

# Cloning and Physical Mapping of RNA Polymerase Genes from *Methanobacterium thermoautotrophicum* and Comparison of Homologies and Gene Orders with Those of RNA Polymerase Genes from Other Methanogenic Archaeobacteria

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The structural genes encoding the four largest subunits of RNA polymerase, A, B', B'', and C, were physically mapped in *Methanobacterium thermoautotrophicum* Winter. The genes formed a cluster in the order B'', B', A, C and had a common orientation. DNA hybridization experiments yielded different degrees of homology between RNA polymerase gene sequences of different species of *Methanobacterium* and *Methanococcus voltae*. No homology was detectable between *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*. From Southern hybridization experiments in which probes of the four genes from *Methanobacterium thermoautotrophicum* Winter and restriction digests of the genomic DNAs of the different methanogens were used, a common gene order of the RNA polymerase genes could be deduced.

Methanogenic bacteria are one of the large groups of archaeobacteria (2). These procaryotes are phylogenetically distinct from the other procaryotes, the eubacteria, as well as from eucaryotic organisms. This was first recognized on analysis of 16S and 18S rRNAs and confirmed on the basis of many other characteristics (2, 23, 24). Most significant in this respect are comparisons of those macromolecular cell components which, like the ribosome, are constituents of all living cells. DNA-dependent RNA polymerases belong to these types of macromolecules. It is assumed that they are all derivatives of a common evolutionary ancestor.

Procaryotes possess only one type of RNA polymerase, while eucaryotic cells contain functionally different RNA polymerases (12). The complexities of the eubacterial, archaeobacterial, and eucaryotic enzymes differ. Eubacterial RNA polymerases have the simplest subunit composition (4). Within the archaeobacteria the halophilic or methanogenic and the mostly sulfur-dependent thermophilic branches of the archaeobacteria also differ in the numbers of their RNA polymerase subunits (6, 26; M. Thomm, Ph.D. thesis, University of Regensburg, Regensburg, Federal Republic of Germany, 1983).

Analyses of immunological cross-reactions of RNA polymerases and their subunits have been performed to obtain insight into their relationships (16, 26; Thomm, Ph.D. thesis). It has been found that the four largest RNA polymerase subunits of methanogenic bacteria show cross-reactions with the enzymes of all the other archaeobacterial groups and in part with both eubacterial and eucaryotic RNA polymerases (26). Interestingly, the cross-reactions with the eubacterial polymerase subunits were weaker than those observed with the eucaryotic enzymes. However, the resulting data are difficult to evaluate quantitatively, since cross-reactivity can be due to polypeptide sequence homology or to more com-

plex structural similarities of proteins such as surface charge, hydrophobicity, or other parameters leading to similar epitopes.

We have been interested in the genetic analysis of the RNA polymerases of methanogens and have focused our interest on the four largest subunits, since they have structures that have been at least partially preserved in their evolution. Here we report results of the physical mapping of the four genes in a strain of *Methanobacterium thermoautotrophicum*, as well as results of the analysis of the sequence homology of RNA polymerase genes and their chromosomal arrangements in other species of different orders of this archaeobacterial group.

## MATERIALS AND METHODS

**Bacterial strains and vectors.** The bacterial strains and vectors used in this study are listed in Table 1. Growth conditions and vector preparation have been previously described (2, 5, 13).

**Enzymes.** Enzymes were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany [FRG]) or Pharmacia (Freiburg, FRG) and used according to the instructions of the manufacturers.

**Immunoreagents.** RNA polymerase-specific antibodies were prepared by a microprocedure (18). BioYeda peroxidase-labeled goat anti-rabbit antibodies were purchased from Renner (Dannstadt, FRG).

**Preparation of cellular DNA.** Preparation of DNA from *Methanococcus voltae* has been described previously (9). DNA from the other methanogens was prepared, beginning with 1 to 5 g of frozen cells. After suspension in 2 to 5 volumes of  $10^{-2}$  M Tris- $10^{-3}$  M EDTA (pH 8; TE), 1% sodium dodecyl sulfate (SDS) and 100  $\mu$ g of pronase per ml of suspension were added; and the mixture was incubated for 30 min at 65°C. The DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) at 65°C and reextracted at 37°C with chloroform-isoamyl alcohol (24:1), with slow rotation during the extraction steps. RNase was then added

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TABLE 1. Bacteria and vectors used in this study

