Adaptation for Growth at Various Saline Concentrations by the Archaeabacterium *Methanosarcina thermophila*

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We report the ability of *Methanosarcina thermophila* TM-1 to adapt and grow in media containing NaCl concentrations of 0.005 to 1.2 M. When adapted to marine NaCl concentrations, this species ceased to produce the heteropolysaccharide outer layer typically formed by species of nonmarine origin. Concomitant with this adaptation, *M. thermophila* ceased to grow as multicellular aggregates and existed solely in single-cell form. The sodium ion concentration was critical for the adaption process, although magnesium ion appeared to contribute to the cell wall stability of single cells. The results suggest that these archaeabacteria possess regulatory systems that enable them to adapt to environments with a wide range of saline concentrations.

The methanogenic archaeabacteria are one of the earliest divergences of extant life forms, and several lines of evidence suggest that these microorganisms represent a third kingdom with characteristics of both eubacteria and eucaryotes (2, 6, 8). The methanogens that use acetate, which include the *Methanosarcina* spp., have a pivotal role in methanogenic food chains, because up to 70% of the methane produced from polymer degradation is derived from the methyl moiety of acetate (4, 7, 11, 16). Despite their relative importance in anaerobic degradation, only limited progress has been made toward understanding the physiology and genetics of these species. The technical difficulties in these studies are largely due to the production of a thick heteropolysaccharide outer layer surrounding most species of *Methanosarcina*. The resilient outer layer promotes the aggregation or clumping of cells, which prevents plating of individual cells for mutant selection, formation of protoplasts, and the gentle cell lysis required for isolation of intact DNA and RNA. Only one marine species, *Methanosarcina acetivorans*, does not produce a heteropolysaccharide outer cell layer (17). All other reported species maintain a heteropolysaccharide layer throughout growth, with the exception of *Methanosarcina mazel*, which hydrolyzes the heteropolysaccharide layer during certain stages of growth (5, 10, 13, 14). We report here the ability of *Methanosarcina thermophila* to grow as single cells and remain viable without production of the heteropolysaccharide outer layer when adapted to marine NaCl concentrations.

*M. thermophila* TM-1 was maintained in basal salts medium that contained less than 0.005 M NaCl (19) for at least 5 years. Sterile media were prepared under an N2–CO2 (4:1) atmosphere by a modification of the Hungate technique (2). Low-saline medium contained the following constituents, in grams per liter (final concentration), in demineralized water: trimethylamine hydrochloride, 4.8; Na2CO3, 3.0; Na2HPO4, 0.60; NH4Cl, 0.50; cysteine hydrochloride·H2O, 0.25; Na2S·9H2O, 0.25; MgCl2·6H2O, 0.10; CaCl2·2H2O, 0.10; and resazurin, 0.001. In addition, 1% (vol/vol) each of vitamin and trace element solutions were added (20). Marine medium consisted of the low-saline medium with the following additions, in grams per liter: NaCl, 23.36; MgCl2·6H2O, 10.07; KCl, 0.76; CaCl2·2H2O, 0.04. The final pH of the media was 6.8. Portions (10 ml) of the media were anaerobically dispensed into culture tubes (16 by 150 mm), and the tubes were sealed with butyl rubber septa secured by aluminum crimp collars. Low-saline and marine cultures were incubated at 55 and 45°C, respectively, unless otherwise indicated. Growth was monitored by measuring the exponential increase in methane production with a gas chromatograph (Varian model 3300) equipped with a silica gel-packed column and a flame ionization detector.

*M. thermophila* formed large multicellular aggregates when grown at 45°C in medium with a low NaCl concentration (Fig. 1A and 2A), as previously described (22, 23). The appearance of the cultures changed when inocula from late-exponential-phase liquid cultures were transferred to marine medium. After a lag of 1 to 3 weeks, an exponential increase in methane production occurred in all cultures; this coincided with an increase in turbidity that resulted from the formation of single cells (Fig. 1B and 2B). When sodium dodecyl sulfate detergent was added to the marine cultures, the single cells lysed and the culture became clear. Only a few multicellular aggregates remained in the cultures. The presence of detergent-sensitive cells of *M. thermophila* is consistent with the loss of the heteropolysaccharide outer layer, since *Methanosarcina* spp. that synthesize heteropolysaccharide walls are not susceptible to lysis by detergents (10). Multicellular aggregates were no longer observed in cultures after a second serial transfer in marine medium. *M. thermophila* grew exponentially as single cells in marine medium and remained viable after 10 serial transfers.

