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Characterization and comparative study of the *rrn* operons of alkaliphilic *Bacillus halodurans* C-125

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Abstract The ribosomal RNA operons (*rrn*) of alkaliphilic *Bacillus halodurans* C-125 were characterized and compared with those of *B. subtilis*. We isolated clones containing *rrn* operons from a lambda phage library of the C-125 chromosome, and the complete nucleotide sequence of each was determined. Eight *rrn* operons were identified by PFGE analysis of the C-125 chromosome digested with *I-Ceu*I. The transcriptional orientation of the *rrn* operons mapped on the chromosome by Southern hybridization analysis was the same as the direction of replication of the chromosome. These operons were designated as *rrnA–H*, starting from the *oriC* locus in clockwise rotation. Sequence and structural analyses of these operons suggested that six of the *rrn* operons in the C-125 chromosome, *rrnA*, *rrnB*, *rrnC-rrnD*, *rrnE*, and *rrnH*, correspond to *rrnO*, *rrnA*, *rrnJ-rrnW*, *rrnI*, and *rrnD* in *B. subtilis*, whereas the other *rrn* operons (*rrnF* and *rrnG*) were specifically observed in C-125. The *rrn* loci were positioned from 0° to 90° on the physical map, with the *oriC* locus assigned the position zero degrees. Two ORFs annotated as *tnpA* and *ykfC*, whose gene products are likely to act as transposases, were found downstream of these six operons. Comparative analysis of the 16S–23S and 23S–5S ITS (internally transcribed sequence) regions of *B. halodurans* C-125 and those of *B. subtilis* revealed that the ITS regions in C-125 were much longer than those in *B. subtilis*. There was no substantial difference in the length of potential promoter sequences in *B. halodurans* and *B. subtilis*.

Key words Alkaliphilic *Bacillus halodurans* C-125 · Genome analysis · *rrn* operons · PFGE

Introduction

The facultatively alkaliphilic *Bacillus halodurans* C-125 (Takami and Horikoshi 1999), formerly called *Bacillus* sp. C-125, can grow well at pH 7–10.5 when sufficient sodium chloride (1%–2%) is present in the medium. During the past two decades, our studies have focused on the enzymology, physiology, and molecular genetics of alkaliphilic microorganisms in an effort to elucidate their mechanisms of adaptation to alkaline environments (Horikoshi 1991). To facilitate further genetic studies of *B. halodurans*, we have constructed an improved physical and genetic map of the C-125 chromosome (Takami et al. 1999a,e). At the same time, we reported analysis of the nucleotide sequence of the region containing the *oriC* locus and that of the major ribosomal protein gene cluster of C-125 (Takami et al. 1999c,d). Also, three independent DNA inserts (15–20 kb) isolated from a lambda phage library have been analyzed to determine their genetic features (Takami et al. 1999d). From this research background, we have been proceeding with systematic sequencing of the genome of strain C-125.

Although the majority of genes in prokaryotic organisms are present each at a single locus on the chromosome, multiple copies of genes and gene clusters such as rRNA operons (*rrn*) have been detected in bacterial genomes (Schmidt 1998). Although multiple copies of *rrn* genes are advantageous to support the high concentrations of ribosomes in rapidly growing cells, the full physiological significance of the multiplicity of *rrn* operons is still unclear. There is considerable variation in the number of *rrn* operons in prokaryotes, ranging from 1 to 13. For instance, Mycoplasma has only 1 *rrn* operon (Amikan et al. 1982), whereas *Clostridium beijerinckii* possesses 13 *rrn* operons (Wilkinson and Young 1995). In the case of *B. subtilis*, 10 *rrn* operons are distributed

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throughout the chromosome (Bott et al. 1984), and the positions of these *rrn* operons have been determined (Jarvis et al. 1988). Before studies on rRNA gene expression systems, maturation of rRNA, and protein synthesis of *B. halodurans* in alkaline conditions are undertaken, characterization of the *rrn* operons in this alkaliphile and comparisons with the nonalkaliphilic strain such as *B. subtilis* are definitely required. In this study, we characterized 8 *rrn* operons in the C-125 chromosome and conducted a detailed comparative analysis with those of *B. subtilis*. This is the first report of an exhaustive analysis of the *rrn* operons in an alkaliphile.

