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Lamina, a Novel Multicellular Form of *Methanosarcina mazei* S-6

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A novel multicellular form of *Methanosarcina mazei* S-6 is described. It was termed lamina, and it formed during the exponential growth phase when packets or single cells were grown in 40 mM trimethylamine and a total concentration of 8.3 to 15.6 mM Ca^{2+} and/or Mg^{2+}, in cultures that were not shaken. A distinct molecular event represented by the increment in expression and a spatial redistribution of an antigen during lamina formation is documented.

Methanosarcinae are the only members of the archaebacterial domain, Archaea (33, 34), for which uni- and multicellular forms have been observed (20, 26, 29, 31), suggesting an underlying complexity in gene regulation not hitherto observed in other archaebacteria. There are other features of methanosarcinae that remind one of higher organisms, such as the cell wall polymer methanochondroitin, which closely resembles eukaryotic chondroitin (14, 15). Also, *Methanosarcina* gene organization, although typically prokaryotic, shows conserved molecules involved in replication, transcription, and translation with a higher degree of homology to those of eukaryotes than to those of eubacteria (4, 33, 37).

Among methanosarcinae, *Methanosarcina mazei* S-6 (20, 21) is one of the better-studied species in pure culture concerning different morphologies—i.e., single cells and small and large packets (1, 12, 26, 27, 35). These morphologies have also been observed in complex ecosystems like anaerobic bioreactors (18). Different morphologies allow analysis from various perspectives. Packets of *M. mazei* S-6 can easily be monitored in different types of anaerobic bioreactors (18, 19), whereas single cells facilitate molecular biologic studies (12). In addition, the multicellular structures in *M. mazei* S-6 afford the opportunity to examine cell-cell interaction in archaebacteria.

The use of monoclonal antibodies has proven to be a powerful tool for examining developmentally regulated antigens in both prokaryotic and eukaryotic systems (5, 10). Investigations of the cell surface's architecture and antigenic mosaic of methanosarcinae have revealed antigens whose expression is associated with morphology (22, 23), and thus these antigens can be useful as markers in the study of temporal gene expression during morphologic changes. A multicellular form of *M. mazei* S-6, which we have termed lamina, and the temporal variations of a cell surface antigen (AgIc) paralleling the morphological rearrangements involved in lamina formation are described here. The word lamina best illustrates the structure's distinctive morphologic feature, i.e., its flat shape observed macro- and microscopically, its thickness being minimal in comparison with its length and width.

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Cells of *M. mazei* S-6 were grown at 37°C in S6-2 alpha medium (1, 3). The basal calcium ($CaCl_2 \cdot 2H_2O$) and magnesium ($MgCl_2 \cdot 6H_2O$) concentrations in the medium were 0.1 g/liter each, corresponding to 0.7 and 0.49 mM, respectively (Ca^{2+} and/or Mg^{2+}, 1.2 mM). For conditions with higher cation concentrations, Ca^{2+} and Mg^{2+} were added to the medium from sterile anoxic stock solutions.

The growth rate of cultures was determined by calculating the proportion of methane present in the total gas produced (2). The inoculum used for experiments to obtain lamina consisted either of packets maintained in 125 mM methanol (M) with 1.2 mM Ca^{2+} and/or Mg^{2+} or of single cells grown in trimethylamine (TMA) with the same cations at 5.4, 9.2, or 13.2 mM.

For the immunohistochemistry studies, cells were fixed in Telleysniczky's solution (1% of 37 to 40% formaldehyde, 90% of 70% ethanol, and 1% glacial acetic acid) for 2 h (28), embedded in 3% Bacto-Agar in phosphate-buffered saline, pH 7.2, and fixed overnight in Telleysniczky's solution. Agar-embedded samples were dehydrated in a graded distilled water-ethanol-xylene series and embedded in paraffin (11). Sections (5 μm) were obtained with a Spencer 820 microtome (American Optical Co., Buffalo, N.Y.), placed on glass slides (Cell Line Associates, Newfield, N.J.), and deparaffinized in a graded xylene-ethanol-distilled water series just before use.

