JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 548–551 Vol. 179, No. 2
0021-9193/97/$04.00+0

Isolation, Sequence, and Expression of the Gene Encoding Halocin H4, a Bacteriocin from the Halophilic Archaeon *Haloferax mediterranei* R4

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Received 11 July 1996/Accepted 1 November 1996

The first gene to encode a haloarchaeal bacteriocin (halocin H4) has been cloned and sequenced from *Haloferax mediterranei* R4. Both the signal sequence in the halocin H4 preprotein and the monocistronic *halH4* gene have some unusual features. The physiology of *halH4* expression reveals that although *halH4* transcripts are present at low basal levels during exponential growth, halocin H4 activity first appears as the culture enters stationary phase. As halocin activity levels increase, so do transcript levels, but then activity levels decrease precipitously while transcript levels remain elevated.

Halocins are haloarchaeal equivalents of eubacterial bacteriocins (2) and were first discovered in 1982 by F. Rodriguez-Valera (24). Although nearly universal in halobacterial rods (17, 32), only three halocins have been characterized in any detail: halocin H4 from *Haloferax mediterranei* R4 (15, 16), halocin H6 from *Haloferax gibbonsii* Ma2.39 (30, 31), and halocin HalR1 from *Halobacterium* sp. strain GN101 (23). In parallel with antibiotic production in the domain *Bacteria* (33), halocins H4, S8, and HalR1 are all initially detected as the cultures leave exponential growth and enter stationary phase (21). Consequently, we chose halocins, beginning with halocin H4, as models to study stationary-phase gene expression in the haloarchaea.

Isolation of the *halH4* gene. Halocin H4 was purified from *H. mediterranei* R4 (ATCC 33500) culture supernatants essentially as described elsewhere (4, 15). The amino-terminal sequences of the secreted protein and of two tryptic fragments (numbers 9 and 17 [Fig. 1]) were used to design inosine-containing degenerate oligodeoxynucleotide primers. Using these primers, we amplified the 5′ end of the halocin H4 gene (*halH4*) by PCR and used the larger product (150 bp) as a probe to recover the gene from an enriched *Hin*dIII plasmid library.

The start site of transcription of the *halH4* gene was determined by primer extension (26). Note that the initiator AUG codon is only 4 bases from the 5′ end of the message (Fig. 1). Similar leaderless transcripts are produced by other haloarchaeal genes, including *bop* (7); *brp* (3); *hop* (5); and *arcA*, *arcB*, and *arcC* (25). Inspection of the DNA sequence upstream of the *halH4* transcriptional start site reveals a box A haloarchaeal promoter hexamer (Fig. 1) that fits the criteria established by Palmer and Daniels (22). The transcriptional terminator is a perfect 11-base inverted repeat separated by a 16-base interval. The resultant stem-loop structure would be stable ($\Delta G^\circ = -24.6$ kcal) and is similar in design to the transcriptional terminator of the *hop* gene (5).

H. mediterranei R4 contains three large plasmids of 490, 320, and 130 kbp, which are identified as pHM 500, 300, and 100, respectively (14) (Fig. 2). These plasmids were separated from the chromosome by contour-clamped homogeneous electric field (CHEF) gel electrophoresis with a Bio-Rad CHEF-DRIII System (Bio-Rad, Richmond, Calif.) as per the manufacturer's suggestions. Plasmids were separated in a 1% agarose gel (0.5× Tris-borate-EDTA; pH 8.0 [26]) by varying pulse times between 25 and 45 s for 20 h. A Southern blot probed with the PCR product showed that the *halH4* gene is encoded on the pHM300 plasmid. The hybridization signal associated with DNA present in the well is most likely due to the presence of open circular pHM300 that did not enter the gel (10, 14, 18). The plasmidal location for halocin H4 is not surprising, given that most bacteriocins are plasmid encoded (2).

