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The *Bradyrhizobium japonicum* $rpoH_1$ Gene Encoding a σ^{32}-Like Protein Is Part of a Unique Heat Shock Gene Cluster Together with $groESL_1$ and Three Small Heat Shock Genes

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The heat shock response of *Bradyrhizobium japonicum* is controlled by a complex network involving two known regulatory systems. While some heat shock genes are controlled by a highly conserved inverted-repeat structure (CIRCE), others depend on a σ^{32}-type heat shock sigma factor. Using Western blot (immunoblot) analysis, we confirmed the presence of a σ^{32}-like protein in *B. japonicum* and defined its induction pattern after heat shock. A *B. japonicum rpoH*-like gene ($rpoH_1$) was cloned by complementation of an *Escherichia coli* strain lacking σ^{32}. A knockout mutation in $rpoH_1$ did not abolish σ^{32} production in *B. japonicum*, and the $rpoH_1$ mutant showed the wild-type growth phenotype, suggesting the presence of multiple *rpoH* homologs in this bacterium. Further characterization of the $rpoH_1$ gene region revealed that the $rpoH_1$ gene is located in a heat shock gene cluster together with the previously characterized $groESL_1$ operon and three genes encoding small heat shock proteins in the following arrangement: $groES_1$, $groEL_1$, *hspA*, $rpoH_1$, *hspB*, and *hspC*. Three heat-inducible promoters are responsible for transcription of the six genes as three bicistronic operons. A σ^{32}-dependent promoter has previously been described upstream of the $groESL_1$ operon. Although the *hspA*-$rpoH_1$ and *hspBC* operons were clearly heat inducible, they were preceded by σ^{70}-like promoters. Interestingly, a stretch of about 100 bp between the transcription start site and the start codon of the first gene in each of these two operons was nearly identical, making it a candidate for a regulatory element potentially allowing heat shock induction of σ^{70}-dependent promoters.

Induction of heat shock proteins (Hsps) after a heat shock or other stress conditions is a universal response common to all known organisms. In general, Hsps assist in protein folding and transport, in assembly of protein complexes, and in protein degradation. Under stress conditions they allow an organism to cope with damage inflicted by sudden environmental challenges, for example by refolding denatured proteins or by their degradation through proteolysis (for recent reviews, see references 16 and 21). Despite the universality of the heat shock response, the mechanism of Hsp induction varies greatly not only between eukaryotes and prokaryotes but also among different bacterial species. In a few known cases, several mechanisms are realized even within one given organism (6, 19).

For a long time *Escherichia coli* served as a paradigm regarding heat shock gene expression in prokaryotes (for reviews, see references 9, 28, and 60). It is well established that rapid induction of Hsps in this species is accomplished by a transient accumulation of σ^{32}, an alternative sigma factor encoded by the *rpoH* gene (54). This sigma factor directs RNA polymerase to so-called heat shock promoters that share a specific consensus sequence (12, 17). Several mechanisms contribute to controlling the concentration of σ^{32} in the cell. Although *rpoH* mRNA levels increase slightly when *E. coli* cells are exposed to high temperatures, transcriptional control of the gene itself plays only a minor role (13, 55). Increased synthesis of σ^{32} upon heat shock is primarily due to elevated translation and stabilization of the protein. Two *cis*-acting elements on the *rpoH* mRNA presumably form a secondary structure that is thought to be responsible for translational repression under nonstress conditions and for induction upon temperature upshift (34, 61). While σ^{32} produced in the absence of stressful conditions is highly unstable (half-life of approximately 1 min), its stability increases at least eightfold after a temperature upshift (54). Recent data indicate that the heat shock proteins DnaK, DnaJ, and GrpE form a complex with σ^{32} and deliver it to a proteolytic system under normal growth conditions (15). HflB (also called FtsH), a membrane-bound ATP-dependent metalloprotease, was identified as a specific protease responsible for σ^{32} degradation (22, 56). According to the "DnaK/DnaJ titration model," DnaK preferentially binds to denatured proteins accumulated under stress conditions and thus releases free σ^{32}, which in turn can associate with RNA polymerase leading to the transcription of heat shock genes (9, 15).

Heat shock gene induction by σ^{32} is just one of several mechanisms occurring in bacteria. During attempts to uncover the mechanism(s) for heat shock gene expression in gram-positive bacteria, a highly conserved *cis*-acting regulatory element was found simultaneously in *Bacillus subtilis* and *Clostridium acetobutylicum* (26, 37, 38, 49, 57). Apparently, this inverted-repeat sequence is more widespread than anticipated and has so far been discovered upstream of more than 50 stress genes or operons from a total of 30 organisms, including gram-negative bacteria (19, 52). Because of its function as a regulatory element, it has been designated CIRCE (for controlling inverted repeat of chaperone expression) (63). Recently, the putative repressor protein in *B. subtilis* which binds to this region was identified (50, 59). Two additional mechanisms account for Hsp induction in *B. subtilis*. The majority of stress genes is induced by an alternative sigma factor, named σ^B. This sigma factor is different from σ^{32} and is regulated by a set of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories, Gaithersburg, Md.
MC4100	F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR*	10
A7448	MC4100 Δ*rpoH30*::*kan zhf-50*::Tn*10* (λ*imm*^{21}pF13-P*groE-lacZ*)	8
JM101	*supE thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	31
S17-1	Sm^r Sp^r *hsdR* RP4-2 *kan*::Tn*7 tet*::Mu, integrated in the chromosome	53
B. japonicum strains		
110*spc*4	Sp^r (wild type)	43
5008	Sp^r Km^r *rpoH*$_1$::*aphII* (*rpoH*$_1$ and *aphII* oriented in opposite directions)	This study
5009	Sp^r Km^r *rpoH*$_1$::*aphII* (*rpoH*$_1$ and *aphII* oriented in the same direction)	This study
Plasmids		
pUC18	Ap^r, P*lacZ*, α LacZ[a]	39
M13mp18		39
pBSL86	Ap^r Km^r	1
pUC-4-KIXX-PSP	Ap^r Km^r (pUC-4-KIXX) *Pme*I-*Swa*I-*Pac*I linker in *Sma*I site of *ble*	23
pSUP202	Ap^r Cm^r Tc^r *oriT* from RP4	53
pRJ5000	Ap^r (pUC18), 1.4-kb *Bam*HI fragment containing *B. japonicum rpoH*$_1$ (*rpoH*$_1$ in α *lacZ*[b] orientation)	This study
pRJ5001	Ap^r (pUC18), 1.4-kb *Bam*HI fragment containing *B. japonicum rpoH*$_1$ (*rpoH*$_1$ opposite to α *lacZ* orientation)	This study
pRJ5008	Ap^r Tc^r (pSUP202) Km^r (pUC-4-KIXX-PSP) *rpoH*$_1$::*aphII* (*rpoH*$_1$ and *aphII* oriented in opposite directions)	This study
pRJ5009	Ap^r Tc^r (pSUP202) Km^r (pUC-4-KIXX-PSP) *rpoH*$_1$::*aphII* (*rpoH*$_1$ and *aphII* oriented in same direction)	This study

[a] α LacZ, subfragment of the LacZ protein which is encoded by the pUC18 plasmid required for α complementation of recombinant strains.
[b] α *lacZ*, gene encoding by the α LacZ protein.

accessory factors. Another class of heat shock genes in this organism is neither CIRCE nor σ^B dependent (for a review, see reference 19).

