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Genes and derived amino acid sequences of S-layer proteins from mesophilic, thermophilic, and extremely thermophilic methanococci

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Abstract Cells of methanococci are covered by a single layer of protein subunits (S-layer) in hexagonal arrangement, which are directly exposed to the environment and which cannot be stabilized by cellular components. We have isolated S-layer proteins from cells of *Methanococcus vannielii* ($T_{opt.}$ = 37°C), *Methanococcus thermolithotrophicus* ($T_{opt.}$ = 65°C), and *Methanococcus jannaschii* ($T_{opt.}$ = 85°C). The primary structure of the S-layer proteins was determined by sequencing the corresponding genes. According to the predicted amino acid sequence, the molecular masses of the S-layer proteins of the different methanococci are in a small range between 59,064 and 60,547 Da. Compared with its mesophilic counterparts, it is worth noting that in the S-layer protein of the extreme thermophile *Mc. jannaschii* the acidic amino acid Asp is predominant, the basic amino acid Lys occurs in higher amounts, and Cys and His are only present in this organism. Despite the differences in the growth optima and the predominance of some amino acids, the comparative total primary structure revealed a relatively high degree of identity (38%–45%) between the methanococci investigated. This observation indicates that the amino acid sequence of the S-layer proteins is significantly conserved from the mesophilic to the extremely thermophilic methanococci.

Key words Archaea · *Methanococcus* · Cell walls · S-layer · Amino acid sequence · Thermophiles

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Dedicated to Prof. Dr. Karl Otto Stetter on the occasion of his 60th birthday

Introduction

Two-dimensional crystalline protein or glycoprotein arrays (S-layers) represent the outermost cell wall layer in about 350 different species of the prokaryotic domains Bacteria and Archaea so far investigated. S-layers consist of single (glyco-)protein species with molecular masses ranging from about 40 to 170 kDa, which form lattices of oblique, tetragonal, or hexagonal architecture. Depending on the growth conditions, some microorganisms can also produce different surface proteins (Boot and Pouwels 1996; König and Messner 1997; Messner and Sleytr 1992). Structural differences may also be found as a consequence of post-translational modifications such as glycosylation, transfer of phosphate and sulfate groups, or proteolytic processing of the proteins (Sleytr 1997; Sára and Sleytr 2000). With a few exceptions, which include *Lactobacilli* and the Archaea *Methanothermus fervidus* and *Methanothermus sociabilis* (Bröckl et al. 1991), S-layer proteins have weakly acidic isoelectric points (Sleytr 1997; Sára and Sleytr 2000).

Depending on their location on the cell surface and their stability, S-layers fulfill quite different functions. They form protective coats, maintain the shape, and direct cell division, function as molecular sieves and attachment sites for extracellular enzymes, and represent virulence factors (Beveridge et al. 1997; Sleytr and Beveridge 1999). Representing the outermost cell envelope layer, they are directly exposed to the often extreme conditions of their environment. As a consequence, intrinsic resistances against environmental stresses such as high salt, acidity, and temperature should be attributed to archaeal S-layers. The molecular mechanisms for this high stability are only poorly understood. Members of the genus *Methanococcus* living in

mesophilic, thermophilic, or extremely thermophilic environments represent an ideal model system for comparative analyses of their S-layers as a focus for thermal adaptation. Hitherto, only the gene sequence of the S-layer protein of the mesophilic methanogen *Methanococcus voltae* has been published (Dharmavaram et al. 1991; Konisky et al. 1994). It was the aim of this study to compare the genes and derived amino acid sequences of S-layer proteins of methanococci living under different thermal conditions.

Materials and methods

Organisms and culture conditions

Methanococcus (*Mc.*) *vannielii* (DSM 1224), *Methanococcus* (*Mc.*) *thermolithotrophicus* (DSM 2095), and *Methanococcus* (*Mc.*) *jannaschii* (DSM 2661) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Cells were cultivated in pressurized serum bottles (H_2:CO_2 = 80:20) using the technique described by Balch et al. (1979). *Mc. vannielii* and *Mc. thermolithotrophicus* were grown in DSM medium 141 (DSMZ) without acetate, yeast extract, and tryptone at 37° and 65°C, respectively. *Mc. jannaschii* was cultivated in DSM medium 282 (DSMZ) at 85°C.