Materials and methods

Bacterial strain and media

Bacillus halodurans C-125 was used as a standard alkaliphilic *Bacillus* strain (Takami and Horikoshi 1999). Strain C-125 was grown aerobically at 37°C in N-II medium (pH 7.5) consisting of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 2% NaCl (Takami et al. 1992).

Preparation and digestion of *B. halodurans* chromosomal DNA with I-*Ceu*I

Bacillus halodurans chromosomal DNA for pulsed-field gel electrophoresis (PFGE) was prepared in agarose plugs using a modified version of the procedure previously described (Takami et al. 1999a). Strain C-125 was grown in 100 ml of N-II medium for 4–5 h until the mid-logarithmic phase of growth was reached. The cells from 1 ml of culture were harvested by centrifugation, washed once in buffer A (10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 50 mM EDTA), resuspended in 500 µl of buffer A (50°C) and mixed with 500 µl of 2% pulsed field certified (PFC) agarose prewarmed at 50°C. The resulting suspension was poured into a mold chamber (BioRad, Hercules, CA, USA). The solidified blocks were immersed in 10 ml of buffer B (10 mM Tris-HCl, pH 8.0, 50 mM EDTA) containing 40 mg of lysozyme and incubated at 37°C for 2 h. After washing the blocks in buffer C (20 mM Tris-HCl, pH 8.0, 50 mM EDTA) twice, they were incubated at 50°C in 10 ml of buffer D (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1.0% *N*-laurylsarcosine sodium salt) containing 10 mg Proteinase K (Gibco BRL, Gaithersburg, MD, USA) overnight. The blocks were washed once in buffer C and then incubated in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 1 h at room temperature. They were then washed three times more in buffer C. The blocks thus prepared were stored immediately in buffer C at 4°C until required for digestion. Agarose blocks containing the chromosomal DNA of strain C-125 were washed in 10 ml of 0.1× TE buffer, equilibrated with 2× and then 1× restriction buffer recommended by the manufacturer at 4°C for 20 min. DNA was digested with 10–15 units of I-CeuI (New England Biolabs, Beverly, MA, USA) at 37°C for 3 h in 300 µl of the restriction buffer.

PFGE analysis

To separate the I-*Ceu*I fragments of the chromosome of strain C-125, PFGE in 1% PFC agarose was carried out using the CHEF Mapper system (BioRad) in 0.5× TBE buffer at 14°C. The separation conditions used to resolve the shorter fragments (5–75 kb) and the larger fragments (50–600 kb) were described previously by Takami et al. (1999a). For separation of fragments in the range of 1–5 Mb, PFGE in 0.8% Chromosomal Grade Agarose (BioRad) was performed in 1× TAE buffer using a pulse time of 35 min, an angle of 106°, a voltage of 2V/cm, and a run time of 74 h. PFC agarose, chromosomal grade agarose, and DNA size markers (*Schizosaccharomyces pombe* chromosomal DNA; *Hansenula wingei* chromosomes) were from Japan BioRad Laboratories (Tokyo, Japan). DNA size markers (lambda ladder PFG marker and low range PFG marker) were purchased from New England Biolabs. To prepare PFGE hybridizing membranes for Southern blotting analyses, PFGE in 1% PFC agarose was also performed in 0.5× TBE buffer at 14°C using a pulse time of 0.47–44.89 s, an angle of 120°, a ramping factor of 0.532, a voltage of 6V/cm, and a run time of 20 h, 18 min. Blotting and hybridization experiments were performed by the previous method (Takami et al. 1999a).