Strain or vector	Relevant genetic characters	G+C content (%)	Source or reference
<i>Escherichia coli</i> SG4044	<i>hsdR hsdM<sup>+</sup> pcI857 Km<sup>r</sup> lon</i>		E. Pfaff, Heidelberg, FRG
<i>Escherichia coli</i> BNN102	<i>hflA150 hsdR hsdM<sup>+</sup> (chr::Tn10)</i>		A. Bach, Heidelberg, FRG (25)
<i>Methanobacterium thermoautotrophicum</i> Winter <sup>a</sup>		49	J. Winter, Regensburg, FRG
<i>Methanobacterium thermoautotrophicum</i> ΔH DSM 1953		49	DSM <sup>b</sup>
<i>Methanobacterium thermoautotrophicum</i> Marburg DSM 2133		48	DSM
<i>Methanobacterium formicicum</i> DSM 1535		41	DSM
<i>Methanosarcina barkeri</i> Fusaro DSM 804		39	DSM
<i>Methanococcus voltae</i> DSM 1537		31	DSM
λgt10			A. Bach, Heidelberg, FRG (7, 25)
pEx31a, -b, -c			E. Beck, Heidelberg, FRG (20)
pUC8			E. Beck, Heidelberg, FRG (22)

<sup>a</sup> The strain was cloned by plating.

<sup>b</sup> DSM, German Collection of Microorganisms, Göttingen, FRG.

at a final concentration of 50 μg/ml, and the mixture was incubated for 30 min at 37°C. Phenol extraction was repeated as described above, followed by extensive dialysis against TE.

**Cloning techniques.** DNA cloning into λgt10 or plasmid vectors was done by standard procedures (5).

**DNA hybridization.** DNA hybridization was performed as described previously (17) after the transfer of electrophoretically separated restriction fragments or application of spots (1 to 5 μg/15 mm<sup>2</sup>) of heat-denatured DNA of approximately 500 base pairs (bp), which was obtained by sonication, to nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, N.H.). Probes were labeled by nick translation (13) or end labeling with [γ-<sup>32</sup>P]ATP, which was prepared as described previously (8).

**Immunodetection techniques.** Bacterial clones producing RNA polymerase antigenic determinant polypeptides from cloned DNA were identified as described previously (10). For further analysis of the produced polypeptides, the positive *Escherichia coli* clones were grown and heat induced. They were lysed by boiling in SDS-polyacrylamide gel (11) sample buffer. *Methanobacterium thermoautotrophicum* Winter cell extracts were prepared by sonication. The cell extracts were separated by SDS-polyacrylamide gel electrophoresis, and the polypeptides were transferred to nitrocellulose filters by electroblotting. Immunodetection of the bound polypeptides (21) exhibiting RNA polymerase-specific antigenic determinants was performed with the antisera specified above.

## RESULTS

### Identification and analysis of expression plasmids carrying fragments of the RNA polymerase genes A, B', B'', and C.

TABLE 2. pEx31 expression vectors containing *Methanobacterium thermoautotrophicum* RNA polymerase genes or fragments thereof

Plasmid	Expressed subunit antigenic determinants	Insert size (kb)
pEx62AC	A, C	2.6
pEx51AB'	A	1.6
pEx42B''	B''	0.2
pEx11B'B''	B', B''	1.0

Plasmids which were isolated from *E. coli* SG4044 colonies carrying pEx31 expression vectors with random *Sau3A* fragment inserts generated by partial digests of *Methanobacterium thermoautotrophicum* Winter DNA are given in Table 2. The pEx31 vector system consisted of three vectors which allow cloning of gene fragments in all three reading frames behind the λ P<sub>L</sub> promoter into a 5'-terminal gene fragment of phage MS2 polymerase, which is preceded by its own ribosome-binding site (20). The colonies were heat induced and were screened with antisera specific for the four RNA polymerase subunits A, B', B'', and C of *Methanobacterium thermoautotrophicum*. The reaction of colonies carrying pEx62AC or pEx11B'B'' with antisera against A and C or B' and B'', respectively, showed that the A and C and the B' and B'' genes are adjacent.