Isolation of the S-layer and S-layer proteins

S-layers were isolated from cells by the procedure described by Nusser and König (1987), slightly modified. The cells were disrupted by sonication, followed by incubation with DNase/RNase and extraction with Triton X-100 (S-layer preparation); the chloroform-methanol extraction step, however, was omitted.

Soluble S-layer proteins released from cells during growth could also be easily isolated from the culture medium in significant amounts. Aliquots (20 ml) of the culture medium (three replicates) were taken from 200-ml cultures at different growth phases. The cells were pelleted by centrifugation (15,000 *g*, 4°C). The proteins in the supernatants were precipitated with two volumes of acetone (–20°C, overnight). The proteins from the cell pellet and culture medium were redissolved in an SDS solution (1%, w/v; 0.5 ml) and aliquots were applied on SDS polyacrylamide gels. The gels were photographed (Polaroid GDS system) and the relative intensities of protein bands were estimated using scanning software (TINA 2.1; Raytest Isotopenmessgeräte, Berlin, Germany).

Polyacrylamide gel electrophoresis

SDS polyacrylamide gels were prepared according to the method of Laemmli (1970). Electrophoresis was carried out at 100 V until the marker dye, Bromphenol Blue, reached the end of the gel. Gels were also run with 4 M urea instead of SDS in the electrophoresis buffer. The gels were stained for protein with Coomassie Blue R-250. The carbohydrate staining was performed using periodate–Schiff reagent (Segrest and Jackson 1972).

Electroelution

For analytical purposes S-layer proteins were eluted from polyacrylamide gel slices with a gel elution apparatus (BioRad Model 422, Munich, Germany). Elution was carried out at 10 mA per tube for 16 h with a 0.4% $(NH_4)_2CO_3$ solution (pH 8.5) containing 0.1% SDS.

Isoelectric focusing

Purity and the isoelectric point (pI) of S-layer proteins were checked with analytical polyacrylamide gels (Precotes 3–10; Serva, Heidelberg, Germany). Large-scale purifications of protein preparations were performed by preparative isoelectric focusing (IEF) (Rotofor cell; BioRad) in the presence of 4 M urea and 2% ampholytes (Servalyte 3–6; Serva) at 15 W and 10°C.

Protein-blotting

Proteins were transferred overnight at 4°C from SDS polyacrylamide gels to polyvinylidene fluoride membrane (PVDF; Millipore no. IPVH 10100) with a tank blot apparatus (Mini transblot electrophoretic transfer cell; BioRad). Blotting was conducted with 48 mM Tris buffer (pK 9.2) containing 39 mM glycine and 0.1% SDS at 30 V and 4°C for 16 h. Before transfer, proteins were visualized on the gels with copper stain (copper staining and destaining kit; BioRad).

Protein sequencing

The N-terminal amino acid sequences of S-layer proteins blotted from SDS polyacrylamide gels onto PVDF membranes were determined by Edman degradation with a protein sequencer (model 464; Applied Biosystems). In order to obtain internal sequences, purified proteins were also digested with specific endoproteinases (peptide mapping set; Boehringer Mannheim, Germany) before electrophoresis and blotting.

DNA sequencing

On the basis of conserved regions at the 5′-ends and within the published presumptive S-layer genes of *Mc. jannaschii* and *Methanococcus* (*Mc.*) *voltae*, oligonucleotide primer (forward: 5′-TGGCAATGAGCTTAAANAAAATCG-3′ and 5′-GACGTTGTTTCAGCTGC-3′, reverse: 5′-CTCTG TCKCCACCAG-3′) were constructed for a PCR sequencing strategy. The template DNA was isolated from the *Mc.*

vannielii and *Mc. thermolithotrophicus* cells with the aid of specific coated magnetic beads (DNA direct system I; Dynal, Hamburg, Germany). In a first sequencing step, about one-third of nucleotide bases of the whole PCR products (approx. 1,400 bp) could be sequenced (ABI PRISM 377; Genterprise, University of Mainz, Germany). In consecutive sequencing steps, the obtained nucleotide sequences were used for the construction of new sequencing primers. By using this strategy we obtained almost the complete sequence of the PCR products. The residual gene sequences in the 3′ and 5′ directions were completed by a two-step PCR using specific internal biotinylated and degenerated flanking primers (Sørensen et al. 1999). Specific PCR products were isolated with the aid of streptavidin-coated magnetic beads and a magnet-holding device (Dynabeads M-280 Streptavidin; Dynal MPC-E/E-1; Dynal, Hamburg, Germany). The PCR assays contained in 100 µl PCR buffer: 50 pmol oligonucleotide primer, 200 nmol $MgCl_2$, 15 nmol dNTP mix, 2.5 units of Taq DNA polymerase, and 2 µl template DNA. The standard PCR was programmed with an average of 35 amplification cycles (Thermocycler, Progene, Techne) as follows: denaturation step 1, cycle 5 min 95°C; 35 further cycles 1.5 min 94°C; 2 min at the appropriate annealing temperature (depending on the primer sequence); extension: 2 min 72°C, last cycle 10 min 72°C.