Monoclonal antibody IC (MAbIC) generated and characterized as described previously (9, 17) was used for immunologic assays. The presence of antigen (AgIc) in sections of *M. mazei* S-6 was detected with MAbIC by immunohistochemistry and indirect immunofluorescence (6, 7, 13). Slides were incubated with MAbIC for 1 h, washed with distilled water, and then underwent a second 1-h incubation with fluorescein-conjugated goat anti-mouse immunoglobulin (Organon Teknika Cappel, West Chester, Pa.). Photomicrographs were taken with a Zeiss Axiophot microscope with bright-field, phase, or differential interference-contrast optics for fresh whole-cell preparations and with UV excitation for indirect immunofluorescence.

Macroscopic comparison of packets and lamina. Packets grown in M and a basal concentration of cations (M 1.2 medium) were transferred for more than 100 passages as stable stock packets that formed large aggregates (Fig. 1a). These packets were inoculated into medium containing 40

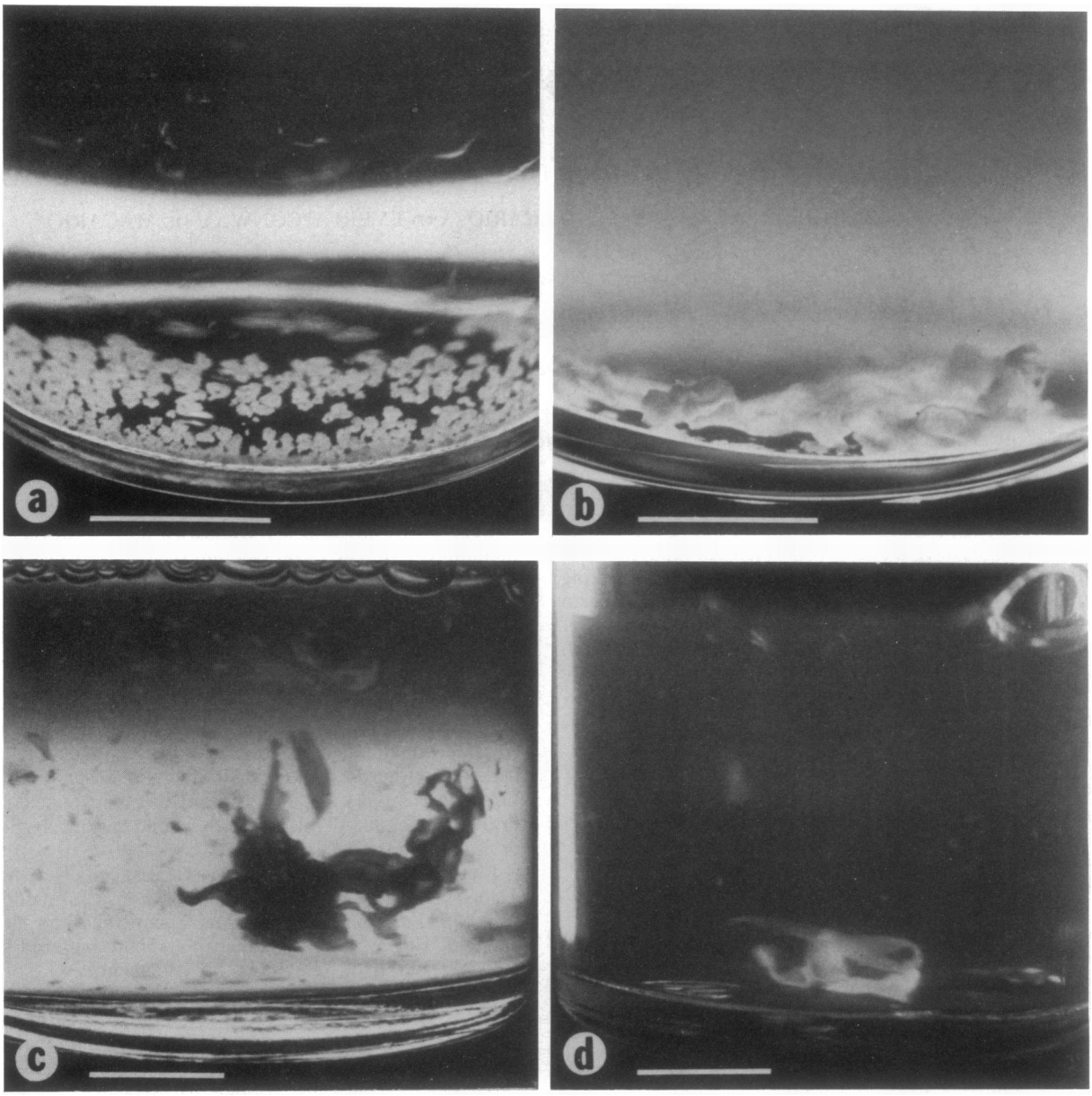

FIG. 1. *M. mazei* S6 forms in culture bottles (frontal view). (a) Packets; (b) lamina, kept in the same medium for several days, at which time dissociated cells and very small packets made the supernatant turbid; (c) lamina being swirled; (d) strip of lamina curled upon itself. Bars, 1 cm.

mM TMA and cations Ca^{2+} and/or Mg^{2+} at 9.2 mM (TMA 9.2 medium) and left undisturbed for 7 days. The lamina formed as a flat sheet (Fig. 1b), and at intervals during culture, the lamina contained gas bubbles and floated to the surface of the medium (Fig. 1c). The lamina that formed from cells that collected around the bottom edge of the bottle appeared as narrow curled-up strips (Fig. 1d). The color of the lamina was a light beige, similar to that of packets. The structural integrity of the lamina was resistant to gentle swirling, and although pieces would break off, it did not disperse as would a film of single cells. In cultures, the lamina could be maintained for days in the same medium.