Isolation and sequencing of *rrn* operons from a lambda phage library of the *B. halodurans* chromosome

A portion of the 16S rDNA region of the chromosome of strain C-125 was amplified by PCR using two primers (27F; 5′-AGAGTTTGATCCTGGCTCAG-3′, 350R; 5′-CTGCTGCCTCCCGTAG-3′), and the PCR product was labeled with digoxygenin (Boehringer, Mannheim, Germany) for use as a hybridization probe. A lambda phage library of the C-125 chromosome constructed in a previous study (Takami et al. 1999b) was screened using the 16S rDNA probe and then positive plaques were purified by four serial plaque hybridizations. The inserts in lambda phage were amplified by PCR by the previous method (Takami et al. 1999b). For sequencing of these inserts, the primer-walking method was used with a DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Assembling and editing of the determined DNA sequences were performed using AutoAssembler Ver. 2.0 (PE Applied Biosystems), and GENETYX-MAC Ver. 10 from Soft-ware Development (Tokyo, Japan) was used for sequence analysis. The sequences of the *B. halodurans rrn* operons have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB031209 for *rrnA*, AB031210 for *rrnB*, AB031211 for *rrnC/D*, AB031212 for *rrnE*, AB031213 for *rrnF*, AB031214 for *rrnG*, and AB031215 for *rrnH*.

Results and discussion

I-*Ceu*I sites on the *B. halodurans* chromosome

We used PFGE analysis of the chromosome digested with I-*Ceu*I to characterize the *rrn* operons, because this enzyme is known to recognize a specific sequence of 26 bases (5′-TAACTATAACGGTCCTAA/GGTAGCGA-3′) within the *rrn* operons in the genomes of *Salmonella typhimurium, Escherichia coli, B. subtilis*, and other bacteria (Liu et al. 1993; Toda and Itaya 1995). As shown in Fig. 1, digestion of the chromosomal DNA of strain C-125 with I-*Ceu*I yielded eight fragments (1I–8I) ranging in size from 6.5 to 3250 kb. The sizes of these fragments were determined through comparison with a series of DNA size markers for PFGE (Fig. 1). The mean total size of the C-125 chromosome was estimated to be 4.25 Mb based on the I-*Ceu*I digestion pattern, as well as the results of a previous study (Takami et al. 1999a), and in total eight *rrn* operons were identified by PFGE analysis of the C-125 chromosome digested with I-*Ceu*I.

Isolation of *rrn* operons from a lambda phage library of *B. halodurans*

A lambda phage library of strain C-125 constructed in lambda DASHII was screened using the DIG-labeled partial 16*S* rDNA as a probe, and clones containing a whole *rrn* operon region were detected. Of approximately 2.6×10^3 plaques, we obtained 71 positive clones. After four serial plaque hybridizations, all plaques showing positive signals were practically pure. The insert DNA of each positive clone was amplified by long accurate PCR to prepare the template for sequence analysis. Partially sequenced clones were grouped into eight groups, and the complete nucleotide sequence of each *rrn* operon and the surrounding region was determined by the primer-walking method. The I-*Ceu*I recognition site was confirmed in each 23S rDNA of the eight *rrn* operons, and these results were consistent with the I-*Ceu*I digestion pattern (Fig. 1). From these results, we were convinced that alkaliphilic *B. halodurans* strain C-125 possesses eight *rrn* loci on the chromosome. Sequence analysis also demonstrated that two of the eight *rrn* operons (*rrnC* and *rrnD*) were closely linked, similar to the case of the *rrnJ-rrnW* operons in *B. subtilis*.

Hybridization experiments for mapping and determination of the transcriptional orientation of the *rrn* operons

Mapping and determination of the transcriptional orientation of each *rrn* operon were performed by Southern hybridization of I-*Ceu*I-digested fragments with specific DNA probes designated from the 5′- and 3′-regions of the operons. These specific probes prepared from the regions upstream (5′-) and downstream (3′-) of *rrn* operon, including no ribosomal specific sequence (1–10 kb upstream or downstream of the operons), allowed us to determine the copy number and orientation of the *rrn* operons. To compare the positions of the *rrn* operons in the C-125 chromosome with those of *B. subtilis*, it was indispensable to know which I-*Ceu*I fragment contained the *oriC* region. A *dnaA* probe (Takami et al. 1999d) was used to identify the *oriC*-containing I-*Ceu*I fragment. In this experiment, the *dnaA* probe hybridized to the 1I fragment. The distance from the I-*Ceu*I site to the *oriC* locus was estimated to be approximately 25 kb by sequencing of the 1I fragment (data not shown).