Thin-section electron micrographs of *M. thermophila* grown as aggregates in low-saline medium showed that cells were surrounded by a thin monolayer, adjacent to the cytoplasmic membrane, and an outer heteropolysaccharide layer (Fig. 2C and E) (23). In contrast, *M. thermophila* grown in marine medium retained the thin monolayer but did not produce the heteropolysaccharide outer layer (Fig. 2D and F). To test for the presence of heteropolysaccharide components, gluconic acid, which comprises 40% of the outer layer of *M. thermophila* (9), was measured by a modification of the carbozole colorimetric assay for uronic acid (3, 10). Cell pellets were obtained by centrifugation at 10,000 × g for 15 min. A 1-ml sample was boiled in concentrated sulfuric acid for 10 min, carboxze was added, and the mixture was boiled for an additional 15 min. Absor-

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with a previous report that divalent magnesium cations are required to maintain the protein cell wall integrity of the marine species *M. acetivorans* (17). When the polyvalent amine spermine was substituted for MgCl₂ in low-saline medium that contained single cells of *M. thermophila*, there was only slight lysis and the cells remained viable. This observation suggests that Mg²⁺ is required to maintain the structural integrity of the single-cell form of *M. thermophila*. Mg²⁺ was also reported to promote the viability of the disaggregated form of *M. mazei* (5). The lack of an Mg²⁺ requirement by aggregated cells grown in low-saline medium suggests that the integrity of the inner wall layer is maintained by the heteropolysaccharide outer layer. The contrasting response to NaCl by cells pregrown in low-saline or marine medium suggests that NaCl is involved in the physiological adaptation for growth under the two conditions. To determine if the adaptation was Na⁺ or Cl⁻ specific or only osmotic, marine-grown *M. thermophila* was inoculated into media with either LiCl, Na₂SO₄, or sucrose substituted for NaCl at equimolar concentrations. Growth was inhibited in medium that contained sucrose, but equivalent growth was observed in medium that contained Na₂SO₄ or NaCl. LiCl supported slightly slower growth of *M. thermophila*. This observation is consistent with the ability of Li⁺ to be substituted for Na⁺ in bioenergetic studies (15). These results suggest that Na⁺ is involved in the transition between the two physiological forms.

The two phenotypically distinct forms of *M. thermophila* were tested for other physiological differences. The maximum temperature for growth of the aggregated form in low-saline medium was 55°C, as previously reported (22), but the maximum temperature for growth of the single-cell form in marine medium was 45°C. The higher temperature maximum of the aggregated form may be due to a stabilizing effect by the heteropolysaccharide outer layer on the protein cell wall. Doubling times of *M. thermophila* grown in low-saline medium at 55°C and in marine medium at 45°C were 6.3 and 5.3 h, respectively. The differences in growth rates between the two forms may reflect slower diffusion of substrate into the large aggregates compared with diffusion into the individual cells and a greater cellular energy requirement for heteropolysaccharide synthesis.

Various concentrations of NaCl were tested for their effect on the growth of low-saline- and marine-adapted forms of *M. thermophila* (Fig. 3). Cells pregrown in marine medium grew optimally at NaCl concentrations between 0.4 and 0.8 M but were inhibited at concentrations above or below these concentrations. However, cells pregrown in marine medium lysed when inoculated into media that contained concentrations of sodium chloride lower than 0.1 M. Spermine (1 mM) was added to the test media to minimize lysis of marine-grown cells inoculated into media that contained low saline concentrations (12). In contrast, clumped cells pregrown in low-saline medium were inhibited by NaCl concentrations above 0.1 M, with no growth occurring at 0.6 M.