**Determination of the gene order.** Western blot analysis of extracts from clones harboring pEx62AC showed a truncated A gene product, whereas the C polypeptide was apparently of identical size as the C subunit detected in

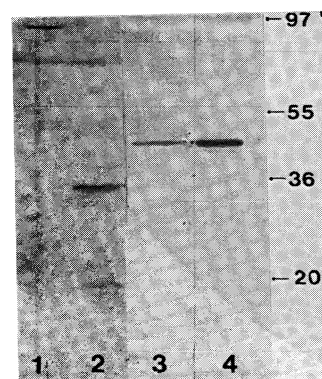
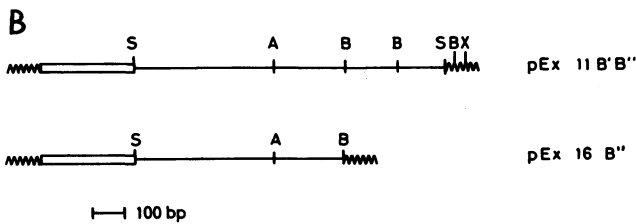
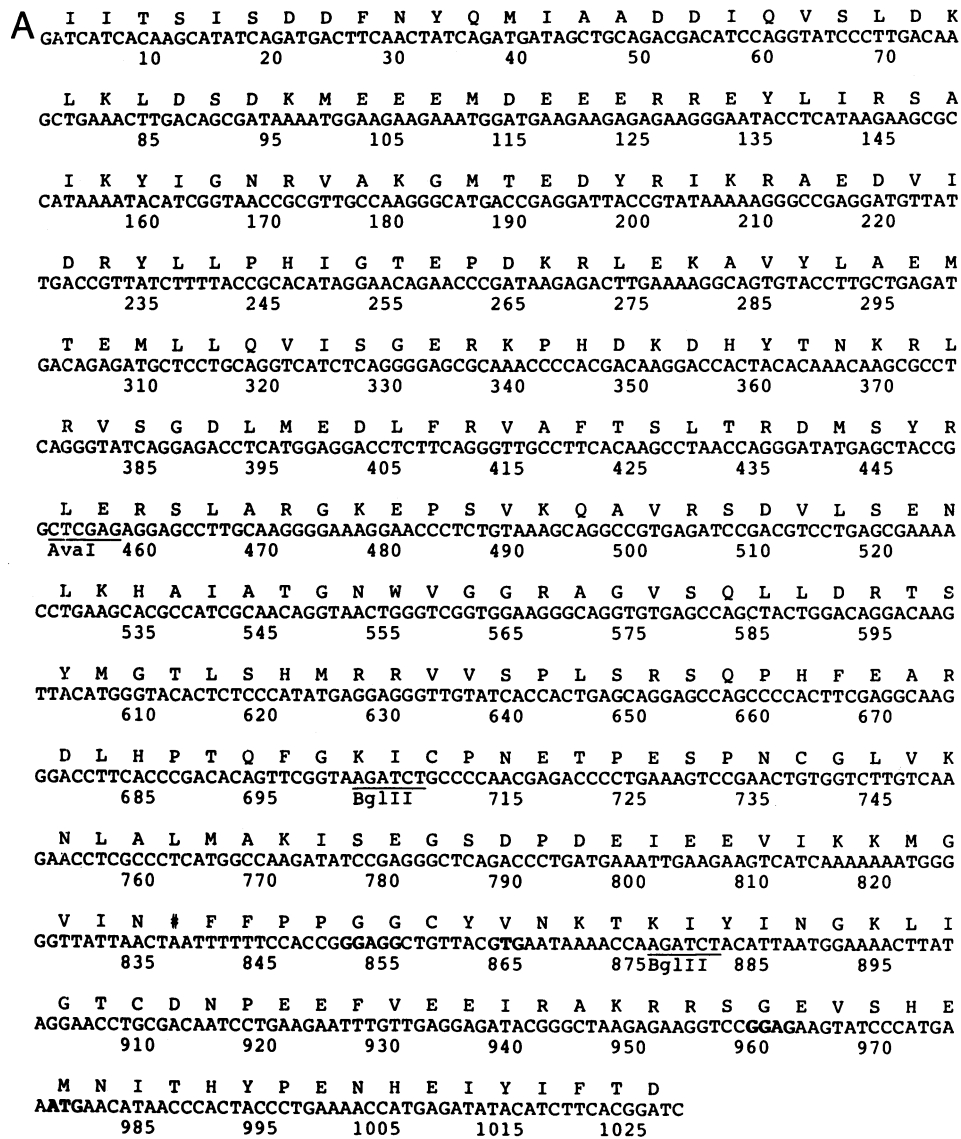


FIG. 1. Identification of polypeptides exhibiting antigenic determinants of subunits A or C of *Methanobacterium thermoautotrophicum* Winter RNA polymerase produced by *E. coli* harboring pEx62AC. Extracts from induced SG4044 cells harboring pEx62AC (lanes 2 and 4) were separated on SDS-polyacrylamide gels next to *Methanobacterium thermoautotrophicum* cell extracts (lanes 1 and 3). The separated polypeptides were transferred to nitrocellulose filters and reacted with antisera against the purified A (lanes 1 and 2) or C (lanes 3 and 4) subunits of the RNA polymerase. The position of markers, whose sizes are given in kilodaltons, is indicated on the right.



