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Immunological Comparison of Ribosomal Proteins from Archaebacteria

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Antisera were raised in rabbits against ribosomal proteins of *Methanobacterium bryantii* and used to analyze immunological relationships to ribosomes from other archaebacteria, from eubacteria, and from yeasts. Cross-reaction could be detected within the methanogens and with a member of the extreme halophiles; the degree of immunological similarity reflected the relationship delineated by 16S ribosomal ribonucleic acid oligonucleotide analysis (Fox et al., Science **209**:457-463, 1980). With the methods and the anti-total-protein sera employed, there was no detectable cross-reaction with ribosomal proteins or ribosomes from *Sulfolobus* sp., eubacteria, or yeast.

On the basis of the comparative analysis of oligonucleotide composition of 16S rRNA, a third evolutionary line of descent, that of the archaebacteria, has recently been proposed by Woese and collaborators (4). This concept has raised considerable interest in the investigation of those cellular components in which the other two lines of descent, eubacteria and eucaryotes, differ, especially in the cell envelope (10) and in the transcriptional and translational apparatus (14, 24). The ribosome deserves particular interest in this connection since many functional and structural differences exist between the eubacterial 70S and the eucaryotic 80S particle (13).

Whereas the ribosomal components of extreme halophiles are well characterized, there is a considerable lack of information on those from the other two archaebacterial groups, the methanogens and thermoacidophiles. An initial electrophoretic characterization has recently been carried out with ribosomal proteins from methanogenic organisms (3). A striking difference found was that many more proteins migrate to the "acidic" side of the Kaltschmidt and Wittmann (9) two-dimensional gel electrophoretic system than do eucaryotic or eubacterial ribosomal proteins. This in general more acidic nature reflects the relationship between extreme halophiles and methanogens as delineated by 16S rRNA sequence studies (4).

In this work, we report immunological studies of ribosomal proteins which were performed to analyze whether it is possible by this criterion to detect any relationships among different archaebacterial genera and species.

MATERIALS AND METHODS

Organisms, media, and growth conditions. The following organisms were used in this study; DSM numbers refer to the strain numbers listed in the *Catalogue of Strains: German Collection of Microorganisms* (2).

Archaebacteria. The archaebacteria used were *Methanobacterium formicicum* DSM 1312, *Methanobacterium bryantii* DSM 863, *Methanobacterium thermoautotrophicum* DSM 1053, *Methanobrevibacter arboriphilus* DSM 1125, *Methanococcus vannielii* DSM 1224, *Methanospirillum hungatei* DSM 864, *Methanosarcina barkeri* DSM 800 (morphotype I), *Methanosarcina barker;* DSM 1232 (morphotype II), *Halobacterium cutirubrum* DSM 669, *Halobacterium halobium* DSM 670, and *Sulfolobus acidocaldarius* DSM 639.

Eubacteria. *Bacillus subtilis* strain 168 DSM 402, *Clostridium butyricum* DSM 552, *Pseudomonas aeruginosa* PAO 303, *Rhodopseudomonas sphaeroides*, *Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis*, and *Escherichia coli* B were used.

Eucaryote. *Saccharomyces cerevisiae* A364A was used.

The media and growth conditions employed for the cultivation of methanogenic bacteria were described recently (3). Halobacteria were cultivated at 37°C in a medium consisting of 1% bacteriological peptone (Oxoid), 25% NaCl, 2% $MgSO_4$, and 0.2% KCl. *Sulfolobus* sp. was grown at 70°C in medium 88, which is cited in the *Catalogue of Strains: German Collection of Microorganisms* (2). For growth of *C. butyricum*, a tryptone-thioglycolate medium (no. 48 in the *Catalogue of Strains: German Collection of Microorganisms*) was used. *P. aeruginosa* and *B. subtilis* were cultivated in a medium containing 0.5% bacteriological peptone (Oxoid), 0.3% meat extract, and 0.5% glycerol. The same medium was employed for *Staphylococcus aureus* except that glucose was substituted for glycerol. *E. coli* and *Streptococcus faecalis* were grown in TGYES medium (1% tryptone [Oxoid], 0.5% yeast extract, 0.5% glucose, 0.5% NaCl). *R. sphaeroides* was cultivated photosynthetically in a medium containing 2.5 g of malic acid, 1.2 g of NH_4Cl, 0.2 g of $MgSO_4$, 0.07 g of $CaCl_2 \cdot 2H_2O$, 0.9 g of K_2HPO_4, 0.6 g of KH_2PO_4, 0.5 g of yeast extract, and 8 ml of the SL6 trace