Recent reports indicate that CIRCE- and σ^{32}-mediated heat shock regulation may occur within a single bacterial species. *Agrobacterium tumefaciens* and *Caulobacter crescentus* belong to this category (5, 29, 44, 51). An even higher level of complexity is observed in *Bradyrhizobium japonicum*, the nitrogen-fixing root nodule symbiont of soybean. Five homologs of the *groESL* heat shock operon were identified in this organism. Three of these five operons are heat inducible. Regulation by the CIRCE element applies to *groESL*$_4$ and probably also *groESL*$_5$. By contrast, *groESL*$_1$ was shown to rely on a σ^{32}-like system (6). Interestingly and uniquely, one copy (*groESL*$_3$) is expressed only under microaerobic conditions and depends on the nitrogen-fixing regulatory protein NifA together with the RNA polymerase containing σ^{54} (14).

The aim of this work was to provide firm evidence for the previously postulated presence of a σ^{32}-like protein in *B. japonicum* and to subsequently clone and characterize the corresponding *rpoH* gene. The gene we isolated was designated *rpoH*$_1$ because we obtained indirect evidence for additional *rpoH* copies in *B. japonicum*. The *rpoH*$_1$ gene was found within a heat shock gene cluster consisting of at least six genes whose transcriptional organization and regulation were studied.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* cells were grown in Luria-Bertani (LB) medium (32) supplemented with ampicillin (200 μg/ml), kanamycin (30 μg/ml), or tetracycline (10 μg/ml) if required. With the exception of *E. coli* A7448, which was grown at 23°C, the growth temperature for all *E. coli* strains was 37°C. *B. japonicum* was grown aerobically at 28°C in PSY medium (43) supplemented with 0.1% (wt/vol) arabinose. The concentrations (in micrograms per milliliter) of antibiotics were as follows: spectinomycin, 100; chloramphenicol (for counterselection against *E. coli* donor strains), 20; kanamycin, 100; and tetracycline, 50.

Western blot (immunoblot) analysis. Crude extracts of *E. coli* and *B. japonicum* cells were prepared, separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to nitrocellulose membranes as described previously (6). Proteins were detected by binding of rabbit anti-*E. coli* σ^{32} serum (kindly provided by B. Bukau, Heidelberg, Germany; 3,000-fold dilution) and using a Chemiluminescence Western Blotting Kit (Boehringer, Mannheim, Germany).

DNA manipulations. Recombinant DNA techniques were performed by standard protocols (46). Chromosomal DNA from *B. japonicum* was isolated as described previously (18). DNA was sequenced by the chain termination method of Sanger et al. (47) with a model 373 DNA sequencer (Applied Biosystems, Foster City, Calif.). Both double-stranded plasmid DNA and single-stranded DNA originating from bacteriophage M13 derivatives were used. The DNA region sequenced and the deduced proteins were analyzed with the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison) (version 8.0) or the NCBI (National Center for Biotechnology Information) BLAST network server. Multiple sequence alignments were generated with the PILEUP program provided by the UWGCG software.

Transcript mapping. RNA isolation and primer extension analysis were performed as described elsewhere (6). The following oligonucleotides were used to determine the transcription start sites upstream of either *hspA*, *rpoH*$_1$, or *hspB*: Sig46 (*hspA*, 3250), 5′-GATAGTTGATCGCCACAGGGGGCTGATG-3′; Sig32 (*hspA*, 3259), 5′-GTCGAAGCCGATAGTTGATCGCCACAGG-3′; Sig16 (*rpoH*$_1$, 3870), 5′-TCGACAGACGGGGCAGGTAGAGCGGCATTG-3′; Sig9 (*rpoH*$_1$, 3904), 5′-ACGGATCTCGACTAGATATTTCGACAGCCC-3′; Sig47 (*hspB*, 5323), 5′-CGACCGCCAAAGGGGAGCGAAATCGTAG-3′; and Sig31 (*hspB*, 5354), 5′-CAAGATCGAACAGACGGTCAAAGCCGATG-3′ (the numbers in parentheses indicate the position of the 5′ end based on the numbering used in the physical map shown in Fig. 2).

Construction of *B. japonicum rpoH*$_1$ mutant strains. To construct *rpoH*$_1$ insertion mutants, the 1.4-kb *Bam*HI insert of pRJ5000 was ligated into the 2.4-kb vector fragment of pBSL86 that had been isolated upon *Bam*HI digestion. The resulting plasmid contained a single *Hin*dIII site located in the *rpoH*$_1$ gene (see the restriction map in Fig. 2). The 1.6-kb *aphII* cassette (Km^r) isolated from pUC-4-KIXX-PSP was inserted into this *Hin*dIII site. Both orientations of the *aphII* cassette with respect to the *rpoH*$_1$ gene were obtained. The 3.0-kb *Eco*RI inserts of these constructs were ligated into *Eco*RI-digested pSUP202 to give plasmids pRJ5008 and pRJ5009, which were then mobilized from *E. coli* S17-1

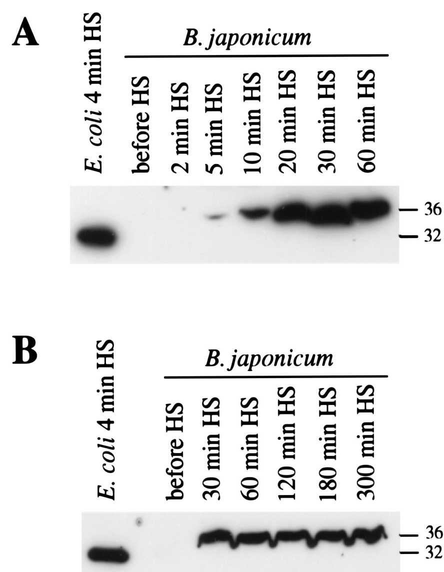

FIG. 1. Immunoblot analysis of *E. coli* and *B. japonicum* extracts by using anti-*E. coli* σ^{32} serum. *E. coli* MC4100 or *B. japonicum* 110*spc*4 cells were grown to mid-exponential phase at 28°C. After a reference sample was taken, the culture was shifted to 43°C and samples were collected at the time points indicated. Crude cell extracts were prepared, separated on an SDS–12% polyacrylamide gel, and subjected to Western blot analysis. Panels A and B represent two independent experiments with different sampling periods. The apparent molecular masses (in kilodaltons) are indicated to the right of the gels. HS, heat shock.

into *B. japonicum* 110*spc*4 for marker replacement mutagenesis as described previously (18).

