JOURNAL OF BACTERIOLOGY, Apr. 1999, p. 2118–2123 Vol. 181, No. 7
0021-9193/99/$04.00+0

Isolation of RNase H Genes That Are Essential for Growth of *Bacillus subtilis* 168

MITSUHIRO ITAYA,[1]* AKIRA OMORI,[1] SHIGENORI KANAYA,[2] ROBERT J. CROUCH,[3] TERUO TANAKA,[1]† AND KANAE KONDO[1]

Mitsubishi-Kasei Institute of Life Sciences, Machida-shi, Tokyo 194-8511,[1] and Material and Life Science, Graduate School of Engineering, Osaka University, Osaka 565-0871,[2] Japan, and Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-2790[3]

Received 29 October 1998/Accepted 28 January 1999

Two genes encoding functional RNase H (EC 3.1.26.4) were isolated from a gram-positive bacterium, *Bacillus subtilis* 168. Two DNA clones exhibiting RNase H activities both in vivo and in vitro were obtained from a *B. subtilis* DNA library. One (28.2 kDa) revealed high similarity to *Escherichia coli* RNase HII, encoded by the *rnhB* gene. The other (33.9 kDa) was designated *rnhC* and encodes *B. subtilis* RNase HIII. The *B. subtilis* genome has an *rnhA* homologue, the product of which has not yet shown RNase H activity. Analyses of all three *B. subtilis* genes revealed that *rnhB* and *rnhC* cannot be simultaneously inactivated. This observation indicated that in *B. subtilis* both the *rnhB* and *rnhC* products are involved in certain essential cellular processes that are different from those suggested by *E. coli rnh* mutation studies. Sequence conservation between the *rnhB* and *rnhC* genes implies that both originated from a single ancestral RNase H gene. The roles of bacterial RNase H may be indicated by the single *rnhC* homologue in the small genome of *Mycoplasma* species.

RNase H (EC 3.1.26.4) endonucleolytically cleaves RNA in RNA-DNA hybrid molecules (26). This activity is present in almost all organisms, from viruses to humans (3). An RNase H gene that encodes bacterial RNase HI (*rnhA*) (2) was first cloned from *Escherichia coli* K-12 by measurement of biochemical activity (21, 31). Subsequently, use of conditional lethal *E. coli rnhA* mutants, very sensitive to the residual levels of RNase H activity in vivo (18), permitted isolation of a second RNase H gene (*rnhB*) from *E. coli* that encodes bacterial RNase HII (13).

Isolation of *rnhA* genes from *Salmonella typhimurium* LT2 (18), *Thermus thermophilus* HB8 (17), *Mycobacterium smegmatis* (34), and the yeast *Saccharomyces cerevisiae* (18) has been reported. The three-dimensional structure has been determined by X-ray crystallographic analysis for *E. coli* RNase HI (25, 41), the heat-stable RNase HI from *T. thermophilus* (12), and the retroviral homologue RNase H domain of human immunodeficiency virus (HIV) reverse transcriptase (28). Extensive mutagenesis of *E. coli* RNase HI (6, 9, 22–24) has been carried out, and a detailed mechanism for the enzyme's catalytic reaction has been proposed (24). Based upon studies of various *E. coli rnhA* mutants, physiological roles of RNase HI (*rnhA*) in DNA replication (1, 4, 16, 27, 35), repair (15), and transcription (36) have been proposed. In contrast to the highly active RNase HI (*rnhA*), constituting more than 90% of the total RNase H activity of *E. coli* (3), RNase HII (*rnhB*), which exhibits only 0.4% of the activity of wild-type RNase HI with poly(rA) · poly(dT) used as a substrate (13), has not been studied in any detail.

By computer-assisted searches of the complete genomes of bacteria, a clearly recognizable homologue of RNase HI (*rnhA*) cannot be found in the *Archaebacteria* or *Mycoplasma* genomes. In contrast, ubiquitous RNase HII (*rnhB*) homologues have been recognized in *Archaebacteria* (33, 42). The lack of obvious bacterial RNase HI (*rnhA*) or RNaseHII (*rnhB*) homologues in the genomes of *Mycoplasma* species (8, 11), along with the viability at low temperature of an *E. coli* mutant strain that lacks both RNase HI and RNase HII (12a), suggests that RNase H is dispensable for cell viability or can be replaced by another enzyme possessing exonuclease activity (29).

We attempted to isolate a functional RNase H gene(s) from the gram-positive bacterium *Bacillus subtilis* by screening a DNA library. Two RNase H genes are present in the *B. subtilis* genome, including the newly characterized *rnhC* gene. Mutational analysis indicated that loss of *rnhB* and *rnhC* renders *B. subtilis* unable to grow, suggesting essential roles for these RNase H genes in cell viability. Discovery of the *B. subtilis rnhC* gene allowed computational identification of the *rnhC* homologue in the *Mycoplasma* genomes, where no RNase H homologue had yet been reported (8, 11).

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Preparation and transformation of competent *E. coli* cells were by the method of Mandel and Higa (32). Preparation and transformation of competent *B. subtilis* cells were as previously described (40). Luria-Bertani (LB) broth was used for growth of *E. coli*. Antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) was used for growth of *B. subtilis*. Bacteria were grown at 37°C unless otherwise mentioned. Antibiotic resistance gene cassettes were prepared from the *E. coli* plasmids listed in Table 1. All plasmids were purified by CsCl-ethidium bromide ultracentrifugation.