Growth conditions for lamina formation. To identify some of the conditions needed for lamina formation, cultures were grown on M, acetate, or TMA, with a range of concentrations of Ca^{2+} and Mg^{2+} from 1.2 to 71.7 mM. The lamina was observed only in medium containing 40 mM TMA and when the total Ca^{2+} and Mg^{2+} concentration was between 8.3 and 15.6 mM, except when the inoculum was from cultures containing higher cation concentrations (see below). The lamina formed regardless of whether Ca^{2+} or Mg^{2+} was the predominant cation or they were present in similar concentrations.

Lamina formation occurred during the exponential phase of culture growth as measured by methane production (data not shown). Cultures inoculated with either single cells grown in TMA medium or packets grown in M medium formed laminae in 2 and 6 days, respectively, but the growth rates were similar. Once formed, the lamina increased in size up to 2 to 3 cm by 1 cm by 2 mm and remained stable for 6

TABLE 1. Lamina formation from the three different forms of *M. mazei* S-6

Inoculum form	Culture age[a]	Medium transferred to		Days to formation[b]	Structure formed[c]
		Substrate	Ca^{2+} and/or Mg^{2+} (mM)		
Lamina in TMA 9.2 medium	12	TMA	9.2	2	Lamina
	16	TMA	9.2	2	Lamina
	11	TMA	9.2	2	Lamina
	11	TMA	1.2	2	Single cells
	11	M	9.2	16	Intermediate packets
	11	M	1.2	11	Reverted packets
Single cells in:					
TMA 13.2 medium	7	TMA	4.6	4	Lamina
	7	TMA	13.2	3	Lamina
TMA 4.6 medium	7	TMA	4.6	—	Single cells
	7	TMA	13.2	3	Lamina
Reverted packets in M 1.2 medium	7	M	1.2	—	Reverted packets
	9	TMA	9.2	3	Lamina
	7	TMA	9.2	4	Lamina

[a] Days of inoculum culture at time of transfer.
[b] —, form did not change.
[c] Intermediate packets look flaky compared with stock packets. Reverted packets look the same as typical stock packets.

to 11 days. Disaggregation into viable single cells occurred after the end of exponential growth.

