A comprehensive study into the molecular methodology and molecular biology of methanogenic Archaea

Marianne Lange a,1, Birgitte K. Ahring a,b,*

a Biocentrum-DTU, Technical University of Denmark, Building 227, DK-2800 Lyngby, Denmark
b School of Engineering and Applied Sciences, University of California at Los Angeles, Los Angeles, CA, USA

Received 17 January 2001; received in revised form 25 June 2001; accepted 12 July 2001
First published online 24 August 2001

Abstract

Methanogens belong to the kingdom of Euryarchaeota in the domain of Archaea. The Archaea differ from Bacteria in many aspects important to molecular work. Among these are cell wall composition, their sensitivity to antibiotics, their translation and transcription machinery, and their very strict demands to anaerobic culture conditions. These differences may, at least partly, be responsible for the delay in availability of genetic research tools for methanogens. At present, however, the research within genetics of methanogens and their gene regulation and expression is in rapid progress. Two complete methanogenic genomes have been sequenced and published and more are underway. Besides, sequences are known from a multitude of individual genes from methanogens. Standard methods for simple DNA and RNA work can normally be employed, but permeabilization of the cell wall may demand special procedures. Efficient genetic manipulation systems, including shuttle and integration vector systems, have appeared for mesophilic, but not for thermophilic species within the last few years and will have a major impact on future investigations of methanogenic molecular biology. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Contents

1. The methanogenic world ................................................. 554
   1.1. Introduction .................................................................. 554
   1.2. Chemistry and stability of cell walls in methanogens ................. 554
   1.3. Sequencing and phylogeny determinations ......................... 557
   1.4. Identification of organisms and enumeration of methanogens ...... 558
2. Transcription in methanogens ............................................ 558
   2.1. Transcription in methanogens ....................................... 558
   2.2. Replication and expression of archaeal DNA and proteins in non-Archaea and vice versa 559
3. Genetic systems in methanogens ........................................... 559
   3.1. Mutagenesis, spontaneous mutations and potential antibiotic markers .... 559
   3.2. Cultivation of methanogens ......................................... 561
   3.3. Natural extra-chromosomal elements of methanogens .............. 561
   3.4. Plasmid constructions for the use in genetic experiments in methanogens and their applications .................................................. 563
   3.5. Transformation methods for methanogens .......................... 565
4. Perspectives ........................................................... 567
   4.1. Present tools .......................................................... 567
   4.2. Questions to be answered ............................................. 567

* Corresponding author. Tel./Fax: +45 (45) 25-6183. E-mail address: brigitte.k.ahring@biocentrum.dtu.dk (B.K. Ahring).

1 Present address: Department of Clinical Immunology, Odense University Hospital, DK-5000 Odense, Denmark.
1. The methanogenic world

1.1. Introduction

The anaerobic microorganisms capable of methanogenesis belong to the kingdom Euryarchaeota in the domain of Archaea [1]. They are characterized by their ability to produce methane under anaerobic conditions. This particular metabolism has provided the methanogens with a unique set of enzymatic pathways, as well as certain coenzymes and cofactors that are not found in other organisms [2].

The methanogens are responsible for the final step of the anaerobic degradation of organic matter. Their natural habitats are anaerobic niches, such as intestines of ruminants, the human digestive system, rice paddies, lake or marine sediments, hot pools and salt ponds [3-5]. Sewage digesters and other biogas reactors are man-made methanogenic habitats, from which many methane producing strains have been isolated [3-6]. The methanogens are found at temperatures from 20°C and up to as much as 98°C [7-9].

Phylogenetically the methanogens are divided into five orders, the Methanobacteriales, the Methanococcales, the Methanospirillales, the Methanopyrales, and the Methanomicrobiales [8]. Most of the work within molecular biology of methanogens has focused on the former three. A phylogenetic tree based on 16S rRNA sequences is pictured in Fig. 1.

In some aspects of their molecular biology, such as their transcription apparatus, the methanogens are closer to Eukarya than to Bacteria, whereas other features of methanogens (and of the Archaea in general) are more similar to Bacteria than to Eukarya, since they are indeed prokaryotes [1]. Some of these characteristic features are listed in Table 1.

The aim of this review is to summarize the development within the field of molecular biology research for this diverse group of methanogenic Archaea. Studies of methanogens making use of genetic manipulation are not yet as numerous and advanced as for many groups of Bacteria, but research within these fields is progressing rapidly because of new tools now becoming available. The intention is not to list what we know or do not know about the specific genes of methanogens, their promoters and regulation, but instead to focus on the techniques used and to summarize the development within the field of molecular research for this diverse group. Relevant features that distinguish the methanogens from other prokaryotes, such as their cell envelope, transcriptional and translational features, and how these affect the research will be treated.

1.2. Chemistry and stability of cell walls in methanogens

The nature of the cell envelope is of great importance in experimental work involving permeabilization, penetration, or breakdown of the barrier between the cytoplasm and the environment. Depending on the aim of the study the treatment of the cell envelope must be severe enough to allow for specific components to enter or leave the cell, yet for some purposes, so gentle that the organism is capable of survival and growth after the manipulations.

The diversity of the archaeal cell walls is great. Even among the methanogens the variance is extensive but a characterizing property of the members of Archaea is the lack of murein (peptidoglycan) in the cell envelope [12].

Characteristics of the cell envelope of some major groups of methanogens are outlined in Table 2, and will be briefly described in the following sections.

1.2.1. Methanobacterium and Methanobrevibacter

Methanobacterium and Methanobrevibacter belong to the family Methanobacteriales and have pseudomurein, the archaeal analogue to bacterial murein, in their cell wall. Pseudomurein and murein have the same overall structure and appearance, but they differ in biosynthesis, bindings and some of the building blocks. The bindings between the sugar moieties of the wall subunits, N-acetyl-D-glucosamine and/or galactosamine and N-acetyl-L-talosaminuronic acid, are of the $\beta(1 \rightarrow 3)$ type as opposed to the $\beta(1 \rightarrow 4)$ type, which is found in bacterial murein [12]. Short peptide chains attached to the carboxyl group of N-acetyl-L-talosaminuronic acid are responsible for the cross-linking of the glycan strands. Since the chemical bonds between some of the components differ, so does the biosynthesis of pseudomurein and murein. Therefore, commonly used antibiotics like penicillin and vancomycin, as well as the enzyme lysozyme and some proteases, have no or only little effect on these methanogens [3,12].

