Immunology of Archaebacteria That Produce Methane Gas

Abstract. *The antigenic map of 17 methanogenic bacteria representing the entire range of available species was determined by multiple assay with antibody probes. Four major clusters of antigenically related strains coincide with the families proposed on the basis of 16S ribosomal RNA analysis. Immunological mapping uncovered relationships not yet shown by other methods and allowed identification and classification of two new bacterial isolates.*

A third primary kingdom encompassing the Archaebacteria has been proposed (*1*). Among these microorganisms are the methanogens, which constitute a biological domain whose distinctive property is the production of methane gas. They are strict anaerobes, different from eubacteria and from nonmethane-producing Archaebacteria, and are thought to have arisen very early on earth (*1–3*). As such, the methanogens may prove useful for elucidating the evolutionary significance of Archaebacteria and the mechanisms of energy conservation and chemical catalysis. Methanogens may become useful in the development of procedures for the conversion of renewable resources from biomass and wastes to the fuel methane (*2–5*).

Methanogens are heterogeneous with respect to morphology and to the macromolecular features that have been used to separate certain prokaryotes into a new kingdom (*2, 3*). Studies on their ultrastructure, physiology, biochemistry, and metabolic pathways have led to their characterization and organization in subgroups (*2, 3, 6*). Analysis of the 16*S* ribosomal RNA (rRNA) of methanogens has led to their classification (*3, 7*); however, alternative proposals have been put forward for their phylogeny (*3, 8*). Additional information about the relationships between different groups of methanogens and the relation of methanogens to other prokaryotes (*1, 3, 8*) can be provided by immunological analyses. Moreover, dissimilarities in the chemistry of the cell walls of methanogens of different families (*6*) suggest that they are antigenically diverse and, thus can be characterized and classified by immunological methods. However, little is known of the immunogenic and antigenic properties of methanogens (*9, 10*).

We determined the antigenic map of 17 methanogens, representing practically all species accepted at the present time, by multiple assay with a set of antibody probes (Fig. 1). For example, the map of *Methanobacterium thermoautotrophicum* strain ΔH shows a weak cross-reaction with antibody to *Methanobacterium bryantii* strain MoH and *Methanobrevibacter smithii* strains ALI and PS, a moderate cross-reaction with antibody to *Methanobacterium thermoautotrophicum* strain GC1, and absence of cross-reaction with the rest of the probes in the set. Organization of all maps in a binary figure revealed four major clusters of cross-reactive methanogens (Fig. 1). These clusters coincide with the families recently proposed on the basis of the analysis of 16*S* rRNA codons (*3*). Although the cross-reactions observed never broke across the barrier dividing families, some intergeneric cross-reactions occurred. The latter, however, were weaker than the intrageneric cross-reactions.

Methanogens differ in immunogenicity as well as in antigenic pattern. One antigenic determinant may be shared by two or more strains, but its immunogenicity (that is, its capacity to elicit antibodies) may be fully expressed in all or in only one of them. In the other strains, this determinant may be equally immunogenic, less immunogenic, or not immunogenic at all, and may be detectable only by its capacity to bind antibody in vitro. The cross-reactions shown in Fig. 1 are, most likely, accounted for by antibodies directed to the dominant determinant of the immunizing strain, that is, the one that elicits the most antibodies with highest affinity. Since the antibody probe used was dilute, the antibodies in the probe were most likely the ones recognizing those strains with a family that share an antigenic determinant that is dominant in the immunizing strain. For example, antibody to *Methanobrevibacter smithii* PS reacts strongly with the immunizing strain PS but not as strongly