The formation of the lamina was repeated more than 80 times with inoculum packets from different stock culture bottles which ranged in age from 6 to 45 days from previous passage and which had been passaged exclusively as packets prior to the experiments. The time needed for lamina formation varied from 6 to 8 days. Lamina formation from single cells was also routinely repeated with different inocula of single cells and laminae (see below).

Transfer of the lamina. Laminae were transferred to several media. First, when transferred to the same TMA 9.2 medium, the pieces which had been sheared in the needle during transfer re-formed into a large lamina within 2 days (Table 1). While transfer of the lamina to TMA 1.2 medium resulted in disaggregation within 2 days and a subsequent lack of single-cell growth, when laminae were transferred to M 9.2 medium, intermediate packets formed by 16 days, resembling typical packets. Transfer of the intermediate packets to TMA medium resulted in lamina re-formation, whereas transfer to M medium resulted in reverted packets (not shown).

Single cells transferred from cultures containing lamina (TMA 13.2 medium) were able to re-form lamina in 3 to 4 days whether placed in TMA 4.6 or TMA 13.2 medium (Table 1). In contrast, single cells newly released from packets in TMA 4.6 medium did not form lamina when transferred to TMA 4.6 medium but did so consistently when transferred to TMA 13.2 medium.

Transfer of the lamina to M 1.2 medium resulted in reverted packet formation by 11 days. These reverted packets continued to grow as packets after 4 days when transferred to the same medium but returned to lamina after 3 days when grown in TMA 9.2 medium (Table 1).

Light microscopy. The lamina appeared as a flat structure with discrete edges made of very small packets and cells firmly enmeshed in a sheet of intercellular connective material (ICMA). Packets and cells could be seen more clearly when sloughed off between rips of the lamina or in disrupted portions of its outer edge (Fig. 2).

Expression of antigen AgIc. The cell surface antigen (AgIC) of *M. mazei* S-6, recognized by a monoclonal antibody (MAbIC) and present on both packets and single cells, showed a different pattern of expression between these morphologic forms. This AgIC is also present in lamina.

Paraffin-embedded sections of packets and lamina, harvested at different ages and stages of lamina formation and prepared for immunohistochemistry, were reacted with MAbIC. The cell surface antigen AgIc was found to be present both in inoculum packets and through lamina formation but showed an increase in expression and a spatial redistribution over the growth period observed. The sections of 21-day inoculum packets at day 1 showed a moderately intense reaction with MAbIC. The positive reaction on the packets was more intense toward the outer edges, appearing as lines and granules of fluorescence (Fig. 3a). In contrast, early in lamina formation, the blankets of confluent packets and individual cells showed an intense, textured reaction with MAbIC (Fig. 3b). By 10 days, the lamina had solidified and the intensely positive reaction was discretely distributed around the cells. The reaction was heterogeneous, being positive in some areas and not in others (Fig. 3c). The negative controls, tested with diluent rather than MAbIC, were negative (Fig. 3d).

Other *Methanosarcina* species. Two other *Methanosarcina* species, *M. barkeri* 227 and *M. vacuolata* Z-761, and one other strain of *M. mazei*, strain LYC, were tested for lamina formation. No lamina was observed under the same conditions tested for *M. mazei* S-6.

The lamina was reproducibly formed and was transferable, indicating that it is a stable structure that can be obtained under defined conditions, including the condition that the culture remain undisturbed.