Enzymes capable of hydrolyzing pseudomurein have, however, been identified in Methanobacterium wolfei and in Methanobacterium thermoautotrophicum Marburg [14]. Endopeptidase-mediated lysis of $M$. thermoautotrophicum Marburg is induced by a phage, ΨM1, whereas the endopeptidase of $M$. wolfei is induced by energy depletion [14]. The pseudomurein endopeptidase of $M$. wolfei was also functional on e.g. $M$. thermoautotrophicum ΔH and others. The degradative potential of the enzyme was exploited.
under protoplast formation and extraction and purification of plasmid and chromosomal DNA, see Section 1.2.6 [12,15].

One of the Methanobacteriales, the thermophilic Methanothermus, has both an inner pseudomurein sacculus and an outer surface layer (S-layer), which is composed of glycoprotein. Crystalline surface layers (S-layers), consisting of protein or glycoprotein, are found in both prokaryotic domains. The S-layer of Methanothermus is believed to provide thermostability and has a different amino acid composition and a higher degree of β-sheeted structure than S-layers of the mesophilic species [3,12].

1.2.2. Methanosarcina

The genus of Methanosarcina comprises members that are very fragile and members that are almost unbreakable [16]. Some have a particularly thick and rigid outer envelope, with a grid made up of N-acetylglactosamine and d-glucuronic (or d-galactouronic) acid polymer, with a minor fraction consisting of d-glucose and d-mannose. This rigid outer layer is similar to animal chondroitin [3,16] and was consequently named pseudochondroitin or methanochondroitin. The methanochondroitin covers in some species a proteinaceous S-layer, which in turn is connected to the cytoplasmic membrane [17,18]. The presence of the outer wall polymer in certain species of Methanosarcina is to some extent dependent on the growth conditions (see below). Some species, e.g. Methanosarcina mazei and Methanosarcina thermophila, undergo morphological changes [19,20], whereas Methanosarcina frisia and Methanosarcina acetivorans merely have the S-layer covering their cytoplasmic membrane [12]. The methanochondroitin shows high resistance towards chemicals such as sodium dodecyl sulfate (SDS) or Triton X-100, and to physical...
disruption [21]. Autolysis, as a consequence of protoplast formation, may occur in substrate-depleted cultures of Methanosarcina barkeri [22], and during the cell cycling of M. mazei, where single cells are released when the methanochondroitin is degraded [19].

1.2.3. Morphology of Methanosarcina

The life cycle of M. mazei has been studied intensively [19,23–30] and consists of three main phases or types: large aggregates or clumps, a cysts stage and single cells. M. mazei S-6 is able to grow in even more morphological states; lamina is a sheet-like structure of individual cells and small aggregates or packets, which was first described in 1992 [31]. Aggregates of M. thermophila may also be triggered to disintegrate and form single cells [20,29,32].

The single cells of Methanosarcina are fragile and lye very easily, for instance in water. Much work has been done to determine the exact conditions that induce the conversion between the morphological forms, but the details have not yet been entirely elucidated. However, the concentrations of the cations Ca$^{2+}$ and Mg$^{2+}$ as well as the concentrations of NaCl and substrate are affecting the

### Table 1

Features of Archaea, Bacteria, and Eukarya*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Archaea</th>
<th>Bacteria</th>
<th>Eukarya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome</td>
<td>Prokaryote</td>
<td>Prokaryote</td>
<td>Eukaryote</td>
</tr>
<tr>
<td>Extra-chromosomal elements</td>
<td>single circular</td>
<td>single circular</td>
<td>several linear</td>
</tr>
<tr>
<td>Operons</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>one class (8–10 subunits)</td>
<td>one class ($\beta\beta\alpha\sigma$)</td>
<td>three classes, complex</td>
</tr>
<tr>
<td>Promoter type</td>
<td>BoxA: TTTAT/AATA [−(24–28)]; BoxB: PyrPur (+1)</td>
<td>−35 (TTGACA); −10 (TATAAT)</td>
<td>upstream activating sequences; −25 (TATA)</td>
</tr>
<tr>
<td>Termination signal mRNA</td>
<td>stem-loop structures, thymidine stretches</td>
<td>stem-loop structures</td>
<td>thymidine stretches</td>
</tr>
<tr>
<td>Intron RNA</td>
<td>unstable, short poly(A) tail</td>
<td>unstable, short poly(A) tail</td>
<td>stable, capped, long poly(A) tail</td>
</tr>
<tr>
<td>Ribosome binding site</td>
<td>in tRNA and rRNA; in mRNA (hyperthermophiles)</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>Initator tRNA</td>
<td>methionine</td>
<td>fornylmetionine</td>
<td>methionine</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>70S, diphtheria toxin-sensitive</td>
<td>70S, diphtheria toxin-resistant</td>
<td>80S, diphtheria toxin-sensitive</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>ether linkage between glycerol and isoprene</td>
<td>ester linkage between fatty acids and glycerol</td>
<td>ester linkage between fatty acids and glycerol</td>
</tr>
<tr>
<td>Cell wall (main component)</td>
<td>see Table 2</td>
<td>peptidoglycan</td>
<td>polysaccharide</td>
</tr>
<tr>
<td>Spore formation</td>
<td>absent</td>
<td>present in some</td>
<td>absent</td>
</tr>
</tbody>
</table>

*Revised from [11].