*Methanobacterium thermoautotrophicum* cell extracts (Fig. 1). Reaction of the same extract with antiserum against MS2 RNA polymerase gave a positive result, with the polypeptide exhibiting A antigenic determinants (data not shown). This indicates that the polypeptide is a fusion product and, therefore, that the A gene is located in front of the C gene in the transcriptional direction.

The insert of plasmid pEx11B'B'' was sequenced (Fig. 2A) and showed an open reading frame reaching from its 5'-terminal end to a stop codon between two *Bgl*III sites. Truncation of the insert by elimination of its 3' part beyond the first *Bgl*III site led to plasmid pEx16B'' (Fig. 2B). Cells transformed with this plasmid expressed only B'' antigenic determinants. This establishes the gene order 5', B'', B', 3'.

FIG. 2. Determination of the order of the genes encoding the B' and B'' RNA polymerase subunits. (A) Sequence of the insert of expression plasmid pEx11B'B''. The sequences of both strands were determined as described by Maxam and Gilbert (15). The expression of the B'' antigenic determinant results from a fusion of the 3'-terminal end of the B'' gene, with the 5'-terminal fragment of the MS2 polymerase gene contained in the vector. The two possible start sites of the B' gene (GTG at position 863 or ATG at position 977) are shown in boldface letters. No further continuous open reading frames were detectable in the sequence. (B) Results of the subcloning experiment to determine the order of the B' and B'' gene sequences of plasmid pEx11B'B''. Plasmid pEx16B'' was obtained after cleavage of pEx11B'B'' with *Bgl*III and religation with the loss of the two 3'-terminal *Bgl*III fragments. The expressed subunit antigenic determinants were identified with antisera applied to induced bacterial colonies harboring the plasmids. Symbols: wavy line, vector sequences; □, MS2 polymerase gene fragment; —, *Methanobacterium thermoautotrophicum* DNA insert.

To locate the identified genes on a longer contiguous DNA fragment, expression plasmids were used to probe a genomic library of *Methanobacterium thermoautotrophicum* Winter DNA created by ligation of a partial *Eco*RI digest into  $\lambda$ gt10. Three positive phages ( $\lambda$ 9,  $\lambda$ 16, and  $\lambda$ 32) were isolated;