In vitro DNA manipulations. Type II restriction enzymes and T4 DNA ligase were obtained from Toyobo (Tokyo, Japan), except for *Not*I (Takara Shuzo, Kyoto, Japan). A Takara exonuclease III (ExoIII) deletion kit was purchased from Takara. DNA manipulations in vitro were done according to procedures described in reference 38 or the manufacturers' instructions, unless specified otherwise. Southern hybridization was carried out with nylon membranes

* Corresponding author. Mailing address: Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194-8511, Japan. Phone: 81-427-24-6254. Fax: 81-427-24-6316. E-mail: ita@libra.ls.m-kagaku.co.jp.

† Present address: School of Marine Science and Technology, Tokai University, Shimizu, Shizuoka 424-8610, Japan.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid[a]	Genotype or insert	Reference or source
B. subtilis strains		
1A1 (= 168 *trpC2*)	*trpC2*	BGSC[d]
CU741	*trpC2 leuC7*	14
OA101	Prototroph isolated from CU741	14
BEST23	CU741 plus *rnhC151*::*cat*	pMIB15-N1 × CU741: Cm
BEST138	OA101 plus *rnhB21*::*neo*	pMIB21LNEO × OA101: Nm
BEST206	1A1 plus *ypdQ44*::*spc*	pRNHA-4 × 1A1: Sp
BEST218	1A1 plus *rnhB21*::*neo*	BEST138 × 1A1: Nm
BEST220	1A1 plus *rnhC151*::*cat*	BEST21 × 1A1: Cm
BEST207	*ypdQ44*::*spc rnhB21*::*neo*	BEST23 × BEST206: Cm
BEST208	*ypdQ44*::spc *rnhC151*::*cat*	BEST138 × BEST206: Nm
E. coli strains		
JA221	F$^-$ *hsdR hsdM*$^+$ *trp leu lacY recA1*	13
MIC3037[b]	F$^-$ *rnhA339*::*cat recC271 hsdR hsdM*$^+$ *trp leu*	15
MIC2067[b]	F$^-$ *rnhA339*::*cat rnhB716*::*kam*	This study
MIC1021[b]	F$^-$ *rnhA-91 recB270*	16
Plasmids		
pRNHA-1	*ypdQ* (0.474-kb insert) in pGEM4	This study
pUC2.2	*ypdQ* (2.2-kb insert) in pUC18	N. Ohtani
pRNHA-4	*ypdQ44*::*spc* in pUC18	This study
pMIB21	*rnhB* in pBR322	This study
pMIB21LNEO	*rnhB21*::*neo* in pBR322	This study
pMIB15	*rnhC* in pBR322	This study
pMIB15-N1	*rnhC151*::cat in pBR322	This study
pGEM4		13
pBEST6	Chimeric plasmid pBR322 and pGEM4	This study[c]
pBEST517A	*spc* cassette	12a
pBEST512	*neo* cassette	14
pBEST4F	*cat* cassette	20

[a] Selection conditions for both *E. coli* and *B. subtilis* were 5-μg/ml chloramphenicol (Cm) and 50-μg/ml spectinomycin (Sp). Selection for neomycin resistance transformants was on 25-μg/ml kanamycin (Km) for *E. coli* and 5-μg/ml neomycin (Nm) for *B. subtilis*.
[b] Temperature-sensitive growth phenotype. Details for construction of MIC2067 will be published elsewhere.
[c] *Ava*I-*Bgl*I fragment of pBR322 and *Pvu*II-*Bgl*I fragment of pGEM4 were ligated to give multiple cloning sites to the pBR322 replicon.
[d] *Bacillus* Genetic Stock Center (Ohio State University, Columbus).

(Nytran 13N; Schleicher & Schuell, Dassel, Germany), as previously described (14).

Complementation of conditional-lethal *E. coli* RNase H mutants. Three *E. coli* mutants—MIC3037, MIC1021, and MIC2067—form colonies at 30°C but not at 42°C. The temperature-dependent growth defect of MIC3037 and MIC1021 was explained as the adverse effect of *recBC* and *rnhA* double mutations at restrictive temperature (16). This temperature-sensitive phenotype was relieved by introduction of the *recBC* or *rnhA* gene (16, 17). The temperature-sensitive growth of MIC2067 resulted from the *rnhA* and *rnhB* double mutation and was relieved only by delivering plasmids that carry an RNase H gene (12a), such as the following: pSK760, *rnhA* from *E. coli* (21); pMIC27, *rnhB* from *E. coli* (13); pMY2051, *RNH1* from *S. cerevisiae* (18); pRET4, *rnhA* from *T. thermophilus* (17). The versatility of the cloning system using MIC3037 and MIC2067 is demonstrated in this study. MIC1021 was used only when chloramphenicol was needed as a plasmid selection marker.

RNase H activity in vitro. A 0.5-ml overnight culture of *E. coli* was harvested in a 1.5-ml microcentrifuge tube. The pellet was suspended in 50 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, sonicated, and centrifuged. The supernatant was transferred to a fresh tube and adjusted to 40 mM Tris-HCl (pH 6.8)–1% sodium dodecyl sulfate (SDS)–50 mM dithiothreitol–5% (vol/vol) glycerol in 100 μl. Ten microliters was boiled for 3 min immediately before loading on an SDS-polyacrylamide gel containing poly([^{32}P]rA) · poly(dT), and the renaturation gel assay was carried out as described previously (13).

Construction of a *B. subtilis* DNA library. Genomic DNA for library construction or analysis by conventional gel electrophoresis was prepared by a liquid isolation method (37). Agarose (1.0%, wt/vol) in TAE solution (50 mM Tris-acetate [pH 8.0], 1.0 mM EDTA) was used for conventional gel electrophoresis at room temperature.