element solution per liter (3).

Preparation of cell-free extracts and isolation of ribosomes, ribosomal subunits, and ribosomal protein. Cells were harvested at the end of exponential growth by centrifugation at 4°C; methanogenic organisms were collected under aerobic conditions. Before centrifugation of *Sulfolobus* cultures, the medium was adjusted to a pH between 5.5 and 6.5 by the addition of 2 M Tris-base. The sedimented cells were washed once in TMNSH buffer (10 mM Tris-chloride [pH 7.5], 10 mM Mg acetate, 30 mM NH_4Cl, and 3 mM 2-mercaptoethanol), centrifuged again, frozen in dry ice-ethanol, and kept at −70°C until use.

For preparation of S30 extracts, the cells were suspended in an approximately equal volume of one of the buffers listed below containing DNase I (Boehringer Mannheim Corp.) at 2 μg/ml. TMNSH was used in all cases except with *Sulfolobus* sp. (replaced by TMKSH: 20 mM Tris-chloride [pH 7.5]–10 mM Mg acetate–200 mM KCl–6 mM 2-mercaptoethanol) and with halobacteria (replaced by 10 mM Tris-chloride [pH 7.0]–4 M KCl–100 mM $MgCl_2$) (22). The suspended cells were broken by passage through a French press cell, and the extracts were clarified by two consecutive centrifugations, first at 10,000 × *g* for 10 min and then at 30,000 × *g* for 30 min (S30 extract).

The S30 extracts (except those from halobacteria and *Sulfolobus* sp.) were diluted with an equal volume of TMNSH buffer containing 1 M NH_4Cl and layered on top of a twofold volume of a 30% sucrose solution made up in TMNSH, with an NH_4Cl concentration of 0.5 M. The ribosomes were sedimented through this cushion by a 3-h centrifugation at 50,000 rpm (60 Ti rotor). The ribosomal pellet was rinsed with and taken up in a small volume of the respective buffer. Aggregates were removed by a 10-min centrifugation at 30,000 × g; after measurement of the absorbance at 260 nm, the ribosomal suspensions were frozen and stored at −70°C.

70S ribosomes from *Sulfolobus* sp. prepared in this way were grossly contaminated, possibly by the cell wall lipoprotein fraction (23). To obtain a reasonably pure 70S fraction in this case, about 300 to 400 A_{260} units (1 unit corresponds to the amount of material in 1 ml giving an absorption at 260 nm of 1.0) of S30 in TMKSH buffer (see above) were layered on linear 10 to 30% sucrose gradients made up in TMKSH buffer with 0.5 M NH_4Cl and centrifuged for 60 min at 40,000 rpm in a VTi 50 rotor. The 70S material was then sampled.

For preparation of ribosomal subunits, the 70S ribosomes were first dissociated by dialysis against TMNSH buffer, with the Mg^{2+} concentration being 1 mM. Subunits were then separated by three consecutive centrifugations in a 10 to 30% linear sucrose gradient in a VTi 50 rotor. Then 90 to 100 A_{260} units of 70S ribosomes were loaded onto each gradient; centrifugation was for 80 min at 40,000 rpm. Other details of the procedure were as described previously (3).