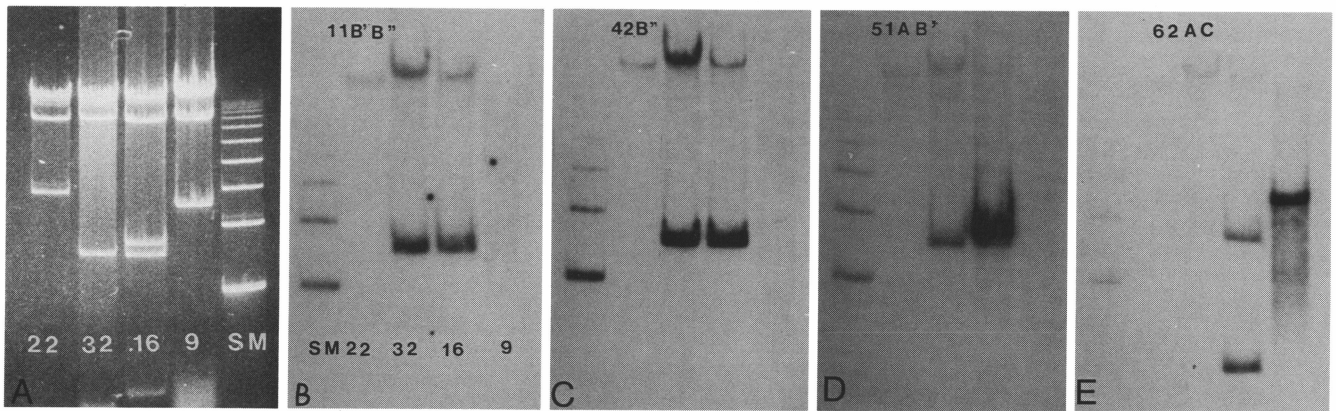


FIG. 3. Identification of sequences coding for RNA polymerase subunits on  $\lambda$  phages carrying *Methanobacterium thermoautotrophicum* genomic DNA inserts. Hybrid phages containing RNA polymerase genes were identified by plaque hybridization. The isolated phage DNAs were digested with *EcoRI* and separated electrophoretically on agarose gels. The fragments were transferred to nitrocellulose. (A) Restriction analysis of the identified phages. (B to E) Results of Southern hybridizations.  $^{32}\text{P}$ -labeled plasmid DNAs served as probes, as indicated in the other panels. Oligomers of a 1,650-bp pEx31 vector fragment were used as size markers (SM), with the smallest fragment being the 1,650-bp monomer. The numbers in the different lanes identify the analyzed phages (compare with Fig. 4). They were identical for all panels, in which hybridization autoradiograms are shown.

and their inserts were analyzed by cleavage with *EcoRI* and Southern hybridization with the labeled plasmids pEx11B'B'', pEx42B'', pEx51AB', and pEX62AC (Fig. 3). Phages  $\lambda 16$  and  $\lambda 32$  contained a common 2.3-kilobase (kb) fragment covering 3'-terminal sequences of gene B' and 5'-terminal B' sequences. The 2.7-kb fragment from  $\lambda 16$  hybridized to both pEx51AB' and pEX62AC, indicating that this fragment contains A-gene sequences. The 0.5-kb fragment of the  $\lambda 16$  insert reacted only with pEX62AC and is therefore located 3' of the 2.7-kb fragment.

Restriction analysis showed that the insert of pEX62AC contained two *EcoRI* sites which were 0.5 kb apart. Since only pEX62AC hybridized to the 4-kb insert of  $\lambda 9$ , this fragment must be located 3' of the 0.5-kb fragment.

Phage  $\lambda 32$  has a 350-bp *EcoRI* insert fragment which, after it was cloned into pUC8, was used as a probe in a subsequent screening of the  $\lambda$ gt10 gene bank and yielded the

overlapping phage  $\lambda 22$ . This explains why none of the four expression plasmids used hybridized to the DNA of that phage. The 4.5-kb insert fragment which did not overlap with  $\lambda 32$  constituted the 5'-terminal segment of the total region shown in Fig. 4. This figure also includes a more detailed restriction map and the deduced positions of the four genes. The gene borders are approximate, based on the molecular weights of the subunits A, B', B'', and C (96,000, 74,000, 59,000, and 50,000, respectively), as determined by SDS-polyacrylamide gel electrophoresis (19), from hybridization data, from the assumed location of the B'-B'' gene border, and from our finding of the 5' end of an open reading frame in the common 350-bp fragment of phages  $\lambda 22$  and  $\lambda 32$ , which we assume to be the 5' end of the B' gene. This assumption is again consistent with the expected size of the gene.

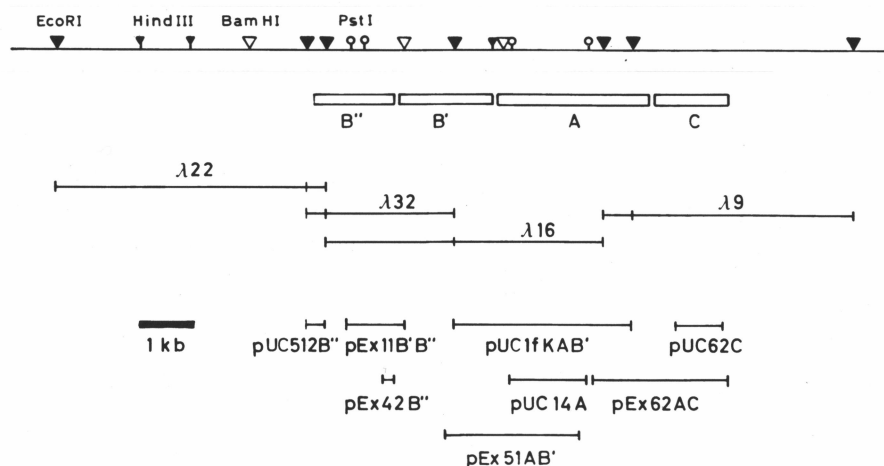


FIG. 4. Genomic region of *Methanobacterium thermoautotrophicum* Winter comprising the structural genes for RNA polymerase subunits A, B', B'', and C. The figure also shows the fragments cloned in the vectors  $\lambda$ gt10, pUC8, and pEx31 used in this study. pUC62C contains a *HpaII* subfragment of the insert of pEX62AC. The border between the B' and B'' genes was determined by sequence analysis (compare Fig. 2A). The localization of the other genes is described in the text.

