries from natural systems (Mullins et al. 1995). These are, therefore, good models for examining genome size in predominant bacterioplankton. Our model organism, *Pseudoalteromonas haloplanktis* (formerly *Alteromonas haloplanktis*; Gauthier et al. 1995), is an obligately aerobic gram-negative rod that is readily isolated from both coastal and open ocean water columns (Baumann et al. 1984). *P. haloplanktis* has also been found in both Atlantic and Pacific Ocean study sites by rDNA analysis, suggesting that it is a widespread and numerically important species in natural systems (Mullins et al. 1995).

Here *P. haloplanktis* is shown to have a moderate-sized genome, measured by pulsed-field gel electrophoresis (PFGE) to be ~3500 kb. We also report that *P. haloplanktis* has a complex genome structure, composed of two large, chromosome-sized replicons: The larger is ~2700 kb and the smaller ~800 kb. These results indicate that it would not be possible to reconstruct the genome of *P. haloplanktis* by assembling contiguous large DNA fragments cloned from environmental DNA samples.

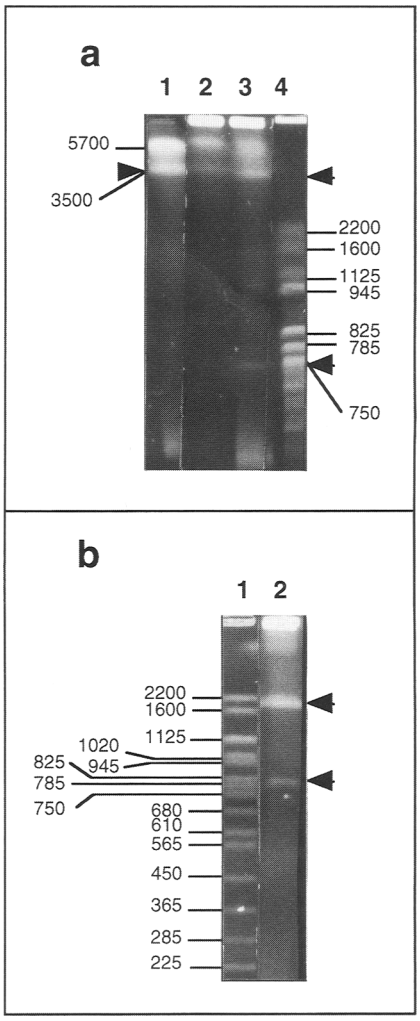

Figure 1 Undigested genomic DNA separated by two different CHEF protocols. Size markers are in kb. Arrowheads indicate linearized genomic elements. (*a*) PFGE protocol A (Table 1). Arrowhead at *left* refers to lane *2*; at *right*, lane *3*. (Lane *1*) *S. pombe* chromosomal DNA (size standard); (lane *2*) *E. coli* AB1157 (high concentration); (lane *3*) *P. haloplanktis* (one-fifth high concentration); (lane *4*) *S. cerevisiae* chromosomal DNA (size standard). (*b*) PFGE protocol B (Table 1). (Lane *1*) *S. cerevisiae* chromosomal DNA (size standard); (lane *2*) *P. haloplanktis* (one-fourth high concentration).

RESULTS

PFGE of Intact *P. haloplanktis* Genomic DNA

We examined the intact genome of *P. haloplanktis* and compared it with that of *Escherichia coli*. For this purpose, we used PFGE, specifically, the contour-clamped homogenous electric field (CHEF) method. During the optimization of CHEF protocols, an ancillary band of ~800 kb was always observed in preparations of *P. haloplanktis* genomic DNA but never in the preparations of *E. coli* genomic DNA. To examine this phenomenon further, we optimized two different run protocols designed to adequately separate this band from the larger replicon (Fig. 1 a,b). The extra band was observed under all conditions examined and during all steps of the optimization process. No bands smaller than the chromosomal DNA were ever seen for *E. coli* genomic DNA.

The relative ethidium bromide fluorescence intensity for the larger *P. haloplanktis* replicon was 14-fold higher than for the smaller replicon—4.1-fold higher than expected from the size

difference alone, assuming a one-to-one stoichiometry.