Gene sequence analysis

The following software and databases were used for the analysis of the nucleotide and amino acid sequences: (1) EMBL, TIGR, BLAST (comparison of amino acid sequences); (2) CLUSTAL W, BLAST 2.0 (alignment of nucleotide and amino acid sequences); (3) ExPASY-Translate tool (translation of nucleotide in amino acid sequences); (4) ExPASY-ProtParam tool, PREDICT and Signal P (predictions about protein structure). The S-layer genes included in the comparative analysis are compiled in Table 1.

Table 1. Comparison of the deduced amino acid sequence of archaeal S-layer proteins[a]

Species with S-layer (glyco-)proteins (growth optimum temperature; accession no.)	*Mc. vol.*	*Mc. van.*	*Mc. lit.*	*Mc. jan.*	*Pc. aby.*	*Pc. hor.*	*Ms. maz.*	*Mb. the.*	*Mt. fer.*	*Ag. ful.*	*St. the.*	*Hb. ha.*	*Hf. vol.*	*Ha. jap.*
Mc. voltae (37°C; M59200)		44 (59)	48 (61)	38 (54)	23 (38)	28 (44)	–	–	–	–	–	–	–	–
Mc. vannielii (37°C; AJ308553)	47 (60)		49 (62)	44 (59)	24 (38)	31 (45)	–	–	–	–	–	–	–	–
Mc. thermolithotrophicus (65°C; AJ308554)	50 (63)	49 (61)		53 (69)	26 (41)	29 (41)	–	–	–	–	–	–	–	–
Mc. jannaschii (85°C; MJ0822)	40 (56)	44 (60)	53 (69)		25 (37)	33 (49)	–	–	–	–	–	–	–	–
Pc. abyssi (97°C; PAB1861)	24 (39)	25 (39)	26 (41)	26 (40)		79 (81)	–	–	–	–	–	–	–	–
Pc. horikoshii (95°C; PH1395)	27 (41)	32 (45)	29 (41)	29 (46)	79 (87)		–	–	–	–	–	–	–	–
Ms. mazei (37°C; S502210)	–	–	–	–	–	–		28 (43)	30 (40)	28 (42)	–	–	–	–
Mb. thermoautotrophicum (65°C; AAB85224)	–	–	–	–	–	–	26 (43)		30 (40)	27 (41)	–	–	–	–
Mt. fervidus (85°C; X58297)	–	–	–	–	–	–	26 (36)	28 (38)		37 (59)	–	–	–	–
Ag. fulgidus (85°C; AF1413)	–	–	–	–	–	–	26 (41)	34 (50)	25 (40)		–	–	–	–
St. marinus (92°C; S68553)	–	–	–	–	–	–	–	–	–	–		–	–	–
Hb. halobium (37°C; P08198)	–	–	–	–	–	–	–	–	–	–	–		83 (83)	52 (66)
Hf. volcanii (37°C; P25062)	–	–	–	–	–	–	–	–	–	–	–	37 (53)		40 (57)
Ha. japonica (37°C; D87290)	–	–	–	–	–	–	–	–	–	–	–	52 (66)	40 (56)	

[a]Alignments of amino acid sequences (BLAST 2.0)
Data are given as numbers of identity or similarity values (brackets) of the aligned regions
– No significant homology found by BLAST 2.0
Genus abbreviations: *Mc.* = *Methanococcus*, *Pc.* = *Pyrococcus*, *Ms.* = *Methanosarcina*, *Mb.* = *Methanobacterium*, *Mt.* = *Methanothermus*; *Ag.* = *Archaeoglobus*, *St.* = *Staphylothermus*, *Hb.* = *Halobacterium*, *Hf.* = *Haloferax*, *Ha.* = *Haloarcula*

