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Functionality of Purified σ^N (σ^{54}) and a NifA-Like Protein from the Hyperthermophile *Aquifex aeolicus*

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The genome sequence of the extremely thermophilic bacterium *Aquifex aeolicus* encodes alternative sigma factor σ^N (σ^{54}, RpoN) and five potential σ^N-dependent transcriptional activators. Although *A. aeolicus* possesses no recognizable nitrogenase genes, two of the activators have a high degree of sequence similarity to NifA proteins from nitrogen-fixing proteobacteria. We identified five putative σ^N-dependent promoters upstream of operons implicated in functions including sulfur respiration, nitrogen assimilation, nitrate reductase, and nitrite reductase activity. We cloned, overexpressed (in *Escherichia coli*), and purified *A. aeolicus* σ^N and the NifA homologue, AQ_218. Purified *A. aeolicus* σ^N bound to *E. coli* core RNA polymerase and bound specifically to a DNA fragment containing *E. coli* promoter *glnHp2* and to several *A. aeolicus* DNA fragments containing putative σ^N-dependent promoters. When combined with *E. coli* core RNA polymerase, *A. aeolicus* σ^N supported *A. aeolicus* NifA-dependent transcription from the *glnHp2* promoter. The *E. coli* activator PspFΔHTH did not stimulate transcription. The NifA homologue, AQ_218, bound specifically to a DNA sequence centered about 100 bp upstream of the *A. aeolicus glnBA* operon and so is likely to be involved in the regulation of nitrogen assimilation in this organism. These results argue that the σ^N enhancer-dependent transcription system operates in at least one extreme environment, and that the activator and σ^N have coevolved.

The special form of bacterial RNA polymerase (RNAP) containing alternative sigma factor σ^N or σ^{54} (σ^N-RNAP), initiates transcription by a mechanism quite distinct from RNAP containing the major σ^{70} sigma factor (35, 39, 48). Transcription initiation by σ^N-RNAP requires the hydrolysis of nucleoside triphosphate, catalyzed by activator proteins bound to upstream activator sequences (enhancer elements). The mechanisms of regulation involving this form of RNAP are among the most sophisticated in bacteria (52).

Since this novel form of RNAP was first recognized in enteric bacteria (25, 29, 47), σ^N has been discovered in many other bacteria, including several proteobacteria, as well as in the gram-positive *Bacillus subtilis* (14) and in *Planctomyces limnophila* (36). Furthermore, σ^N appears to be encoded by the genomes of the hyperthermophile *Aquifex aeolicus* (15), the spirochete *Borrelia burgdorferi* (18), and the obligate intracellular pathogens *Chlamydia trachomatis* (51) and *Chlamydia pneumoniae* (31).

The σ^N proteins from *Klebsiella pneumoniae* and *Escherichia coli* have been the subject of much genetic and biochemical analysis (e.g., see references 8, 20, and 23). Although considerable progress has been made towards understanding the mechanisms by which σ^N performs its function, to date no high-resolution structural data are available for this system. Thermophilic homologues of mesophilic proteins have often been shown to be tractable for structural studies. For example, the crystal structures of the histidine kinase domain of CheA from *Thermotoga maritima* and the core RNAP from *Thermus aquaticus* have recently been elucidated (4, 57). The publication of the complete genome sequence (15) of *A. aeolicus*, a hyperthermophilic bacterium capable of growth at temperatures as high as 95°C, provides a potentially valuable resource for the study of thermostable proteins.

A tRNA-binding protein has previously been purified from *A. aeolicus* (41), and Klenk et al. (33) have demonstrated activity and thermostability in the RNAP holoenzyme from its relative *Aquifex pyrophilus*. Characteristics of the hyperthermophile *A. aeolicus* pose important questions about the evolutionary origins and relationships of the bacteria. According to 16S ribosomal DNA (rDNA)-based phylogenies, the order *Aquifecales* (of which *A. aeolicus* is a member) represents the deepest branch of the bacterial evolutionary tree (7). Thus, it has been argued that by studying these organisms we can gain insights about the original bacterial ancestor. In particular, the hyperthermophilic nature of the deepest-branching organisms has been cited to support the hypothesis of a hyperthermophilic origin for the bacteria (1). However, several protein-based phylogenetic analyses, including that of the RNAP β and β′ subunits, have thrown into question the status of the *Aquifecales* as the deepest-branching group (e.g., see reference 33). Based on sequences of σ^{70} sigma factors, *Aquifecales* appears to be an early-branching member of the proteobacteria (22).

In this paper we describe the heterologous overexpression and purification of the σ^N and show its core RNAP-binding activity and sequence-specific DNA binding. We also purified a NifA-like protein from *A. aeolicus* and demonstrate activator-dependent transcription activity of a holoenzyme containing *A. aeolicus* σ^N in vitro. We predict possible functions of the σ^N-RNAP mode of transcription in *A. aeolicus*.

MATERIALS AND METHODS

Computer-based analysis. DNA sequences were examined for potential σ^N-dependent promoters using SEQSCAN (B. T. Nixon: http://www.bmb.psu.edu/seqscan/seqform1.htm). Similarity searches were performed using the PSI-BLAST server (2).