Sizing of the *P. haloplanktis* Genome

P. haloplanktis genomic DNA was digested with the restriction endonucleases *Not*I (Fig. 2a,b) and *Sfi*I (Fig. 2 c,d). *Not*I digestions yielded 16 observable DNA fragments ranging from 24.7 kb to 711 ± 8.7 kb. *Sfi*I cut the DNA into 20 fragments ranging from 31.0 kb to 586 ± 18.1 kb (Table 2, below). No single set of conditions were able to

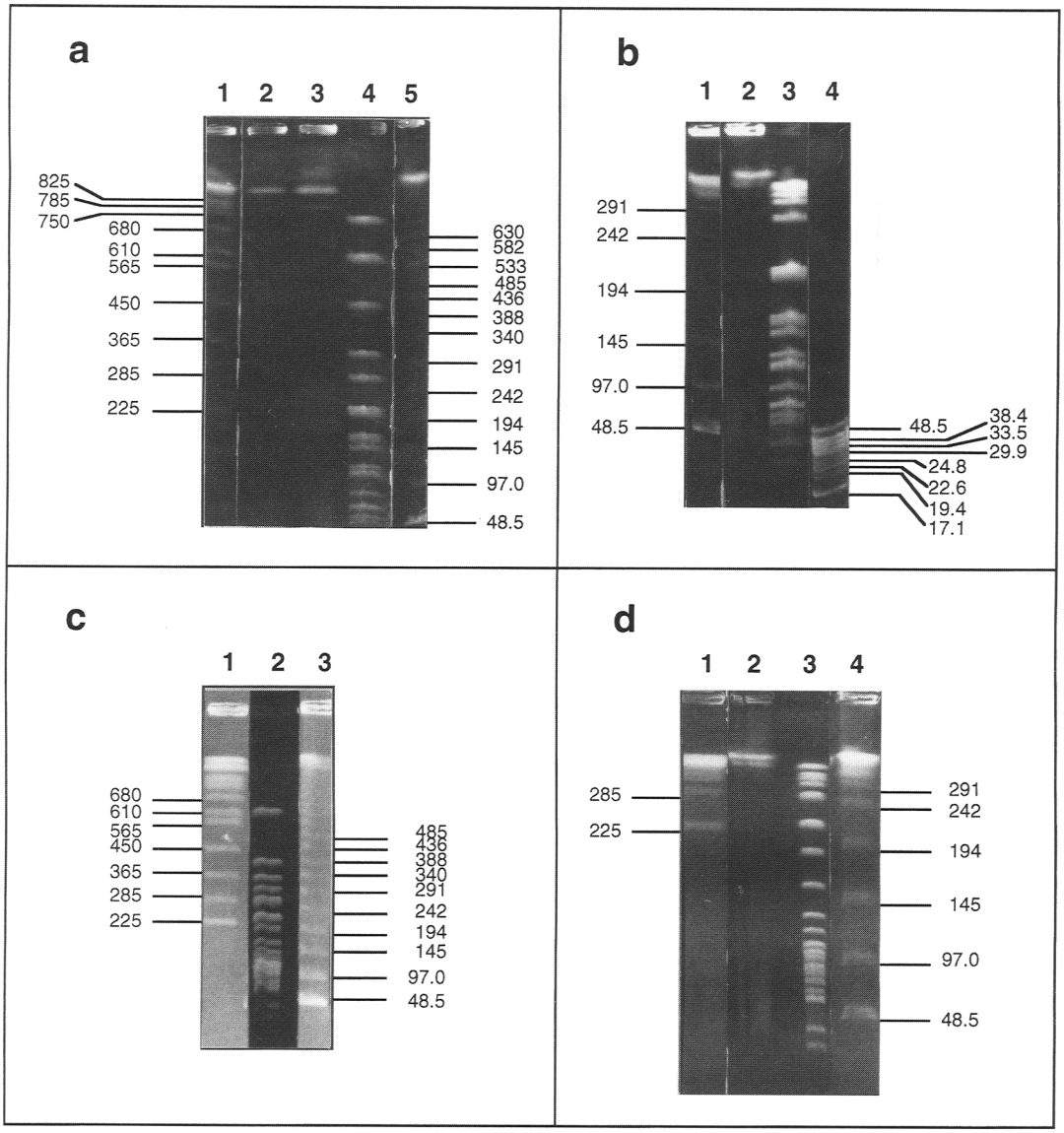

Figure 2 Sizing of the *P. haloplanktis* genome by digestion with *Not*I and *Sfi*I restriction endonucleases. Size standards are in kb. All *P. haloplanktis* lanes are one-fifteenth high concentration. (*a*) *Not*I large fragments. PFGE protocol C (Table 1). (Lane *1*) *S. cerevisiae* chromosomal DNA (size standard); (lane *2*) *P. haloplanktis* (untreated); (lane *3*) *P. haloplanktis* (*Not*I digestion buffer without enzyme); (lane *4*) *P. haloplanktis* (*Not*I digest); (lane *5*) bacteriophage λ concatemers (size standard). (*b*) *Not*I small fragments. PFGE protocol D (Table 1). (Lane *1*) Bacteriophage λ concatemers (size standard); (lane *2*) *P. haloplanktis* (*Not*I digestion buffer without enzyme); (lane *3*) *P. haloplanktis* (*Not*I digestion); (lane *4*) high-molecular-weight markers (size standard). (*c*) *Sfi*I large fragments. PFGE protocol C (Table 1). (Lane *1*) *S. cerevisiae* chromosomal DNA (standard size); (lane *2*) *P. haloplanktis* (*Sfi*I digest); (lane *3*) bacteriophage λ concatemers (size standard). (*d*) *Sfi*I small bands. PFGE protocol E (Table 1). (Lane *1*) *S. cerevisiae* chromosomal DNA (size standard); (lane *2*) *P. haloplanktis* (*Sfi*I digestion buffer without enzyme); (lane *3*) *P. haloplanktis* (*Sfi*I digest); (lane *4*) bacteriophage λ concatemers (size standard).