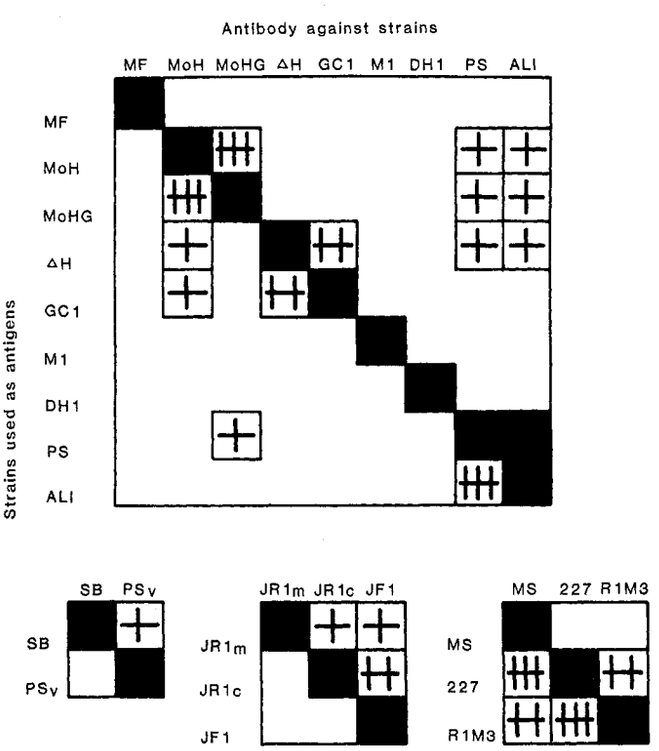

Fig. 1. The strains of *Methanobacterium* tested were *formicicum* (MF), *bryantii* (MoH and MoHG), and *thermoautotrophicum* (ΔH and GCl); those of *Methanobrevibacter* tested were *ruminantium* (M1), *arboriphilus* (DH1), and *smithii* (PS and ALI); those of *Methanococcus* were *vannielii* (SB) and *voltae* (PSv); those of *Methanogenium* were *marisnigri* (JR1m) and *cariaci* (JR1c); that of *Methanospirillum* was *hungatei* (JF1); and those of *Methanosarcina* were *barkeri* (MS, 227, and R1M3). All strains (*12*) have been described (*3*), except GC1 and ALI (which we isolated) and *Methanosarcina* strain R1M3 (isolated by M. P. Bryant). All strains were grown in M1 medium (*13*) or media recommended by Balch *et al.* (*3*) in an atmosphere of H_2 and CO_2 (50:50 or 80:20; pressure, 3 bars), except for *Methanosarcina barkeri* 227, which was grown as described (*14*). The anaerobic techniques have been described (*3, 15*). Bacteria were harvested by centrifugation and suspended in Formalin (4 percent by volume) in 0.85 percent NaCl. Rabbits were given two 1.5-ml doses (1 month apart) of a bacterial suspension (A_{660} = 0.6; 1 cm path) in phosphate-buffered saline, *p*H 7.2, emulsified in Freund's complete adjuvant. Serums were obtained 2 weeks after the second injection. Indirect immunofluorescence (*16*) was measured on heat-fixed smears with fluorescein isothiocyanate–labeled goat immunoglobulin to rabbit γ-globulin. Controls included (i) serum from rabbits injected only with Freund's complete adjuvant or serum from normal uninjected rabbits; (ii) omission of antiserum to methanogen; (iii) omission of labeled goat immunoglobulin; and (iv) omission of both. All controls were consistently negative. Each slide was coded and read by two independent observers. Antiserums were titrated against the immunizing methanogen, and the highest dilution giving a 4+ immunofluorescence reaction was used to determine the antigenic map of all strains and to detect cross-reactions. All 17 strains were tested with the 17 antiserums but only the reactive combinations are shown in a scale of 1+ to 4+ according to the immunofluorescence intensity as follows: 1+, +; 2+, ++; 3+, +++; and 4+, ■.

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with *Methanobrevibacter smithii* ALI, and only weakly with *Methanobacterium bryantii* MoH and MoHG, and *Methanobacterium thermoautotrophicum* ΔH. Such a pattern indicates that these last-mentioned four strains share a determinant which is immunodominant in PS but not in ALI, MoH, MoHG, and ΔH. This idea is supported in the case of strains MoH and ΔH by their failure to elicit antibodies that cross-react with strain PS. Other similarities in the bacterial-wall composition and architecture (*6*) may also be involved in determining cross-reactions.

Multiple assay with antibody probes proved useful for identification and classification of two new isolates, designated *Methanobacterium thermoautotrophicum* GC1 and *Methanobrevibacter smithii* ALI. The strain GC1 is morphologically and physiologically similar to the type strain *Methanobacterium thermoautotrophicum* ΔH, and the immunological typing supports the inclusion of GC1 in the same species as ΔH. Although strain ALI is morphologically and physiologically similar to both *Methanobrevibacter ruminantium* strain M1 and *Methanobrevibacter smithii* PS, it is more closely related immunologically to *Methanobrevibacter smithii* PS.