DNA fragments (>4 kb) of a partial *Sau*3A digest of strain OA101 genomic DNA were isolated from a low-melting-point agarose gel and ligated in the *Bam*HI site of plasmid pBR322 (19). Competent MIC3037 cells were incubated under transformation conditions (18) and spread on LB plates containing ampicillin (100 μg/ml) at 30°C. Transformants that were tetracycline sensitive were grown in LB medium containing ampicillin at 30°C. Aliquots of 96 cultures were collected and each was designated a "Bsu club." Thirty-two Bsu clubs containing 2,955 independent colonies were obtained. Based on an insert size of 5 kb (data not shown), the library should contain, on average, three copies of each DNA sequence (30).

Determination of the nucleotide sequence. To obtain deletion clones of plasmid pMIB15-3 or pMIB21F at average intervals of 250 bp from the T7 promoter, the ExoIII digestion method (10) was applied. The sequences of the two clones were determined by dideoxy chain termination sequencing with ^{35}S-labeled nucleotides with the T7 promoter-primer as the sequence primer. Alignment of sequences was done with GENETYX, version 7.0.

Overexpression of RNase H enzymes in *E. coli*. For *B. subtilis* RNase HII expression in *E. coli*, the 984-bp *Eco*RI-*Bam*HI segment from a deletion derivative of pMIB21F (see Fig. 2) was inserted in the *Xba*I site 25 bp downstream of the T7 promoter of pBEST6, yielding pMIB2106. The plasmid was introduced into *E. coli* BL21(DE3) (39) and selected by ampicillin (100 μg/ml). The resultant transformant was grown in LB medium containing ampicillin at 37°C. Isopropyl-β-D-thiogalactopyranoside was added at a final concentration 0.4 mM to the culture when it was at an approximate A_{590} of 0.5, with incubation continuing for 4 h. Aliquots were removed at hourly intervals and analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Cloning of two functional RNase H genes from *B. subtilis* 168. The *B. subtilis* DNA library was constructed in *E. coli* MIC3037 (*rnhA339*::*cat rnhB*$^+$ *recC271*) and subdivided into 32 groups designated Bsu clubs (see Materials and Methods). The library was appropriate for direct screening of the RNase H gene(s) by temperature-sensitive complementation assay and activity in the gel assay, because the host strain, MIC3037, did not grow at the restricted temperature (42°C) and lacks the major *E. coli* RNase HI (*rnhA*) activity, as shown in lane 2 of Fig. 1. Promoters of *B. subtilis* genes normally function in

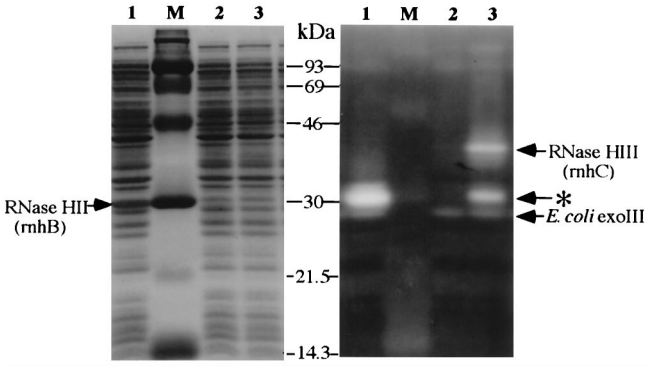

FIG. 1. Renaturation gel assay for *B. subtilis* RNase HII (*rnhB*) and RNaseHIII (*rnhC*) expressed in *E. coli*. Lysates from MIC3037 strains carrying pMIB21 (lane 1), pBR322 (lane 2), and pMIB15 (lane 3) were run. Preparation of lysates and assays in the gel are described in Materials and Methods. Cleared areas in the righthand panel are positions where RNase H proteins degraded radiolabeled substrate. MIC3037 (*rnhA* mutant) lacks major RNase HI activities, and RNase HII (*rnhB*) activities cannot be detected by this assay (13). Only activities by ExoIII (*E. coli* exoIII) are visible and indicated. The band in lane 3 indicated by an asterisk may be a degradative product of RNase HIII (*rnhC*). Size markers (lane M) are as follows: 93, phosphorylase b; 69, bovine serum albumin; 46, ovalbumin; 30, carbonic anhydrase; 21.5, trypsin inhibitor; 14.3, lysozyme.

E. coli. Therefore, no special sequences were employed for gene expression. By the two independent screenings described below, two clones were positive for both assays.

(i) Each Bsu club was spread after appropriate dilution on an LB plate containing ampicillin (100 μg/ml), and colonies were selected at 42°C. Plasmid DNAs isolated from candidate colonies were analyzed by digestion with *Hin*dIII, *Eco*RI, and *Pst*I. A total of 100 independent clones were classified into 14 groups. The 14 representative clones were examined for RNase H activity in the renaturation gel assay and for the ability to suppress temperature-sensitive growth of MIC2067 (*rnhA339*::*cat rnhB716*::*kam*). One clone, pMIB15, exhibited an RNase H activity of approximately 40 kDa (Fig. 1) and suppressed the temperature-sensitive phenotype of strain MIC2067 (Fig. 2).

(ii) Lysed Bsu clubs were examined directly in the gel assay for screening of RNase H activity. When activity was detected, all 96 colonies in the club were examined to isolate the positive clone. After screening of the 32 Bsu clubs, RNase H activity was detected in 2 clubs, from which three independent clones were obtained. Only one clone, pMIB21, giving an approximately 30-kDa product in the gel assay (Fig. 1), also suppressed the temperature-sensitive phenotypes of both MIC3037 and MIC2067 at 42°C (Fig. 2). The other two clones did not complement the temperature-sensitive growth of any *E. coli* mutants.