Strains, plasmids, and DNA. Plasmids are listed in Table 1. Samples of genomic DNA from *A. aeolicus* and expression strain *E. coli* C41(DE3) were kind gifts from R. Huber and J. Walker, respectively (15, 40).

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TABLE 1. Plasmids used in this study

Plasmid	Relevant feature	Source	Reference
pFC60	*E. coli glnHp2* wild-type promoter	B. Magasanik	10
pFC60-m11	*E. coli glnHp2*-m11 mutant promoter	B. Magasanik	10
pDJS42.12	pET29b+::*A. aeolicus rpoN*	Our collection	This work
pDJS48.8	pET29b+::*A. aeolicus AQ_218*	Our collection	This work
pMTHσN	pET28b+::*K. pneumoniae rpoN*	Our collection	19

Oligonucleotides, enzymes, and PCR. PCR was carried out using standard protocols with BioTaq (Bioline) or, where proofreading activity was required, Bio-X-Act thermostable DNA polymerase (Bioline). *E. coli* core RNAP was purchased from Epicentre Technologies.

Cloning. The sequences of *rpoN*, encoding σ^N, and *nifA*, encoding AQ_218, were amplified from *A. aeolicus* genomic DNA by PCR using primers (Table 2, sets A and B) which introduced *Nde*I sites immediately upstream of the start codons and *Bam*HI sites immediately downstream of the stop codons. These restriction sites were exploited to clone the PCR-amplified fragments into pET29b+ (Novagen). The inserts in the resulting plasmids (pDJS42.12 and pDJS48.8) were verified by DNA sequencing.

Proteins, overexpression, and purification. Overexpression and purification of *K. pneumoniae* σ^N from pMTHσ^N has been described previously (19). For *A. aeolicus* σ^N and AQ_218, cultures of C41(DE3)(pDJS42.12) and C41(DE3)(pDJS48.8) were grown at 37°C until mid-log phase, when they were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 to 16 h at 25°C. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 5% glycerol containing a protease inhibitor cocktail (Boehringer). Cells were disrupted by two passages through a French press. Following centrifugation at 20,000 × *g* for 40 min, the overexpressed protein was found predominantly in the soluble fraction, which was then heated to 80°C for 10 min and again centrifuged at 20,000 × *g* for 40 min. The *A. aeolicus* protein remained in the soluble fraction and was purified (to more than 95% homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by affinity chromatography on a heparin-Sepharose column and eluted with a NaCl gradient, using fast-protein liquid chromatography (Pharmacia). The purified proteins were dialyzed into 50 mM Tris-HCl (pH 8.0)–50 mM NaCl–50% glycerol–1 mM dithiothreitol (DTT) for storage at −20°C.

Core-binding assays. Core-binding assays were carried out as described previously (20) but using a Tris–3-(cyclohexylamino)-1-propanesulfonic acid (Tris-CAPS) buffer system (38) at pH 9.5.

Promoter DNA fragments. The wild-type *E. coli glnHp2* and mutant *glnHp2*-m11 fragments (approximately 250 bp) were amplified from pFC60 and pFC60-m11, respectively, using the primers listed in Table 2 (set C). Three putative *A. aeolicus* promoter DNA fragments, *dhsU*, *glnB*, and *nirB*, were amplified from genomic DNA by PCR using primers in Table 2 (sets D to F). Each of these fragments contained the ATG start codon plus about 240 bp of upstream sequence. All promoter DNA fragments were radioactively end labeled using T4 polynucleotide kinase as described in reference 8. Two additional fragments were amplified from the *A. aeolicus glnB* region (Table 2, sets G and H). These two fragments differed from each other in that the shorter fragment lacked a 56-bp sequence from the upstream end (see Fig. 4).

Protein-DNA-binding assays. Binding of σ^N and holoenzyme to radioactively labeled promoter DNA fragments was detected by a gel mobility shift assay (8, 9). Holoenzyme was prepared by mixing *E. coli* core RNAP and σ^N subunit (from *K. pneumoniae* or *A. aeolicus*) in a 1:2 molar ratio. Assay reactions included concentrations up to 1 μM of the σ^N protein or 100 nM holoenzyme (see figure legends for details), 25 nM labeled DNA, and 680 μg of salmon sperm DNA ml^{-1} in buffer (40 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 10% [vol/vol] glycerol, 100 nM NaCl, 250 mM KCl, and 1 mM DTT). For AQ_218 binding to DNA, except where stated otherwise, assays contained 100 nM AQ_218 protein (with respect to monomer), 25 nM labeled DNA, and up to 204 μg of salmon sperm DNA ml^{-1} in TAPS buffer (50 mM Tris-acetate [pH 8.0], 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 1 mM DTT, 3.5% [wt/vol] polyethylene glycol 8000).