Ribosomal proteins were extracted from ribosomes and ribosomal subunits as described by Hardy et al. (6).

Immunological procedures. (i) Preparation of antisera. The time scheme of immunization was essentially identical to that described by Hennecke et al. (7). Two rabbits each were immunized with 120 A_{260} units of 30S or 50S ribosomal subunits from *Methanobacterium bryantii*. Before injection, the antigens were mixed in a 1:1 (vol/vol) ratio with Freund adjuvant; the complete form of adjuvant was used in the first three injections, and the incomplete form was used for the booster injection. Blood was collected from the ear vein of the animals before and after immunization, and the sera were prepared as described previously (7). The anti-30S sera and the anti-50S sera were then mixed in a 1:1 (vol/vol) ratio and used for the preparation of a crude immunoglobulin fraction by three consecutive ammonium sulfate precipitation steps (33% saturation at room temperature, pH 8.0). The sediment of the last precipitation step was taken up in one-fourth of the original volume (1 mM potassium phosphate buffer [pH 8.0] containing 0.9% NaCl).

For the immuno-double-diffusion experiments, the immunoglobulin G fraction was isolated. For this purpose, 10 ml of the crude immunoglobulin preparation was first dialyzed overnight against 5 liters of 0.1 M Tris-chloride, pH 8.0, and then chromatographed on a Sephadex G-150 column (7 by 40 cm). The peak containing immunoglobulin G was pooled, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and lyophilized. The protein was then taken up in 1 ml of 0.9% NaCl, centrifuged to remove any undissolved material, and used for the immunodiffusion experiments.

(ii) Immuno-double-diffusion. Immuno-double-diffusion experiments were carried out as described by Ouchterlony (16) with the modifications necessary for analysis of ribosomal proteins. The purified immunoglobulin G fraction was used. The plates contained 1.5% agarose in Veronal buffer (pH 8.6)–0.75 M LiCl–1 mM NaN_3 (20, 21). They were incubated for 24 to 48 h at 4°C, and the precipitin lines were photographed, using a circular light source.

(iii) Quantitative immunoprecipitation. Quantitative immunoprecipitation experiments were performed by the procedure developed by Geisser et al. (5). The crude immunoglobulin fraction was employed, and controls were run with the antibodies alone, the antigens alone, or the antigens together with the nonimmune serum. The quantitation of the precipitate was done by protein measurement with the Folin phenol reagent (11) or by RNA determination as described by Schneider (18).

(iv) Modified immunoelectrophoresis on cellulose acetate. Ribosomal proteins were separated on cellulose acetate strips by the method of Stöffler (20) as modified by Zubke et al. (25). Between 25 and 50 μg of 30S and 50S total protein was applied when proteins were directly localized by staining; when proteins were stained after immunofixation, between 3 and 12 μg was applied. The crude immunoglobulin fraction was used for immunofixation in each case.

RESULTS

Antibodies were raised in rabbits against 30S and 50S subunits of ribosomes from *Methanobacterium bryantii* and pooled to an anti-70S serum. Cross-reaction was then tested with ribosomal proteins from various other archaebacteria, from several eubacteria, and from *Saccha-*

romyces cerevisiae by using three different immunological techniques.