Hybridization experiments with I-*Ceu*I digests were carried out using several 5′- and 3′- region probes. Probes *rrnA*-5′ and *rrnA*-3′ hybridized to 1I and 7I, *rrnB*-5′ and

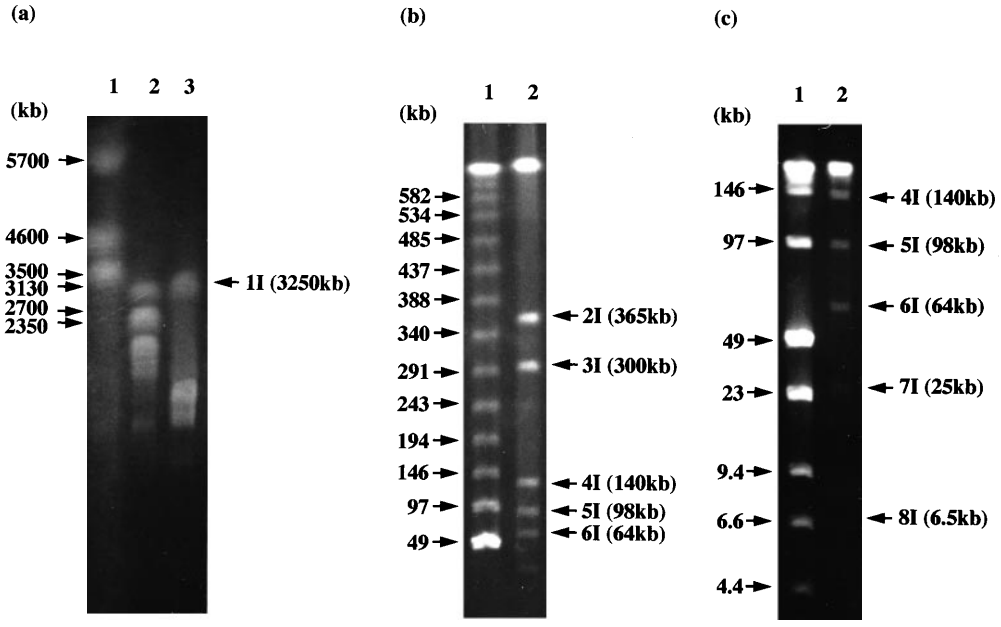

Fig. 1a–c. Separation of the I-*Ceu*I fragments of the *Bacillus halodurans* chromosome. **a** Separation of fragments ranging in size from 1 to 5 Mb using a pulse time of 35 min, an angle of 106°, a voltage of 2V/cm, and a run time of 74 h. **b**, **c** Separation of fragments from 50 to 600 kb and from 5 to 75 kb (Takami et al. 1999a). Separation patterns of fragments obtained by complete I-*Ceu*I digestion are shown in *lane 3* of **a** and *lane 2* of **b** and **c**. Size markers above 1 Mb shown in **a** are *Hansenula wingei* chromosomes (*lane 2*) an *Schizosaccharomyces pombe* chromosomal DNA (*lane 1*). The size marker used for 50–600 kb (*lane 1* in **b**) was the lambda ladder PFG marker; that used for the range below 75 kb (*lane 1* in **c**) was the low range PFG marker

Table 1. Lengths of ITS and potential promoter regions in *rrn* operons of *Bacillus halodurans*

rrn operon	Length of ITS (bases)		Length of potential promoter region (bases)
	16*S*–23*S*	23*S*–5*S*	
rrnA	684	408	238
rrnB	851	305	172
rrnC	998	410	368
rrnD	807	305	193
rrnE	682	304	260
rrnF	727	408	173
rrnG	971	408	171
rrnH	851	408	256