Lamina is different from other multicellular forms found in methanosarcinae, which consist of a more homogeneous population of cells in globular packets (16, 20, 26, 29, 31, 35). Lamina is a flat sheet or meshwork of connected miniature packets and individual cells of different sizes and shapes, many of which seem to be actively dividing. These are held together by a fibrillar ICMA in a tertiary structure holding

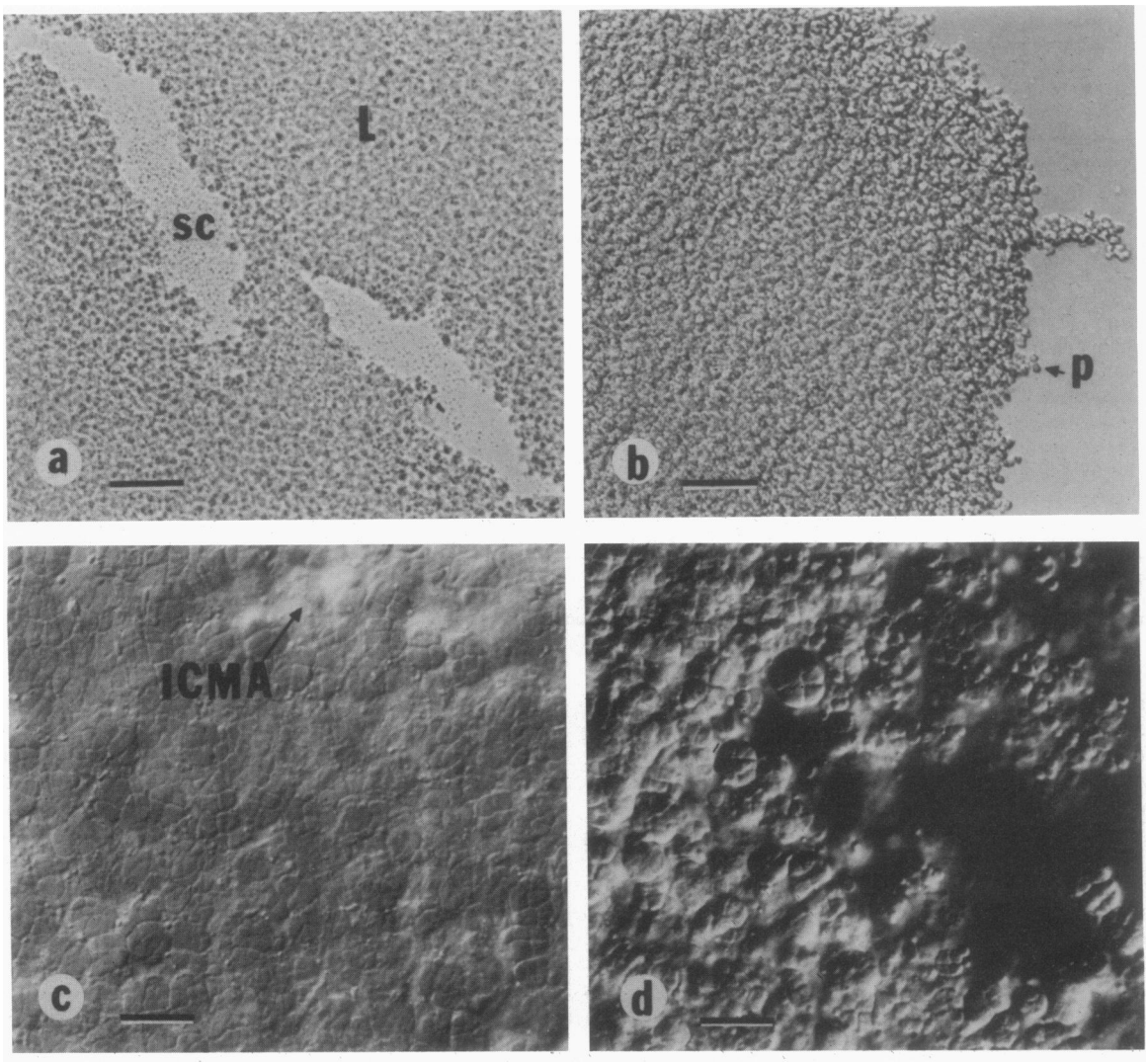

FIG. 2. Panoramic view of lamina (L) showing two rips and outer edge with sloughed off cells (sc) and very small packets (p). (a) Bright field; (b) differential interference-contrast. Higher magnification shows the compact texture of an intact lamina with ICMA firmly holding very small packets and cells (c), which can be distinguished at a disrupted edge (d). Bars, 50 μm (a and b) and 10 μm (c and d).

everything together and making it resistant to disruption. Although the molecular mechanism of lamina formation from packets or single cells is yet to be determined, it can be hypothesized that the concentration range of Ca^{2+} or Mg^{2+} between 8.3 and 15.6 mM is optimal for facilitating cell-ICMA-cell interactions. This cation concentration may also be optimal for inhibition of the synthesis or the activity of disaggregatase (the enzyme involved in packet disaggregation [36]) or both. Studies of other prokaryotic and eukaryotic systems also demonstrate that cation-dependent cell interactions operate only within a certain range of cation concentrations (8, 24, 30).

The lamina formed only when the substrate was TMA, which contains amine groups that M and acetate do not have. What effect amine groups may have is not clear, but there appears to be a relationship between the metabolism of TMA and lamina formation, which is perhaps stabilized by critical concentrations of cations. The lamina was formed during the exponential growth phase of the culture, indicating that its formation is not a starvation response. Its disaggregation after the end of exponential growth could be caused by nutrient exhaustion, a pH shift, a buildup of metabolic waste products, such as ammonia from TMA, or depletion of a factor that inhibits disaggregatase, since the lamina could be maintained intact by transfer to fresh media.

Since the lamina does not form when cultures are shaken, cell physical contact with the ICMA may trigger a critical signal. During lamina formation, extended cell contact may stimulate the production of factors not present otherwise. The continued presence of such factors (residual gene products) in the cells might explain why reverted packets and single cells from the lamina are able to revert to lamina in half the time required for stock packets and single cells that have never been in the lamina form.

When the lamina is formed exclusively from single cells, there is a buildup of ICMA where there was none before, and only at later stages do incipient packets begin to appear, each likely the progeny of individual single cells. Furthermore, laminae also occur in complex ecosystems as part of microbial consortia (19). The presence of laminae in these