Deletion analysis of the insert in pMIB21 or pMIB15 located the RNase H gene as shown for pMIB21F and pMIB156-2 (Fig. 2a).

Nucleotide and amino acid sequences of the *rnhB* gene. The nucleotide sequence of the insert (1,685 bp) of pMIB21F was determined as described in Materials and Methods (data not shown). One open reading frame (ORF) was found that encodes a protein of 255 amino acid residues (calculated molecular mass, 28,204 Da; pI, 5.52), consistent with the estimated size of the RNase H activity (approximately 30 kDa) from the gel assay (Fig. 1). Plasmid pMIB2106, described in Materials and Methods, overexpressed the gene product in *E. coli* BL21 (DE3) (data not shown). The expressed protein was subjected to automated sequence analysis in a gas-phase protein sequencer (model 477A; Perkin-Elmer Applied Biosystems), and the N-terminal 15 amino acids, MNTLTVKDIKDRLQE, were identical to those predicted from the nucleotide sequence (Fig. 3). Searches of the *B. subtilis* genome database (33a) revealed an ORF designated *rnh* (accession no. BG12666) encoded by nucleotides 1,676,850 to 1,677,614 (30). The *rnh* gene had a similarity of 63% (amino acid) to that of *E. coli* RNase HII

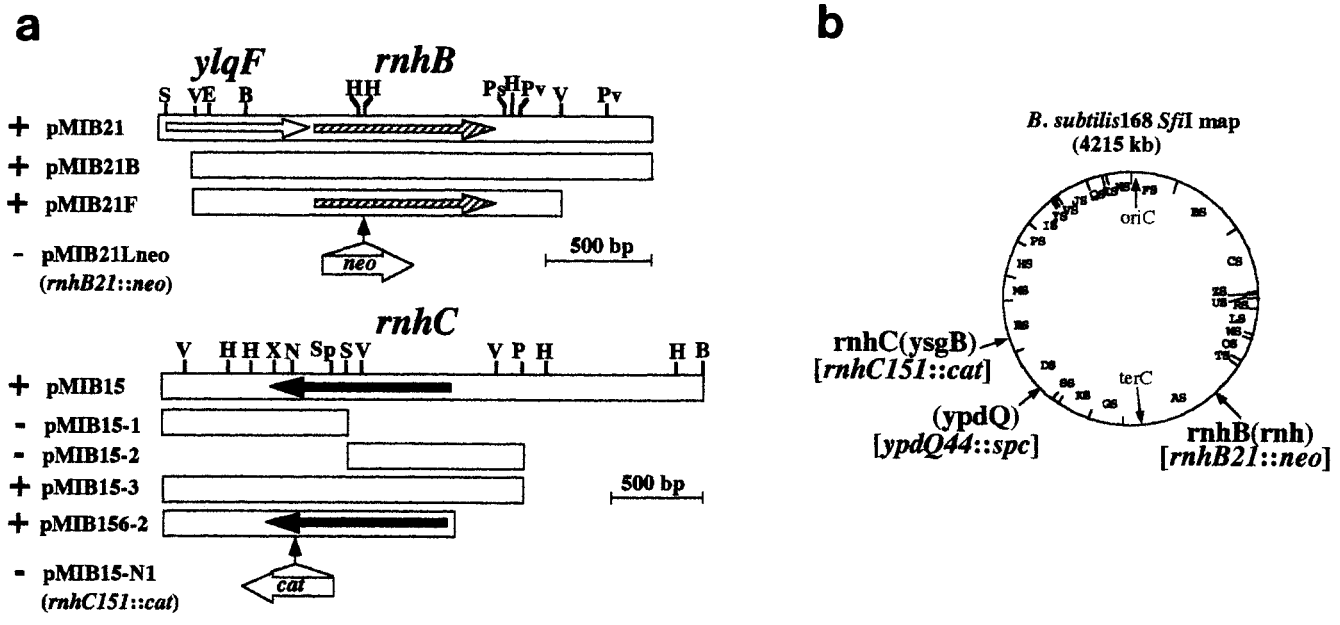

FIG. 2. Cloned fragment carrying RNase H genes from *B. subtilis* 168. (a) Plus and minus signs indicate temperature-sensitive complementation of the *E. coli rnh*-deficient mutants MIC3037 and MIC2067 and RNase H activity in the gel assay. Restriction enzymes are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Sp, *Sph*I; V, *Eco*RV; X, *Xho*I. Antibiotic resistance genes were inserted in the site indicated by the vertical arrow. Scales are 500 bp for each clone. All fragments are oriented from left (distal to *oriC*) to right (distal to *terC*). (b) Locations of the three genes cloned in this study in the *B. subtilis* physical map (19). Gene names cited in reference 30 are in parentheses. Mutations are shown in brackets.