separate all resulting bands from either digest, so several protocols designed to separate different DNA size ranges were developed (Table 1). We analyzed seven gels with a total of 20 lanes containing *Not*I-digested *P. haloplanktis* genomic DNA and five gels with a total of 13 lanes containing *Sfi*I-digested *P. haloplanktis* DNA. Although these PFGE protocols were optimized, not every band within the appropriate size range for a protocol was visible in every lane owing to variability in loading and other interexperimental changes. Therefore, we averaged all of the sizes obtained for each band (Table 2). Many of the bands were sized with more than one size standard; each measurement was counted separately in the number of bands analyzed (Table 2). The total genome size was 3512 ± 112 kb as determined with *Not*I and 3468 ± 54.1 kb as determined with *Sfi*I.

We analyzed five gels with a total of 20 lanes containing undigested *P. haloplanktis* genomic DNA. Although there was high variability in the relative position of the band corresponding to the larger replicon (measured as 2977.6 ± 789.5 kb), the smaller replicon consistently gave a size of 800 ± 9.32 kb. By inference, the expected size of the large replicon is ~2690 kb. The larger band varied in position relative to the size standards depending on run conditions. This is probably because its size is between the largest band on the *Saccharomyces cerevisiae* size standard (2200 kb) and the smallest band on the *Schizosaccharomyces pombe* size standard (3500 kb), and, therefore, minor variations in either of these markers, sample loading, or other error could lead to large variations in the size estimate for this band.

Table 1. PFGE Protocols

Protocols[a]		Run time (hr)	Voltage (V/cm)	Switch time (sec)
A[b]	Step 1	24	1.5	2500
	Step 2	24	1.8	1200 → 600
	Step 3	24	2.4	600 → 300
	Step 4	24	3.6	300 → 90
	Step 5	8	4.8	90 → 5
B	Step 1	24	4.8	5 → 90
	Step 2	24	3.6	90 → 300
C	Step 1	24	4.8	90 → 5
D	Step 1	24	4.8	15
E	Step 1	16	4.8	15
	Step 2	8	4.8	9

[a]All gels were run at 12°C in a CHEF DR-II system from BioRad.
[b]All gels were run in 1.0% IBI Ultrapure Agarose, except for run protocol A, which was run in 0.85% IBI Ultrapure Agarose.