In vitro σ^N transcription activity assays. Transcription assays were performed as described previously (20) except that supercoiled pFC60 and pFC60-m11 plasmid DNAs, containing the wild-type *E. coli glnHp2* and mutant *glnHp2*-m11 promoters, were used as templates. The σ^N-RNAP activator PspFΔHTH (30) is a useful test activator, since it is constitutively active without a requirement for covalent modification and is stable. It lacks DNA-binding activity and exerts its activity while in solution. The assays were performed at 30, 37, and 48°C and contained 50 nM RNAP holoenzyme assembled from the *E. coli* core and a sixfold excess of σ^N. Template DNA (10 nM) was preincubated with holoenzyme for 15 min in STA buffer (25 mM Tris-acetate) [pH 8.0], 8 mM Mg acetate, 10 mM KCl, 1 mM DTT, 3.5% [wt/vol] polyethylene glycol 6000) with 4 mM GTP, 100 nM activator protein (*A. aeolicus* NifA-like or *E. coli* PspFΔHTH [30]), and 0.1 mM UTP before heparin, ATP, CTP, and radioactively labeled UTP (1.5 μCi) were added. Since the initial three nucleotides of the transcript are TGT, this preincubation would favor the formation of stable initiated complexes.

RESULTS AND DISCUSSION

The genome of *A. aeolicus* contains an open reading frame (ORF) apparently encoding a σ^N protein and five ORFs predicted to encode NtrC/NifA-family transcriptional activators (15), designated AQ_218, AQ_164, AQ_230, AQ_1117, and AQ_1792. ORFs AQ_164, AQ_230, and AQ_1117 resemble the NtrC/AtoC subfamily of activators, members of which contain the receiver component of a two-component signal transduction system, whereas AQ_218 and AQ_1792 closely resemble the VnfA/NifA subfamily (unpublished data), whose members lack the conserved aspartate residue which undergoes phosphorylation in the two-component signal transduction system (43).

Purification of *A. aeolicus* σ^N and NifA proteins. ORFs AQ_599 and AQ_218, encoding *A. aeolicus* σ^N and NifA proteins, respectively, were cloned, and the proteins were overproduced and purified using the T7 system (see Materials and Methods). The purified σ^N was recognized by anti-*E. coli* σ^N antiserum in Western blots (data not shown).

***A. aeolicus* σ^N binding to core RNAP.** The purified σ^N from *A. aeolicus* bound *E. coli* core RNAP, as demonstrated by a gel mobility shift assay (Fig. 1A). The band comprising free core RNAP was depleted, and new species formed upon addition of the *A. aeolicus* σ^N. The lower degree of mobility of the *A. aeolicus* σ^N-*E. coli* core complex compared to that of the *K. pneumoniae* σ^N-*E. coli* core complex may be explained by the relatively high pI of the *A. aeolicus* protein (predicted to be 8.15, versus 4.60 for *K. pneumoniae* σ^N; also compare Fig. 1, lanes 2 and 6). Also, whereas the *K. pneumoniae* σ^N-*E. coli* core complex ran as a single band on the gel, the *A. aeolicus* σ^N-*E. coli* core complex ran as at least two discrete bands, suggesting more than one conformation, a phenomenon that has previously been reported for several mutant *K. pneumoniae* σ^N-*E. coli* core complexes (45, 54).

Sequence-specific DNA-binding activity of *A. aeolicus* σ^N. The purified *A. aeolicus* σ^N bound to the *E. coli glnHp2* wild-type promoter with significantly greater affinity than the mutant *glnHp2*-m11, which has a single base substitution at position −13 (Fig. 1B). This DNA-binding activity was resistant to high levels of nonspecific competitor DNA (680 ng ml^{-1}), further supporting the sequence specificity of the interaction. In contrast to the σ^{70} family of sigma factors, the *A. aeolicus* σ^N, like those of the enteric bacteria (5), clearly has its DNA-binding determinants available without requiring core binding.

In vitro transcription activity. The heterologous RNAP holoenzyme, containing *A. aeolicus* σ^N and *E. coli* core subunits, supported activator-dependent transcription from the wild-type *E. coli glnHp2* promoter in vitro, giving a transcript of the same length as that from the holoenzyme containing *K. pneumoniae* σ^N (see Fig. 1C). When the *E. coli* activator protein PspFΔHTH was added to the transcription assays in place of

TABLE 2. Oligonucleotide PCR primers

Primer set	Forward primer[a]	Reverse primer[b]	Description
A	NNNCATATGTTAAATCAGAGATTAGAAGTAAGG	NNNGGATCCTTAAATCCTTCTTTCCCTTGAGGAGGG	*A. aeolicus rpoN* coding sequence
B	NNNCATATGGATTTAAAGGTAGAGATCGAAACT	GTGCGACAGAACGGATCCTTACTC	*A. aeolicus nifA* coding sequence
C	CCACATCATCACACAATCG	CAGACTTCATAGCATTTTCC	*E. coli glnHp2* and *glnHp2*-m11 promoters
D	GGCGTAAACTATTTTTGTAATC	ACCCATACTTCTTACCTCCATT	*A. aeolicus dhsU* upstream region
E	CTTCCTCGCCGTGTTAGAA	TCATCTTCCTTCCTCCTTGTAAGTT	*A. aeolicus glnB* upstream region
F	CCCGTGGGAACACCTTCGAGT	CATTCTTTCACTCCTCCTGAG	*A. aeolicus nirB* upstream region
G	AAACACGAAAAATTTCCATACCTT	GAGGGCGTCCTTCACTTCGTCG	*A. aeolicus glnB* region 147-bp fragment
H	GGAAATTAAGCTGATTTAGTACC	GAGGGCGTCCTTCACTTCGTCG	*A. aeolicus glnB* region 203-bp fragment

[a] *Nde*I site is underlined.
[b] *Bam*HI site is underlined.