(i) Ouchterlony immuno-double-diffusion. Cross-reaction was first assessed qualitatively by immuno-double-diffusion on plates, using an approximately 40-fold-concentrated immunoglobulin G preparation. Figure 1A shows the strong reaction with 70S ribosomal proteins of *Methanobacterium bryantii*, *Methanobacterium thermoautotrophicum*, and *Methanobrevibacter arboriphilus*. Spur formation could be observed between *Methanobacterium thermoautotrophicum* and *Methanobacterium bryantii* as well as between *Methanobrevibacter arboriphilus* and *Methanobacterium bryantii*, whereas no spurs were apparent between *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilus*. This could mean that those determinants reacting with immunoglobulins directed against *Methanobacterium bryantii* ribosomal proteins are (nearly) identical in these organisms. A weak reaction was obtained with ribosomal proteins from *Methanosarcina barkeri*, *Methanococcus vannielii*, and *H. halobium*, whereas none was detected with those from *Methanospirillum hungatei* (Fig. 1A and B). Although it is difficult to judge, there seems to be no major spur formed between *Methanosarcina barkeri* and *Methanococcus vannielii* precipitin lines. There was no reaction of the antibodies directed against *Methanobacterium bryantii* ribosomes with ribosomal proteins from *S. cerevisiae*, *S. acidocaldarius*, or *E. coli* under conditions in which proteins from the extreme halophile *H. halobium* were reacting (Fig. 1C). Figure 1D, finally, demonstrates that the cross-reaction with *Methanosarcina barkeri* ribosomal proteins was mainly due to reaction with 50S and not 30S determinants.

(ii) Quantitative immunoprecipitation. Figure 2A and B show the results of quantitative immunoprecipitation of antibodies directed against ribosomes from *Methanobacterium bryantii* with those from other methanogenic organisms and from *Sulfolobus* sp. With the exception of data for *Sulfolobus* sp., the data reflect the cross-reaction pattern obtained by the immuno-double-diffusion experiments. There was a high degree of immunological relatedness to *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilus*. Ribosomes from *Methanobacterium formicicum* reacted as efficiently as those from the homologous system, whereas there was only a low amount of precipitate formed with *Methanosarcina barkeri* and *Methanococcus vannielii*. No precipitate was obtained with ribosomes from any of the other organisms tested.

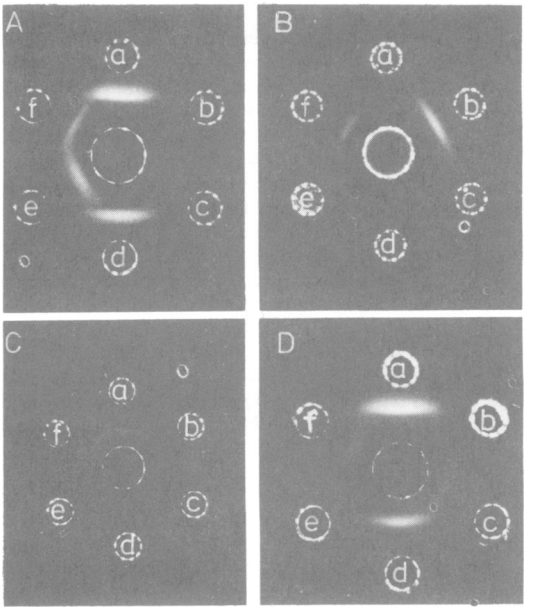

FIG. 1. *Immuno-double-diffusion of immunoglobulin G directed against 70S ribosomes from Methanobacterium bryantii and total 70S (if not mentioned otherwise) ribosomal proteins. Center wells contained 2.3 mg of immunoglobulin protein. (A) (a and d) 6.25 μg of ribosomal protein from Methanobacterium bryantii, (b) 75 μg from Methanococcus vanniellii, (c) 75 μg from Methanosarcina barkeri, (e) 75 μg from Methanobacterium thermoautotrophicum, and (f) 75 μg from Methanobrevibacter arboriphilus. (B) (a) 75 μg of ribosomal protein from H. halobium, (b) 6.25 μg from Methanobacterium bryantii, (c) 75 μg from Methanococcus vannielii, (d) 75 μg from Methanosarcina barkeri, (e) 75 μg Methanospirillum hungatei, and (f) 75 μg from Methanobrevibacter arboriphilus. (C) (a) 75 μg of ribosomal protein from H. halobium, (b) 75 μg from E. coli, (c) 75 μg from Saccharomyces sp., and (d) 75 μg from Methanospirillum hungatei; (e) 37.5 μg of total 50S protein from Sulfolobus sp.; and (f) 37.5 μg of 30S protein from Sulfolobus sp. (D) (a) 6.25 μg of ribosomal protein from Methanobacterium bryantii, (b) 75 μg from Methanosarcina barkeri, (c) 75 μg from Methanococcus vannielii, and (d) 75 μg from Methanobrevibacter arboriphilus; (e) 37.5 μg of 30S protein from Methanosarcina barkeri; and (f) 37.5 μg of 50S protein from Methanosarcina barkeri.*