16S rDNA Hybridization to *P. haloplanktis* Genomic DNA

To determine whether the ancillary band seen during the PFGE of undigested total *P. haloplanktis* genomic DNA harbored rRNA operons, we hybridized a *P. haloplanktis* 16S rDNA PCR amplicon to a Southern blot of *E. coli* and *P. haloplanktis* total genomic DNA. As expected, there was strong hybridization to the single *E. coli* chromosomal DNA band. Visually, there was no hybridization to the smaller *P. haloplanktis* replicon, but there was obvious hybridization to the larger one (data not shown).

To determine whether the difference in hybridization intensity could simply be a reflection of the variance in the amount of DNA in the bands, a photograph of the ethidium bromide-stained gel corresponding to the blot was digitized and the pixel intensity of the two bands was quantitated. The larger replicon stained ~14-fold more intensely than the smaller replicon. After hybridization with the 16S rDNA probe, the phosphorimage of the blot was analyzed. We found that the hybridization to the larger replicon was a minimum of 40 times and a maximum of 200 times greater than the hybridization to the smaller replicon, depending on the value of the background subtraction. The difference in hybridization intensity was much greater than the difference in ethidium bromide staining, implying that the hybridization difference was attributable to factors other than the difference in the amount of DNA in the bands. This large difference in hybridization to the two bands is unlikely to be attributable to unequal transfer of the two bands during Southern blotting because, theoretically, the smaller replicon should transfer more efficiently than the larger. However, hybridization was stronger to the larger band than the smaller band.

*Not*I Digestion of the Larger Replicon

Two-dimensional PFGE (Cole and Saint Girons 1994) was used to further verify that the two

Table 2. Genome Size of *A. haloplanktis* as Determined by *Not*I and *Sfi*I Digestion

*Sfi*I			*Not*I		
band	size (kb)	number of bands analyzed	band	size (kb)	number of bands analyzed
A	586 ± 18.1	12	A	711 ± 8.70	27
B	395 ± 9.67	13	B	580 ± 8.55	29
C	345 ± 10.1	17	C	438 ± 6.42	30
D	312 ± 10.8	19	D	327 ± 6.10	31
E	266 ± 7.61	25	E	277 ± 4.89	31
F	226 ± 7.77	23	F	217 ± 7.21	20
G	192 ± 6.25	11	G	171 ± 2.42	14
H	162 ± 4.99	12	H	159 ± 1.91	13
I	140 ± 9.96	10	I	130 ± 1.29	14
J	123 ± 7.47	8	J	119 ± 1.53	13
K	109 ± 2.67	8	K	93.2 ± 1.69	13
L	104 ± 2.66	6	L	73.0 ± 1.22	13
M	98.3 ± 2.06	7	M	60.8 ± 1.64	5
N	91.7 ± 2.87	6	N	54.7 ± 0.55	4
O	84.0 ± 3.16	6	O	33.5 ± ND[a]	1
P	76.3 ± 2.14	7	P	24.7 ± ND	1
Q	67.2 ± 1.79	9			
R	61.0 ± 1.79	6			
S	42.0 ± ND	1			
T	31 ± ND	1			
Total	3512 ± 112	ND[a]	Total	3468 ± 54.1	ND[a]

[a]ND: no data.

bands constituted separate replicons. The first dimension was undigested *P. haloplanktis* total genomic DNA (e.g., Fig. 1b). The bands constituting the two replicons and a higher molecular weight band often seen just below the well (JBW) were excised from this gel, digested with *Not*I, and run in a second dimension (Fig. 3). We were unable to obtain enough DNA from the smaller replicon to see the digested fragments in the second dimension. However, we did visualize both the JBW digest and the larger replicon digest. The banding pattern for these digests was identical (Fig 3, lanes 4,6), demonstrating that these two bands are different conformers of the larger replicon.

Comparison of the banding pattern of the *Not*I-digested 2700-kb replicon to *Not*I-digested total genomic DNA revealed a band of ~650 kb that was present in the total genomic DNA digest but was missing from the large-replicon-alone digest (Fig. 3, lanes 3,4,6, see arrowhead). This band accounts for ~80% of the size of the smaller replicon. Presumably, the other 20% is found in lower molecular weight bands that we were unable to detect.