β-Galactosidase assay. Recombinant *E. coli* strains were grown overnight in LB medium supplemented with ampicillin plus 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and β-galactosidase activity was measured as previously described (32).

Nucleotide sequence accession number. The nucleotide sequence of the *B. japonicum rpoH*$_1$ gene region has been deposited in the GenEMBL database under accession no. U55047.

RESULTS

Heat-induced synthesis of σ^{32}-like protein in *B. japonicum*. A heat-inducible protein band in *B. japonicum* cross-reacting with an antibody raised against *E. coli* σ^{32} has been identified (6). In order to further define the kinetics of heat shock induction, we performed Western blot (immunoblot) analyses using extracts from cells harvested before and at different time points after a shift from 28 to 43°C. *E. coli* MC4100 (heat shocked at 43°C for 4 min) served as a control (Fig. 1). Extracts of *B. japonicum* cells collected before or 2 min after heat shock did not yield any signal. At 5 min after the shift, a faint signal which reached its maximum between 20 and 30 min after heat shock was detected (Fig. 1A). The *B. japonicum* band showed a higher apparent molecular mass in an SDS-polyacrylamide gel than the *E. coli rpoH* product. A separate experiment indicated that under continuous heat shock conditions the intensity of the signal in *B. japonicum* was kept at an elevated level for at least 5 h (Fig. 1B). Whether this steady-state level is due to permanent de novo synthesis or high protein stability in *B. japonicum* cannot be distinguished at present. Preliminary data indicate, however, that the intensity of the heat-inducible band remained constant in extracts from cells kept at 28 or 0°C subsequent to heat treatment (36).

Isolation of a *B. japonicum rpoH* gene. Assuming that a σ^{32}-like protein of *B. japonicum* would be functional in *E. coli*, we attempted to complement the σ^{32}-deficient *E. coli* A7448 with cloned *B. japonicum* DNA. In strain A7448 (kindly provided by A. Oppenheim, Jerusalem, Israel), the *rpoH* region corresponding to the amino acid residues 23 to 268 of σ^{32} is replaced by a kanamycin resistance gene (8, 62). In addition, the A7448 strain carries a P*groE-lacZ* fusion integrated into the chromosome which serves as suitable reporter for σ^{32} activity. Chromosomal DNA of *B. japonicum* was completely digested with *Bam*HI and ligated into the appropriately digested pUC18 vector. After transformation into *E. coli* A7448 and growth at 23°C on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and IPTG, some clones yielded blue colonies, which suggests that they produced a functional σ^{32} protein. Plasmid pRJ5000 isolated from one

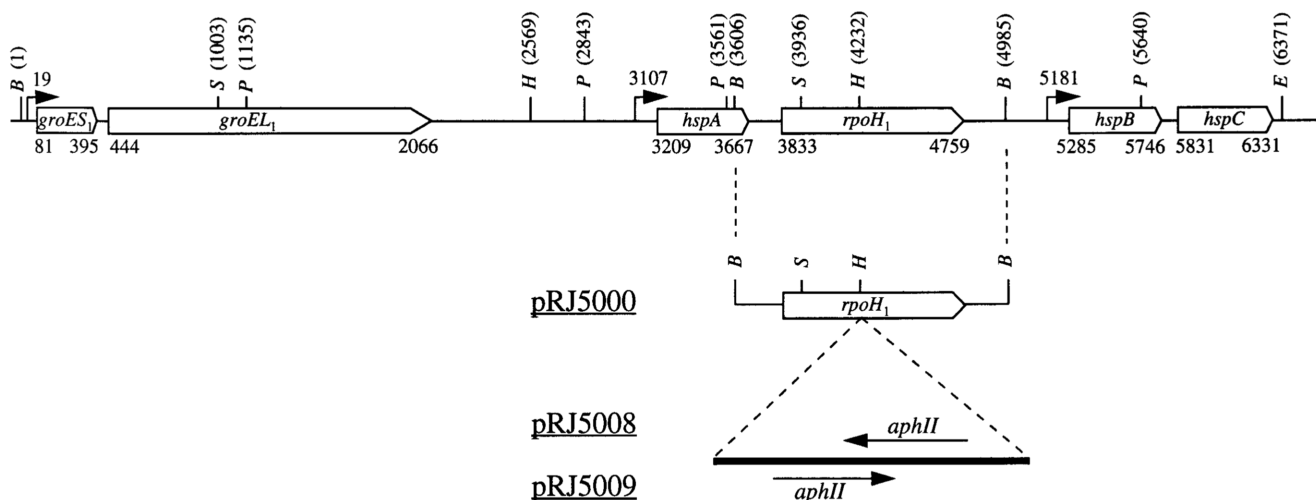

FIG. 2. Physical map of the *B. japonicum rpoH*$_1$ gene region. Numbers indicate the nucleotide positions of open reading frames, transcription start sites, or recognition sites of the following restriction enzymes: *Bam*HI (*B*), *Eco*RI (*E*), *Hin*dIII (*H*), *Pst*I (*P*), and *Sac*I (*S*). The solid arrows represent transcription start sites determined by Babst et al. (*groESL*$_1$) (6) or in the present study (*hspA* and *hspB*). The inserts of pRJ5000, pRJ5008, and pRJ5009 are presented below the physical map. Arrows indicate the orientation of the *aphII* cassette with respect to the *rpoH*$_1$ reading frame.