A. aeolicus AQ_218, transcription levels were very low (PspFΔHTH is a good activator of RNAP containing *K. pneumoniae* σ^N). Furthermore, there was only a very low level of transcription activation by AQ_218 with a holoenzyme containing *K. pneumoniae* σ^N. This requirement for both *A. aeolicus* σ^N and *A. aeolicus* activator for appreciable levels of transcription but tolerance of heterologous core subunits suggests that the *A. aeolicus* σ^N and activators have coevolved and might directly interact with each other during activation of transcription initiation. Transcription could not be detected from the *glnHp2*-m11 promoter, demonstrating promoter-specific interaction (data not shown).

The transcriptional activity of the RNAP holoenzyme containing *A. aeolicus* σ^N was lower than that of the holoenzyme containing *K. pneumoniae* σ^N. It is possible that at the relatively low temperatures at which the assays were performed (30, 37, and 48°C, while the optimal growth temperature for *A. aeolicus* is about 85°C) the thermophile proteins are in a "frozen" state that is kinetically unfavorable to the conformational changes required for transcription initiation. Indeed, activity increased with temperature over the range tested. Higher temperatures were not used, since 48°C is close to the upper temperature limit for *E. coli* RNAP activity (55).

Many σ^N-dependent promoters have integration host factor binding sites (26). The *A. aeolicus* genome encodes no obvious homologues of *himA* or *himB*, and so involvement, if any, of a sequence-specific DNA bending protein in *A. aeolicus* σ^N-dependent transcription is presently unclear. Also, the fact that purified *A. aeolicus* NifA did not require additional factors for the activation of transcription suggests its activity might be negatively regulated in vivo.

We were unable to rigorously demonstrate that nucleoside hydrolysis by *A. aeolicus* NifA is required for transcription initiation due to the apparent instability of open complexes using the heterologous RNAP holoenzyme (data not shown). However, based on similarity of central domain sequences in the *A. aeolicus* NtrC/NifA family activators to those of well-studied members of this family (48), we believe that the mechanism of activation is likely to be similar.

Identification of potential σ^N-dependent promoters in the *A. aeolicus* genome. RNAP containing σ^N transcribes from promoters with a consensus sequence of YTGGCACGRN NNTTGCW with the highly conserved GG and GC motifs at −24 and −12, respectively, relative to the +1 transcription start site (3). The *A. aeolicus* genome sequence was examined for potential σ^N-dependent promoters. Six good matches to the consensus were found in predicted noncoding regions immediately upstream of predicted coding regions (Fig. 2). Four of the six predicted coding regions showed significant similarity to genes of known function and are designated *glnB*, *fhp*, *dhsU*, and *nirB*. The remaining two predicted coding regions encode the hypothetical proteins AQ_087 and AQ_1119.

In *A. aeolicus*, it appears that *glnB*, which encodes regulatory protein PII, is the first gene in an operon with *glnA*. This *glnBA* operon structure is found in several other bacteria, including *Azospirillum brasilense* (16) and *Rhodobacter sphaeroides* (58). It is well documented that *gln* genes are transcribed by σ^N-RNAP in several proteobacteria (37), where their transcription is activated by the nitrogen-regulatory protein C (NtrC). However, in the gram-positive *B. subtilis*, transcriptional regulation is not dependent on the σ^N homologue, σ^L (28). It should also be noted that *glnA*, encoding glutamine synthetase, is found in the genomes of *Thermotoga maritima* (44) and *Mycobacterium* spp. (13), which have been completely sequenced, and yet no homologues of σ^N have been identified.