### Table 2

Structure of cell envelope of methanogens

<table>
<thead>
<tr>
<th>Genera</th>
<th>Strain</th>
<th>Cell envelope</th>
<th>Morphology</th>
<th>Susceptibility to detergents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanosarcina</td>
<td>M. mazei S-6 (single cells)</td>
<td>SL*</td>
<td>single cells, pseudosarcina</td>
<td>sensitive to SDS and hypoosmotic solutions</td>
<td>[12,13]</td>
</tr>
<tr>
<td></td>
<td>M. acetivorans</td>
<td>CM</td>
<td>cysts, aggregates, pseudosarcina</td>
<td>resistant to SDS</td>
<td>i.b.</td>
</tr>
<tr>
<td></td>
<td>M. mazei S-6 (aggregates)</td>
<td>MC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanococcus</td>
<td>M. voltae</td>
<td>SL</td>
<td>irregular cocci</td>
<td>lysis in hypoosmotic solution</td>
<td>i.b.</td>
</tr>
<tr>
<td>Methanosaeta</td>
<td>M. concilii</td>
<td>PS</td>
<td>sheated rods</td>
<td>resistant to SDS</td>
<td>i.b.</td>
</tr>
<tr>
<td>Methanothermus</td>
<td>Methanothermus fervidus</td>
<td>SL, CM</td>
<td>rods</td>
<td></td>
<td>i.b.</td>
</tr>
<tr>
<td>Methanobacterium</td>
<td>M. thermoautotrophicum,</td>
<td>PM, HP, CM</td>
<td>rods</td>
<td>forms protoplasts with lysozyme in presence of sorbitol</td>
<td>i.b.</td>
</tr>
<tr>
<td></td>
<td>Methanobrevibacter, Methanopyrus</td>
<td>CM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations used: SL, surface layer (S-layer); CM, cytoplasmic membrane; PS, polysaccharide; MC, methanochondroitin; HP, heteropolysaccharide; PM, pseudomurein. Idea from [13], see Section 1.2 for details.
1.2.4. Methanosaeta and Methanospirillum

Methanosaeta and Methanospirillum have resistant fibrillar sheet layers, made up by protein and glycoprotein, surrounding an inner wall. The proteinaceous layers cover several individual rods (5–10 for surrounding an inner wall. The proteinaceous layers cover several individual rods (5–10 for Methanospiroplasma TM-1 and M. mazei S-6, but did not have any effect on aggregates of Methanosaeta strain SMC or M. barkeri 227 [26]. The disaggregatase produced by M. mazei S-6, however, did not disintegrate aggregates of M. mazei LYC [25].

1.2.5. Methanococcus

A diverse group of methanogens has S-layers covering the cytoplasmic membrane. The genus Methanococcus has an S-layer consisting of protein subunits, whereas the members of the genera Methanogenium, Methanoculleus, Methanomicrobium, Methanoplanus, and Methanolobus have S-layers of glycoprotein subunits. The S-layers fall apart after a 30 min incubation in 2% SDS at 100°C, and the cells are sensitive to low osmolarity solutions [3,12,33].

1.2.6. Extraction of nucleic acids

For certain methanogens cell lysis happens readily, but for others it may be challenging to gain access to the nucleic acids and other intracellular components. Jarrell et al. [33] reported a disruption method for resistant methanogens including crushing the pelleted culture in liquid nitrogen in order to obtain access to the cell components. This method is applicable to all strains of methanogens and gives good yields of high quality DNA, compared to DNA resulting from e.g. French press treatment [33]. Bead-beating is another powerful method which has been used e.g. in ribosomal RNA extraction methods in phylogenetic characterizations of environmental samples [35]. Methanococcus cells were broken by sonication in the study by Klein and Schnorr [36]. Stettler and Leisinger lysed a culture of Methanococcus jannaschii [43] and Methanococcus TM-1 [44].

The complete genomes, complemented by the numerous reports on individual genes or clusters of genes, have long proven that standard sequencing techniques are fully applicable for methanogens along with techniques such as Northern blotting and Southern blotting. Consequently, methanogenesis is one of the first complete biochemical pathways amenable to comparison at the gene level [45].

A large proportion of genes are unique to methanogens or share little or no homology to sequences in the public databases [44,45] and their functions will have to be determined experimentally, if at all possible. Many other open reading frames (ORFs) identified in sequencing projects will be ascribed a function merely from database comparisons with known genes from other organisms.

by SDS treatment [36,37] or by freezing at −70°C followed by thawing on ice in 10 mM EDTA [38]. Lysis of single cells of Methanosarcina can be achieved simply by resuspending cells in water (see Sections 1.2.2 and 1.2.3), whereas methods as the one described by Jarrell et al. [33] must be used for the aggregated form.

As soon as the methanogenic cell is lysed the actual purification of DNA and RNA may be done by standard methods [39–42].

Complete lysis is of course only useful for assays requiring DNA, RNA or protein. Under in situ experiments, such as fluorescent in situ hybridization for identification purposes (see Section 1.4) or in situ mRNA PCR (Section 2.1) which rely on retention of certain components inside the cell, the envelope should be only permeabilized, but the cells need not be able to survive after the treatment. In transformation experiments, however, the nature and degree of the disruption of the permeability barrier are of significance for survival of the target cell, and must be gentle enough to render the manipulated cell viable. For a few methanogenic species successful transformations have been carried out. Throughout this paper we will refer to transformation experiments and they will be described in detail in Sections 3.4 and 3.5.

Finally, it must be emphasized that the cell wall also provides an important shield against e.g. antibiotic or other toxic compounds. Therefore, resistance of methanogens to certain antibiotics may simply be caused by low permeability of the substance; this phenomenon will be dealt with in Section 3.1.3.
1.3.2. Ribosomal RNA and functional genes

The majority of methanogenic species have been described since 16S rRNA sequences were introduced as a phylogenetic tool for this very group. Therefore, the methanogens are very well characterized with respect to 16S rRNA [4,8,46]. Approaches such as length polymorphism and single strand conformation polymorphism analysis on the variable region of the 16S rRNA have been applied to analyze strains of methanogenic Archaea [47]. These PCR-based techniques are useful and rapid. DNA for amplification is obtained simply by boiling the cultures for 15 min in a lysis buffer [47]. Even for strains with a rigid cell wall, such as aggregates of \textit{M. mazei}, sufficient DNA for PCR amplification is recovered by boiling [48].