DISCUSSION

Digestions of *P. haloplanktis* total genomic DNA with *Not*I or *Sfi*I indicated a genome size of ~3500 kb (Fig. 2; Table 2). This genome size was consistently observed over a large number of repetitions and measurements. Although it has been suggested that marine bacteria have small genomes (Robertson and Button 1989; Button et al. 1993), the genome size of *P. haloplanktis* falls near the middle of the range for bacteria, which range from 600 kb (*Mycoplasma genitalium*) to 9450 kb (*Myxococcus xanthus*) as measured by PFGE (for review, see Cole and Saint Girons 1994). The most abundant marine bacteria, unlike *P. haloplanktis*, cannot be cultured by traditional methods (Giovannoni et al. 1990); therefore, *P. haloplanktis* may not be typical of marine bacterio-

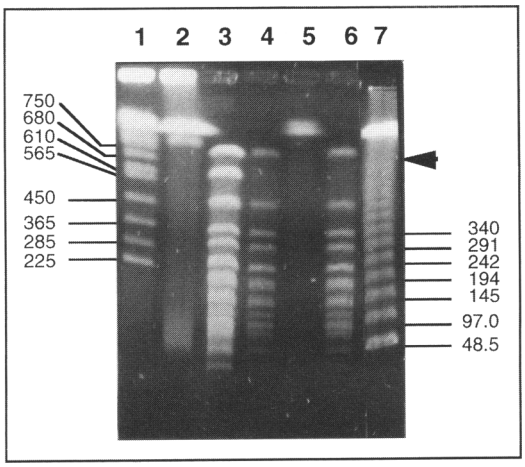

Figure 3 Two-dimensional PFGE of *P. haloplanktis* DNA. Size standards are in kb. First dimension: PFGE protocol B (Table 1). Second dimension: PFGE protocol C (Table 1). (Lane *1*) *S. cerevisiae* chromosomal DNA (size standard); (lane *2*) *P. haloplanktis* (*Not*I digestion buffer minus enzyme, one-fifteenth high concentration); (lane *3*) *P. haloplanktis* (*Not*I digested, one-fifteenth high concentration); (lane *4*) JBW band (*Not*I digested, one-fifth high concentration); (lane *5*) 2700-kb replicon (*Not*I digestion buffer minus enzyme, one-fifth high concentration); (lane *6*) 2700-kb replicon (*Not*I digested, one-fifth high concentration); (lane *7*) bacteriophage λ concatemers (size standard). Lane *3* contains an ~650-kb band that is not present in lanes *4* and *6* (arrowhead).

plankton. However, there is evidence that *P. haloplanktis* has a ubiquitous distribution in marine systems, and, therefore, it is one of the best examples of a marine bacterioplankton species available for study in culture (Mullins et al. 1995).

Three DNA bands consistently were observed by PFGE of *P. haloplanktis* undigested total genomic DNA (Fig. 1a,b), whereas two bands were visualized with *E. coli* AB1157 (Fig. 1a). Only one band was expected for *E. coli* AB1157, because this organism has only one chromosome and does not carry any plasmids. The band seen just below the well (JBW) had an apparent size much greater than the reported size of the *E. coli* K-12 chromosome (4700 bp; Smith et al. 1987). Because large circular DNA migrates only poorly through pulsed-field gels (Chu and Gunderson 1991; Gunderson and Chu 1991), we hypothesized that the JBW bands observed in both *E. coli* and *P. haloplanktis* lanes were circular conformers of the chromosome. This was confirmed by two-dimensional PFGE, which demonstrated that the JBW band and the 2700-kb band had identical restriction fragment banding patterns (Fig. 3, lanes 4,6).

The presence of the third band in the *P. haloplanktis* undigested genomic DNA implied the presence of a second large replicon. This conclusion was confirmed by comparisons to *Not*I digests of *P. haloplanktis* total genomic DNA, which revealed a band that was missing in digests of the 2700-kb or JBW bands (Fig. 3, see arrowhead). This band accounted for ~80% of the size of the smaller replicon as determined by PFGE of undigested *P. haloplanktis* DNA. Another piece of evidence indicating the presence of a second large replicon was 16S rDNA hybridization, which was much stronger for the larger replicon than for the smaller. Although part of the definition of a chromosome is that it carries essential housekeeping genes (Holloway 1993), the lack of a gene encoding 16S rRNA does not exclude a chromosomal function for the smaller *P. haloplanktis* replicon. Other housekeeping functions may yet be ascribed to the 800-kb replicon.