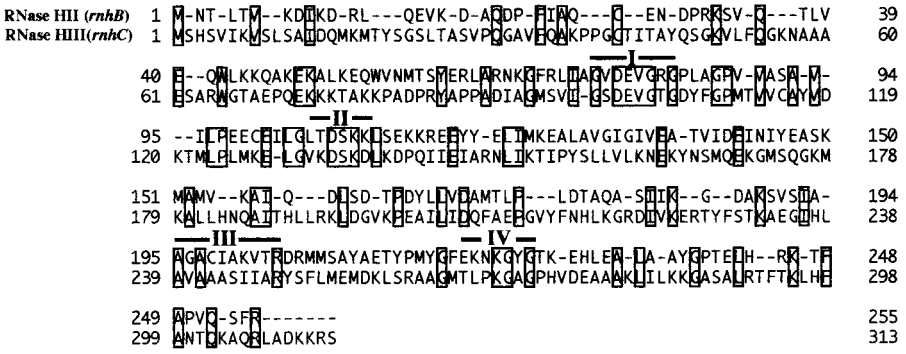

FIG. 3. Alignment of the two RNase H sequences of *B. subtilis* 168. The sequence comparison between *B. subtilis rnhB* and *rnhC* is shown. Identical amino acids are boxed, with overall similarity of 20.0%. The four conserved motifs suggested for the *rnhB* (33, 42) are indicated as I through IV.

(*rnhB*). Consequently, the *rnh* gene is designated *rnhB*, encoding *B. subtilis* RNase HII.

Nucleotide and amino acid sequences of the *rnhC* gene. The nucleotide sequence of the insert (1,248 bp) of pMIB156-2 (Fig. 2a) was also determined. One ORF was found that encodes a protein of 313 amino acid residues (calculated molecular mass, 33,915 Da; pI, 10.07), consistent with the estimated size of the RNase H activity (approximately 40 kDa) from the gel assay (Fig. 1). The ORF corresponds to the *ysgB* gene (accession no. BG12324) encoded by nucleotides 2,925,133 to 2,926,071 of the *B. subtilis* genome; the function of *ysgB* is unknown (30). The amino acid sequence is shown in Fig. 3. The *ysgB* gene product had little similarity to *E. coli* RNase HI (*rnhA*). Although the overall similarity between *B. subtilis rnhB* and the *ysgB* gene is 20.0%, there are a few well-conserved regions between the two, as indicated in Fig. 3. The *ysgB* gene, therefore, is designated *rnhC*, encoding *B. subtilis* RNase HIII.

Overexpression of the RnhC product in *E. coli* BL21(DE3) with a pBEST6 vector was unsuccessful. However, a similar expression plasmid constructed with a PCR-amplified DNA fragment overproduced a product whose amino acid sequence was identical to that of the *ysgB* gene (35a).

RNase HI (*rnhA*) homologue in the *B. subtilis* genome. On searching the entire *B. subtilis* genome for proteins related to *E. coli* RNaseHI (*rnhA*), a single sequence with 29% homology, *ypdQ* (accession no. BG11608), encoded by nucleotides 2,309,611 to 2,310,056 of the *B. subtilis* genome (30) was found. The *ypdQ* gene was cloned as a 474-bp DNA fragment amplified by using the forward primer 5′-ACCTCGCCATTAGGA TGAAC and the reverse primer 5′-TGCAGCCAAAAAAAT GATACC from genomic DNA of *B. subtilis* 1A1. PCR was performed in 20 cycles with a GeneThermoUnit GTU1605 (Taitech, Tokyo, Japan). The PCR fragment was inserted in pGEM4, resulting in pRNHA-1, but this plasmid was unable to suppress the temperature-sensitive growth of any of the *E. coli rnh* mutants in Table 1. Possibly this protein is not expressed well in *E. coli* mutants and/or has lower levels of RNase H activity than that needed to give suppression in vivo. This is consistent with the observation that no *rnhA* homologue was obtained in screening for the Bsu clubs. The *ypdQ* gene encodes a protein of 132 amino acid residues (calculated molecular mass, 14,529 Da; pI, 5.60). The product expressed in *E. coli* is being characterized (35a).

RNase H genes are conserved in related *B. subtilis* species. Southern hybridization to *Sfi*I and *Not*I fragments by three clones, pRNHA-1, pMIB21F, and pMIB15-3 (data not shown), placed these clones in the *B. subtilis* physical map shown in Fig. 2b. The same DNA probes gave clear signals to genomic DNAs from closely related *B. subtilis* species, *B. subtilis* W23 and *Bacillus natto* IFO1212 (data not shown). The results indicated that all three genes are conserved in these two strains.

RNase H-deficient mutant of *B. subtilis*. Mutations of each of the three *B. subtilis* genes were constructed in *E. coli* plasmids. The *ypdQ* gene (*rnhA* homologue) was disrupted by insertion of a spectinomycin resistance gene cassette (*spc*) in the unique *Bsm*I site of pUC2.2, resulting in pRNHA-4. The *spc* cassette was prepared by *Sma*I digestion of pBEST517A (Table 1). Similarly, the *rnhB* gene in pMIB21F was disrupted by insertion of a neomycin resistance gene cassette (*neo*) in the internal *Hin*dIII site, resulting in pMIB21Lneo (Fig. 2a). The *neo* cassette gene was prepared by *Hin*dIII digestion from pBEST512 (14). The *rnhC* gene in pMIB156-2 was disrupted by insertion of a chloramphenicol resistance gene cassette (*cat*) in the unique *Not*I site after being blunt ended, resulting in pMIB15-N1 (Fig. 2a). The *cat* gene was prepared by *Sma*I digestion of pBEST4F (20).

Mutations carried in these plasmids were introduced into the *B. subtilis* genome by gene replacement (40). Single mutants were selected by appropriate antibiotic resistance: spectinomycin for the *ypdQ* mutant, neomycin for the *rnhB* mutant, and chloramphenicol for the *rnhC* mutant at 30°C (Table 1). The genomic structures of these mutants were verified by Southern analysis by using the parental plasmids as probes (data not shown). The *rnhC* mutant BEST220 (*rnhC151*::*cat*) gave colonies slightly smaller than the other two single mutants.