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Eco     ...............MADKMQ SLALAPVGGL DSYIRAANAW PMLSADEERA LAEKLHYHGD    46
Pae     ..............MTTSLQP VHALVPGANL EAYVHSVNSI PLLSPEQERE LAERLFYQQD    47
Mxa     ..................MQA SNSFSSPDSL STYLSEINQY PLLTQPQEQE LSKRFRA.GD    42
Ccr     ................MAVNS LSVMSPDGGL SRYLTEIRKF PMLSKDEEFM LAQRWKEHQD    45
Atu     ...............MARNSL PTITAGEAGL NRYLDEIRKF PMLEPQEEYM LGKRYAEHGD    46
Bja     ................MFNAA LPAPSVDAGL SKYLVEIRKF PLLTPEQELA YARRWREHRD    46
Con                                    L   Y        P L    E   l  r     D
                                                 1.2

Eco     LEAAKTLILS HLRFVVHIAR NYAGYGLPQA DLIQEGNIGL MKAVRRFNPE VGVRLVSFAV   106
Pae     LEAARQMVLA HLRFVVHIAK SYSGYGLAQA DLIQEGNVGL MKAVKRFNPE MGVRLVSFAV   107
Mxa     LAAGHQLVTA NLRFVVKVAY EYRSYGLKMS DLIQEANIGL MKAVQKFDPD KGIRLISYAV   102
Ccr     PQAAHKMVTS HLRLVAKIAM GYRGYGLPIG EVISEGNVGL MQAVKKFEPE KGFRLATYAM   105
Atu     RDAAHKLVTS HLRLVAKIAM GYRGYGLPIG EVVSEGNVGL MQAVKKFDPE RGFRLATYAM   106
Bja     RDAAYHLVTS HLRLVAKIAM RYRGYGLPIA EIVSEGNIGL MQAVRRFDPD RGVRLATYAM   106
Con       Aa   v   hLR V  iA   Y gYGL        EgN GL M AV  F P   G RL   A
                   2.1                   2.2                  2.3

Eco     HWIKAEIHEY VLRNWRIVKV ATTKAQRKLF FNLRKTKQRL GWFNQDE... ..VEMVAREL   161
Pae     HWIKAEIHEF ILRNWRIVKV ATTKAQRKLF FNLRSQKKRL AWLNNEE... ..VHRVAESL   162
Mxa     WWIRAYIQNC ILKNWSLVKL GTTQAQRRLF FSLARTRREL EKMGAGDANV VNAEEIARKL   162
Ccr     WWIRASIQEY ILRSWSLVKM GTTAAQKKLF FNLRKAKSQI AAFQEGDLHP DQVSQIATKL   165
Atu     WWIKASIQEY ILRSWSLVKM GTTANQKRLF FNLRRLKGRI QAIDDGDLKP EHVKEIATKL   166
Bja     WWIRASIQEY ILRSWSLVKI AASASQKKLF FKLRRAKSAI SALQDGDLRP EQVRLIAERL   166
Con      Wi A I e  iLr W  VK   tt  Q  LF F Lr  k                 v   A  L
                   2.4               RpoH box                  3.1

Eco     GVTSKDVREM ESRMAAQDMT FDLSSDDDSD SQPMAPVLYL QDKSSNFADG IEDDNWEEQA   221
Pae     GVEPREVREM ESRLTGQDMA FDPAADADDE SAYQSPAHYL EDHRYDPARQ LEDADWSDSS   222
Mxa     NVKASEVREM EQRMGGRDLS LDAPM.GEDG DATHLD..FV ESESVSAVDE VADRQQANLT   219
Ccr     GVLDSEVISM NRRLSGPDAS LNAPLR.RDG ES.EWQDWLA DEEQVSQETR VAEDEEKSLR   223
Atu     QVSEEEVISM NRRLHG.DAS LNAPIKASEG ESGQWQDWLV DDHE.SQEAV LIEQDELETR   224
Bja     KVAERDVVAM DRRLRG.DAS LNVPIHD.ED EGGQTLDWLV DPAPTC.EIT LAEEQEAKQR   223
Con      V    V  M   R    D
                                                   3.2

Eco     ANRLTDAMQG LDERSQDIIR ARWLDEDNKS TLQELADRYG VSAERVRQLE KNAMKKLRAA   281
Pae     SANLHEALEG LDERSRDILQ QRWLSEE.KA TLHDLAEKYN VSAERIRQLE KNAMSKLKGR   281
Mxa     RELVQRALRR LDPRERFIIE QRVMGDA.EM TLSELGEHFG FSRERARQLE IRAKDKLKLA   278
Ccr     MSLLEEAMVE LTDRERHILT ERRLKDD.PT TLEELAAQYG VSRERVRQIE VRAFEKLQKT   282
Atu     RRMLAKAMGV LNDRERRIFE ARRLAED.PV TLEELSSEFD ISRERVRQIE VRAFEKVQEA   283
Bja     RLALANALAN LNARERNIFT ARWLNEE.ST TLEELAAEYG VSRERVRQIE ERAFQKVKAA   282
Con        l  A    L  R r I    R l       TL eL       S ER RQ E   A  K
                     4.1                       4.2

Eco     IEA                               284
Pae     ILG                               284
Mxa     LVTLMAEAGV DESTLNA                295
Ccr     MREAAIAKNM VDA                    295
Atu     VQKEALEAAR ALRVVDA                300
Bja     MLTSRHEANG PPSSRAKEMK QGVARA      308
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FIG. 3. Alignment of deduced amino acid sequences of six different σ^{32}-like proteins. Amino acids are presented in the one-letter code. Numbers at the end of each line indicate the amino acid positions relative to the start of each protein. Representative σ^{32}-like proteins of *E. coli* (Eco) (25), *P. aeruginosa* (Pae) (8, 33), *M. xanthus* (Mxa) (SigC [4]), *C. crescentus* (Ccr) (44, 58), *A. tumefaciens* (Atu) (35), and *B. japonicum* RpoH$_1$ (Bja) are compared. In the consensus sequence (Con), amino acids which are identical in all six proteins are defined by capital letters, and amino acids present in five of six proteins are indicated by lowercase letters. Numbers below the sequences identify regions which are conserved in all members of the σ^{70} family (27). RpoH box defines a conserved region exclusively found in σ^{32} proteins (35). The dots indicate gaps introduced to maximize alignment.

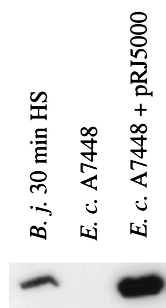

FIG. 4. Immunodetection of a σ^{32}-like protein in *B. japonicum* and in recombinant *E. coli* A7448. The *B. japonicum* (*B. j.*) extract is the same as in Fig. 1A. *E. coli* (*E. c.*) A7448 was grown to mid-exponential phase in LB medium containing 0.5 mM IPTG. Western blot analysis using anti-*E. coli* σ^{32} serum was performed as described in Materials and Methods.

such clone contained a 1.4-kb *Bam*HI fragment (Fig. 2). DNA sequence analysis revealed an open reading frame coding for a protein with highest similarity to σ^{32}-like proteins of *A. tumefaciens*, *C. crescentus*, and *Zymomonas mobilis* (62.5, 59.6, and 57.4% identical amino acids, respectively) (35, 44, 58). The deduced *B. japonicum* protein shared less but still significant similarity with the development-specific sigma factors SigB and SigC of *Myxococcus xanthus* (41 and 43% identity, respectively) (3, 4) and with σ^{32} proteins of *E. coli* and closely related organisms (approximately 39% identity) (25, 35). The cloned *B. japonicum* gene was designated *rpoH*$_1$ because work currently in progress in our laboratory indicates that additional *rpoH* homologs exist in *B. japonicum* (36) (see Fig. 9 and Discussion). An amino acid sequence alignment of the deduced RpoH$_1$ protein and representative σ^{32}-like proteins from other organisms is shown in Fig. 3. The calculated molecular masses of RpoH$_1$ (35,041 Da) and *E. coli* σ^{32} (32,381 Da) are consistent with their migration in an SDS-polyacrylamide gel (Fig. 1).