The transition between the single-cell and aggregated-cell forms was also characterized in further detail. After transfer into media containing 0.2 to 0.4 M NaCl, the low-saline-adapted cell aggregates formed single cells after approximately 7 days. *M. thermophila* that was adapted to growth in marine medium with the concomitant loss of the heteropolysaccharide outer layer could be conditioned to grow in low-saline medium by serial transfers in media that contained decreasing NaCl concentrations. However, the growth rates were slower and the formation of viable cell aggregates from single cells was only occasionally observed.

**FIG. 1.** Cultures of *M. thermophila* grown in low-saline (A) and marine (B) media. Cells incubated in the low-saline medium at 55°C for 5 days formed clumps that settled to the bottom of the tube, leaving a clear supernatant. Cells incubated in the marine medium at 45°C for 3 days became optically dense with growth and remained suspended in the medium.
Attempts to reproducibly obtain aggregates from single cells under various physiological conditions were unsuccessful. To demonstrate that the cultures did not contain a mixed population of single cells and aggregates, cultures of aggregates that contained low-saline medium were pregrown at 55°C, which would kill any single cells present in the population. When a 10% inoculum of this culture was transferred to marine medium and incubated at 45°C, the transition to single cells was observed. These results demonstrate that the single cells formed from the aggregated cells of *M. thermophila*.

Most bacteria require a rigid cell wall to prevent lysis when their internal osmotic pressure is high relative to the external environment. One role of the heteropolysaccharide layer produced by *M. thermophila* grown in low-saline medium may be prevention of cell lysis under these condi-

**FIG. 2.** Phase-contrast and thin-section electron micrographs of *M. thermophila*. Phase-contrast micrographs of *M. thermophila* show the large multicellular clumps that form in low-saline medium (A) and the single-cell form that is observed in marine medium (B); bars, 10 μm. Thin-section electron micrographs of cells grown in low-saline medium show the close association of cells in multicellular clumps (C), while cells grown in marine medium lack the heteropolysaccharide outer layer (D); bars, 5 μm. High-magnification thin-section electron micrographs of the cell-medium interfaces of low-saline-grown (E) and marine-grown (F) cells show the cytoplasmic membrane (CM), the cell wall layer (CW), and, in the case of low-saline-grown cells, the heteropolysaccharide layer (HL); bars, 0.1 μm. Phase-contrast micrographs were made with an Olympus BH-2 microscope. Thin-section electron micrographs were prepared as previously described (18). Cells were fixed with 2% glutaraldehyde and 2% osmium tetroxide, dehydrated in a graded series of aqueous ethanol mixtures, and embedded in plastic resin for sectioning. Electron micrographs were made with a Hitachi H7000 electron microscope.
FIG. 3. Effect of NaCl concentration on the growth rates of *M. thermophila* pregrown in low-saline (○) and marine (●) media. Data were prepared as described in the text with the following modifications. K$_2$CO$_3$ and KH$_2$PO$_4$ were substituted for Na$_2$CO$_3$ and Na$_2$HPO$_4$, respectively, at equimolar concentrations. Spermine (1 mM) was added to stabilize the single-cell form at low saline concentrations (12). The final Na$^+$ concentration included Na$_2$S contained in the medium. Low-saline inoculum was pregrown to late exponential phase at 55°C; marine inoculum was pregrown to late exponential phase at 45°C. Results are the means of triplicate cultures.

The results of this study indicate that *M. thermophila* has regulatory mechanisms that allow this species to grow optimally in a wide range of saline concentrations. The results also suggest that synthesis of the heteropolysaccharide layer is regulated by *M. thermophila* in response to these different environments. Preliminary evidence indicates that some strains of *M. Barkeri* and *M. mazei* also adapt to high saline concentrations with concomitant loss of the heteropolysaccharide outer layer. The physiological adaptation to high saline concentrations, in conjunction with the absence of the heteropolysaccharide outer layer, is consistent with the close phylogenetic relationship between *M. acetivorans*, the marine species, and the other *Methanosarcina* spp. of non-marine origin (19). These characteristics indicate that environmental niches for this group may be less restricted than previously assumed. Studies are currently being conducted to determine how widespread this phenomenon is among the *Methanosarcina* spp.

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