Phylogenetic investigations of methanogens based on mRNA or DNA have also been attempted by Springer et al. [46]. A 490-bp fragment of the \textit{A} subunit of the gene encoding methyl coenzyme M (CoM) reductase (\textit{mcr}], which is involved in the final step of the CH$_4$ synthesis and one of the best known catabolic enzymes among anaerobes, was sequenced in 25 members of the family \textit{Methanosarcinaeae} [46]. The phylogenetic picture derived from these sequences was similar to the 16S rRNA-based tree, although there were some discrepancies in the relative distances between species [46]. To our knowledge no other studies involving as many sequences, as the above mentioned, have been performed solely on methanogen genes.

The increasing number of 23S rRNA sequences, which will provide the basis for even higher specificity in primer design than 16S rRNA, submitted to the databases, will not leave much room for analyses of functional genes, such as \textit{mcrI}, as a phylogenetic tool. It has been argued that basing phylogenetic analysis only on the sequences of the functionally related 5S, 16S or 23S rRNA may not reveal anomalies, which may be found by comparison with independent genes [46]. For analyses of the complete methanogenic pathway, genes, and genomes see Reeve et al. [45,49–51].

1.4. Identification of organisms and enumeration of methanogens

For identification and determination of methanogen numbers in the environment, particularly the sequences of 16S and 23S rRNA/DNA are providing valuable information and supporting powerful techniques, such as in situ or membrane probing [52].

1.4.1. Whole cell or filter studies with nucleic acid probes

Membrane studies using total RNA extracted from environmental samples, hybridized with labeled oligonucleotide probes with varying specificity for analyses of methanogenic populations, were introduced by Raskin et al. [53,54]. Other studies have been published using this technique [35,52,55]. In this particular field, the technical possibilities for methanogens equal those of other groups of organisms, although RNA extraction from environmental samples of e.g. manure or sludge may require special precautions [35].

Fluorescently labeled nucleic acid probes may also be applied in whole cell hybridizations. A critical step in this procedure is the permeabilization of the cell wall and membrane in order to allow the probe to reach the ribosomal targets [55]. The advantages over membrane procedures are that there is no need for RNA extraction from environmental samples, and spatial distribution of the organisms can be elucidated. The large number of available methanogen 16S rRNA sequences means that probes are readily obtainable. The lack of published work indicates, however, that the procedures are still causing some troubles.

1.4.2. Nucleic acid vs. immunology for in situ studies

The nucleic acid-based techniques are quickly gaining terrain compared to immuno-based techniques, which have been used for the past 15 years. Conway de Macario and Macario (e.g. [56,57]) did much of the development within the area of immuno-methodology. Several approaches are possible for rapid identification and quantification of methanogens in environmental samples using immunological probes as outlined by Sørensen [58].

Although the antibody probes are not able to reach the same detailed level of specificity as nucleic acid probes, and the production of antibodies is laborious and requires that the immunizing strain is isolated, some qualities are superior. Binding of antibodies to the cell happens on the cell surface, leaving no need for permeabilization of the cells, as opposed to hybridization with nucleic acid probes, which takes place inside the cell. The signal obtained with nucleic acid probes is dependent on the level of ribosomal RNA in the cell, which is related to the physiological state of the cell [59,60]. A quantitative in situ oligo-hybridization is probably not possible with organisms as versatile as the methanogens, where probe accession varies between species and even between cells within a single species [55]. However a qualitative hybridization is surely within reach.

2. Transcription in methanogens

2.1. Transcription in methanogens

The subunit structure of RNA polymerases of Archaea is more similar to those of Eukarya than to the bacterial polymerase, but both in Bacteria and Archaea there is only one polymerase [43,44,61,62]. The activity of the methanogenic RNA polymerase depends strongly on the presence of a promoter sequence of high homology to the TATA box recognized by one of the eukaryal RNA polymerases [61].
In 1990, Frey et al. [63] found that the ability of the methanogenic RNA polymerase to transcribe genes was dependent on components contained within a protein fraction, and later these transcription factors were purified (for a review, see [62]). The archaeal transcription factors are, at least in the tested cases, not species-specific, but functional in other Archaea, and a high degree of conservation within this group of molecules has been found among the Archaea and Eukarya [62]. In vitro transcription is a useful technique which has been used in several studies [64–66]. Several transcription factors including a TATA binding initiation factor were identified in both fully sequenced methanogen genomes [43,44].

Transcription and identification of transacting DNA binding factors (transcription factors) have been studied by mobility shift assays, in which specific PCR-synthesized DNA fragments are incubated with protein extracts from cells grown at different conditions and the DNA–protein complexes, as well as unbound components are then run in an acrylamide gel. In this way transacting factors have also been identified in methanogens. Some of these are preferentially synthesized and/or bound under certain conditions such as heat-shock [67] or during N-starvation [68].

A more recent application for studying gene transcription is in situ reverse transcription PCR, or in situ mRNA PCR, that was used successfully in e.g. Salmonella typhimurium [69] and human cells (for a review, see [70]). We have succeeded in adapting this technique for the use in M. mazei S-6. The in situ work was performed on the M. mazei S-6 single-cell morphology. A series of thermal cycles was sufficient to permeabilize the cell; however, the permeabilization was more efficient when the cells were treated with a 0.5 mg ml⁻¹ lysozyme solution, prior to PCR amplification of specific targets inside the cell [42]. We used the approach to analyze the transcription of the stress-induced gene dnaK and found that an increase in the fluorescence of the individual cells could indeed be observed after heat-shock.

2.2. Replication and expression of archaeal DNA and proteins in non-Archaea and vice versa

Several methanogenic genes have been isolated, sequenced and analyzed due to their ability of complementing mutations in Escherichia coli. The corresponding proteins are for example members of the amino acid or purine biosynthesis pathways [71]. Also large amounts of genes originating from methanogens are replicated on cloning vectors in E. coli at sufficient levels for e.g. sequencing purposes (e.g. [43,44,72]). Incompatibility in gene expression may, however, arise. The differences in gene structures, sequences, and codon usage may explain the experimental difficulties sometimes encountered when methanogenic genes are to be expressed in non-methanogens and vice versa.