A second operon that appears to have a σ^N-RNAP-depen-

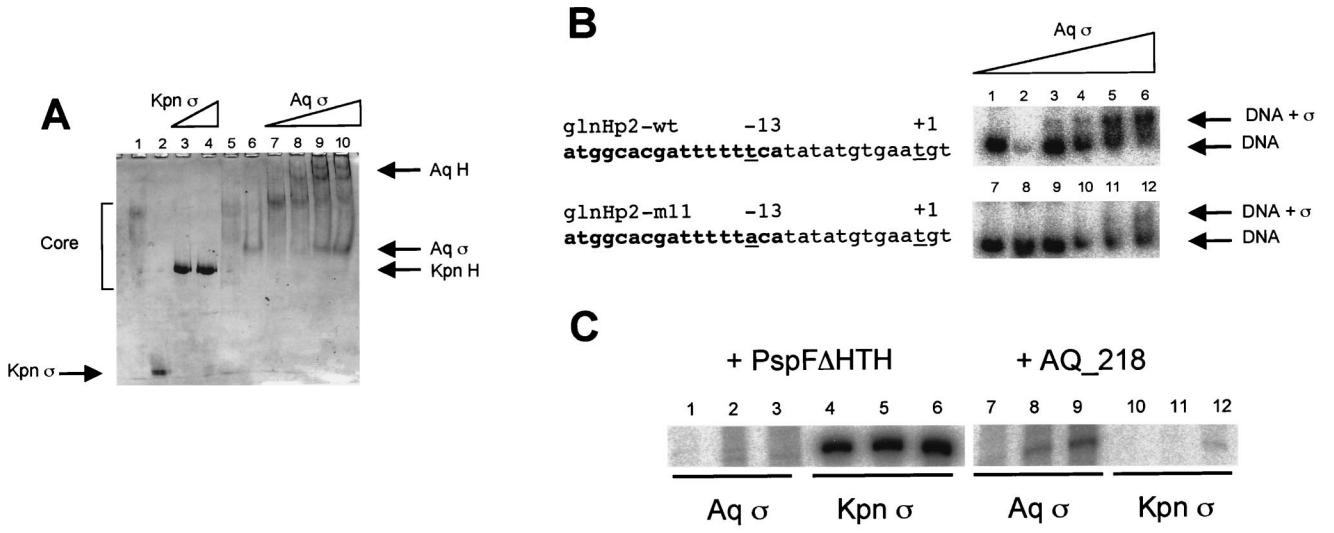

FIG. 1. Activity of purified *A. aeolicus* σ^N protein. (A) Core RNAP binding. Reaction mixtures contained 1 μM *E. coli* core RNAP and 0.5, 1, 2, and 3 μM *A. aeolicus* σ^N (lanes 7 to 10, respectively). Lane 6, 3 μM *A. aeolicus* σ^N and no core; lanes 1 and 5, 1 μM *E. coli* core RNAP and no σ^N; lanes 3 and 4, 1 μM *E. coli* core RNAP plus *K. pneumoniae* σ^N (0.5 and 1 μM, respectively); lane 2, 3 μM *K. pneumoniae* σ^N and no core. The positions of core, *K. pneumoniae* σ^N-RNAP holoenzyme (Kpn H), *K. pneumoniae* σ^N (Kpn σ), *A. aeolicus* σ^N-RNAP holoenzyme (Aq H), and *A. aeolicus* σ^N (Aq σ) are indicated. (B) Sequence-specific binding of purified *A. aeolicus* σ^N protein to wild-type *E. coli glnHp2* (*glnHp2*-wt) and mutant *glnHp2*-m11 promoter DNA. The nucleotide sequences of the promoters are shown with the transcription start site (+1) and site of the mutation (−13) underlined. DNA-binding reaction mixtures contained 0 to 1 μM σ^N. Positions of free DNA (DNA) and bound DNA (DNA + σ) are indicated. Assays contained 0 (lanes 1 and 7), 0.5 (lanes 2 and 8), 1 (lanes 3 and 9), 2 (lanes 4 and 10), 4 (lanes 5 and 11), and 5 (lanes 6 and 12) μM *A. aeolicus* σ^N. (C) Activator-dependent transcription activity of σ^N-RNAP holoenzymes. Transcription from the *E. coli* wild-type *glnHp2* promoter was assayed using holoenzymes containing either *K. pneumoniae* σ^N (lanes 4 to 6 and 10 to 12) or *A. aeolicus* σ^N (lanes 1 to 3 and 7 to 9) in the presence of either *E. coli* PspFΔHTH activator protein (lanes 1 to 6) or *A. aeolicus* protein AQ_218 (lanes 7 to 12). Reactions were carried out at 30°C (lanes 1, 4, 7, and 10), 37°C (lanes 2, 5, 8, and 11), and 48°C (lanes 3, 6, 9, and 12).

dent promoter contains genes designated *fhp*, *cynS*, *glnBi*, *nasA*, and *narB*. Each of the ORFs designated *fhp* (flavohemoprotein), *cynS* (cyanate hydrolase), *nasA* (nitrate transporter), and *narB* (nitrate reductase) shows similarity to genes associated with nitrate reduction (reviewed in reference 42). The *glnBi* ORF appears to encode a protein similar to the PII and GlnK family of nitrogen regulatory proteins.

A potential σ^N-dependent promoter is found in the *A. aeolicus* genome upstream of *dhsU*, predicted to encode a flavocytochrome C sulfide dehydrogenase (10). It appears that *dhsU* is part of an operon with *soxF* and *fccB′*, which are predicted to encode a Rieske-I iron-sulfur protein and a sulfide dehydrogenase flavoprotein, respectively (49, 50). Therefore, this operon is almost certainly involved in sulfur respiration.

In *A. aeolicus* we have identified a potential σ^N-dependent promoter upstream of *nirB*, predicted to encode the large subunit of cytoplasmic NADH-dependent nitrite reductase, whose function is detoxification of nitrite formed as a result of nitrate respiration (12). It appears that *cobA* and *trpD2* may also be in the *nirB* operon, and they are predicted, respectively, to encode products involved in cobalamin and tryptophan biosynthesis (17, 27). Although nitrate respiration has not been demonstrated in *A. aeolicus*, it has been observed in the close relative *A. pyrophilus* (7). Previously, σ^N-RNAP had been implicated in the regulation of nitrite reductase in *Pseudomonas stutzeri* (24). ORFs AQ_087 and AQ_1119 have no significant similarity to sequences of known function. A PSI-BLAST search using AQ_1119 as a probe revealed significant similarity to an ORF of unknown function, AF0913, in the genome of the archaeon *Archaeglobus fulgidus*.