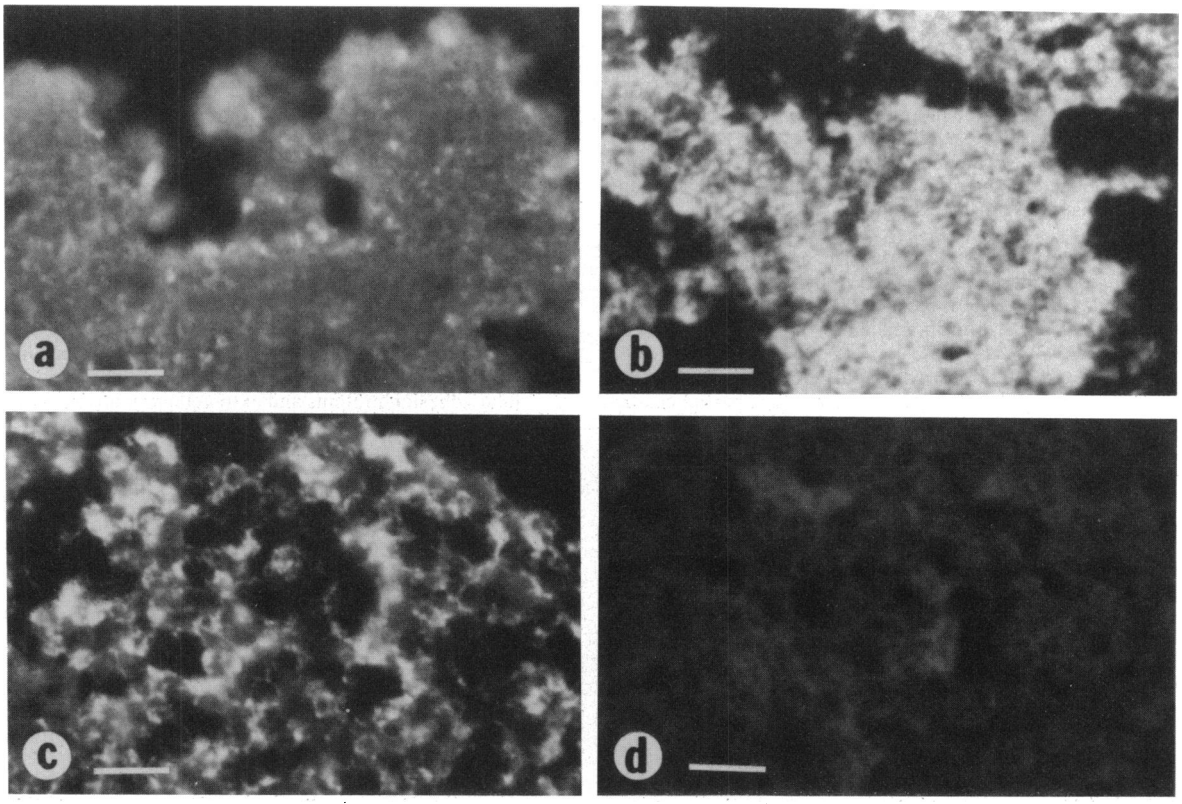

FIG. 3. Mapping of the spatial distribution of AgIc in fixed sections of *M. mazei* S6 at progressive stages of the lamina formation as shown by indirect immunofluorescence using MAbIC. (a) Day 1 inoculum packets; (b) day 7 lamina; (c) day 10 lamina; (d) negative control (day 10 lamina, no MAbIC added). Bars, 10 μm.

ecosystems can be explained only if it is a physiologic structure evolved by methanosarcinae to better adapt to certain environmental conditions.

The cell surface and the amount and content of the extracellular matrix change with time, suggesting biochemical changes likely controlled by differential gene expression. One indication of this may be seen in the pattern of expression of the surface protein antigen AgIC. Its presence and function have yet to be determined, but its temporal variations and distinctive distribution patterns in the lamina make it a promising candidate for further study.

Alteration of culture conditions has been shown to induce developmental changes, such as in *Dictyostelium discoideum*, in which there are several factors, both endogenous (pH and NH_3, cyclic AMP, and Ca^{2+} concentrations) and exogenous (ionic composition of the medium, temperature, and nutrient availability), that stimulate development (32). In the gram-negative, soil-dwelling bacterium *Myxococcus xanthus*, nutrient depletion induces a developmental response that results in the formation of a complex fruiting body containing myxospores (10, 30). In *Caulobacter crescentus*, another gram-negative bacterium, response to chemotactic signals initiates a developmental program resulting in the formation of structurally different progeny: swarmer and stalk cells (25).

It would be interesting to study an archaebacterial model that might display rudimentary developmental pathways in which response to a signal could result in the decision to switch morphology and perhaps a host of other characteristics, accompanied by changes in gene expression, all occurring under anaerobiosis. Previous studies with *Methanosarcina* species have shown that switching between packets and single cells can be manipulated by changing the culture conditions (31, 35), but *M. mazei* S-6 is unique in that the morphologic conversions occur in the same medium, as shown in this report. In addition, monoclonal antibodies are already available and can be used to monitor the temporal expression of antigens throughout these morphologic transformations.

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