Functional characterization of the *B. japonicum rpoH*$_1$ gene product. The fact that pRJ5000 could lead to activation of the P*groE-lacZ* fusion present in *E. coli* A7448 demonstrated that *rpoH*$_1$ codes for a functional σ^{32}-like protein. In order to quantify this activity, we performed β-galactosidase assays. β-Galactosidase activity of strain A7448 containing pRJ5000 was approximately 4,700 U, whereas the same host containing the pUC18 vector or pRJ5001 (same as pRJ5000 but with the same 1.4-kb *Bam*HI insert in reverse orientation) generated only 15 or 220 U, respectively. Western blot analysis confirmed the presence of a σ^{32}-like protein in the strain containing pRJ5000 (Fig. 4). As expected, no cross-reacting band was detected in the host strain A7448.

Cloning and sequencing of the DNA region encompassing *rpoH*$_1$. Tentative primer extension experiments using oligonucleotides complementary to the 5′ end of *rpoH*$_1$ (Sig9 and Sig16; see Materials and Methods) did not result in a signal immediately upstream of the gene, suggesting that *rpoH*$_1$ is located in an operon and preceded by at least one more gene. This prompted us to analyze the DNA region upstream and downstream of the gene. A cosmid library was hybridized with the 1.4-kb *Bam*HI fragment from pRJ5000, and overlapping fragments covering the region extending from the *Bam*HI site at position 1 to the *Eco*RI site at position 6371 in Fig. 2 were subcloned. The DNA sequence of the entire region was established on both strands. Surprisingly, the sequencing analysis revealed that *rpoH*$_1$ is located downstream of the previously identified *groESL*$_1$ operon whose transcription is dependent on σ^{32} (6). Hence, the *rpoH*$_1$ region can be assigned to the same location on the *B. japonicum* chromosome map, namely, 223 min (6, 23). The completed *groESL*$_1$ DNA sequence included in this work is the third fully sequenced *groESL* operon of *B. japonicum* in addition to the previously sequenced *groESL*$_2$ and *groESL*$_3$ operons (14).

Besides *groESL*$_1$, three other open reading frames (*hspA*, *hspB*, and *hspC*) encoding small Hsps were found to be associated with *rpoH*$_1$. HspA and HspB share 73% identical amino acids and are thus quite similar. Moreover, they show striking homology to the inclusion body-associated proteins IbpA and IbpB of *E. coli* (between 37 and 50% identical amino acids) (2) and to the global stress protein GspA of *Legionella pneumophila* (34% identity in the case of HspA, 40% in the case of HspB) (24). An alignment of the amino acid sequences of these proteins is shown in Fig. 5A.

The deduced product of *hspC* shows the most homology to a class of small heat shock proteins mainly found in plants, either in the cytosol or in chloroplasts. Prokaryotic homologs in *C. acetobutylicum* and *Stigmatella aurantiaca* have also been identified (20, 48). Figure 5B presents a comparison of *B. japonicum* HspC with the prokaryotic proteins and one member each of cytosolic and chloroplast plant proteins. Although the overall sequence similarity between the HspA- or HspB-like and HspC-like proteins is poor, a short motif in the C-terminal region (A. . .nGL/VL; underlined in Fig. 5) appears to be common.

Transcriptional analysis of the *rpoH*$_1$ gene region. A σ^{32}-dependent promoter upstream of *groESL*$_1$ has previously been reported (6). For the reasons described above, we thought that *rpoH*$_1$ was transcribed from a promoter located in front of the upstream gene. Therefore, we performed primer extension experiments with oligonucleotides complementary to the 5′ end of *hspA* (Sig32 and Sig46 [see Materials and Methods]). In fact, we detected a transcription start site upstream of *hspA* that was preceded by a −35/−10 region with striking sequence similarity to σ^{70}-dependent promoters. The result from the experiment performed with primer Sig46 is shown in Fig. 6 (left panel) and Fig. 7. Despite being transcribed from an apparent σ^{70}-like promoter sequence, no mRNA was detected under non-heat shock conditions. Instead, the transcript was clearly heat inducible. Very similar results were obtained for *hspB* (primers Sig31 and Sig47; Sig31 results shown in Fig. 6, right panel). A comparison of both promoter regions is presented in Fig. 7. In addition to the −10 and −35 sequences, there is in both cases an AAAAT box around position −50 which conforms well to the UP element known to stimulate transcription of σ^{70}-like promoters (45) (Fig. 7). Most notably, the ~100-bp sequences between the transcriptional and translational start sites of *hspA* and *hspB* are almost identical (Fig. 7).

Analysis of *B. japonicum rpoH*$_1$ mutants. *rpoH*$_1$ mutants were constructed by insertion of a kanamycin resistance cassette into its central *Hin*dIII site (Fig. 2 and Materials and Methods). The genomic structures of *B. japonicum* 5008 and 5009, which differ only with respect to the orientation of the inserted resistance gene, were verified by Southern blot hybridization using different restriction enzymes (e.g., in *Bam*HI digests the 1.4-kbp fragment occurring in the wild type was shifted to 3.0 kbp in *B. japonicum* 5008 and 5009 because of the inserted kanamycin cassette). However, Western blot analysis revealed that both strains were still able to produce σ^{32}-like protein after heat shock (Fig. 8A). In addition, primer extension experiments showed that the mutation had little if any effect on transcription of *groESL*$_1$, which is a potential target of σ^{32} (Fig. 8B). These data suggest that additional σ^{32}-like pro-

A

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HspA     ..MRTYNISP LWRSTIGFDR VFDLVDAARH T.AGEANYPP CNVERLSDDR YRISLALAGF    57
HspB     .MRTTYDFAP LWRSTIGFDR LFDLVDAAQQ A.GTEDNYPP CNVERLSEDR YQISLAVAGF    58
IbpA     ..MRNFDLSP LYRSAIGFDR LFNHLE.NNQ S.QSNGGYPP YNVELVDENH YRIAIAVAGF    56
IbpB     ..MRNFDLSP LMRQWIGFDK LANALQNAGE S.QS...FPP YNIEKSDDNH YRITLALAGF    54
GspA     MNTTSLSLTP LLRHSVGFER FNDLFESMRN ADDSTYAYPA YDIEKHGEDN YMITMAVPGF    60
Con1              P L R  iGFdr                   yPp  n E       Y I  A aGF