Immunological typing revealed other interesting features in relation to the classification of methanogens by Balch *et al.* (*3*). Although their 16*S* rRNA oligonucleotide catalogs showed no difference between *Methanosarcina barkeri* strains MS and 227, the strains are immunologically different. In contrast, while *Methanobacterium formicicum* strain MF, *Methanobrevibacter ruminantium* M1, and *Methanobrevibacter arboriphilus* strain DH1 are closely related to one or another of the Methanobacteriaceae according to the 16*S* rRNA catalog, they do not cross-react with any member of that family. The cross-reactions between *Methanospirillum hungatei* strain JF1 antibody and the two *Methanogenium* strains is noteworthy because the latter are marine forms that require NaCl for growth and differ in morphology from JF1. Our immunological evidence supports the family relationships of the genera indicated by the 16*S* rRNA catalog. Some pairs of cross-reactive strains [such as *Methanobacterium bryantii* MoH and MoHG (Fig. 1)] display similar patterns of reactivity. In such cases, antigenic identity can be confirmed or ruled out by using our antibody probes at other levels of sensitivity or by standard serial dilution analysis and selective adsorptions of the antiserums.

Our data support the recent classification of methanogenic bacteria based on information produced by molecular biology techniques (*3, 7*). Although those techniques are important for establishing phylogenetic relationships among taxa, immunological methods offer a simple and quick alternative for identification of new isolates and their assignment to a family and, in most cases, to a genus. Quantification of antigenic relatedness and elucidation of the molecular basis of the antigenicity of the methanogens' cell wall, by means of antibody probes, including monoclonal (*11*), should furnish valuable information for a critical analysis of the evolutionary significance of methanogens, including the "adaptive sense" (*8*) of their heterogeneity.

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Lower Eocene and Paleocene Gentianaceae: Floral and Palynological Evidence

Abstract. *Lower Eocene flowers with* Pistillipollenites macgregorii *pollen represent the earliest megafossil evidence of the Gentianaceae. The Paleocene occurrence of* P. macgregorii, *the fossil's modern floral structure, and suggested trends in the evolution of pollen in the Gentianaceae indicate a considerably earlier origin for the family. Floral morphology typical of bee-pollinated flowers provides the earliest, albeit indirect, fossil evidence of bees.*

Flowers allied with the Gentianaceae have been discovered in the Wilcox Formation (Lower Eocene) of northeastern Texas. The flowers have a 22-mm diameter, sympetalous, open, funnelform-salverform, seven-lobed corolla (Fig. 1a).

Pollen preserved within the anthers matches an unusual type of dispersed pollen: *Pistillipollenites macgregorii* (*1, 2*). Pollen grains isolated from the flowers are 22 μm in diameter, triporate, and are distinguished by gemmate ornamentation (Fig. 1, b and c). Pores are round to lolongate and are flanked by two elongate processes (Fig. 1c), possibly resulting from the fusion of gemmae, or are surrounded by discrete gemmae (*2*). Exine is intectate (Fig. 1b), except under the larger gemmae where there are short columellae (Fig. 1d). In areas with no ornamentation, the exine is 0.3 μm in thickness, and the largest gemmae are up to 2.4 μm in height. Although several staining techniques have been used on the fossil pollen, it is impossible to distinguish between the endexine and the ektexine or to determine whether the endexine is preserved at all.

Pistillipollenites has not been previously assigned to an extant family because it is a unique grain with no exact modern analog (*1, 2*). Pollen with similar gemmate ornamentation is now known in a few species of four extant families; namely, the Boraginaceae (in several species of the genus *Tournefortia*) (*3*), the Euphorbiaceae (in several species of the genus *Zimmermannia*) (*4*), the Papilionoideae (in one species of *Herpyza*) (*4*), and in the Gentianaceae (in four species of the genus *Macrocarpaea*) (*5–8*). Pollen of *Zimmermannia* is tricolporate (*4*); pollen of *Herpyza* has apertures independent of gemmae and a granular

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