(iii) Modified immunoelectrophoresis on cellulose acetate. To study whether the interactions obtained in the immunodiffusion and immunoelectrophoresis experiments were specific, ribosomal proteins were separated by electrophoresis on cellulose acetate strips and stained after immunofixation (Fig. 3). Distinct bands were obtained with *Methanobacterium formicicum*, *Methanobacterium thermoautotrophicum*, *Methanobrevibacter arboriphilus*,

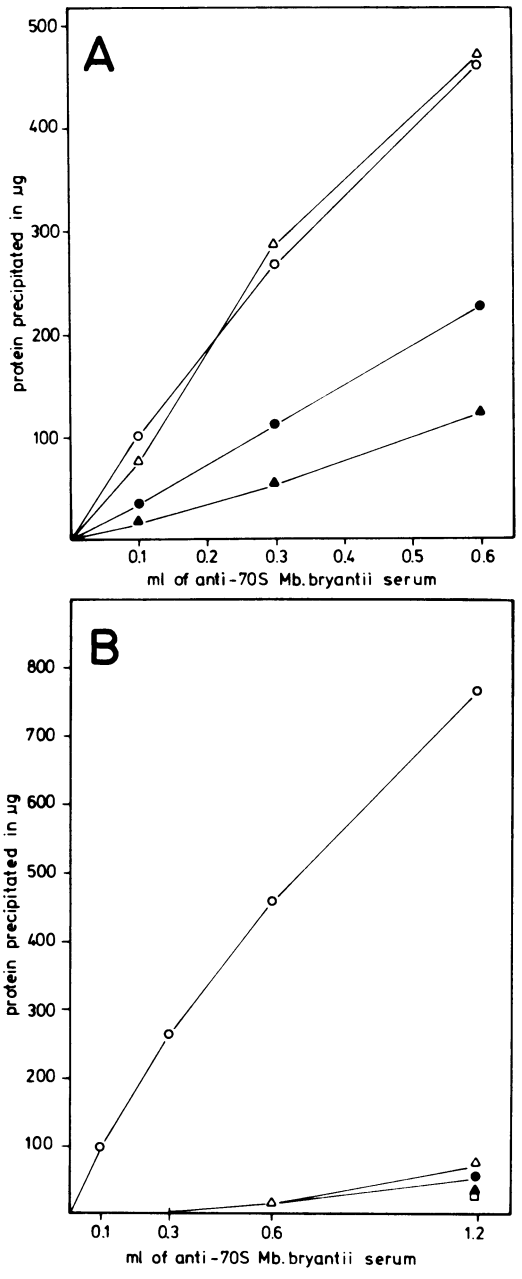

FIG. 2. *Quantitative immunoprecipitation of 3 A_{260} units of 70S ribosomes. Ribosomes (A) from Methanobacterium bryantii* (○), *Methanobacterium formicicum* (△), *Methanobacterium thermoautotrophicum* (●), *and Methanobrevibacter arboriphilus* (▲), *and (B) from Methanobacterium bryantii* (○), *Methanosarcina barkeri DSM 800* (△), *Methanosarcina barkeri DSM 1232* (●), *Sulfolobus acidocaldarius* (▲), *and Methanococcus vannielii* (□) *precipitated by antiserum directed against Methanobacterium bryantii 70S ribosomes.*

Methanosarcina barkeri, and *Methanococcus vannielii*. Total 70S protein from *Sulfolobus acidocaldarius* contained a reactive protein migrating close to the sample application site. The following evidence, however, indicated that this was due to the fact that the *Sulfolobus* preparation contained a "sticky" protein which was not washed from the cellulose acetate after the immunofixation step: (i) this band was not visible when 30S and 50S subunit protein was used instead of 70S total protein, and (ii) it was visible on control electropherograms, i.e., on cellulose acetate strips which had not been treated with antiserum.