HspA     SPDEITVTAE QSVLTIEG.. RKGEKGRRDF VYRGISSRPF KRQFGLAAHV RVEGARFDNG   115
HspB     SADEIAITAE QSVLTVEG.. RKSEKQQREF LYQGISSRPF KRQFNLADYV QVKGASFDNG   116
IbpA     AESELEITAQ DNLLVVKG.A HADEQKERTY LYQGIAERNF ERKFQLAENI HVRGANLVNG   115
IbpB     RQEDLEIQLE GTRLSVKG.T PEQPKEEKKW LHQGLMNQPF SLSFTLAENM EVSGATFVNG   113
GspA     QESDLNIMVQ NDQLRVSGRI QEKETKESEY LHRGIVTRAF EQTFRLADHM KVTGAEIKKG   120
Con1           i       L v G     e      l  Gi  r F    F LA     V GA   nG

HspA     LLQIELVREI PDAMKPRRIP IDNLAASDVQ QIEREAA               152
HspB     LLQIELVREI PEAMKPRRIS ISGSSASNVR QIDGKAA               153
IbpA     LLYIDLERVI PEAKKPRRIE IN                               137
IbpB     LLHIDLIRNE PEPIAAQRIA ISERPALN                         141
GspA     LLSISLIREI PEEAKPRIIP IKSISDQESN KKSKTIEPES KKKISN    166
Con1     LL I L R i Pe  kprrI  I
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B

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Phy      MACKTLTCSA SPLVSNGVVS ATSRTNNKKT TTAPFSVCFP YSKCSVRKPA SRLVAQATGD    60

Phy      NKDTSVDVHV SNNNQGGNNQ GSAVERRPRR MALDVSPFGL LDPMSPMRTM RQMMDTMDRL   120
Gma      .......... .......... .......MSL IPNFFGGRRN NVFDPFSLDV WDPFKDFPFP    33
Sau      .......... .......... ......MADL SVRRGTGSTP QRTREWDPFQ QMQELMNWDP    34
Cac      .......... .......... .......... ......MFGM VPFRRNNNGL MRREDFFDKM    24
Bja      .......... .........M GIKDLIPWNN GGREVGIHRG ADVNPFLTLH REMNRMFDEV    41

Phy      FEDTMTFPGS RNRGTGEIRA PW DIKDDEN EIKMRFDMPG LSKEEVKVSV EDD.VLVIKG   178
Gma      NTLSSASFPE FSRENSAFVS TRVDWKETPE AHVFKADIPG LKKEEVKVQI EDDKVLQISG    93
Sau      FELANHPWFA NRQGPPAFVP A.FEVRETKE AYIFKADLPG VDEKDIEVTL TGDRV.SVSG    92
Cac      FDNFFSDDFF PTTTFNGNAG FKVDIKEDDD KYTVAADLPG VKKDNIELQY ENN.YLTINA    83
Bja      FRGFDLAPFG SSRGLSGLGW PQIDIDETDK EVRITAELPG LEEKDVSLEI ANG.VLSISG   100
Con2     f                       d  e        adlPG                vl i g

Phy      EHKKEESGKD DS....WG.R NYSSYDTRLS LPDNVDKDKV KAELKNGVLL ISIPKTKVEK   233
Gma      ERNVEKEDKN DT..WHRVER SSGKFMRRFR LPENAKVEQV KASMENGVLT VTVPKEEVKK   151
Sau      KREREKREES ER..FYAYER TFGSFSRAFT LPEGVDGDNV RADLKNGVLT LTLPKRPEVQ   150
Cac      KRDDIVETKD DNNNFVRRER SYGELRRSF. YVDNIDDSKI DASFLDGVLR ITLPKKVKGK   142
Bja      EKKSESEDKA RR....FSER YYGRFERRIP L.EGIDEDKV SAAFKNGVLT ITVPKSAEAK   155
Con2         e   k          eR   g   r    l    d   v  A   nGVL   t PK    k