The observed 14-fold difference in the ethidium bromide fluorescence of the large and small replicons was greater than the value of 3.4 predicted from the ratio of the estimated molecular weights. This can be accounted for in three ways: (1) The 2700-kb replicon is always found in at least fourfold stoichiometry over the 800-kb replicon; (2) there were measurement errors owing to overlap between the JBW band and larger replicon; (3) double-stranded breaks are more likely to occur in the larger replicon because of its 3.4-fold greater size. Possibility 3 is the most likely explanation. The bands we observed are probably linearized versions of circular molecules. Assuming that the larger replicon is 3.4 times more likely to have fortuitous double-stranded breaks leads to an estimate of a 1:1.2 stoichiometry between the two replicons from the ethidium bromide fluorescence data. It also implies a circular nature for both bands in vivo, which is supported by the observation of different conformers of the larger *P. haloplanktis* replicon described above.

Suzuki and colleagues (1994) used PFGE to examine the genome of *P. haloplanktis* and several other, closely related organisms. Although they were unable to size the genomes of *P. haloplanktis* or *Pseudoalteromonas espejiana*, they reported the size of the genomes of *Pseudoalteromonas nigrifaciens* (2040 kb), *Pseudoalteromonas* sp.

M-1 (2240 kb), and *Shewanella putrefaciens* (2383 kb). The larger replicon of *P. haloplanktis* (~2700 kb) is close to the size range of the chromosomes of other *Pseudoalteromonas* spp. These data suggest that other organisms of the same genus do not carry the second (800-kb) genomic element. However, they did not report the banding pattern for undigested total genomic DNA of these species; therefore, it is unknown whether these congeners carry a second large replicon. Unlike Suzuki and colleagues (1994), we encountered no endogenous DNase activity that leads to random digestion of DNA in *P. haloplanktis*.

The *P. haloplanktis* genome organization is complex, consisting of two large, independent replicons. The nature of these replicons and their function is unknown. Many prokaryotes carry large genetic elements that code for important metabolic functions. These have been called megaplasmids because they resemble plasmids in transfer functions, copy number variability, and lack of essential housekeeping functions (Holloway 1993). Recently, however, multiple chromosomes have been described in five different bacterial species. The size of the "extra" chromosomes range from 350 kb in the case of *Leptospira interrogans* (Zuerner 1991; Zuerner et al. 1993) to 2500 kb in the case of *Pseudomonas cepacia* 17616 (Cheng and Lessie 1994) (Table 3). A mathematical model has been proposed that explains the selective advantage of maintaining the genome in multiple, smaller replicons rather than a single, larger replicon (Stouthamer and Kooijman 1993).

The line separating megaplasmids from chromosomes is vague; therefore, in this study we use the term "replicon." One current definition of a chromosome is an independent genetic element that carries essential housekeeping genes, is consistently present in all strains of a species, is nontransferable, and is present in a constant stoichiometric relationship with other chromosomal elements (Holloway 1993). By these criteria, neither the smaller replicon nor the larger replicon of *P. haloplanktis* were proven to be chromosomes.

P. haloplanktis is the first marine bacterium shown to have a complex genome structure consisting of more than one large genetic unit. The relevance of unusual genome structures such as those seen in this study to the functioning of cells in nature is not understood. They may have considerable implications in the study of microbial ecology, including measurement of genome sizes, gene copy number estimates, and determination of metabolic capabilities of bacterioplankton.

METHODS

Strains and Growth Conditions

P. haloplanktis was obtained from the American Type Culture Collection (catalog no. 14393). *E. coli* AB1157 was a gift from L. Walter Ream (Dept. of Agricultural Chemistry, Oregon State University, OR). *P. haloplanktis* was cultured at 30°C in 100 ml of 1/2 SWPYGR medium [20 ml/liter of Hutner's mineral base (Atlas 1993), 0.25 g/liter of D-glucose, 0.25 g/liter of Difco peptone, 0.25 g/liter of Difco yeast extract, 0.25 g/liter of ribose, 0.25 ml/liter of VA vitamin solution (Atlas 1993), 500 ml/liter of 0.2-μm filter-sterilized seawater]. Cells were grown to a density of of ~2×10^8 cells/ml and were pelleted by centrifugation at 5000*g* for 5 min at 4°C.