On the other hand, 30S and 50S ribosomal proteins from *Methanosarcina barkeri* displayed at least one immunoreactive band on the electropherogram, which indicates that a specific interaction had taken place in immunodiffusion and immunoprecipitation.

Comparison of the pK_i values of ribosomal proteins from archaebacteria. One of the characteristics of the ribosomal proteins from methanogenic organisms is that they generally are more acidic than those from eubacterial ribosomes, as judged by their migration in the Kaltschmidt and Wittmann (9) electrophoretic system (3, 15) or by sequence analysis of the "A" protein (13). A parallelism was observed between the number of proteins migrating to the acidic side on Kaltschmidt and Wittmann gels and the degree of 16S rRNA relatedness between methanogenic bacteria and halophiles (3), which might be a reflection of the relatively close phylogenetic relation between one group of the methanogens and the extreme halophiles (12). The actual range of isoelectric points, however, has not been determined. To this end, the relative mobility of 70S ribosomal proteins from archaebacteria, eubacteria, and *Saccharomyces* sp. was analyzed by cellulose acetate electrophoresis (Fig. 4). (i) Ribosomal proteins from eubacteria (lanes 1 through 6 and 14) under the experimental conditions migrated to the left part of the electropherograms; (ii) ribosomal proteins from methanogenic organisms (lanes 7 to 12, 15, and 16) were generally more distributed in the central part, i.e., on the average, they were more acidic; (iii) ribosomal proteins from halophiles were by far the most acidic ones, with only a few proteins migrating to the cathode; and (iv) the ribosomal protein patterns of *H. halobium* and *H. cutirubrum* were identical.

Although cellulose acetate electrophoresis and polyacrylamide electrophoresis were carried out at the same pH, a considerably higher number of proteins migrated to the anode in the polyacrylamide gel. This different relative mo-