ITS, internally transcribed sequence

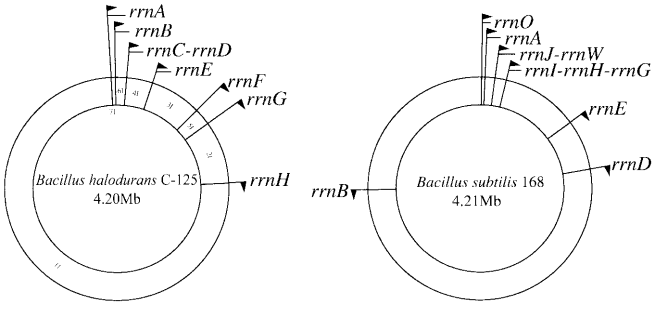

Fig. 2. Location of 8 *rrn* operons in the *B. halodurans* chromosome and comparison with that of 10 *rrn* operons in the *B. subtilis* chromosome. The transcriptional orientation of the *rrn* operons is symbolized by a *black flag*

rrnB-3′ to 7I and 6I, *rrnC*/D-5′ and *rrnC/D*-3′ to 6I and 4I, *rrnE*-5′ and *rrnE*-3′ to 4I and 3I, *rrnF*-5′ and *rrnF*-3′ to 3I and 5I, *rrnG*-5′ and *rrnG*-3′ to 5I and 2I, and *rrnH*-5′ and *rrnH*-3′ to 2I and 1I. The 6.5-kb 8I fragment was found to be located between 6I and 4I, indicating that the linkage of these fragments was 6I–8I–4I. A series of hybridization experiments revealed that the linkage of the I-*Ceu*I fragments of the C-125 chromosome was 1I–7I–6I–8I–4I–3I–5I–2I–1I– (Fig. 2). These operons were designated as *rrnA–H*, as shown in Fig. 2; each is symbolized by a black flag starting from the *oriC* locus in clockwise rotation. The transcriptional orientation of the eight *rrn* operons mapped on the chromosome by Southern hybridization analysis was the same as the direction of replication of the chromosome. Considering that highly expressed genes are usually transcribed in the same direction as the replication forks, avoiding head-on collision with the replication forks (Brewer 1988), it seems likely that the *B. halodurans* chromosome is essentially replicated by a bidirectional replication mechanism from the *oriC* locus to the *terC* locus.

Structural analyses of the *rrn* operons and comparison between *B. halodurans* and *B. subtilis*

The determined sequences of the regions upstream and downstream of each *rrn* operon were searched for ORFs by the previous method (Takami et al. 1999b). Two ORFs identified in the region upstream of *rrnA* (Fig. 3) showed no significant similarity to any protein so far reported. Another two ORFs, similar to *yaaC* and *guaB* in *B. subtilis*, were identified in the region downstream of *rrnA* in *B. halodurans* (Fig. 3). It seems that *rrnA* in *B. halodurans* corresponds to *rrnO* in *B. subtilis*, as similar ORFs were found just downstream of *rrnO*, although the distance from *oriC* to *rrnA* is 15 kb longer than in the case of *rrnO*. It has been reported that there are three ORFs of unknown function downstream of *gyrA* instead of *rrnO*, which is found in the same region of *B. subtilis* (Takami et al. 1999d). On the other hand, it was found that *rrnB*, *rrnC-rrnD*, *rrnE*, and *rrnH* in *B. halodurans* correspond to *rrnA*, *rrnJ-rrnW*, *rrnI*, and *rrnD* in *B. subtilis*, but counterparts of *rrnH*, *rrnG*, and *rrnB* in *B. subtilis* were not found in *B. halodurans* (Fig. 3). In the region upstream of *rrnF*, two ORFs (*ydcI* and *ydck* in *B. subtilis*) and tRNA genes were identified. In *B. subtilis*, those genes are not linked to any *rrn* operon. Two ORFs of *ygaH* and *ygxA* were commonly observed upstream of *rrnH* in *B. halodurans* and *rrnD* in *B. subtilis*, as shown in Fig. 3. On the other hand, another ORF that is partially similar (with 31% identity) to p-type ATPase gene was found between *ygaH* and *ygxA* in *B. halodurans*. The *yvgQ* gene located at 291° in the *B. subtilis* chromosome was found upstream of the *rrnG* operon in *B. halodurans*. An ORF similar to *nasA* (31°), located in a region separate from the *rrn* operons in the *B. subtilis* chromosome, was found downstream of *rrnG* in *B. halodurans*. Thus, these two *rrn* operons (*rrnF* and *rrnG*) seem to be specifically observed in *B. halodurans* (Fig. 3).