2.2.1. Codon preferences

Codons, such as AUA, AGA, and AGG, are frequently found in methanogens but rarely in E. coli [71]. The high level of As and Ts in the third position, often found in methanogens, may pose a problem for proper expression in other organisms. The Methanococcus genome is an example of this. The circular chromosome has a G+C content of only 31.4% [7,44,71,73,74]. Six archaeal recombinant and plasmid-encoded proteins were to be expressed in E. coli. Four were from M. jannaschii and two from Pyrobaculum aerophilum. The coding regions contained several codons that are rarely found in E. coli. These proteins were only poorly expressed until another plasmid was co-introduced, encoding the rare codon-specific tRNAs [74].

Another codon-specific feature is the presence of in-frame amber codons in a number of methanogen genes [75]. A mechanism is believed to be present in methanogens capable of overruling the stop signal, but such codons may cause problems when recombinant proteins are to be expressed in other organisms and decrease translation efficiency.

2.2.2. Restriction systems

Several restriction, modification, and DNA repair systems have been recognized in methanogenic species [43,44,76–80], and these may affect the stability of foreign DNA.

2.2.3. Concluding remarks on gene expression

The experiences from expression of methanogenic genes in Bacteria may be used when the reverse process is required. A simple check in the available databases of codon preference of the target organism may give an idea of the possibility of gene expression when e.g. choosing suitable reporter genes or resistance markers for genetic studies of methanogens. So far, only a limited number of foreign genes have been expressed in methanogens. Puromycin transacetylase from Streptomyces or β-galactosidase from E. coli can be functionally expressed in methanogens [81,82], and have been used to develop transformation methods. Recombinant genes in methanogens will be discussed in Sections 3.4 and 3.5.

3. Genetic systems in methanogens

3.1. Mutagenesis, spontaneous mutations and potential antibiotic markers

Mutants and antibiotic resistance, mutagenesis and genes encoding the resistant phenotype are prerequisites for elucidation of gene function, for transformation, and for the development of transformation vectors.

Spontaneous mutations have been selected for by 6-mercaptoturidine, 5-fluorouracil, 5-fluorodeoxyuridine, 8-aza-guanine, 6-azaguanine, sodium 2-bromoethanesulfonate...
(BES), neomycin, and puromycin [83–86]. ICR191 (a frameshift mutagen), 2-bromo-2'-deoxyuridine, ethyl methanesulfonate (EMS), and UV irradiation were used as randomly mutagenizing agents in other studies [87–89]. Site-directed mutagenesis has also been employed, and will be described in Section 3.1.2.

3.1.1. Stability and efficiency of mutagenesis

Jain and Zeikus [88] reported that the acetate auxotrophs induced by EMS were stable whereas the same phenotype, induced by UV illumination, reverted within two to four transfers. Photocatalyzed repair of UV-induced DNA damage was found to take place in *M. thermautotrophicum* [90]. It is not clear if this is the cause of the reversion of mutants, referred to by Jain and Zeikus [88]. Olson et al. [91] also confirmed the activity of visible light on methanogens. The growth of members of four methanogenic families was inhibited and the number of viable cells decreased significantly when they were exposed to visible light, mainly the blue end of the spectrum [91].

Exposure to mutating agents such as EMS and UV reduces the number of viable cells drastically as found in studies by Yang et al. [92] and Haas et al. [84] and a relatively large fraction of the surviving microbes was mutated. High lethality caused by such mutagenesis methods is not unique to methanogens.

Knox and Harris [83] found that mutants that had only a low level of resistance reverted more readily than mutants with a higher resistance level. Likewise when the resistance marker is carried on e.g. a plasmid and integrated into the genome, a background of colonies exhibiting a low level of resistance to the antibiotic are found. Patel et al. [93] found that the diameter of *Methanococcus voltae* colonies with spontaneously developed resistance was much smaller than that of colonies of true transformants. Thus, the frequency of spontaneous mutation events, the colony size, and the reversion rate are factors that are indicative of the nature of the resistance and must be taken into consideration when picking mutant strains or choosing an appropriate drug.

3.1.2. Site-directed mutagenesis

Site-directed mutagenesis and insertional mutations have been used in four studies, with *Methanococcus maripaludis* [82,94,95]. The *nifH* (nitrogen fixation) gene was targeted by insertion mutagenesis, in a study where a transposon-based system was developed [95], and genes encoding hydrogenases were inactivated by the insertion of a large plasmid fragment [94]. Lastly the promoter of the *nifH* was altered specifically by PCR, inserted on a plasmid, which was subsequently transformed into the organism and incorporated in the genome by recombination [82]. Gene-specific or directed mutagenesis have also been used in *M. acetivorans* C2A [96,97]. In bacterial systems, efficiencies of directed mutagenesis are often above 80% [39] and there is no doubt that with the progress within the field of transformation (see Section 3.5) and other molecular techniques, site-directed mutagenesis will also become the method of choice in methanogenic systems.

3.1.3. Permeability of the cell envelope and antibiotic resistance

BES is a competitive methyl CoM (CH₃ScoM) analogue, and the resistance towards this chemical is, theoretically, caused by the development of resistance within the methanogenesis enzymes. *M. barkeri* 227, however, became resistant due to an altered and heritable permeability of the cell and not due to mutated enzymes involved in methanogenesis [98,99]. Two other genera reacted differently from *M. barkeri* 227 to the exposure to BES: a strain of *M. hungatei* was extremely sensitive to BES, and no resistant colonies appeared, whereas a strain of *Methanobacterium formicicum* was resistant without any prior selection [98], indicating the severe differences in cell envelope structure among methanogens.

Also the resistance of *Methanococcus vannielii* to A2315-A and neomycin was caused by changes in cell permeability. In an in vitro experiment the poly(U) dependent translation by ribosomes prepared from resistant *M. vannielii* strains was inhibited to the same extent as the wild-type, indicating that in vivo the antibiotic never reaches the target ribosome in the resistant strains [84].