Features of the halocin H4 protein. The *halH4* gene encodes a single polypeptide (Fig. 1). The calculated molecular mass of the secreted (mature) form of halocin H4 is 34.9 kDa, which is approximately 7 kDa larger than the 28 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). The preprotein, complete with a 46-amino-acid signal peptide (Fig. 1), has a molecular mass of 39.6 kDa. The use of a signal sequence to transport the halocin out of the cell is in direct contrast to the lysis proteins used by most colicins and the vast majority of other bacteriocins (6). Bacteriocins from gram-positive bacteria and some colicins do possess cleavable leader peptides, but their method of secretion is novel, since these leader peptides do not conform to typical signal sequences (12).

The signal sequence for halocin H4 has two unusual features (Fig. 1). First, the amino-terminal *n* region is highly charged (+6) and is very long (18 residues). Similar *n* regions have been found in the signal peptides of a halolysin (13) and of a xylanase from *Streptomyces halstedii* (27). Second, the polar *c* region is atypically long at 14 residues but begins with a glycine residue (residue 33), which is typical in prokaryotes (11). The hydrophobic *h* region is typical, and the residues surrounding the cleavage site follow von Heijne's −3, −1 rule (34).

Correlation of halocin H4 activity and *halH4* expression. Figure 3 correlates growth phase with halocin H4 activity and *halH4* transcript levels. *H. mediterranei* R4 was grown aerobi-

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ACCTATATGTATTTTATTATAATATAAAAGTGATAATTTTTTCGGAAAAATATAAATATTTGCTCTGAATTTAAGTATGCAATAAGAATTATGTATCACGAGTCACATTCGGGAAGTGT    -1

1                                                  10                                                  20                  30
M   S   K   D   R   D   G   R   R   T   S   R   R   G   T   L   K   K   I   G   G   F   S   L   G   A   L   S   F   G
ATG TCG AAA GAC AGA GAT GGG AGA AGG ACA AGT CGG CGA GGC ACG TTA AAG AAA ATC GCC GGT TTC AGT CTC GGA GCG CTT AGT TTC GGG    90
<---------------------------- n region ----------------------------> <------------------- h region ------------------

                                                   40                                                  amino terminus  60
A   V   G   R   T   Q   A   A   T   G   S   S   V   T   T   A   D   I   A   P   P   G   P   N   G   D   P   K   S   V
GCA GTC GGA CGA ACT CAA GCG GCG ACC GGC TCA TCC GTT ACG ACC GCT↑GAT ATC GCA CCT CCC GGA CCG AAC GGA GAC CCG AAG AGT GTT   180
-----> <---------------------- c region ---------------------->

                                peptide fragment #9                                                 80          peptide fragment #17    90
Q   I   D   D   K   Y   T   G   A   E   M   Y   G   E   G   D   F   R   V   G   L   G   T   D   L   T   M   Y   P   P
CAG ATA GAT GAT AAA TAC ACC GGA GCC GAG ATG TAC GGC GAG GGT GAC TTC AGA GTC GGT CTC GGA ACT GAC CTG ACG ATG TAT CCG CCC   270

                                                  100                                                 110                 120
V   Y   R   E   S   L   G   N   G   S   G   G   W   E   F   D   F   T   V   C   G   S   T   A   C   R   F   V   D   S
GTG TAC CGT GAG AGT CTT GGA AAT GGA AGC GGG GGT TGG GAA TTC GAC TTC ACC GTT TGT GGG TCC ACT GCC TGT CGA TTT GTG GAC AGT   360

                                                  130                                                 140                 150
N   G   D   V   K   E   D   D   K   A   K   E   M   W   W   Q   E   I   N   F   N   D   I   N   Q   D   L   Y   S   R
AAC GGT GAC GTC AAA GAG GAC GAC AAG GCG AAA GAA ATG TGG TGG CAG GAA ATT AAC TTC AAC GAC ATA AAT CAG GAT TTA TAC AGT CGG   450

                                                  160                                                 170                 180
N   D   S   D   W   V   G   S   T   P   A   D   T   Q   P   E   F   D   Y   T   D   F   A   L   A   R   D   G   V   T
AAC GAT TCC GAC TGG GTC GGG TCG ACC CCT GCC GAT ACC CAA CCG GAG TTC GAT TAC ACC GAC TTT GCG CTC GCT CGG GAC GGA GTG ACG   540