Lysis and Deproteination

Cells were embedded in agarose plugs as described by Smith and Condemine (1990), with minor modifications. Briefly, cells were embedded in 0.7% InCert agarose (FMC, Rockland, ME) or IBI Ultrapure low melting point agarose (IBI, New Haven, CT). These plugs were treated with a lysis buffer [50 mM NaCl, 10 mM Tris (pH7.5), 100 mM ethylene diamine tetraacetic acid (EDTA) (pH 8.0), 0.5% NaSarkosyl (Sigma); and 1 mg/ml of lysozyme (Sigma)] overnight at 37°C. The buffer was then changed to freshly made DB 0.5 [0.5 mM EDTA (pH 8.0); 1.0% NaSarkosyl (Sigma); 0.5 mg/ml of proteinase K (Sigma)] and further incubated for 24 hr at 37°C. Treated plugs were washed for at least 24 hr with at least three buffer changes in 0.5 M EDTA (pH 8.0). Agarose plugs could be stored in 50 mM EDTA (pH8.0) at 4°C for up to 6 months with no detectable degradation of the DNA. Our baseline concentration (called "high concentration") was 2.5×10^{10} cells/ml. We used various dilutions of this baseline for different applications as described.

Restriction Digestion

Plugs were equilibrated in 1 ml of Tris-EDTA (TE) for 15 min at room temperature, followed by two equilibrations of 15 min each in 300 ml 1× restriction buffer plus 0.1 mg/ml of acetylated bovine serum albumin (BSA). Forty units of enzyme were added to the buffer, and the mix was incubated overnight at 37°C with gentle agitation. The reaction was stopped by the addition of 0.5 M EDTA (pH 8.0) to a final concentration of 50 mM. The plugs were stored in this solution at 4°C until use, up to 1 week.

PFGE of DNA

PFGE was performed in a CHEF-DRII apparatus (Bio-Rad, Hercules, CA) in 0.5× Tris/borate/EDTA buffer (TBE) at 12°C. PFGE protocols are described in Table 1. *S. pombe*, *S. cerevisiae*, concatemers of bacteriophage λ DNA, and high-molecular-weight DNA markers (Bio-Rad) were used in

Table 3. Examples of Prokaryotic Organisms That Have Multiple Large Replicons Including the Sizes of Each Replicon

Organism	Genome size: total (kb)	Genome size: individual replicons (kb): chromosome(s)	Genome size: individual replicons (kb): megaplasmid(s)	Notes	Reference
Rhodobacter sphaeroides	3960	Chromosome I: 3046 Chromosome II: 914	None	Multiple chromosomes first discovered and best studied	Suwanto and Kaplan 1989, 1992
Agrobacterium tumefasciens C58	5750	Circular chromosome: 3000 Linear chromosome: 2100	Cryptic plasmid: 450 Ti plasmid: 200	Interdomain genetic transfer	Allardet-Servent et al. 1993
Leptospira interrogans	4750 to 4950	Chromosome 1: 4400 to 4600 Chromosome 2: 350	None	Spirochete, human and animal pathogen	Zuerner 1991; Zuerner et al. 1993
Brucella melitensis 16M	3250	Chromosome I: 2100 Chromosome II: 1150	None	Human and animal pathogen	Michaux et al. 1993
Pseudomonas cepacia 17616	7000	Chromosome I: 3400 Chromosome II: 2500 Chromosome III: 900	Cryptic plasmid: 170	Bioremediative organism	Cheng and Lessie 1994
Rhizobium meliloti	6500	Chromosome: 3400	pSym-b: 1700 pSym-a: 1400	Nodulating organism	Sobral et al. 1991
Haloferax mediterranei	3840	Chromosome: 2900	pHM500: 490 pHM300: 320 pHM100: 130	*Archaeon*	Lopez-Garcia et al. 1994
Alcaligenes eutrophus	ND[a]	Chromosome: ND[a]	pHG1: 450 pMOL28: 163 pMOL30: 238	Bioremediative organism	Mergeay et al. 1985; Freidrich et al. 1986
Anabaena spp. Strain PCC 7120	7110	Chromosome: 6400	α Megaplasmid: 410 β Megaplasmid: 190 γ Megaplasmid: 110	Provided first evidence of genetic transfer between cyanobacteria	Bancroft et al. 1989; Muro-Pastor et al. 1994