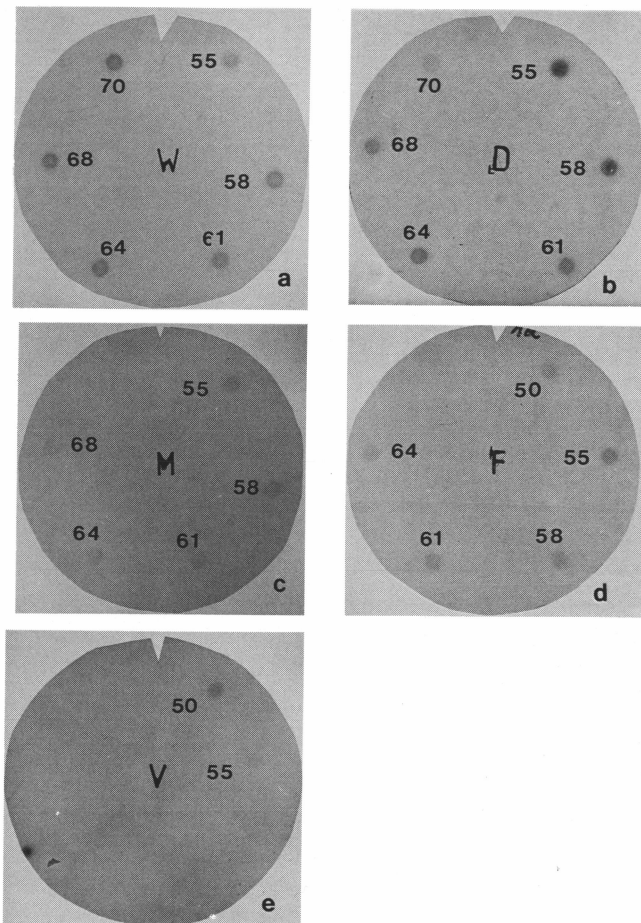


FIG. 5. Detection of hybrids between the plasmid probe pUC1fkAB' containing fragments of the RNA polymerase genes A and B' of *Methanobacterium thermoautotrophicum* Winter and total DNA of other methanogens. The genomic DNAs were spotted and fixed onto nitrocellulose filters after denaturation. The probes were then hybridized to the filters in 750 mM NaCl-75 mM sodium citrate-30% formamide at 37°C for 16 h. After rinsing with 0.3 M NaCl-0.03 M sodium citrate-0.1% SDS at room temperature, the filters were cut into sections, and the sections were washed with the same buffer 3 times for 10 min each at the indicated temperatures (in degrees Celsius). Autoradiography was performed with the dried filters, which were reconstituted from the sectors. (a) *Methanobacterium thermoautotrophicum* Winter; (b) *Methanobacterium thermoautotrophicum*  $\Delta$ H; (c) *Methanobacterium thermoautotrophicum* Marburg; (d) *Methanobacterium formicicum*; (e) *Methanosarcina voltae*.

All four genes were oriented in the same direction; i.e., they were transcribed from left to right.

The expression plasmids that were used to establish the phage maps were subsequently used to probe genomic DNA fragments of *Eco*RI, *Bam*HI, *Hind*III, and *Ava*I digests of genomic DNA and yielded the expected corresponding hybridizing fragments (data not shown). This indicates that the arrangement of the genes on the chromosome is the same as that deduced from the analysis of the phage inserts and that they are single-copy genes.

**Phylogenetic relationships.** In spite of their common energy metabolism, the different orders of methanogenic bacteria are only distantly related. This is obvious from the  $S_{AB}$  values obtained by 16S rRNA oligonucleotide comparison. The three orders *Methanococcales*, *Methanobacteriales*,

and *Methanomicrobiales* have low mutual  $S_{AB}$  values (between 0.2 and 0.3) (2). We were interested in determining the degree of conservation of RNA polymerase genes among methanogenic bacteria, both with respect to the nucleotide sequence and the gene order. As a measure for sequence homology, we performed DNA-DNA hybridization experiments using plasmid pUC1fkAB' as a probe. This plasmid contains a relatively large fragment of the A gene and part of the B' gene from *Methanobacterium thermoautotrophicum* Winter. The A subunit has previously been shown (26) to be homologous to the rpoO21 subunit of yeast RNA polymerase and to the  $\beta'$  subunit of *E. coli* RNA polymerase. Because of this conservation, an A-gene probe appeared to be suitable for the determination of homology among the polymerases of different methanogens, which can be taken as an indicator of their evolutionary relationships.