3.1.4. Potential genetic markers

The sensitivity of Archaea to ribosome-targeted antibiotics is variable. This is caused partly by differences in cell permeability, as discussed above, and partly by the inter-domain variability in ribosome structure, which is not found in Bacteria and Eukarya, but prevalent even among methanogens [100]. For instance was *M. formicicum*, but not *M. Vannielii*, sensitive to tetracycline [100]. In a study by Pecher and Böck [101], the inhibiting concentrations of several protein synthesis inhibitors were tested. Large variations in the inhibiting concentrations were found in the four species of methanogens included in the test [101]. For example, 2 μg ml⁻¹ hygromycin was sufficient to inhibit *M. mazei*, whereas more than 500 μg ml⁻¹ did not affect *Methanobacterium bryantii* or *M. vannielii* [101]. Puromycin, hygromycin, fusidic acid, and pseudomonic acid were all tested for their ability to work on in vitro systems. All of these worked in vitro as well as in vivo [102]. Londei et al. [103] also tested the in vitro effect of pulvomycin, kirromycin and fusidic acid on methanogen ribosomes, and found that kirromycin was not effective on methanogens and fusidic acid only partly inhibited the synthesizing capacity of the ribosomes [103].

Several antibiotic compounds are promising from the point of view that the resistance genes have already been cloned and are, therefore, available for cloning studies. However, a cloned resistance gene from one organism is not always directly transferable. *M. vannielii* is sensitive to both chloramphenicol and chloramphenicol-3-acetate.
Consequently, the resistance marker chloramphenicol transacetylase, which confers resistance to this drug in Bacteria, will not work in \textit{M. vanillii} [84].

At present, puromycin resistance, and the \textit{pac} cassette, which was made available by Gernhardt and co-workers, is the most frequently used selective marker in methanogens, since it is working in several organisms (e.g. [81, 104, 105]). In recent studies also colonies of \textit{M. acetivorans} C2A resistant to BES and fluoracetic acid have been described as a result of transposon-based mutagenesis [96] and to pseudomonic acid by mutating and inserting the responsible gene from \textit{M. barkeri} Fusaro [97].

Previously the lack of suitable antibiotic markers has been one of the reasons for the relatively slow introduction of transformation systems in methanogens. With the availability of several potential selective markers this problem is overcome.

3.2. Cultivation of methanogens

Methanogens require an environment with a reducing potential less than $-330 \text{ mV}$ in order to grow [7]. This of course also applies for methanogenic cultures on solid media, and does impede this part of the work. Therefore, equipment such as anaerobic jars and an anaerobic glove box are necessary. Despite the inconveniences of anaerobic equipment such as anaerobic jars and an anaerobic glove box are necessary. Despite the inconveniences of anaerobic plating methanogens this step is necessary, and in all but one of the transformation experiments mentioned in Section 3.5 transformants were isolated from colonies propagated on solid media. With the right conditions for culturing on solid media, it is possible to achieve plating efficiencies of about 90\% for \textit{Methanococcus} [107, 108], and 80\% for \textit{Methanosarcina} [107] with whole cells. Under protoplast regeneration of \textit{Methanococcus}, between 50 and 60\% efficiency was achieved compared to direct count [93].

3.2.1. Roll-tubes vs. plates

An alternative to cultivating methanogens on plates is the modified roll-tube technique [109, 110]. This technique, which neither demands incubation in canning jars or inoculation in an anaerobic chamber, is practical since methane content as well as pressure increases or decreases in the headspace are measurable. We are routinely using the methods in the isolation of new strains and in the cause of our transformation experiments [6, 111]. Picking single colonies from roll-tubes, however, is a laborious job, but is also possible to perform outside of the anaerobic chamber.

For hyper-thermophilic methanogens as well as for other hyper-thermophiles growth on solid media is sometimes done in bottles. It is important to get rid of some excess water before incubation at temperatures of 65–70°C, and incubation is done with the solidified medium facing down to avoid water collecting on the colonies [112].

3.2.2. Factors influencing plating efficiency

Successful recovery of colonies and the size of the colonies are strongly dependent on volume of agar, concentration of H\textsubscript{2}S and cysteine, and hydration of the media [107, 108]. An illustration of how plating conditions should be optimized for both the strains in question and the available equipment is found in the study by Apolinario and Sowers [107]. The numbers of colony forming units on plates for \textit{M. thermophila} and \textit{M. maripaludis} relative to total cell counts were determined as functions of Na\textsubscript{2}S concentration. The optimal concentration of Na\textsubscript{2}S for \textit{M. thermophila} peaked sharply at 0.02 g l$^{-1}$ Na\textsubscript{2}S$\cdot$9H\textsubscript{2}O, corresponding to 0.2\% (v/v) \textit{H}_{2}S, whereas the efficiency of \textit{M. maripaludis} plating continued to increase up to at least 1.6\% (v/v) \textit{H}_{2}S. At 3.2\% (v/v) \textit{H}_{2}S no growth occurred. Apolinario and Sowers [107] refer to another study by Sowers et al. [29], where the incubation jar was made of metal instead of glass. The optimal H\textsubscript{2}S concentrations for \textit{M. thermophila} were much higher compared to the concentration determined by Apolinario and Sowers [107], and it is believed that the metal reduced the actual concentration of H\textsubscript{2}S in the jar atmosphere. Also the type of agar is of importance [113]. Transformed \textit{M. thermoautotrophicum} \textit{AH} colonies grow on medium solidified by Gel-lan gum, but not when noble agar was used. Jones et al. [108] reported that the colony size of \textit{Methanococcus} decreased when the agar concentration was increased. This is a problem when plating thermophilic cultures, where the agar content must be increased in order to enable solidification at high temperatures.

3.2.3. Biochemical considerations

The strongly reduced media necessary for growing strict anaerobic organisms may cause a problem using pH-sensitive dyes for example to detect sugar fermentation [114]. 5-Bromo-4-chloro-3-indolyl-$\beta$-galactopyranoside, that is commonly used in reporter systems with $\beta$-galactosidase for e.g. \textit{E. coli}, is an example of a substrate that may be affected in the ability to become oxidized under reducing conditions. Therefore the cultures may have to be removed from their anaerobic conditions in order for the reaction to take place [114], which, however, may kill or severely inhibit anaerobic organisms.