All prokaryotes with multiple chromosomes reported to date are presented. Examples of organisms with megaplasmids are also presented.
[a]ND: not determined.

various commbinations as size markers. Gels were stained with ethidium bromide (10 μg/ml) and photographed. Photographs were digitized with a Hewlett-Packard ScanJet IIc digital scanner. Sizing of the bands in the digitized images was performed with the FragmeNT analysis program (Molecular Dynamics, Sunnyvale, CA). In all figures, data in an individual panel are derived from a single gel.

Two-dimensional PFGE

The first dimension of the two-dimensional PFGE (Cole and Saint Girons 1994) was electrophoresis of agarose plugs containing *P. haloplanktis* total genomic DNA using protocol A or B (Table 1). End lanes were cut away from the rest of the gel, stained with ethidium bromide (10 μg/ml), and photographed with a ruler. The end lanes were used as templates for excision of unstained bands. Following excision, the unstained bands were digested with the restriction endonuclease *Not*I as described above. The second dimension was electrophoresis of these plugs with PFGE protocol C (Table 1) after restriction digestion.

Southern Blotting

Gels were photographed with a ruler and then blotted by the alkaline transfer method (Sambrook et al. 1989) onto Zeta-probe membranes (Bio-Rad). Blots were baked for 30 min at 80°C and then UV cross-linked with 1200 µJ in an Ultra-Lum UVC-515 ultraviolet cross-linker (Ultra-Lum, Inc., Carson, CA) to fix the DNA to the membrane.

Probe Synthesis

rDNA probes were made by PCR amplification (Saiki et al. 1988) of *P. haloplanktis* rDNA. DNA was extracted from a single colony by boiling for 5 min in 10 ml of a TAPS buffer [5 mM TAPS (*N*-tris{hydroxymethyl} methyl-3-aminopropanesulfonic acid; Sigma), 50 mM KCl, 0.1% Tween 20 (Sigma)], which assisted in cell lysis. Small-subunit ribosomal primers for the domain Bacteria (*sensu* Woese), 27F (AAGGAGGTGATCCANCCRCA) and 1518R (AGAGTTTGATCMTGGCTCAG) were used (Giovannoni 1991). The reaction conditions were 0.2 mM dNTPs (Stratagene, La Jolla, CA), 0.2 mM each primer, 10% acetamide, 6 mM $MgCl_2$, 1 ml of TAPS lysis supernatant, 1× buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100], and 2.5 units of *Taq* polymerase (Promega, Madison, WI) in a final reaction volume of 100 µl. Cycles were 96°C, 1 min; 55°C, 1.5 min; 72°C, 3 min; for 35 cycles. All reactions were performed on an MJ Research PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA).

Probe Labeling and Hybridization

Probes were radioactively labeled with a random priming DNA labeling kit (U.S.Biochemical, Cleveland, OH) by the manufacturer's instructions with [α-^{32}P]dCTP (3000 Ci/mmole; DuPont, Boston, MA). Blots were prehybridized by the membrane manufacturer's recommendations in a Techne HB-1 hybridization oven (Techne Incorporated, Princeton, NJ). Hybridization was carried out at 65°C overnight with 5×10^7 to 10×10^7 cpm of probe. Washes were performed by the membrane manufacturer's recommendations (Bio-Rad) with a 70°C stringent wash. Hybridization was visualized with a Molecular Dynamics PhosphorImager and ImageQuant software.

Analysis of rDNA Hybridization and Ethidium Bromide Stain Intensity

A photograph of the ethidium bromide-stained gel, taken prior to blotting and probing, was digitized with a Hewlett-Packard ScanJet IIc digital scanner. Pixel intensities for the ethidium bromide-stained bands were measured and compared using NIH Image. A similar procedure was performed for the digitized phosphorimage of the blot.

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