Two kinds of unique ORFs were observed downstream of six *rrn* operons in *B. halodurans*, compared with *B. subtilis*. As shown in Fig. 3, the first ORF, showing similarity to the *tnpA* gene product (transposase) produced by *Bordetella parapertussis* (Van der Zee et al. 1993), was located downstream of *rrnE* and *H*. The second one, which is similar to the *ykfC* gene product of *E. coli* (Blattner et al. 1997), was located downstream of *rrnB*, *C/D*, *F*, and *G* (Fig. 3). The *tnpA* gene has been identified also in lambda clone no. 3 from the *Bacillus halodurans* chromosome (Takami et al. 1999a) and in *Sse*8387I-linking clone q (Takami et al. 1999e). On the other hand, the *ykfC* gene was identified in *Asc*I-linking clone G (Takami et al. 1999a). The *ykfC* gene, the function of which is unknown, is located in between b0257 (putative transposase) and the IS5 transposase gene in *E. coli* (Blattner et al. 1997). The *rrnE* in *B. halodurans* possesses the structure that is deleted with *rrnH* and *rrnG* downstream of *rrnI* in *B. subtilis* (Fig. 3). Two ORFs of *ygaI* and *ygzA* were located downstream of *rrnD* in *B. subtilis*, while in *B. halodurans* these two ORFs were not found downstream of the *rrnH* operon. Instead, the *tnpA* gene was commonly observed downstream of the *rrnE* and *rrnH* operon. These structural differences suggest that this kind of transposable element may be involved in gene transposition in the C-125 chromosome, as mentioned previously (Takami et al. 1999e).

Within each of the *rrn* operons, there is an intergenic spacer region designated as the ITS (internally transcribed sequence), separating the 16S and 23S rRNA genes or the