3.3. Natural extra-chromosomal elements of methanogens

Extra-chromosomal elements and other mobile genetic elements may be useful in the construction of functional genetic vector systems, and have been identified in several methanogens. In the following sections plasmids, phage-like particles and insertion elements of methanogens and their importance for molecular research and the development of transformation systems will be discussed.

3.3.1. Natural plasmids

Plasmids have been identified in both thermophilic and mesophilic methanogenic organisms but are not present in all strains. No plasmids were identified in \textit{M. thermoauto-
trophicum ΔH, whereas two were found in M. jannaschii [43,44]. A selection of natural plasmids is listed in Table 3.

Only twice has the copy number been estimated. The plasmid, pC2A from M. aceticivorans, is naturally present in approximately six copies per cell [115] and pFZ1 of Methanobacterium thermoformicum Z-245 is assumed to be present in four copies [116]. No high-copy number plasmids have been described.

Plasmids were identified in two strains of M. thermoautotrophicum and in two strains of M. thermoformicum. Common for these are backbones including palindromic sequences believed to be involved in replication and maintenance of the plasmid [76,118]. Regions with structural relationship to insertion sequences (ISs) of Bacteria and Archaea were identified [118].

The potential for construction of transformation vectors based on natural plasmids of methanogens was proven by Metcalf et al. [104]. The plasmid from M. aceticivorans, pC2A, was completely sequenced [104]. It contained several restriction sites for known endonucleases and four ORFs, two of which showed homology to genes encoding replication initiation proteins known from phages and certain plasmids, repA, and for recombinases, ssrA, as well as repeat sequences possibly involved in plasmid replication were identified [104].

The plasmid pURB500 from M. maripaludis has also been used in a shuttle vector construction [119]. The natural plasmid contains 18 ORFs and only one of these was ascribed a putative function, showing slight homology to an integrase of Weeksella zoohelcum [119]. Extensive regions of direct and imperfect repeats were found in the regions ORFLESS1 and ORFLESS2, and therefore, these regions are believed to be related to replication origin. A large region of the pURB500 appeared to be required for replication in M. maripaludis [119].

3.3.2. Phage-like particles and insertion elements

In species of Methanococcus, Methanobacterium, and Methanobrevibacter phage or virus-like particles have been identified, see Table 4. The viruses of M. thermoautotrophicum Marburg, ΨM1 and ΨM2, respectively, contain genes, which are responsible for packing the viral DNA [124,125]. ΨM1, which efficiently encapsulates multimers of plasmid DNA as well as chromosomal DNA in a non-specific manner, successfully transduces mutant strains of Methanobacterium, and has been used in genetic transduction studies, see Section 3.5.2 [126].

M. voltae A3 contains up to 30 virus-like particles (A3 VLP) per infected cell. Hybridization showed that virus integrated on the genome. The authors were unable to demonstrate that the viruses were infectious but assume that a suitable strain was missing, or that the virus requires a helper phage [127]. Obviously, the lack of infectivity eliminates the use of the phage for mutant analysis and genetic engineering. No evidence of phage DNA was found under the sequencing of the M. thermoautotrophicum ΔH or M. jannaschii [43,44].

Insertion elements (IS) or simple mobile genetic elements have been identified in some methanogens, see Table 5. Methanogenic ISs have not been used for genetic experiments yet, but the principle of transposon insertion has been used [95,96].

Table 3
Natural methanogenic plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Organism</th>
<th>Size (kb)</th>
<th>Sequence (accession no.)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pME2001</td>
<td>M. thermoautotrophicum Marburg</td>
<td>4.439</td>
<td>X1720</td>
<td>[120,121]</td>
</tr>
<tr>
<td>pME2200</td>
<td>M. thermoautotrophicum ZH3</td>
<td>6.2</td>
<td>partly sequenced</td>
<td>[117]</td>
</tr>
<tr>
<td>pFV1</td>
<td>M. thermoformicum THF</td>
<td>13.513</td>
<td></td>
<td>[76,77]</td>
</tr>
<tr>
<td>pFZ1</td>
<td>M. thermoformicum Z-245</td>
<td>11.014</td>
<td></td>
<td>[77,78]</td>
</tr>
<tr>
<td>pFZ2</td>
<td>M. thermoformicum FTF</td>
<td>11</td>
<td></td>
<td>[78]</td>
</tr>
<tr>
<td>pURB800</td>
<td>M. maripaludis</td>
<td>8.3</td>
<td>U47023</td>
<td>[119,122]</td>
</tr>
<tr>
<td>pURB900</td>
<td>Methanococcus st. AG86</td>
<td>20</td>
<td></td>
<td>[123]</td>
</tr>
<tr>
<td>pURB800</td>
<td>M. jannaschii</td>
<td>64</td>
<td>L77118</td>
<td>[43,123]</td>
</tr>
<tr>
<td>pURB801</td>
<td></td>
<td>18</td>
<td>L77119</td>
<td>[43,123]</td>
</tr>
<tr>
<td>pC2A</td>
<td>M. aceticivorans C2A</td>
<td>5.467</td>
<td>U78295</td>
<td>[104,115]</td>
</tr>
</tbody>
</table>

Table 4
Phage-like particles of methanogens

<table>
<thead>
<tr>
<th>Virus</th>
<th>Organism</th>
<th>Size (kb)</th>
<th>Shape</th>
<th>Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3 VLP</td>
<td>M. voltae A3</td>
<td>23</td>
<td>circular, complexed with low molecular mass protein</td>
<td>temperate*</td>
<td>[127,128]</td>
</tr>
<tr>
<td>ΨM1</td>
<td>M. thermoautotrophicum Marburg</td>
<td>30.4</td>
<td>polyhedral capsid with tail</td>
<td>lytic</td>
<td>[125,128]</td>
</tr>
<tr>
<td>ΨM2</td>
<td>M. thermoautotrophicum Marburg</td>
<td>23.25</td>
<td>a spontaneous deletion mutant of ΨM1</td>
<td>lytic</td>
<td>[128]</td>
</tr>
<tr>
<td>ΨF3</td>
<td>M. thermoautotrophicum FF3</td>
<td>36</td>
<td>polyhedral capsid with tail</td>
<td>lytic</td>
<td>[128]</td>
</tr>
<tr>
<td>ΨF1</td>
<td>Methanobacterium sp.</td>
<td>85</td>
<td>polyhedral capsid with tail</td>
<td>lytic</td>
<td>[128]</td>
</tr>
<tr>
<td>PG</td>
<td>Methanobrevibacter smithii G</td>
<td>50</td>
<td>n.d.</td>
<td>lytic</td>
<td>[128]</td>
</tr>
</tbody>
</table>

*Adapted from [128].
3.4.2. The origin of the pac cassette, its use, and derivatives was additionally surrounded by the ISM. voltae used in methanogens have been reported. A selection of these, all containing the pac cassette, is listed in Table 6.