Construction of double mutants by genetic crosses of the single mutants was successful except for *rnhB* and *rnhC*. Attempts to introduce the *rnhB21*::*neo* mutation of BEST218 into BEST220 (*rnhC151*::*cat*) resulted in no neomycin-resistant transformants. Other markers unrelated to RNase H, *proB* or *leuB*, could be introduced into BEST220, giving approximately 10^3 transformants per microgram of donor DNA. In contrast, introduction of the *rnhC151*::*cat* mutation of BEST220 into BEST218 (*rnhB21*::*neo*) resulted in strains that formed very tiny colonies on selection plates containing chloramphenicol at frequencies similar to those of *proB* or *leuB* transfer. However, these tiny colonies did not produce viable colonies when restreaked on fresh plates, even in the absence of antibiotics. Introduction of the *ypdQ* mutation gave no phenotype, regardless of what other mutations were present in the recipient strain. All of the viable mutants were able to grow in the temperature range of 25 to 50°C, as does the wild-type strain. The results are interpreted to indicate that loss of both RNase HII (*rnhB*) and RNase HIII (*rnhC*) renders *B. subtilis* inviable. From the formation of colonies of inviable cells in the BEST220 × BEST218 cross, it can be interpreted that low

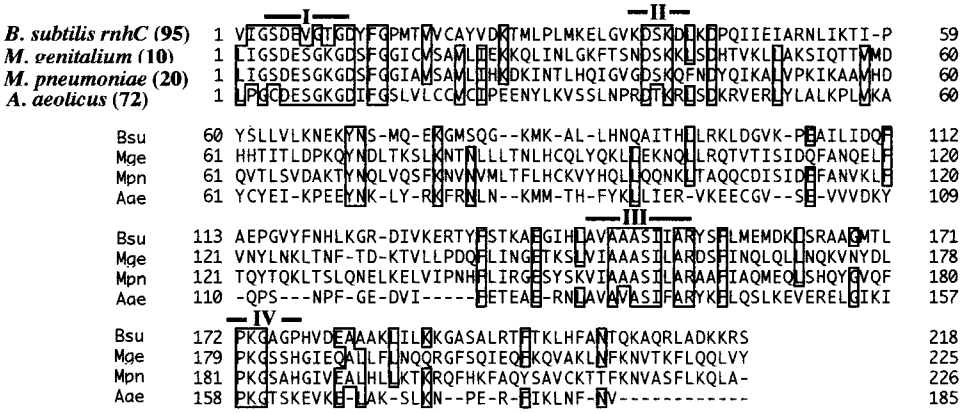

FIG. 4. Newly identified sequences that are similar to *B. subtilis* RNase HIII (*rnhC*). The three ORFs homologous to the *B. subtilis rnhC* sequence are aligned. The four conserved regions shown in Fig. 3 (I through IV) are indicated. Accession numbers are in the text. The numbers of N-terminal amino acid residues omitted in the alignment are shown in parentheses.

levels of RnhC allow for consecutive cell divisions. The number of cell divisions may be enough to form visible (tiny) colonies. In the other cross, BEST218 × BEST220, RnhB was rapidly degraded or diluted out before producing sufficient numbers of cells.

***rnhC* homologues in the database.** On searching the current database for sequences related to *B. subtilis rnhC*, homologues were detected in various bacterial and eukaryotic genomes. However, most had been already designated *rnh* or *rnhB* in the database due to the slight similarity between *rnhB* and *rnhC*, as indicated in Fig. 3, with the exception of three genes. Two of these were from *Mycoplasma* species, the putative gene from *Mycoplasma genitalium* (accession no. MG199) (8, 33a) and the putative gene from *Mycoplasma pneumoniae* (accession no. C09_orf143b) (11, 33a), and the third was from the hyperthermophilic bacterium *Aquifex aeolicus* (accession no. aq_1768) (5, 33a). Their alignment with *B. subtilis rnhC* is shown in Fig. 4. As the *rnhB* gene in *A. aeolicus* was already reported (accession no. aq_1955) (5), it seems likely that this hyperthermophilic bacterium has an *rnhC* homologue and an *rnhB* homologue, although no obvious *rnhA* homologue was detected. It should be mentioned that an *rnhC* homologue was not found in the *E. coli* genome (33a).

DISCUSSION

Two distinctive RNase H genes of *B. subtilis* 168. Two clones encoding functional RNase H genes were isolated from a strain of the gram-positive bacterium *B. subtilis*. The two genes, *rnhB* and *rnhC*, were obtained based on the abilities to complement *E. coli* RNase H mutants in vivo (Fig. 2a) and to exhibit RNase H activity in vitro (Fig. 1). The temperature-sensitive *E. coli* strain MIC2067 newly adopted in this study was more specific for in vivo RNase H activity than MIC2067 newly adopted in this study was more specific for in vivo RNase H activity than MIC3037 (12a), and the MIC strains in Table 1 can discriminate the *rnh* genes in vivo if the genes are correctly expressed. In addition to these functional *B. subtilis* RNase H genes, a gene, *ypdQ*, that has some similarity to *rnhA* in the *B. subtilis* genome was also identified. However, the *ypdQ* clone was negative for all RNase H criteria. It remains to be demonstrated whether the failure of the *ypdQ* gene to suppress the temperature-sensitive phenotypes of MIC strains is due to either incorrect expression or low activity in *E. coli*. Alternatively, as YpdQ lacks the basic protruding region characteristic of *E. coli* RNase HI (25, 41), it may require an additional polypeptide for RNase H activity, similar to that observed for RNase H of HIV reverse transcriptase (28). It may be worth testing the *ypdQ* homologues observed in related *B. subtilis* species for enzyme activity.