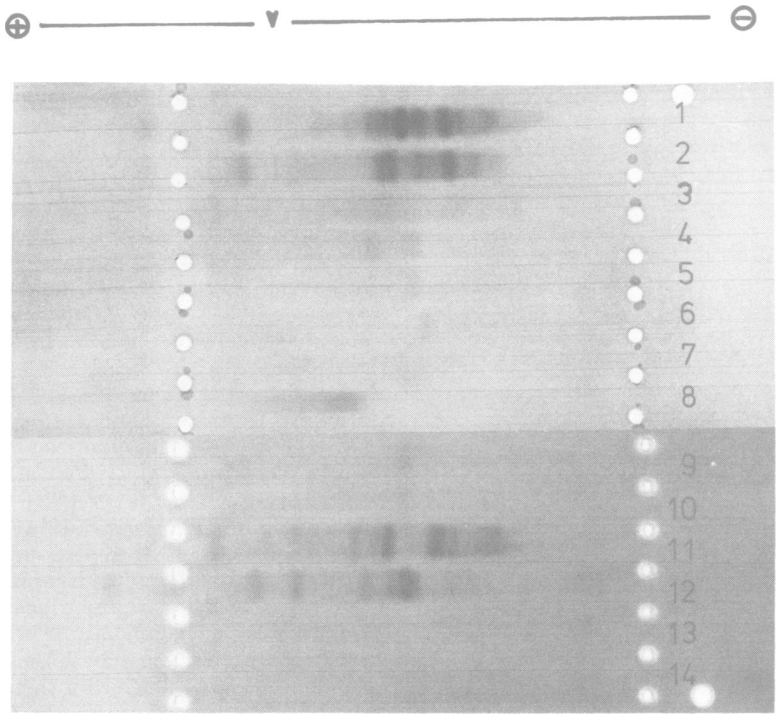

FIG. 3. *Modified immunoelectrophoresis of ribosomal proteins. Lanes 1 to 8 contained 6.25 μg of total 70S protein from Methanobacterium bryantii (1), Methanobacterium formicicum, (2), Methanobacterium thermoautotrophicum (3), Methanobrevibacter arboriphilus (4), Methanosarcina barkeri DSM 800 (5), Methanosarcina barkeri DSM 1232 (6), Methanococcus vannielii (7), and Sulfolobus acidocaldarius (8). Lanes 9 to 14 contained 3 μg of protein each of 50S (lane 9) and 30S (lane 10) subunits from Methanosarcina barkeri DSM 1232, 50S (lane 11) and 30S (lane 12) ribosomal proteins from Methanobacterium bryantii, and 50S (lane 13) and 30S (lane 14) ribosomal proteins from Sulfolobus acidocaldarius.*

bility could be due to electroendosmotic effects on the cellulose acetate support (17).

DISCUSSION

One of the main questions to be answered was whether the genealogy of archaebacteria, as outlined by 16S rRNA oligonucleotide analysis (4), is reflected in the ribosomal protein relationship. In other words, is it possible to support the RNA sequence data by more conventional methods, such as the immunological comparison of proteins? For this purpose, we have plotted the immunoprecipitation results in terms of an "immunological distance" dendrogram (Fig. 5). It is based on the assumption that the amount of immunoprecipitate (which consists of ribosomes plus antibody molecules bound to surface antigenic determinants) is a measure of the number of common determinants between *Methanobacterium bryantii* and the other organisms and thereby reflects their relative phylogenetic distance. This assumption is certainly an oversimplification since it does not take into consideration ribosomal determinants which were antigenically silent. Despite the apparent weakness of this approach, it is interesting to note that an almost complete correspondence to the 16S rRNA sequence results (4) was obtained, supporting by these means the rRNA data as an experimental tool for phylogenetic analysis of organisms.

A specific cross-reaction of antibodies directed against *Methanobacterium bryantii* ribosomal proteins with ribosomal proteins from *H. halobium* could be demonstrated. This agrees with the rRNA data published by Fox et al. (4) and supports the view that members of the extreme halophiles are more related to the order *Methanobacteriales* than other methanogenic organisms, e.g., *Methanospirillum* species. It will be interesting to investigate whether the cross-reaction within the methanogens and also with extreme halophiles is due to the fact that a few proteins are more conserved than others or whether different proteins are responsible in each case. The (at least partial) fusion of precip-