We employed dot blot hybridization to determine the stability of hybrids between the plasmid probe and homologous and heterologous genomic DNAs of different methanogens as a measure for sequence homology.

Total DNAs of *Methanobacterium thermoautotrophicum*  $\Delta$ H, Winter, and Marburg; *Methanobacterium formicicum*; *Methanococcus voltae*; or *Methanosarcina barkeri* were spotted onto nitrocellulose filters after heat denaturation. The filters were then incubated with the labeled probe under low-stringency conditions, which was necessary because of the different G+C contents (especially the low G+C content of *Methanococcus voltae* DNA) and the expected mismatches due to sequence divergence. Hybridization was achieved with all but the *Methanosarcina barkeri* DNA, which did not bind the probe under any of the conditions tested (750 mM NaCl, 75 mM sodium citrate, 10 to 50% formamide, 37°C). In the other cases the hybrids were subsequently washed at increasing temperatures until they dissociated (Fig. 5). The hybrids were found to melt at temperatures above  $T_m - 23^\circ\text{C}$  for *Methanobacterium thermoautotrophicum*  $\Delta$ H,  $T_m - 25^\circ\text{C}$  for *Methanobacterium thermoautotrophicum* Marburg,  $T_m - 26^\circ\text{C}$  for *Methanobacterium formicicum*, and  $T_m - 31^\circ\text{C}$  for *Methanococcus voltae*.

The melting temperatures were calculated for the salt concentration of the washing buffer and the different G+C contents of the strains used (Table 1), according to the following equation (1):

$$T_m = 81.5 + 16.6(\log M) + 0.41(\text{percent G+C}) - 0.72(\text{percent formamide}),$$

with  $M$  being the molar concentration of the monovalent cations. The results are consistent with the phylogenetic relationships previously deduced for the strains used here on the basis of rRNA oligonucleotide comparisons (2) or genomic DNA-DNA hybridization (3).

The RNA polymerases of methanogens (Thomm, Ph.D. thesis) are typical for the methanogenic-halophilic branch of the archaeobacteria and differ from their eubacterial counterparts, as they consist of more subunits, the larger part of which occurs once per polymerase molecule. As described above, the structural genes for the four largest subunits A, B', B'', and C are adjacent in the genome of *Methanobacterium thermoautotrophicum* Winter and are single-copy genes. They are likely to be organized in a common transcriptional unit, since their 5' to 3' orientations are identical. To find out whether the order of these four RNA polymerase genes is generally preserved in methanogens, we carried out DNA hybridization experiments using plasmids containing

TABLE 3. Hybridization of probes containing RNA polymerase gene fragments from *Methanobacterium thermoautotrophicum* Winter with restriction fragments of genomic DNA from other methanogenic bacteria

Organism and probe <sup>a</sup>	Size (kb) of the following restriction fragments:										
	<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	<i>Kpn</i> I	<i>Bst</i> EII	<i>Bgl</i> II	<i>Acc</i> I	<i>Cl</i> aI	<i>Pst</i> I	<i>Sal</i> I	<i>Sma</i> I
<i>Methanobacterium thermoautotrophicum</i> ΔH											
DNA probe											
pEx11B'B''	5.7	2.7	2.4	5	7.2						
pUC1fkAB'	2.5, 5.7	2.7	6.6	1.6, 4.7, 5	7.2						
pUC14A	2.5	2.3	6.6	— <sup>a</sup>	—						
pUC62C	1.6	2.3	6.6	5	7.2						
<i>Methanobacterium thermoautotrophicum</i> Marburg											
DNA probe											
pEx11B'B''	9.2	7	2.6								
pUC1fkAB'	9.2	7	8.6								
pUC14A	—	7	8.6								
pUC62C	1.5	4.2	8.6								
<i>Methanobacterium formicicum</i>											
DNA probe											
pEX11B'B''	4.2	16	—			4.4					
pUC512B''	—	—	2.7			—					
pEx42B''	—	—	2.7			—					
pUC1fkAB'	6	16	3.1			10					
pUC62C	6	2	3.1			10					
<i>Methanosarcina voltae</i>											
DNA probe											
pEx11B'B''			5				3	4	1.8, 4.5	6.5	3.5, 4.7
pUC1fkAB'			1.2				1.6, 3	1.4, 1.9	4.5	6.5	4.7
pEx51AB'			—				1.6, 3	1.4, 1.9	—	—	—
pUC62C			2.3, 3				4.4	2.6	4.5	6.5	4.7

<sup>a</sup> —, Not determined.

various sequences of the genes encoding the four largest RNA polymerase subunits of *Methanobacterium thermoautotrophicum* Winter to probe electrophoretically separated restriction fragments of cellular DNAs from other methano-

gens after their immobilization to nitrocellulose. The gene orders could be deduced from the hybridization patterns (Table 3 and Fig. 6). For all strains the results are consistent with the common gene order B'', B', A, and C.