Are RNase H proteins essential for *B. subtilis*? Because the *E. coli* K-12 genome seems not to have an *rnhC* homologue, *rnhA* and *rnhB* double mutants such as MIC2067 completely abolish RNase H. Although MIC2067 shows a temperature-sensitive growth phenotype, double mutants of a certain *E. coli* genetic background grow normally (12a). In contrast, the *rnhB* and *rnhC* double mutant of *B. subtilis* 168 is unable to form viable colonies. These observations suggest that RNase H-requiring biological processes in *B. subtilis* are different from those suggested for *E. coli* (1, 4, 15, 16, 27, 35, 36). Distinctive pI values of the two *B. subtilis* RNase H genes (5.5 for *rnhB* and 10.1 for *rnhC*), compared with those of *E. coli* (9.7 for *rnhA* and 6.9 for *rnhB*), may imply different roles or substrate recognition properties in vivo. Alternative explanations include the acquisition of a suppressor mutation in laboratory strains of *E. coli* resulting in complete loss of RNase H proteins. Isolation of a *B. subtilis rnhB* and *rnhC* double mutant that has a suppressor mutation(s) is underway.

The requirement for RNase H in *B. subtilis* raises the question of whether RNase H is dispensable for bacteria. Complete DNA sequences are known for two *Mycoplasma* genomes, in which no homologue of *rnhA* and *rnhB* was reported, indicating that RNase H is dispensable (29). However, the presence of homologues to the newly characterized *rnhC* in these *Mycoplasma* species (Fig. 4) may imply that the single bacterial RNase H of the smallest genomes performs a function similar to the two RNase H proteins of *B. subtilis*.

The genome of *A. aeolicus*, a hyperthermophilic hydrogen-oxidizing bacterium, has both *rnhB* and *rnhC* but lacks an *rnhA* homologue. The bacterium grows at 95°C, similar to primordial forms of life (5). This suggests that the two *rnh* genes of *A. aeolicus* represent an ancestral structure of the two present *B. subtilis* RNase H genes.

ACKNOWLEDGMENTS

We thank A. Ogura, K. Matsui, K. Fujita, and M. Murayama for technical help. We especially thank N. Ohtani for kindly communicating data prior to publication.

REFERENCES

1. **Asai, T., and T. Kogoma.** 1994. D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. J. Bacteriol. **176:**1807–1812.
2. **Carl, P. L., A. D. Bloom, and R. J. Crouch.** 1972. Isolation and mapping of a mutation in *Escherichia coli* with altered levels of ribonuclease H. J. Bacteriol. **144:**28–35.
3. **Crouch, R. J.** 1990. Ribonuclease H: from discovery to 3D structure. New Biol. **2:**771–777.
4. **Dasgupta, S., H. Masukata, and J. Tomizawa.** 1987. Multiple mechanisms for initiation of ColE1 DNA replication: DNA synthesis in the presence and absence of ribonuclease H. Cell **24:**1113–1122.
5. **Deckert, G., P. V. Warren, T. Gaasterland, et al.** 1998. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature **392:**353–358.
6. **Doi, N., T. Yomo, M. Itaya, and H. Yanagawa.** 1998. Insertion of foreign random sequences of 120 amino acid residues into an active enzyme. FEBS Lett. **427:**51–54.
7. **Frank, P., C. Braunshofer, and U. Wintrersburger.** 1998. Yeast RNase H(35) is the counterpart of the mammalian RNase HI, and is evolutionary related to prokaryotic RNase HII. FEBS Lett. **421:**23–26.
8. **Fraser, C. M., J. D. Gocayne, O. White, et al.** 1995. The minimal genome complement of *Mycoplasma genitalium*. Science **270:**397–403.
9. **Haruki, M., E. Noguchi, A. Akasako, M. Oobatake, M. Itaya, and S. Kanaya.** 1994. A novel strategy for stabilization of *Escherichia coli* ribonuclease HI involving a screen for an intragenic suppressor of carboxyl-terminal deletions. J. Biol. Chem. **269:**26904–26911.
10. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
11. **Himmelreich, R., H. Hilbert, H. Plagens, E. Pirkl, B.-C. Li, and R. Herrmann.** 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res. **24:**4420–4449.
12. **Ishikawa, K., M. Okumura, K. Katayanagi, S. Kimura, S. Kanaya, H. Nakamura, and K. Morikawa.** 1993. Crystal structure of ribonuclease H from *Thermus thermophilus* HB8 refined at 2.8 A resolution. J. Mol. Biol. **230:**529–542.

12a. **Itaya, M.** Unpublished data.