                                                  190                                                 200                 210
L   A   L   T   A   L   N   P   A   M   G   S   L   A   L   G   A   T   Y   F   L   S   D   M   V   N   W   I   A   S
CTC GCT CTC ACG GCA CTC AAC CCC GCA ATG GGG AGT CTT GCA CTC GGT GCC ACG TAC TTC CTC AGC GAC ATG GTG AAC TGG ATT GCG AGC   630

                                                  220                                                 230                 240
Q   H   E   D   D   S   S   L   K   R   K   W   D   Y   D   G   L   S   G   P   L   Y   A   D   S   S   T   Y   L   L
CAG CAC GAA GAC GAC AGT TCG CTC AAG AGA AAA TGG GAT TAC GAC GGG CTA AGT GGG CCG TTG TAC GCC GAT TCG TCG ACG TAC CTA CTG   720

                                                  250                                                 260                 270
A   R   D   E   M   T   S   N   S   Y   E   S   F   T   I   D   N   I   A   V   A   F   P   E   F   P   V   R   T   K
GCA CGC GAC GAG ATG ACT TCG AAC TCG TAC GAA TCA TTC ACG ATC GAT AAC ATC GCC GTT GCC TTC CCA GAG TTC CCC GTC CGG ACC AAG   810

                                                  280                                                 290                 300
Y   Y   V   T   F   T   A   P   D   D   P   S   T   Q   S   I   S   T   L   E   E   E   G   I   Y   R   V   P   A   T
TAC TAC GTC ACA TTC ACT GCG CCG GAT GAC CCG TCA ACG CAG TCG ATA TCT ACG CTC GAA GAG GAG GGA ATC TAC CGA GTG CCC GCT ACG   900

                                                  310                                                 320                 330
E   V   A   A   A   R   P   P   G   S   R   R   S   K   S   A   A   D   E   M   V   Y   V   A   D   P   K   K   F   I
GAA GTG GCT GCG GCC AGA CCA CCG GGG TCC CGA CGT TCC AAA TCG GCA GCC GAC GAG ATG GTG TAC GTT GCC GAT CCG AAG AAG TTC ATA   990

                                                  340                                                 350                 360
E   V   E   P   V   K   N   P   S   I   P   D   R   I   Y   E   E   I   E   Q   K   K   K   Q   R   S   R   K   Q   AMB
GAG GTC GAG CCG GTG AAG AAC CCA AGT ATC CCG GAC CGA ATC TAC GAG GAG ATA GAG CAA AAA AAG AAA CAA CGG AGT AGG AAA CAG TAG  1080

TTACTCGTCCTTCTGTAGCGGTGCCTCTACCCAGGCAGAGTCGGAGGCACCGCTCCGGCGAAGCTT                                                    1146
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FIG. 1. Nucleotide and amino acid sequences of the *halH4* gene. Nucleotides are numbered on the right side of the sequence. Amino acid residue numbers are located above the sequence. Halocin H4 is encoded by a 1,080-bp open reading frame that corresponds to a polypeptide of 359 residues. A putative box A promoter hexamer at positions −27 to −22 is underlined. Solid arrow, the start site of transcription; italics, signal peptide amino acid sequence; broken arrows, the *n*, *h*, and *c* regions of the signal sequence; thick vertical arrow, cleavage site between the signal sequence and the amino terminus of the secreted (mature) form of halocin H4; underlining, amino acid sequences of the amino terminus, tryptic peptide no. 9, and tryptic peptide no. 17 as determined by amino acid sequencing; boldface, hydrophobic residues from an internal hydrophobic segment of 32 amino acid residues (residues 178 to 209); horizontal arrows under the DNA sequence, the putative transcriptional terminator.