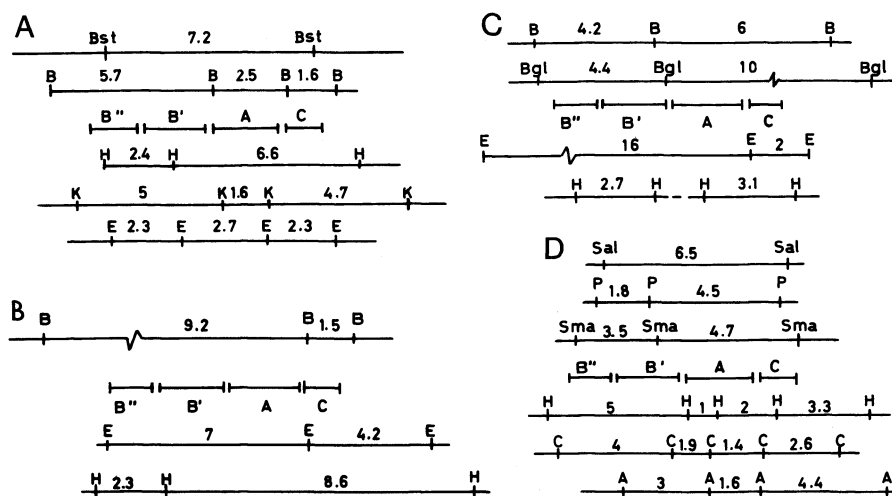


FIG. 6. Arrangements of restriction fragments in the genomic regions comprising the RNA polymerase genes homologous to A, B', B'', and C of *Methanobacterium thermoautotrophicum* Winter in *Methanobacterium thermoautotrophicum* ΔH (A), *Methanobacterium thermoautotrophicum* Marburg (B), *Methanobacterium formicicum* (C), and *Methanosarcina voltae* (D). The assumed gene order is indicated in the centers of the figures in analogy to the one determined in *Methanobacterium thermoautotrophicum* Winter (compare Fig. 3). The arrangement of the fragments was deduced from the hybridization data given in Table 3. Relative orders of the restriction sites of different enzymes (i.e., complete physical maps) cannot be given since hybridization to double digests was not performed. Abbreviations of restriction sites: A, *Acc*I; B, *Bam*HI; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I.

## DISCUSSION

The genes encoding the four largest subunits of RNA polymerase formed a cluster on the chromosome of *Methanobacterium thermoautotrophicum* Winter. All four analyzed genes were transcribed from the same DNA strand, which suggests that they belong to a common transcription unit. This would facilitate their synthesis in an equal stoichiometry, which is found in the enzyme. The possibility of a transcription unit comprising the four genes reminds us of the situation known in *E. coli*, in which two of the core polymerase subunits,  $\beta$  and  $\beta'$ , are also transcribed into a polycistronic messenger (14). Sequence analysis of the genes described here will show whether the homologies seen in immunological analyses also apply to the gene arrangements in the different prokaryotes.

The stabilities of the DNA-DNA hybrids between the *Methanobacterium thermoautotrophicum* Winter RNA polymerase gene probe and homologous sequences of other methanogenic bacteria correlate with the phylogenetic distances of the employed strains (2, 3). Thus, it is not surprising that hybridization of an RNA polymerase gene probe of a *Methanobacterium* species with functionally homologous DNA of a *Methanosarcina* species should be impossible and that only weak hybridization is observed with *Methanococcus* DNA. These results, on the other hand, demonstrate that the immunological relationship of homologous proteins, as found among the RNA polymerases in all orders of the methanogens, need not reflect an equal sequence homology of their genes.

In view of the evolutionary distance between the different orders of the methanogenic bacteria, the conservation of the arrangement of the analyzed RNA polymerase genes in *Methanococcales* species and *Methanobacteriales* species may be interpreted in terms of its functional relevance. Analysis of the transcription pattern of the genes will be interesting in this respect.

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