To date, σ^N-RNAP has been implicated in the transcriptional regulation of such diverse functions as degradation of xylene and toluene, transport of dicarboxylic acids, pilin synthesis, nitrogen fixation, hydrogen uptake, flagellar assembly, arginine catabolism, alginate production, rhamnolipid production, acetoin catabolism, mannose uptake, proline iminopeptidase activity, nitrogen assimilation, nitrate respiration (reviewed in references 3, 35, and 39), pathogenesis (34), development (32), and RNA modification (21). It is also predicted to be involved in the transcriptional regulation of other σ factors (46, 53). Here, we have extended the range of potential functions further to include sulfur respiration and have

Consensus	YTGGCACGrNNNTTGCW
dhsU	**ttggcacgaaaattgca**ataaatacaacgaacaaaaatggaggtaagagt**ATG**
fhp	**ttggcacgctttttgca**attagtttgagtgaaggaggtgaaaag**ATG**
glnB	**ttggcacggaaattgca**taataacttacaaggaggaaggaag**ATG**
nirB	**ttggcacgtttttttgca**agagtttcctcaggaggagtgaaag**ATG**
Aq_087	**ttggcacggagtttgca**attaaaaaatgtgaaaaccaaaaggaggtgtgaagc**ATG**
Aq_1119	**ttggcacactacttgca**tttatccgttcaggaggtgagagat**ATG**

FIG. 2. Identification of putative *A. aeolicus* σ^N-dependent promoters. Close matches to the consensus σ^N-binding motif were identified immediately upstream of *dhsU*, *glnB*, *nirB*, AQ_087, and AQ_1119 ORFs in the *A. aeolicus* genome sequence. Putative σ^N-binding motifs are shown in boldface, as are the ATG start codons. Possible ribosome binding sites are underlined.

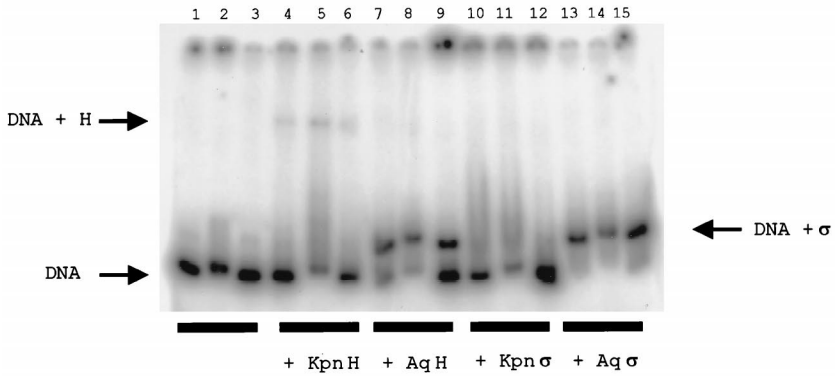

FIG. 3. Binding of RNAP holoenzyme and σ^N to radioactively labeled 240-bp DNA fragments containing the *dhsU* (lanes 1, 4, 7, 10, and 13), *glnB* (lanes 2, 5, 8, 11, and 14), and *nirB* (lanes 3, 6, 9, 12, and 15) upstream regions was carried out in the presence of high concentrations of competitor DNA (680 μg of salmon sperm DNA ml^{-1}). Lanes 1 to 3, DNA only (no protein); lanes 4 to 6, holoenzyme with *K. pneumoniae* σ^N; lanes 7 to 9, holoenzyme with *A. aeolicus* σ^N; lanes 10 to 12, *K. pneumoniae* σ^N; lanes 13 to 15, *A. aeolicus* σ^N. The positions of free DNA (DNA), holoenzyme-DNA complex (DNA + H), and σ^N-DNA complex (DNA + σ) are indicated.

provided evidence that σ^N-RNAP is probably involved in nitrogen assimilation and nitrate respiration in a hyperthermophile.

σ^N- and σ^N-RNAP holoenzyme binding to putative *A. aeolicus* σ^N-dependent promoters. We tested σ^N and σ^N-RNAP holoenzyme binding to 240-bp fragments containing noncoding sequences upstream of *A. aeolicus dhsU*, *glnB*, and *nirB* (Fig. 3) in the presence of high levels of competitor DNA. *A. aeolicus* σ^N bound to all three fragments, reducing the electrophoretic mobility of the labeled DNA (Fig. 3, lanes 13 to 15). *K. pneumoniae* σ^N clearly also bound the *dhsU* and *glnB* upstream fragments (Fig. 3, lanes 10 and 11), albeit less stably than did *A. aeolicus* σ^N, and only very weakly bound the *nirB* upstream fragment (lane 12). The RNAP holoenzyme containing *K. pneumoniae* σ^N bound to all three fragments to produce species with much lower degrees of electrophoretic mobility (Fig. 3, lanes 4 to 6). However, the binding of the RNAP holoenzyme containing *A. aeolicus* σ^N appeared to give rise to complexes with mobility similar to that of those arising from σ^N and DNA alone (lanes 4 to 6). The amount of stable holoenzyme-DNA complex formed was below the limit of detection by this assay. The very low levels of DNA-holoenzyme complex formation, perhaps partly due to the heterologous nature of the system, might explain the relatively low levels of the transcription (Fig. 1C) on the *glnHp2* promoter by RNAP containing *A. aeolicus* σ^N.