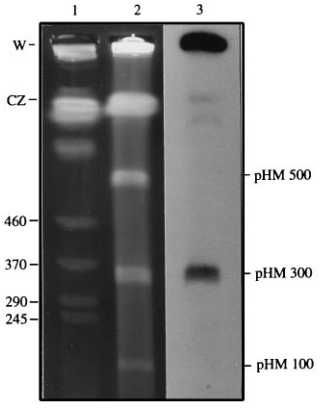

FIG. 2. CHEF gel electrophoresis of total genomic DNA from *H. mediterranei* R4 and corresponding Southern blot. Lanes: 1, yeast chromosome markers (sizes are indicated); 2, *H. mediterranei* R4 genomic DNA; 3, Southern blot of lane 2 probed with ^{32}P-labeled random-primed probes from the 150 bp PCR product. W, well; CZ, compression zone. Locations of the three large plasmids from *H. mediterranei* R4 (pHM) are indicated.

cally at 37°C in a shaking water bath at 300 rpm in American Type Culture Collection medium 1176 (1), with yeast extract-tryptone (5 g of yeast extract, 8 g of tryptone, 2.5 g of NaCl per liter [19]) substituted for yeast extract. *Halobacterium salinarium* NRC817, which was used as an indicator for halocin H4 activity assays, was grown aerobically as described above at 41°C in complex medium (28), with yeast extract (3 g/liter) and tryptone (5 g/liter) substituted for peptone. Activity from culture supernatants (10 μl) was quantified on fresh lawns (less than 1 week old) of *H. salinarium* NRC817 by using serial twofold critical-end-point dilutions to extinction (23). Activities are reported in arbitrary units (AU) and are defined as the reciprocal of the first dilution at which all trace of inhibitory activity disappears.

Halocin H4 activity was first detected as the culture began its transition into stationary phase (optical density at 600 nm [OD_{600}] = 1.18) and reached its maximal level (128 AU) 8 h later at the midpoint between exponential phase and stationary phase (OD_{600} = 4.15); then, within 2 h, the activity rapidly declined to 16 AU. Halocin H4 activity remained at 16 AU for the ensuing 12 h, then increased twofold to 32 AU, and re-

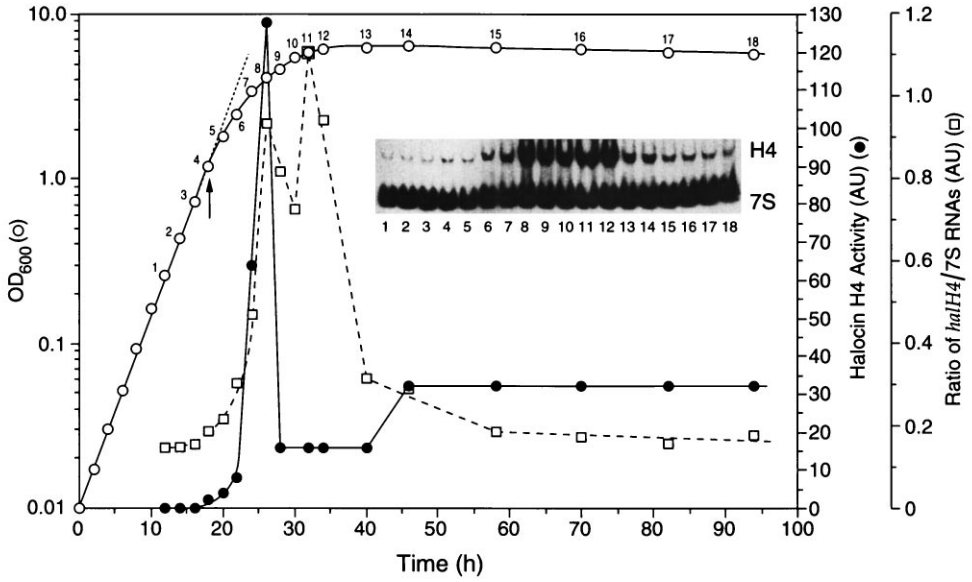

FIG. 3. Expression of the *halH4* gene in *H. mediterranei* R4. Growth curve (○), halocin activities (●), and ratios of *halH4*/*7S* transcript levels (□) as quantified by a PhosphorImager are shown. Arrow, onset of halocin H4 activity; broken line, extrapolation of the exponential growth portion of the curve (g = 2.75 h). Inset, autoradiograph of the Northern blot of total RNA probed with ^{32}P-labeled probes from the *halH4* and 7S RNA genes. The 18 samples (lanes 1 to 18) correspond to the 18 time points on the growth curve.

mained at this level for the rest of the experiment. A very similar activity profile is seen with colicin V, in which activity reached a maximum during the transition to stationary phase and then decreased precipitously to low levels (29).