13. **Itaya, M.** 1990. Isolation and characterization of a second ribonuclease H (RNase HII) of *Escherichia coli* K12 encoded by the *rnhB* gene. Proc. Natl. Acad. Sci. USA **87:**8587–8591.
14. **Itaya, M.** 1993. Stability and asymmetric replication of the *Bacillus subtilis* 168 chromosome. J. Bacteriol. **175:**741–749.
15. **Itaya, M., and R. J. Crouch.** 1991. A combination of RNase H (*rnh*) and *recBCD* or *sbcB* mutations in *E. coli* K-12 adversely affects growth. Mol. Gen. Genet. **227:**424–432.
16. **Itaya, M., and R. J. Crouch.** 1991. Correlation of activity with phenotypes of *Escherichia coli* partial function mutants of *rnh*, the gene encoding RNase H. Mol. Gen. Genet. **227:**433–437.
17. **Itaya, M., and K. Kondo.** 1991. Molecular cloning of a ribonuclease H (RNase HI) gene from an extreme thermophile, *Thermus thermophilus* HB8: a thermostable RNase H can functionally replace the *Escherichia coli* enzyme *in vivo*. Nucleic Acids Res. **19:**4443–4449.
18. **Itaya, M., D. McKelvin, S. K. Chatterjie, and R. J. Crouch.** 1991. Selective cloning of genes encoding RNase H from *Salmonella typhimurium*, *Saccharomyces cerevisiae* and *Escherichia coli rnh* mutant. Mol. Gen. Genet. **227:** 438–445.
19. **Itaya, M., and T. Tanaka.** 1991. Complete physical map of the *Bacillus subtilis* 168 chromosome constructed by a gene-directed mutagenesis method. J. Mol. Biol. **220:**631–648.
20. **Itaya, M., I. Yamaguchi, K. Kobayashi, T. Endo, and T. Tanaka.** 1990. The blasticidin S resistance gene (*bsr*) selectable in a single copy state in the *Bacillus subtilis* chromosome. J. Biochem. (Tokyo) **107:**799–801.
21. **Kanaya, S., and R. J. Crouch.** 1983. DNA sequence of the gene coding for *Escherichia coli* ribonuclease H. J. Biol. Chem. **258:**1276–1281.
22. **Kanaya, S., C. Katsuda, S. Kimura, T. Nakai, E. Kitakuni, H. Nakamura, K. Katayanagi, K. Morikawa, and M. Ikehara.** 1991. Stabilization of *Escherichia coli* ribonuclease H by introduction of an artificial disulfide bond. J. Biol. Chem. **266:**6038–6044.
23. **Kanaya, S., C. Nakai, A. Konishi, H. Inoue, E. Ohtsuka, and M. Ikehara.** 1992. A hybrid ribonuclease H. A novel RNA cleaving enzyme with sequence-specific recognition. J. Biol. Chem. **267:**8492–8498.
24. **Kashiwagi, T., D. Jeanteur, M. Haruki, M. Katayanagi, S. Kanaya, and K. Morikawa.** 1996. Proposal for new catalytic roles for two invariant residues in *Escherichia coli* ribonuclease HI. Protein Eng. **9:**857–867.
25. **Katayanagi, K., M. Miyagawa, M. Matsushima, M. Ishikawa, S. Kanaya, M. Ikehara, T. Matsuzaki, and K. Morikawa.** 1990. Three dimensional structure of ribonuclease H from *E. coli*. Nature **347:**306–309.
26. **Keller, W., and R. J. Crouch.** 1972. Degradation of RNA.DNA hybrids by ribonuclease H and DNA polymerases of cellular and viral origin. Proc. Natl. Acad. Sci. USA **69:**3360–3364.
27. **Kogoma, T., N. L. Subia, and K. Meyenberg.** 1985. Function of ribonuclease H in initiation of DNA replication in *Escherichia coli* K-12. Mol. Gen. Genet. **200:**103–109.
28. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:**1783–1790.
29. **Kooin, E. V., A. R. Mushegian, and P. Bork.** 1996. Non-orthologous gene displacement. Trends Genet. **12:**334–336.
30. **Kunst, F., N. Ogasawara, and A. M. Albertini, et al.** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
31. **Maki, H., T. Horiuchi, and M. Sekiguchi.** 1983. Structure and expression of the *dnaQ* and the RNase H genes of *Escherichia coli*: overlap of the promoter regions. Proc. Natl. Acad. Sci. USA **80:**7137–7141.
32. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
33. **Mian, S. I.** 1997. Comparative sequence analysis of ribonucleases HII, III, II PH and D. Nucleic Acids Res. **25:**3187–3195.

33a. **Microbial Genome WWW Home Page.** [Online.] National Institute of Genetics. http://ddbjs4d.genes.nig.ac.jp:8880/ [29 August 1998, last date accessed.]

34. **Mizrahi, V., P. Huberts, S. S. Dawes, and L. R. Dudding.** 1993. A PCR method for the sequence analysis of the *gyrA*, *polA*, and *rnhA* gene segments from mycobacteria. Gene **136:**287–290.
35. **Ogawa, T., G. G. Pickett, T. Kogoma, and A. Kornberg.** 1984. RNase H confers specificity in the dnaA-dependent initiation of replication at the unique origin of the *Escherichia coli* chromosome *in vivo* and *in vitro*. Proc. Natl. Acad. Sci. USA **81:**1040–1044.

35a. **Ohtani, N., and S. Kanaya.** Unpublished data.

36. **Quinones, A., C. Kucherer, R. Piechocki, and W. Messer.** 1987. Reduced transcription of the *rnh* gene in *Escherichia coli* mutants expressing the SOS regulon constitutively. Mol. Gen. Genet. **206:**95–100.
37. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
38. **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.
39. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
40. **Tanaka, T., and N. Kawano.** 1980. Cloning vehicles for the homologous *Bacillus subtilis* host-vector system. Gene **10:**131–136.
41. **Yang, W., W. A. Hendrickson, R. J. Crouch, and Y. Satow.** 1990. Structure of ribonuclease H phased at 2 Å resolution by MAD analysis. Science **249:** 1398–1405.
42. **Zhang, Y.-B., S. Ayalew, and S. A. Lacks.** 1997. The *rnhB* gene encoding RNase HII of *Streptococcus pneumoniae* and evidence of conserved motifs in eukaryotic genes. J. Bacteriol. **179:**3828–3836.