It is noteworthy that *A. aeolicus* σ^N appeared to bind the *A. aeolicus* DNA fragments more tightly than did *K. pneumoniae* σ^N (compare lanes 10 to 12 with lanes 13 to 15 in Fig. 3). This was also the case for binding on the *E. coli glnHp2* promoter DNA fragment (data not shown). We speculate that a stronger DNA-binding activity may be advantageous to the thermophile in overcoming the kinetic effects of high temperature.

DNA-binding activity of *A. aeolicus* NifA-like protein (AQ_218). The NtrC/NifA family σ^N-dependent transcriptional activators bind to enhancerlike elements, usually located up to 200 bp upstream of the transcription start site (35, 48). Therefore, we tested whether purified protein AQ_218 bound to 240-bp fragments containing noncoding sequences upstream of *A. aeolicus dhsU*, *glnB*, and *nirB*. In a gel mobility shift assay, AQ_218 protein bound to all three DNA fragments to give complexes with reduced mobility (band B in Fig. 4A). The stability of these complexes was very sensitive to the concentration of competitor DNA (salmon sperm DNA). Binding to the *glnB* fragment appeared to be significantly more resistant to the presence of competitor DNA than was binding to the *dhsU* and *nirB* fragments. Furthermore, binding to the *glnB* upstream sequence gave rise to an additional species that was not observed in assays containing *dhsU* and *nirB*. Therefore, we examined the *glnB* upstream DNA sequence and its interaction with AQ_218 in more detail.

NifA and VnfA are involved in the transcriptional regulation of the nitrogenase complex in azotrophic proteobacteria. Since *A. aeolicus* lacks recognizable *nifHDK* genes, the two NifA-like proteins (AQ_218 and AQ_1792) presumably perform a different function in this organism. Two canonical NifA binding sites (6), $TGTN_{10}ACA$, are found centered around 105 bp upstream of the *glnBA* operon of *A. aeolicus* (Fig. 4B), so it is possible that one (or both) of the NifA homologues is involved in the regulation of nitrogen assimilation rather than of nitrogen fixation. The second helix (H_2 in Fig. 4B) of the NifA helix-turn-helix (HTH) motif is believed to be responsible for recognition of specific DNA sequences. Although at present we cannot predict DNA-protein-binding affinities from sequence alone, the high degree of similarity between the putative HTH motifs of AQ_218 and known NifA sequences (Fig. 4B) suggested that they might recognize similar DNA sequences. Therefore, we tested the binding of AQ_218 to two *A. aeolicus* DNA sequences which differed in that one (of 203 bp) contained the two $TGTN_{10}ACA$ motifs and the other (of 147 bp) was truncated so that it lacked a 56-bp sequence containing these motifs (see Materials and Methods; Fig. 4C). Under the conditions of this binding assay, the *A. aeolicus* protein AQ_218 bound to the 203-bp fragment to form species A and B (Fig. 4C) but failed to form these complexes with the 147-bp fragment lacking the putative NifA binding motifs. Therefore, we conclude that the *A. aeolicus glnB* upstream region contains determinants for sequence-specific DNA binding by AQ_218 and that this protein is likely to be involved in positive regulation of the *glnBA* operon in *A. aeolicus*.

The occurrence of several potential σ^N-dependent promoters and five ORFs potentially encoding σ^N-dependent activators supports the proposition that the enhancer-dependent σ^N-RNAP mode of transcription is functional in *A. aeolicus*. Direct binding of *A. aeolicus* promoter specificity factor σ^N to these sequences supports this conclusion. We present indirect evidence that the σ^N-RNAP holoenzyme is involved in sulfur respiration, nitrogen assimilation, reduction of nitrate, and nitrite reductase activity, as well as having at least two unknown functions, in *A. aeolicus*. Furthermore, based on a dem-