RNA extraction and Northern (RNA) blotting have been described previously (28). The blot shown in the inset to Fig. 3 was probed simultaneously with ^{32}P-labeled random-primed probes (8) synthesized from a 335-bp internal *Eco*RV-*Sal*I fragment of the *halH4* gene and from an 850-bp *Sal*I fragment containing the 7S gene from *H. salinarium* (20). The 7S gene was constitutively transcribed, and its transcript served as an internal control to which *halH4* transcript levels were normalized (9). Transcript levels from the *halH4* and 7S genes were quantified with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The phosphor screen was exposed to the blot for 21 h and then scanned at a pixel size of 200 μm. A rectangle of fixed area was drawn around each band and around a blank area of the blot for background control, and the background value was subtracted from the signal for each band.

Transcripts from *halH4* were first detectable in the three earliest samples from mid-exponential phase (OD_{600} = 0.26 to 0.73), representing an average basal level of 0.149 ± 0.004 AU. In contrast, halocin H4 activity was not detectable during this time. Beginning with time point 4 (Fig. 3), *halH4* transcript levels rapidly increased by sixfold over the subsequent 10 h, with a concomitant parallel increase in halocin H4 activity. Transcript levels fluctuated between 0.722 and 1.101 AU (4.8- and 7.4-fold above basal levels, respectively; time points 9 to 12) for the subsequent 8 h and then returned rapidly to an average basal level of 0.171 ± 0.012 AU (average of time points 15 to 18). This type of fluctuation has been seen in transcripts from two other haloarchaeal genes, *brp* and *bat*, when their transcripts were at maximal levels as a result of induction by low oxygen tension and high light intensity (28).

Significance. Halocin genes will serve as a tractable model for isolating ancillary regulatory proteins involved in stationary-phase gene expression outside those needed for the basal transcription apparatus. Expression of *halH4* is made even more interesting due to the lack of correlation between halocin H4 activity and *halH4* transcript levels at two points. First, transcripts are present during exponential growth but halocin activity is not detected. Second, when transcript levels are at their highest, halocin activity is constant at submaximal levels. These data suggest that the synthesis of halocin H4 is regulated posttranscriptionally, and the elucidation of this posttranscriptional regulatory system will be interesting. Finally, halocins will also provide models for studying at least one type of protein export in the haloarchaea.

Nucleotide sequence accession number. The DNA sequence for the *halH4* gene and the protein sequence for halocin H4 have been assigned GenBank accession number U16389.

This work was supported by a grant-in-aid for research from Sigma Xi to J.C., by a Visiting Professorship for Women from the National Science Foundation (HRD-9253008) to K.J.D., by a Cowden Microbiology Fellowship to E.M.O., and by grants from the Arizona Disease Control Research Commission (no. 3-028), the National Institutes of Health, and Northern Arizona University Organized Research to R.F.S.

We thank Mary Betlach and Francisco Rodriguez-Valera for providing unpublished amino acid sequences for halocin H4, Mary Betlach for critical reading of the manuscript, Felix Gropp for supplying the 7S clone, Ramón Santamaría Sanchez for providing information regarding the signal sequence for the xylanase gene, Rachel Skvirsky for providing unpublished data regarding colicin V activity profiles, Debbie Kendall for helpful discussions about signal sequences, Ricardo Amils for information regarding pHM300, Paul Jackson for providing oligodeoxynucleotide primers, Paul Blum for help with DNA sequencing, George Turner and Justin Oliver for help with phosphor imaging, and Eric LaHood and Virginia Coryell for help with primer extension analysis.

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