Results and discussion

Purification of S-layer proteins

After separation of the solubilized S-layer proteins from cells of *Mc. vannielii*, *Mc. thermolithotrophicus*, and *Mc. jannaschii* with SDS-PAGE, the prominent bands of 60, 82, and 80 kDa, respectively, were further investigated. When the eluted S-layer protein (80-kDa band) of *Mc. jannaschii* was further purified with preparative IEF, it showed an apparent molecular mass of 90 kDa on SDS-PAGE. The occurrence of protein bands with different apparent molecular masses from a single S-layer (glyco-)protein on SDS polyacrylamide gels is also known for, e.g., *Methanothermus fervidus* (Nusser et al. 1988) and *Sulfolobus acidocaldarius* (Michel et al. 1980). In order to obtain pure protein preparations the protein bands were electroeluted from SDS gels.

S-layer proteins have a tendency to form insoluble aggregates in the absence of detergents, which hampers the purification of large amounts of protein by column chromatography. As visualized by SDS-PAGE (data not shown), the soluble S-layer protein (82-kDa band) of *Mc. thermolithotrophicus* had already appeared in the synthetic culture medium by the early exponential growth phase. The identity of the protein band obtained from the culture medium was confirmed by sequencing of its N-terminus (Fig. 1). The concentration of the 82-kDa protein band increased in the culture medium with exponential growth and accumulated in the stationary growth phase probably due to cell lysis. Its relative amount was up to 42% of the total extracellular proteins in the culture medium after 86 h of growth. In addition, soluble S-layer proteins of *Mc. jannaschii* (60- and 80-kDa bands) and *Mc. vannielii* (60-kDa band) were released into the culture medium (data not shown).

Determination of the N-terminal amino acid sequences of S-layer proteins

The N-terminal amino acid sequences of the S-layer proteins isolated from cells of the three methanococci; *Mc. jannaschii* (90-kDa band), *Mc. thermolithotrophicus* (82-kDa band), and *Mc. vannielii* (60-kDa band) were sequenced (Fig. 1). In the case of *Mc. jannaschii*, the sequencing was only successful after purification of the soluble S-layer protein (90-kDa band) from the culture medium by preparative IEF. This was also true for an additional S-layer band (60 kDa) of *Mc. jannaschii*. The reason for this observation is not clear. Both protein bands (60, 90 kDa) focused in a pH range between 3.5 and 4.6 (data not shown). When the S-layer of *Mc. jannaschii* was boiled in SDS sample buffer for 60 min, only one band (82 kDa) was visible on SDS-polyacrylamide gels, while in the case of *Methanothermus* (*Mt.*) *fervidus*, the original two glycoprotein bands originating from one glycoprotein (Nusser et al. 1988) were obtained. In the case of other Archaea (e.g., *Sulfolobus acidocaldarius* strain DG6), two protein bands were obtained from purified S-layer preparations (Grogan 1996). The 60- and 90-kDa protein bands of the S-layer protein isolated from the culture medium of *Mc. jannaschii* showed identical N-termini. The same was true for the 82-kDa protein isolated from cells or the culture media of *Mc. thermolithotrophicus*. Generally, the sequences obtained from the S-layer proteins of the methanococci share a great similarity, with the exception that the N-terminus of *Mc. jannaschii* starts with glutamic acid (Fig. 1).

Isolation and sequencing of the S-layer genes

A gene-bank search showed that the N-terminal amino acid sequence obtained in this study from the two protein bands (60 and 90 kDa) of the soluble S-layer protein isolated from the culture medium of *Mc. jannaschii* (E-V-T-T-S-G-F-S-D-Y; Fig. 1) fitted perfectly with a presumptive S-layer coding region within the complete sequenced genome of this species (Bult et al. 1996) leading to the identification of the function of this gene. From this result it can be concluded that the proposed function of the ORF is correct and that both protein bands are at least very similar.