Phy      .KVTDVEIK                                   241
Gma      PDVKAIEISG                                  161
Sau      PKRIQVASSG TEQKEHIKAY PAPAEPGLAA PLGWPGFS    188
Cac      DNGRRIDIH                                   151
Bja      .NVRRIAINR NG                               166
Con2            i
```

FIG. 5. Amino acid sequence comparison of small heat shock proteins. (A) Alignment of the deduced HspA and HspB proteins of *B. japonicum*, IbpA and IbpB from *E. coli* (2), and GspA from *L. pneumophila* (24). In consensus sequence Con1, amino acids which are identical in all five proteins are defined by capital letters, and amino acids present in four of five proteins are indicated by lowercase letters. (B) Comparison of the deduced HspC protein of *B. japonicum* (Bja) with a small chloroplast Hsp from *Petunia hybrida* (Phy) (11), a small cytosolic Hsp from soybean (*Glycine max* [Gma]) (42), SP21 from *S. aurantiaca* (Sau) (20), and Hsp18 from *C. acetobutylicum* (Cac) (48). A consensus sequence (Con2) was determined as in panel A. The dots indicate gaps introduced to maximize alignment.

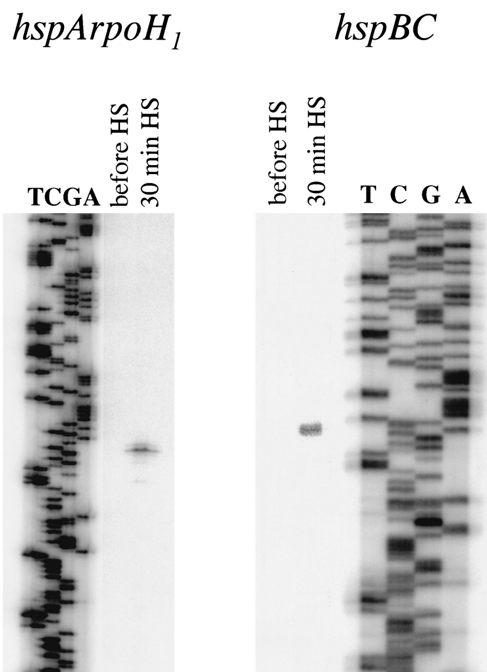

FIG. 6. Determination of the transcription start sites of the *hspA-rpoH*$_1$ and *hspBC* operons by primer extension mapping. Total RNA was isolated from *B. japonicum* cells harvested before and 30 min after a heat shock (HS) from 28 to 43°C. The extension reactions were performed with primers Sig46 (*hspA*) and Sig31 (*hspB*); the same primers were used for the corresponding sequencing reactions shown at the left- and rightmost lanes of the gels, respectively. The primer extension product and the corresponding sequencing reaction were run side by side on the same gel. Different PhosphoImager (Molecular Dynamics) settings were used for each reaction. The transcription start sites and deduced promoter regions are presented in Fig. 7.

teins may be involved in the regulation of heat shock genes in *B. japonicum*. In fact, Southern blot hybridizations using the *rpoH*$_1$ gene as a probe revealed at least two additional bands in chromosomal DNA of *B. japonicum* (Fig. 9) (see also Discussion).

DISCUSSION

From data presented here and in a previous study (6), we learned that the heat shock response in *B. japonicum* is complex and includes numerous regulatory factors. The *groESL*$_3$ operon which is subject to oxygen control via the σ^{54} RNA polymerase and its activator NifA is not considered in this context (14). Babst et al. (6) found that three of the five *groESL* operons in *B. japonicum* are heat inducible. A highly conserved inverted-repeat structure (CIRCE) accounts for induction of *groESL*$_4$ and *groESL*$_5$. Transcription from the *groESL*$_1$ promoter was shown to be dependent on σ^{32} in a recombinant *E. coli* strain. Here we confirmed the inferred existence of a σ^{32} homolog in *B. japonicum* by immunological and genetic evidence. The phenotypes of *rpoH*$_1$ mutants in combination with the results of Southern blot analyses indicate the presence of additional *rpoH* genes in *B. japonicum*. The protein band cross-reacting with σ^{32} antibodies may therefore be a composite consisting of different RpoH species. We found that the induction pattern of this band after heat shock correlates well with the kinetics of *groESL*$_1$ induction which had been measured on the transcriptional level (6). Maximal induction of the σ^{32}-like protein(s) was reached between 20 and 30 min after heat shock, and this preceded the attainment of maximal *groESL*$_1$

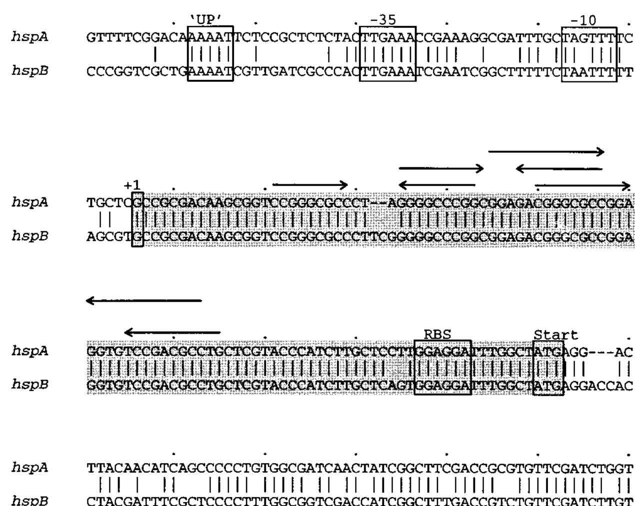

FIG. 7. Sequence comparison of the promoter regions of the *B. japonicum* *hspA-rpoH*$_1$ and *hspBC* operons. The −10 and −35 regions, the transcriptional start sites (+1), the putative ribosome binding sites (RBS), the translational start sites (Start), and a putative UP element (45) are shown in boxes. The proposed regulatory element in the transcribed, untranslated region is shaded. Inverted repeats common to both sequences are indicated by arrows. Identical amino acids are indicated by vertical lines.

mRNA levels observed at 35 min after heat shock. The CIRCE-dependent *groESL*$_4$ expression kinetics, by contrast, showed a transient induction pattern. (Note that in this study the heat shock condition was at 43°C, whereas the previous mRNA measurements had been performed with cells heat shocked at 39°C [6]; however, since the same article states that *groESL*$_1$ induction is maximal at 43°C, we argue that both results are comparable.) Thus, our findings further substantiate the σ^{32} dependence of *groESL*$_1$ expression in *B. japonicum*. The continuously enhanced levels of a σ^{32}-like protein band in

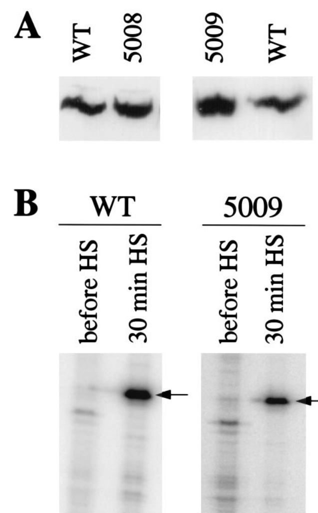

FIG. 8. Analysis of *B. japonicum rpoH*$_1$ mutants. (A) Immunodetection of a σ^{32}-like protein in the *B. japonicum* wild type (WT) and in mutant strains 5008 and 5009. The cells were heat shocked from 28 to 43°C for 30 min and subjected to Western blot analysis as described in Materials and Methods. (B) Heat shock (HS) induction of the *groESL*$_1$ transcript (arrow) in *B. japonicum* wild type and mutant 5009. Primer extension experiments with oligonucleotide 702 (6) were performed as described in Materials and Methods.

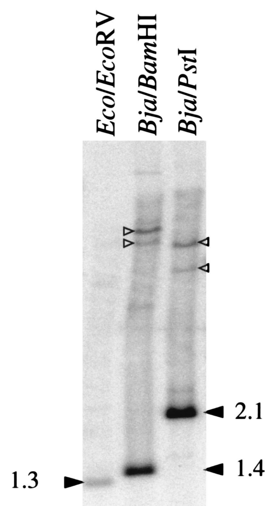

FIG. 9. Southern blot hybridization of genomic DNAs from *E. coli* (*Eco*) and *B. japonicum* (*Bja*). The 1.4-kbp *Bam*HI fragment from pRJ5000 containing $rpoH_1$ was radiolabeled and used as a probe. The hybridization was performed under low-stringency conditions (57°C, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). The sizes of the *E. coli rpoH* fragment and *B. japonicum* $rpoH_1$ fragments are indicated. Additional bands are marked by small open triangles. The numbers to the sides of the gel are kilobase pairs.

B. japonicum are in marked contrast to the results found for *E. coli*, in which the level of σ^{32} (and that of *groESL* transcripts) is only transiently induced during heat shock and declines to almost preshocked levels within 15 min (for a review, see reference 60). This clearly indicates that σ^{32}-dependent regulation in these two organisms is different. Whether an unusually high stability of the *B. japonicum* σ^{32} (see Results) or other mechanisms account for this difference remains to be elucidated. Although preliminary data indicate that the σ^{32}-like protein(s) of *B. japonicum* may be particularly stable, a region implicated in DnaK- and DnaJ-mediated turnover of σ^{32} in *E. coli* is also present in $RpoH_1$ of *B. japonicum*. This so-called RpoH box is uniquely conserved among σ^{32}-like factors and binds to DnaK with high affinity (30, 35).