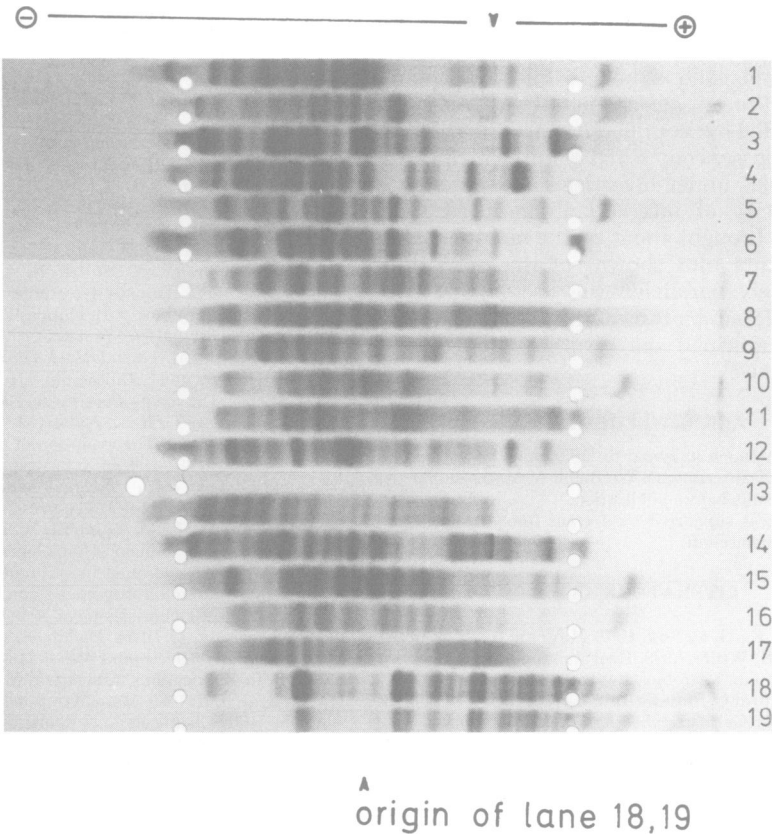

FIG. 4. *Cellulose acetate electrophoresis of 50 μg of total 70S proteins from B. subtilis (1), Staphylococcus aureus (2), P. aeruginosa (3), R. sphaeroides (4), Streptococcus faecalis (5), C. butyricum (6), Methanosarcina barkeri DSM 800 (7), Methanosarcina barkeri DSM 1232 (8), Methanospirillum hungatei (9), Methanobacterium formicicum (10), Methanobacterium thermoautotrophicum (11), Methanococcus vannielii (12), Saccharomyces cerevisiae (13), E. coli (14), Methanobacterium bryantii (15), Methanobrevibacter arboriphilus (16), Sulfolobus acidocaldarius (17), H. halobium (18), and H. cutirubrum (19). Proteins were stained without prior immunofixation.*

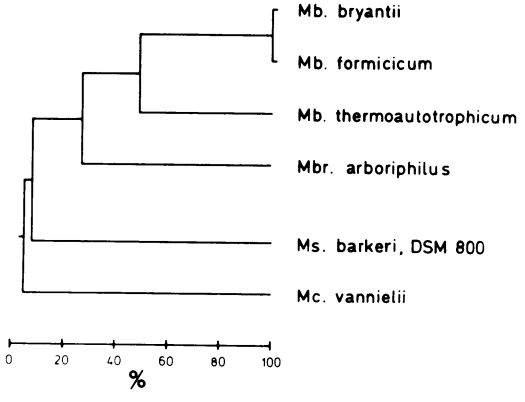

FIG. 5. *Dendrogram of relative immunological relationship between Methanobacterium bryantii and other methanogenic organisms. The amount of immunoprecipitate formed between the antibodies directed against 70S ribosomes from Methanobacterium bryantii and ribosomes from Methanobacterium bryantii is taken as 100, and the precipitates in the heterologous systems are taken as fractions thereof. The dendrogram is based on the assumption that the number of antigenic determinants on ribosomes saturable by antibodies is a reflection of phylogenetic distance. The dendrogram also is based on experiments in which there was no cross-reaction of antiserum against Methanosarcina barkeri ribosomes with ribosomes from the other species listed in the dendrogram (unpublished data).*

itin lines in the immunodiffusion experiments (Fig. 1) between *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilus* and between *Methanococcus vannielii* and *Methanosarcina barkeri* favor the assumption of a few conserved ribosomal proteins. It remains to be demonstrated whether they always are components of the 50S subunit, as in

Methanosarcina barkeri (Fig. 1 and 3).

A caveat connected with an immunological analysis of this kind is the possibility of contamination of the antigen preparation, which can never be ruled out completely since the contaminant may possess much higher antigenicity than the molecules under investigation. Support for the specificity of interaction measured here, however, is brought about by the fact that RNA measurements (not shown) of the precipitate quantitatively paralleled the protein determinations and that analysis of three-times-purified subunits confirmed the results obtained with 70S ribosomes.

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