The alignment of the nucleotide sequences of the S-layer genes of *Mc. jannaschii* (Bult et al. 1996) and the already sequenced S-layer gene of *Mc. voltae* (Dharmavaram et al. 1991; Konisky et al. 1994) revealed a high degree of similarity. On the basis of conserved regions upstream and near the 3′ and 5′ ends of the S-layer genes, oligonucleotide primers were constructed for a PCR-based amplification and sequencing of the major part of the S-layer gene sequences of *Mc. vannielii* (1,476 nucleotides) and *Mc. thermolithotrophicus* (1,211 nucleotides). The missing N- and C-terminal sequences were completed by a second PCR step using internal biotinylated specific and degenerated flanking primers. Specific PCR products were isolated with the aid of streptavidin-coated magnetic beads. The obtained sequences (Fig. 1) of the S-layer genes showed a G + C content of 39.0 mol% and 36.9 mol% for the two species, respectively. The G + C content of the S-layer genes is higher than that of the total DNA of *Mc. vannielii* (33 mol%) and *Mc. thermolithotrophicus* (34 mol%).

Comparison of the S-layer genes

Based on data from the literature, we propose signal sequences for transcription and translation of the S-layer genes of methanococci (for accession numbers, see Table 1). The BRE and TATA boxes correspond largely to the proposed consensus sequences of methanogenic Archaea (Thomm 1996). In contrast to *Mc. jannaschii*, several tandem promoters have been described for the S-layer gene of *Mc. voltae* (Kansy et al. 1994). The proposed ribosome binding site 5′-AGGAGAU-3′, usually located 3–9 nucleotides in front of the translation start point (Dalgaard and Garrett 1993), was found to be complementary to a region at the 3′ terminus of the 16S rRNA of *Mc. jannaschii*. Translation of *Mc. jannaschii* and *Mc. thermolithotrophicus* is supposed to terminate with eight and three stop codons, respectively. A series of stop codons is a common feature of methanogenic