The deduced protein sequence of $RpoH_1$ conforms well to the features of related proteins from proteobacteria of the α subgroup (35). With 308 amino acids, the protein is significantly larger than the *E. coli* σ^{32}, which consists of 284 amino acids. This is partially due to a five-amino-acid insertion around position 150 in region 3.1 that is also present in the *A. tumefaciens*, *C. crescentus* (Fig. 3), and *Z. mobilis* proteins. In addition, an extended C terminus contributes to the size difference between RpoH proteins from α and γ proteobacteria. A rather unusual property of $RpoH_1$ is the calculated isoelectric point of 10.48. By contrast, the corresponding values for *E. coli* σ^{32} and even for the most closely related proteins of *A. tumefaciens* and *C. crescentus* are 5.78, 6.56, and 6.72, respectively. Whether this unique feature of $RpoH_1$ has any structural or functional implication is unknown at this time. Similarly, it is not known why SigB and SigC from *M. xanthus* are closer relatives of $RpoH_1$ than of *E. coli* σ^{32}. Both of these sigma factors are developmentally expressed and play an essential role in the unique life cycle of *M. xanthus* (3, 4). Despite their high similarity to RpoH proteins, they are not able to complement the temperature-sensitive growth phenotype of an *E. coli* Δ*rpoH* strain (35). The same applies to *B. japonicum* $RpoH_1$ which was not able to confer a significant growth advantage on *E. coli* A7448 at 28 or 37°C in liquid culture (36). However, the complementation experiment demonstrated that $RpoH_1$ was able to generate β-galactosidase from the *E. coli groE-lacZ* promoter present in strain A7448. In the light of this observation, it would be interesting to test whether *M. xanthus* SigB and SigC allow the formation of β-galactosidase activity in *E. coli* A7448.

The finding that $rpoH_1$ is clustered with five additional heat shock genes was unexpected. This genomic arrangement may reflect a functional connection between these genes. Initially it was logical to propose that $RpoH_1$ is responsible for induction of $groESL_1$. However, three independent lines of evidence now indicate that this inference was an oversimplification. First and most importantly, $rpoH_1$ insertion mutants still allowed the heat shock induction of $groESL_1$. Second, the same mutants did not show an apparent growth defect and still produced σ^{32}-like protein. Third, hybridization experiments performed with the $rpoH_1$ gene as a probe revealed that additional *rpoH*-like genes might exist in *B. japonicum* (Fig. 9). In fact, we recently succeeded in the isolation of two further DNA regions which, when introduced into *E. coli* A7448, complemented the *rpoH* defect of that strain in a manner similar to that of the *B. japonicum* $rpoH_1$ gene (36). A detailed molecular characterization of these two DNA regions is currently in progress. Our preliminary observations point out that several σ^{32}-like proteins contribute to the complex heat shock regulation in *B. japonicum.*

Equally puzzling is the association of $rpoH_1$ with three other heat shock genes. To our knowledge, the *B. japonicum hspA-*$rpoH_1$ arrangement is the first example of an *rpoH* gene organized in an operon. However, Nakahigashi et al. (35) failed to detect a promoter-like sequence upstream of the *rpoH* genes in *A. tumefaciens* and *Z. mobilis*, which may indicate that these genes are also part of an operon. On the basis of the following observations, we infer that *hspB* is organized in an operon with *hspC*. (i) Both genes are separated by only 85 nucleotides. (ii) No consensus promoter sequence could be identified between *hspB* and *hspC*. (iii) While two potential stem-loop terminator regions are located downstream of $rpoH_1$ (data not shown), no such feature was found downstream of *hspB*, which makes rho-independent termination between *hspB* and *hspC* unlikely. (iv) We were not able to determine a transcription start site immediately upstream of *hspC* by primer extension. Unfortunately, the longstanding technical difficulties with RNA isolates from *B. japonicum* prevented the performance of Northern (RNA) blot analyses to support this assumption.

The functions of the proteins homologous to *hspA*, *hspB*, and *hspC* are not known. The *hspA*- and *hspB*-homologous *ibpA* and *ibpB* genes of *E. coli* form a heat-inducible, σ^{32}-dependent bicistronic operon. The IbpA and IbpB proteins accumulate during overproduction of certain eukaryotic proteins in *E. coli* and are tightly associated with inclusion bodies (2). Expression of another homolog of HspA and HspB, namely, the global stress protein GspA of the pathogen *L. pneumophila*, is regulated by a σ^{70}- and σ^{32}-like promoter. GspA belongs to a family of macrophage-induced proteins in this organism and is believed to play a role in intracellular infection and in protection against different kinds of stress (24). Similarly, little is known of the small Hsps that are homologous to HspC. The corresponding cytosolic plant proteins form large aggregates in the perinuclear region of heat-shocked cells and are thought to be involved in protection of untranslated RNA (40). The prokaryotic members of this class (SP21 and Hsp18) are induced during developmental changes in *S. aurantiaca* (20) and during a metabolic shift or by heat treatment in *C. acetobutylicum* (41, 48). Although all small Hsps mentioned in this context do not exhibit significant over-

all similarity, they share a short stretch of conserved hydrophobic amino acids in their C-terminal regions (see underlined sequences in Fig. 5). This motif may facilitate protein-protein interactions, leading to the observed aggregates or allowing recognition of hydrophobic patches from denatured proteins.

A potentially very interesting feature of the *rpoH*$_1$ gene region is the conserved sequence found between the transcriptional and first translational start sites of the *hspA-rpoH*$_1$ and *hspBC* operons. Database searches did not uncover similar sequences in other organisms. The corresponding promoters almost perfectly match the consensus sequences for $-35/-10$-type promoters from constitutively expressed genes in *E. coli* and *B. japonicum* (7). Nevertheless, unstressed *B. japonicum* cells did not synthesize detectable levels of *hspA* or *hspB* mRNA as determined in primer extension experiments. Transcription of these genes clearly required heat shock induction. It seems likely that the conserved sequence downstream of the *hspA* and *hspB* promoters plays a role in converting an inherently constitutive promoter to a heat-inducible promoter. The placement of this sequence in the nontranslated 5′ end of the transcript and the overlapping potential stem-loop structures (Fig. 7) suggest that certain mRNA secondary structures might contribute to regulation. Alternatively, the conserved region might serve as a protein binding site on the DNA level, by analogy with regulation occurring at the CIRCE element (50, 59). Obviously, a more detailed analysis of the putative regulatory element is required to resolve this matter.

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