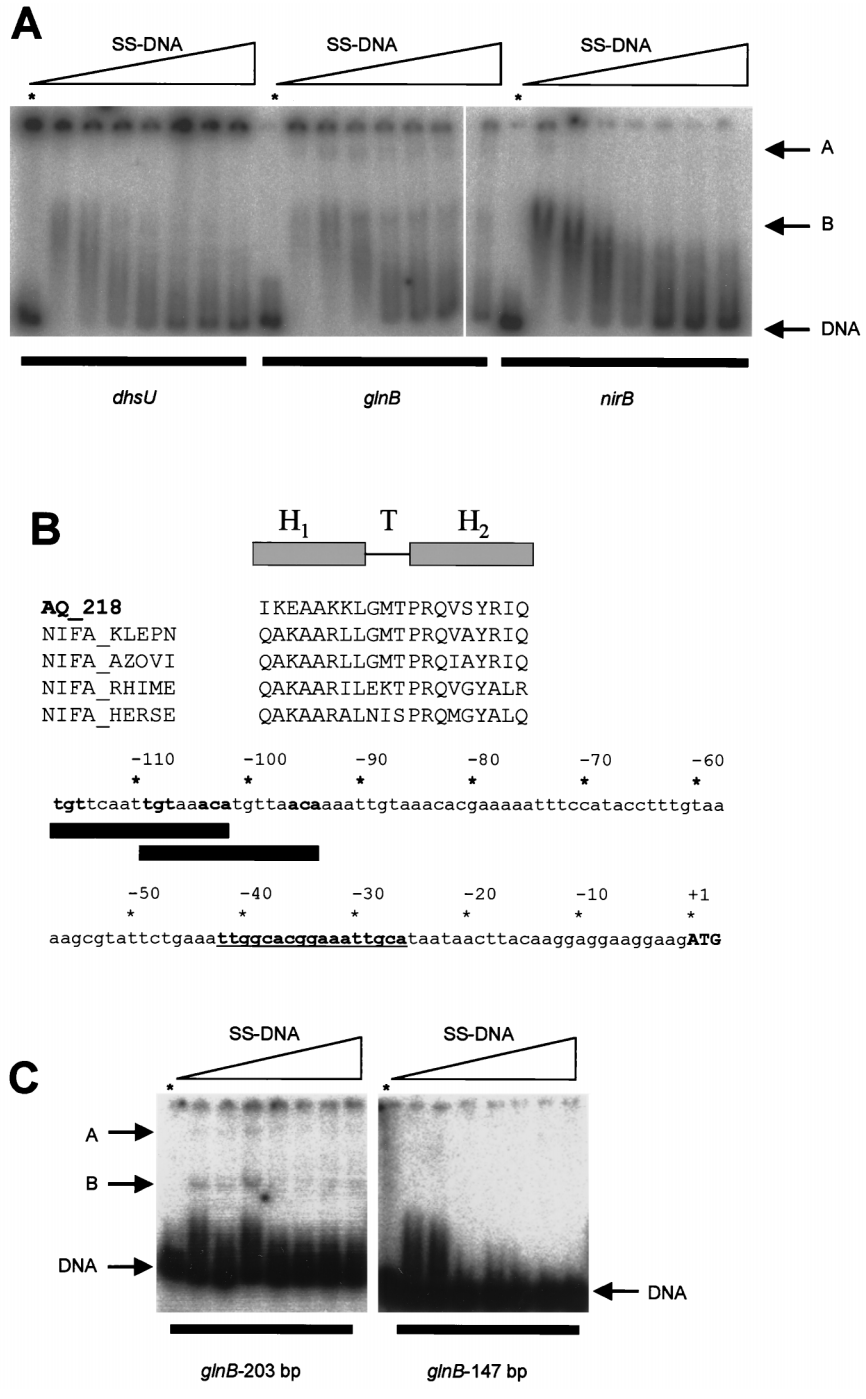

FIG. 4. DNA-binding activities of *A. aeolicus* NifA-like protein (AQ_218). (A) Binding to radioactively labeled 240-bp DNA fragments containing the *dhsU*, *glnB*, and *nirB* upstream regions. Assays were carried out in the presence of various concentrations of competitor DNA (17, 34, 68, 102, 136, 170, and 204 μg ml^{-1}). Lanes marked with an asterisk contained DNA only (no protein). (B) Comparison of the amino acid sequences of the putative HTH motifs of AQ_218 and those of *K. pneumoniae* (NIFA_KLEPN), *Azotobacter vinelandii* (NIFA_AZOVI), *Sinorhizobium meliloti* (NIFA_RHIME), and *Herbaspirillum seropedicae* (NIFA_HERSE) and the DNA sequence upstream of the *A. aeolicus glnB* gene. Two matches to the NifA-binding motif $TGTN_{10}ACA$ are marked with thick solid lines. The σ^N-binding site is highlighted in boldface, as is the ATG start codon. (C) Binding to radioactively labeled *A. aeolicus glnB*-region DNA fragments. These two fragments were 147 and 203 bp, the shorter fragment lacking the −141 to −86 (with respect to the start codon) sequence GGAAATTAAGCTGATTTAGTACCTTTGTTCAATTGTAAACATGTTAACAAAATTGT (with the $TGTN_{10}ACA$ motifs underlined) at the upstream end. Assays were carried out in the presence of varying concentrations of competitor DNA (17, 34, 68, 102, 136, 170, and 204 μg of salmon sperm DNA [SS-DNA] ml^{-1}). Lanes marked with an asterisk contained DNA only (no protein).

onstrated specific DNA-binding activity, we propose that AQ_218, a NifA-like protein from *A. aeolicus*, is involved in the regulation of the *glnBA* operon. Clearly, the NifA subfamily of proteins is unlikely to have arisen twice independently, so there are two possible explanations for these proteins' occurrence both in *A. aeolicus* and in the proteobacteria: (i) horizontal gene transfer between these bacteria occurred, or (ii) the lineage containing *A. aeolicus* split from the proteobacteria

after the evolution of the NifA subfamily. Since NifA has not been found in any bacteria other than *A. aeolicus* and azotrophic proteobacteria, this would suggest that the split was much more recent than is implied by the 16S rDNA data and is more consistent with protein-based models of bacterial evolution (e.g., see references 22 and 33). The RNAP holoenzyme containing purified *A. aeolicus* σ^N appears to be silent for transcription, and the *A. aeolicus* NifA-like protein (AQ_218) overcomes this inhibition to allow transcription, as is the case for all homologous mesophile systems so far studied. It seems that the interactions that silence the polymerase, and others that allow enhancer dependence and which are known to critically involve σ^N region I sequences (20), are at least partly intact when *A. aeolicus* σ^N combines with *E. coli* core RNAP. To the best of our knowledge, this is the first report of transcriptional activation using components from a hyperthermophilic bacterium.

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