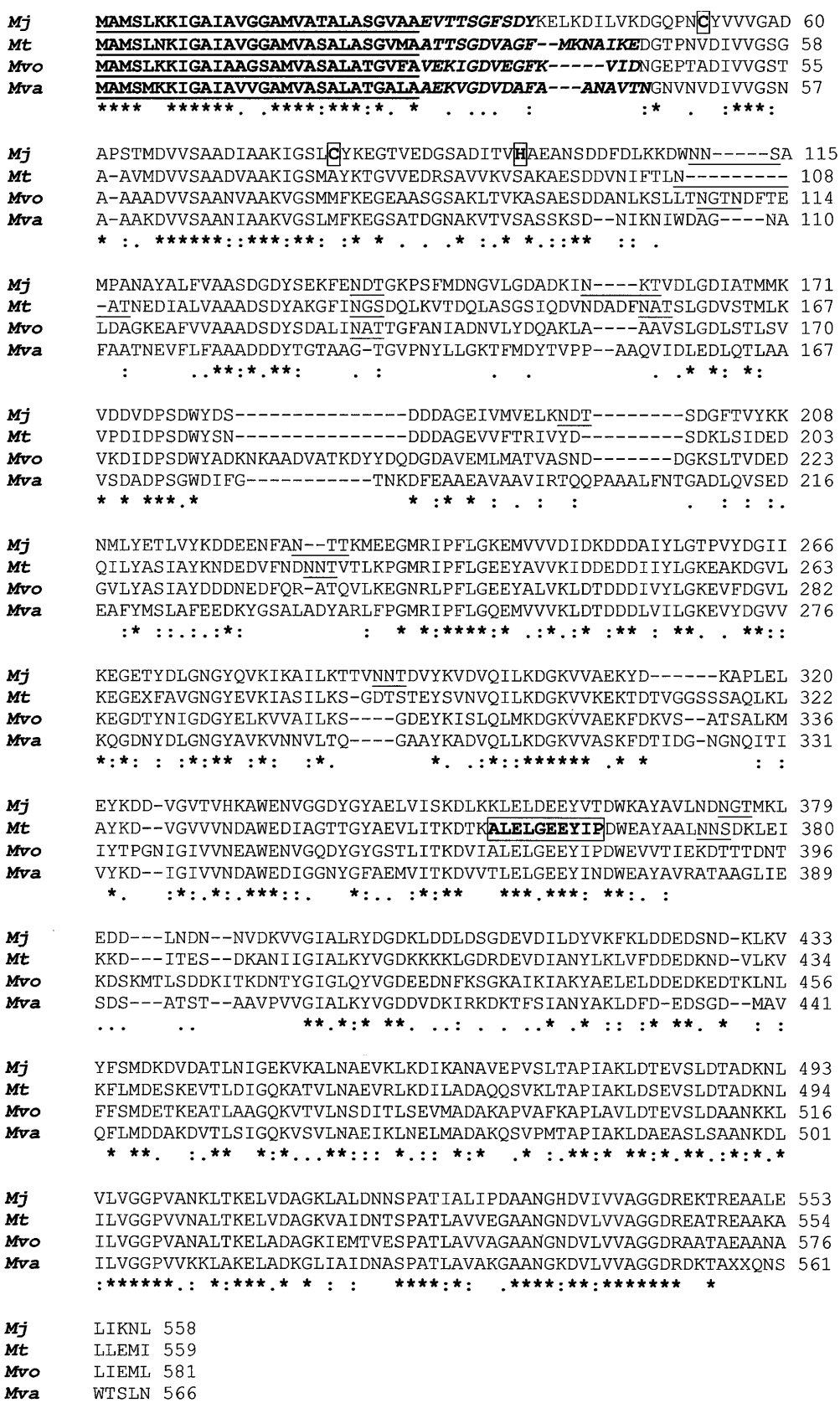

Fig. 1. Alignment of amino acid sequences of the S-layer proteins from *Methanococcus jannaschii* (*Mj*), *Methanococcus thermolithotrophicus* (*Mt*), *Methanococcus voltae* (*Mvo*), and *Methanococcus vannielii* (*Mva*). *Asterisk* denotes identical residues in the alignment; *colon* conserved substitutions; *period* semi-conserved substitutions. The presumptive leader peptides are in *bold* and *underlined*; N-terminal amino acid sequences of purified proteins are in *bold italics* (sequence for *Mc. voltae* as published by Dharmavaram et al. (1991); sequon structures are *underlined*; cysteine and histidine residues in the S-layer amino acid sequence of *Mc. jannaschii* are in *bold* and indicated by a *frame*. The N-terminal sequence of a Lys-C fragment of the S-layer protein of *Mc. thermolithotrophicus* is in *bold* and indicated by a *frame*

Archaea (Dalgaard and Garrett 1993). The data from the literature (Bult et al. 1996) show that in the case of *Mc. jannaschii* the stop codons are followed by a poly A/poly T sequence (nucleotides 1,729–1,877), which probably leads to the formation of a hairpin and termination of transcription.

Comparison of the amino acid composition of the S-layer proteins

Despite the high overall homology of the nucleotide sequences of the genes of the methanococcal S-layer proteins, the deduced amino acid composition displayed some noteworthy differences (Table 2). In the mesophilic (*Mc. voltae*, *Mc. vannielii*) and thermophilic (*Mc. thermolithotrophicus*) methanococci, e.g., the nonpolar amino acid Ala is the most abundant amino acid, whereas in the extreme thermophile *Mc. jannaschii* it is the acidic amino acid Asp. In the latter species, Lys residues mainly localized in nonconserved positions are also found in significantly higher amounts compared with the mesophilic ones (Table 2, Fig. 1). The occurrence of the amino acids Cys and His are characteristic for the S-layer protein of *Mc. jannaschii* and they are not present in the other methanococci.

Although no glycan residues could be detected by PAS staining in the S-layer proteins of methanococci, two potential N-glycosylation sites were found in the mesophilic methanogen *Mc. voltae*, five in the thermophilic methanogen *Mc. thermolithotrophicus*, and eight in the extreme thermophilic species *Mc. jannaschii*, whereas sequon structures (N-glycosylation sites) were missing in *Mc. vannielii* (Fig. 1). The sequon structures are located at nearly the same positions in the methanococcal S-layer proteins (Fig. 1). The S-layer glycoproteins of Archaea, e.g., *Halobacterium halobium* (Lechner and Sumper 1987), can also be O-glycosylated. Since O-glycosylation sites are not conserved, predictions about putative O-glycosylation sites cannot be made in the case of methanococci.