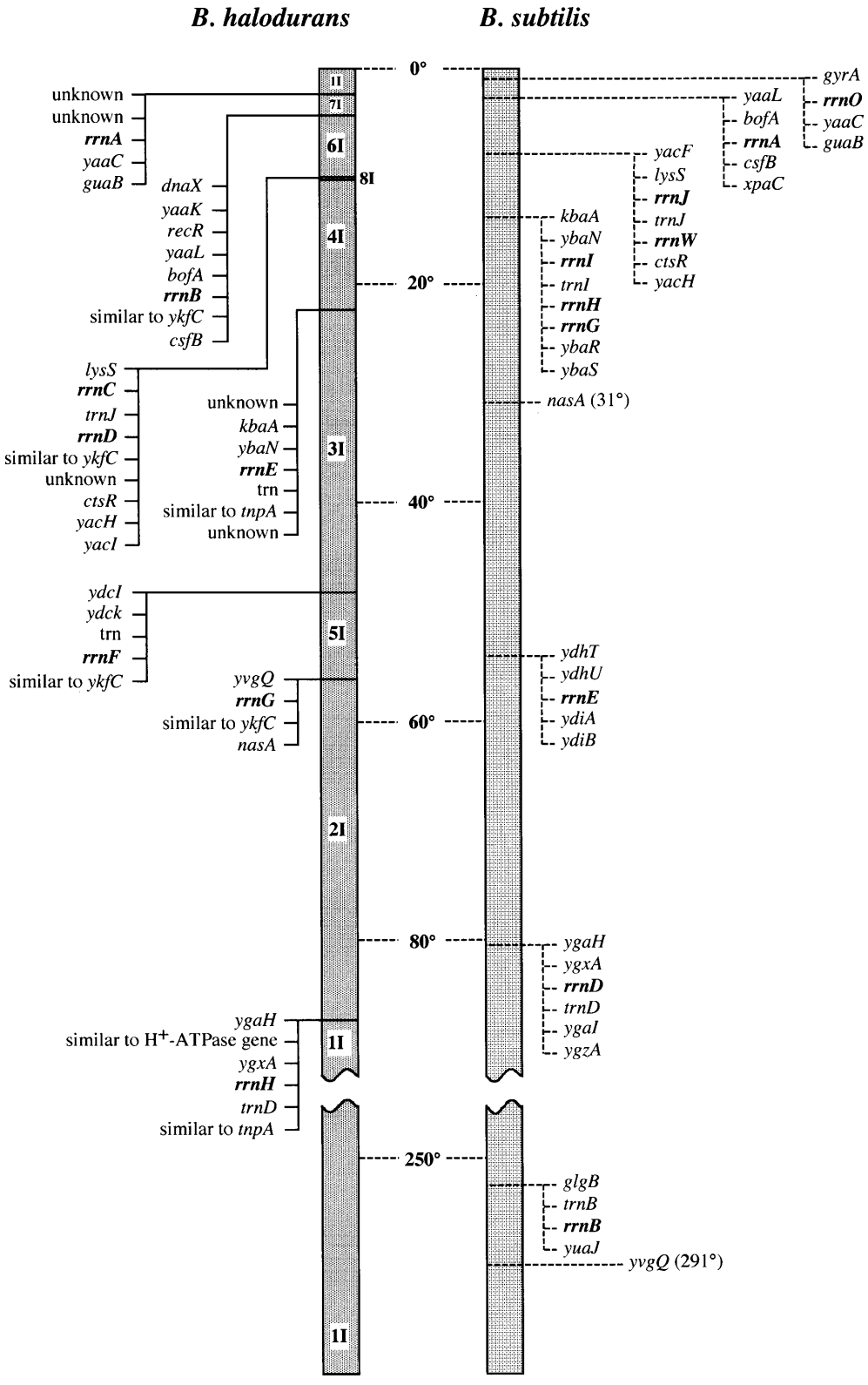

Fig. 3. Analysis of *rrn* operons and their flanking regions on the chromosome of *B. halodurans* and comparison with the genetic map of *B. subtilis*. Each *rrn* operon of *B. halodurans* was mapped on the linkage map of I-*Ceu*I fragments (*I*). The scale of 360°, beginning with zero at the *dnaA* locus, is based on the *B. subtilis* map (Biaudet et al. 1996). The *solid lines* and *dashed lines* represent the *B. halodurans* and the *B. subtilis* chromosome, respectively

23S and 5S rRNA genes, and the ITS is frequently used as one of the criteria for identification of microorganisms (Gürtler and Stanisich 1996). As shown in Table 1, the length of the 16S–23S ITS and the 23S–5S ITS of the *rrn* operons varied from 682 to 998 bases and from 304 to 410 bases, respectively. In *B. subtilis*, the length of the 16S–23S ITS (165–347 bases) and that of the 23S–5S ITS (57–113 bases) were much shorter than those of *B. halodurans*. Although the physiological significance of the differences in length of the ITS is unclear, longer ITS regions are

characteristic of the *rrn* operons of *B. halodurans*. Secondary structures of the ITS regions between these species are also different. Maturation of rRNA requires several exoribonucleases such as RNase T and RNase III recognizing a specific sequence in the ITS and the secondary (or tertiary) structures (Zhongwei et al. 1999). Thus, differences in the maturation mechanism between *B. halodurans* and *B. subtilis* are also of biochemical interest.

We also compared the length of potential promoter sequences in *B. subtilis* and *B. halodurans*. A potential promoter region was defined as a nucleotide sequence extending from a position upstream of the 16S rDNA to the stop codon of the ORF adjacent to the 16S rDNA. As shown in Table 1, the length of the potential promoters varied from 171 to 368 bases in *B. halodurans*, compared to *B. subtilis*, which had potential promoter sequences varying from 173 to 368 bases in length. No substantial differences in potential promoter regions were detected. In *B. subtilis*, two SigA (RpoD)-dependent *rrnB* promoters have already been estimated using a gel retardation assay with *E. coli* RNA polymerase (Wellington and Spiegelman 1993). Similar *rrn* promoters will be detected in *B. halodurans*; these studies are currently being conducted.

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