The overall amino composition (Table 2) of methanococcal S-layer proteins is characterized by the predominance of nonpolar amino acids, followed by polar and acid amino acids, and a lower content of basic ones. An increase in

Table 2. Amino acid (aa) composition of selected archaeal S-layer proteins (mol%)

aa	*Mc. voltae*	*Mc. vannielii*	*Mc. thermolithotrophicus*	*Mc. jannaschii*
Ala	14.0	16.3	12.2	9.9
Arg	0.5	1.1	1.3	0.7
Asn	4.6	5.5	5.0	5.6
Asp	11.3	10.1	11.6	14.0
Cys	–	–	–	0.4
Gln	1.4	2.5	1.6	0.5
Glu	7.3	4.2	6.3	6.5
Gly	7.4	8.3	7.5	7.2
His	–	–	–	0.5
Ile	4.6	5.1	6.4	4.8
Leu	8.8	7.4	7.9	8.6
Lys	8.1	7.1	8.8	10.0
Met	2.1	1.5	2.0	2.7
Phe	2.5	3.2	1.8	1.8
Pro	1.6	2.1	1.6	2.3
Ser	5.5	5.1	6.3	4.1
Thr	6.7	5.1	5.2	5.2
Trp	0.5	0.9	0.5	0.7
Tyr	3.4	2.8	2.9	4.1
Val	9.6	10.4	11.1	10.4
Nonpolar[a]	48.5	52.8	48.5	45.0
Polar[a]	24.2	24.4	23.5	23.3
Acidic[a]	18.6	14.3	17.9	20.5
Basic[a]	8.6	8.2	10.1	11.2
Aliphatic Index[b]	94.16	95.41	100.14	92.42
Hydropathicity[b]	–0.091	0.088	–0.079	–0.296
pI[b]	4.15	4.29	4.30	4.27
Helix[c] (%)	36.1	45.1	26.7	22.3
Sheet[c] (%)	27.1	19.3	27.5	25.3
Loop[c] (%)	36.3	35.6	45.8	51.4
Molecular mass (Dalton)	59,707	59,064	59,225	60,547

– Not found
[a]Mol(%), calculated according to Karlson (1982)
[b]Calculated using the ProtPARAM tool
[c]Predicted using the PHD program
For genus abbreviations see Table 1

basic residues is a common feature of the thermophilic and extremely thermophilic species compared with their mesophilic counterparts. The S-layer glycoproteins of the extreme thermophilic methanogens, *Mt. fervidus* and *Methanothermus* (*Mt.*) *sociabilis*, have a high content of Asn instead of Asp (Bröckl et al. 1991). The S-layer proteins of the two mesophilic methanococci have more nonpolar residues, resulting in a higher degree of hydropathicity (Kyte and Doolittle 1982) than the corresponding (extreme) thermophilic proteins.

The calculated acidic isoelectric point (4.27) of the S-layer polypeptide from *Mc. jannaschii* is in agreement with the results of the preparative IEF (see above). The S-layer proteins of *Mc. vannielii* and *Mc. thermolithotrophicus* also focused at a similar acidic pH (data not shown). In contrast, the calculated pIs of the S-layer proteins (see accession numbers in Table 1) of *Mt. fervidus*, *Methanobacterium* (*Mb.*) *thermoautotrophicum*, and *Methanosarcina* (*Ms.*) *mazei* are in the range 8.47–8.9.

A prediction of the deduced secondary structure indicated a higher content of helical structures in the S-layer proteins of the mesophilic species (*Mc. voltae*, *Mc. vannielii*) than in *Mc. thermolithotrophicus* and *Mc. jannaschii*, which in turn exhibit more loops (Table 2).

Comparison of the deduced amino acid sequences of the S-layer proteins

Pairwise alignments [BLAST 2.0] of the primary amino acid sequences deduced from the nucleotide sequence of the methanococcal S-layer proteins suggested the existence of leader peptides (Fig. 1). The proposed leader peptides of *Mc. vannielii*, *Mc. thermolithotrophicus*, and *Mc. jannaschii*, consisting of 28 amino acids, are almost identical. Originally, the leader peptide of the S-layer protein of *Mc. voltae* has been determined to consist of 12 amino acids (M-V-A-S-A-L-A-T-G-V-F-A) (Dharmavaram et al. 1991). We suggest that the nucleotide sequence upstream of the proposed leader sequence may be a coding region and may not represent the ribosome-binding site as described by the authors. The additional amino acids seem to be part of the leader peptide, which is presumably of the same size as that of the S-layer protein of *Mc. jannaschii*. The presumptive leader peptides of the S-layer proteins showed the typical characteristics of a signal sequence, with a hydrophilic domain (7 amino acids) and a hydrophobic domain (21 amino acids). The occurrence of Ala or Gly residues at the peptide cleavage site is regarded as typical for 88% of prokaryotic signal sequences. This is consistent with the assumptions of the Signal-P program. This model predicts a cleavage site at an alanine residue, 1–5 positively charged amino acid residues at the amino terminus (N-region), a hydrophobic core of 7–15 amino acids (H-region), and a region of 3–7 polar amino acids at the C-terminus.

Furthermore, the alignments revealed a notable degree of homology between the S-layer genes of the mesophilic up to the extremely thermophilic methanococci (see Fig. 1; Table 1), especially at the N- and C-termini. In addition, a peptide sequence (A-L-E-L-G-E-E-Y-I-P) obtained after proteolytic digest of the 82-kDa protein band from *Mc. thermolithotrophicus* with the endoproteinase Lys-C was conserved in the *Mc. voltae* gene. Surprisingly, the S-layer genes of the methanococci shared a significant homology with the presumptive S-layer genes of the extreme thermophilic heterotrophs *Pyrococcus abyssi* and *Pyrococcus horikoshii* (Table 1).

The S-layer proteins of *Ms. mazei* (mesophilic) and the Gram-positive methanogens *Mb. thermoautotrophicum* (thermophilic), *Mt. fervidus* (extreme thermophilic), and *Mt. sociabilis* (extreme thermophilic) possess a significant degree of similarity and they have the conserved sequence I-Q-(E/A)-A-I-D in common. The S-layer proteins of these species and that of the sulfate-reducing *Archaeoglobus fulgidus* (extreme thermophilic) shared a high degree of similarity. No relationship between the latter species and the halobacteria was found with the methanococci or with the extreme thermophilic sulfur-dependent species *Staphylothermus marinus*. The lack of a relationship between methanococci and halobacteria is not surprising, since halobacteria are adapted to life at high salt concentrations.

In our study we found an increase in charged residues and a reduction in polar residues in the S-layer proteins of the thermophilic and hyperthermophilic species as compared to their mesophilic counterparts. As the overall hydrophobicity was even higher in the mesophilic strains, it does not seem to play a major role for adaptation to higher temperatures in the case of *Mc. thermolithotrophicus* and *Mc. jannaschii*. Thus, the increase in charged amino acids, especially of lysine, as found in the S-layer proteins of *Mc. thermolithotrophicus* and *Mc. jannaschii* could contribute to their increased thermal stability. Similarly, an increase in charged residues can be observed in the S-layer proteins of *Ms. mazei* (mesophilic) > *Mb. thermoautotrophicum* (thermophilic) > *Mt. fervidus* (extreme thermophilic), and *Ag. fulgidus* (extreme thermophilic) (see corresponding accession numbers in Table 1). Interestingly, the S-layer glycoprotein of *Mt. fervidus* contains high amounts of Asn (Bröckl et al. 1991) and a basic isoelectric point. A significant feature of the S-layer protein from *Mc. jannaschii* is the occurrence of cysteine, which has been detected in only few S-layer proteins (Sára and Sleytr 2000). Intramolecular disulfide-bridges may be another factor involved in the thermal stability of this surface protein.

The apparent molecular masses of S-layer (glyco-)proteins determined by PAGE may differ from those predicted by the amino acid sequence (Bröckl et al. 1991; Sumper 1993; Wakai et al. 1997). This is also true for the methanococci, but the predicted amino acid sequence indicated similar molecular masses from 59,064 to 60,547 Da for the S-layer proteins of the methanococci and a relatively high degree of identity (38%–45%).

In general, only minor structural differences were observed in the primary and secondary structures of the S-layer proteins of mesophilic and extreme thermophilic methanococci. One important point to consider is that the ancestor of *Methanococcales* was probably a thermophile (Keswani et al. 1996). This pattern supports the hypothesis that mesophily is a modern adaptation and that thermo-

philic structures are still conserved in mesophilic proteins, especially S-layer proteins. An exchange of an amino acid in mesophilic proteins may then be simply the result of a relaxation of selection against this amino acid, which may be of importance in the extreme thermophilic counterparts (McDonald et al. 1999). Further three-dimensional investigations are required in order to obtain more information about the changes brought about by the differences in the primary structure of the methanococcal S-layer proteins and thus their thermostabilization. The first attempts to get three-dimensional crystals from S-layer proteins have been successful (Evrard et al. 1999).

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