Table 5

<table>
<thead>
<tr>
<th>Insertion element</th>
<th>Organism</th>
<th>Size (bp)</th>
<th>Copies per cell</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR-1</td>
<td><em>M. thermoaotrophicum</em></td>
<td>1501</td>
<td>4-5</td>
<td>[118]</td>
</tr>
<tr>
<td>ISM1</td>
<td><em>M. smithii</em></td>
<td>1381</td>
<td>8-10</td>
<td>[129]</td>
</tr>
<tr>
<td>ISAMJ1-A</td>
<td><em>M. jannaschii</em></td>
<td>703 (family*)</td>
<td>10 (chromosome), 1 (large ECE³)</td>
<td>[43]</td>
</tr>
<tr>
<td>ISAMJ1-B</td>
<td><em>M. jannaschii</em></td>
<td>358-360 (family)</td>
<td>2</td>
<td>[43]</td>
</tr>
<tr>
<td>ISAMJ1-C</td>
<td><em>M. jannaschii</em></td>
<td>265 (family)</td>
<td>18</td>
<td>[43]</td>
</tr>
</tbody>
</table>

¹ISs of *M. jannaschii* are clustered in three families.
²ECE, extra-chromosomal element.

3.4. Plasmid constructions for the use in genetic experiments in methanogens and their applications

In this section the plasmid constructions designed for development of transformation methods and for genetic investigations will be described. The transformation methods, as such, will be discussed in Section 3.5.

3.4.1. The first attempts

The first attempt to produce shuttle vectors for methanogens was based on the plasmid pME2001 isolated from *M. thermoaotrophicum* [121]. The constructs all contained a replication system for methanogens and *Bacteria* or yeast as well as the ampicillin resistance gene for selection in the latter two. No marker was included for selection in the methanogen. The constructions ranged from 8 to 17.4 kb and were able to support the synthesis of polypeptides in *E. coli*, but successful introduction of DNA into the methanogenic species was not reported [121]. No further uses of these constructs were published.

Subsequently, several plasmid constructions for the use in methanogens have been reported. A selection of these, all containing the pac cassette, is listed in Table 6.

3.4.2. The origin of the pac cassette, its use, and alternatives

*M. voltae* was the first methanogen for which a functional integration vector with an antibiotic marker was developed. In 1990, Gernhardt and co-workers [81] reported the construction of an integration vector to be used in *M. voltae*. The vectors, pMip1 and pMip2, carried resistance markers for both the bacterial and the archaeal species, namely the β-lactamase (*amp*) gene and the puromycin transacyethylase gene (*pac*), respectively. In order to ensure the expression of the puromycin resistance in the methanogen, the promoter and terminator fragments from the highly expressed methyl CoM reductase (*mcr*) transcription unit were joined to the pac gene, which originated from *Streptomyces alboniger* [130]. The pac cassette was additionally surrounded by the his genes of *M. voltae* in order to introduce homologue sites enabling genomic integration [81].

Puromycin resistance was contained on two integration vectors for *M. maripaludis*, pJLA5 and pJLA6, respectively, designed by Argyle et al. [106]. Neomycin as an alternative to puromycin has only been used in this study.

Another alternative to puromycin was published in a study by Boccazzi et al. [97] in which the generation of selectable markers for resistance to pseudomonic acid is described. By cloning, expression in *E. coli*, and mutagenesis of the isoleucyl-tRNA synthetase gene (*ile5*) from *M. barkeri* Fusaro by hydroxylamine followed by transformation of *M. aceticivorans* pseudomonic acid-resistant colonies were obtained [97].

3.4.3. Other vectors for Methanococcus

A transposon-based approach was developed for *M. maripaludis* by inserting the pac cassette into the transposon Mud, resulting in the transposon Mudpur on the plasmid pMudpur. The transposon was inserted into MmpA1, a *λ* genomic library clone of *M. maripaludis* [95].

Sandbeck and Leigh [131] cloned DNA fragments of 3 and 4.7 kb, from wild-type *M. maripaludis*, into pMip1 and used the resulting vectors, pKAS100 and pKAS102, respectively, as integration vectors. Interestingly, pKAS102 behaved as a shuttle vector when high concentrations of puromycin were applied. Also when the cultures were maintained on relatively high concentrations of puromycin, tandem repeats seemed to arise, as determined by Southern blot analysis [131].

3.4.4. Insertional mutations

To produce insertional mutations in specific genes in *M. voltae*, two integration vectors, pvhc1.1 and pfr2.2, were developed based on the pUCBM21 vector (a derivative of pUC19) for replication in *E. coli*, with additional inserts of the pac cassette and fragments of 1.17 and 2.1 kb of the genomic hydrogenase operons. The plasmids integrated on the genome by a single cross over event and could be found as tandem repeats or as a single copy on the genome, respectively [94].

The same basic vector, only with an internal region of a flagellin gene, *flaA*, as homologue region resulted in pKJ19. The vector integrated into both the *flaA* region and *flaB*2, limiting the region of homology to 150 bp with an identity of 87% thus indicating that only short homologue stretches are required to support genomic insertion [132].
3.4.5. Reporter genes for *M. voltae*

The *uidA* gene encoding the reporter enzyme β-glucuronidase was placed under control of three different promoter regions in three different reporter cassettes, one was from the *hmvA* gene, which encodes a histone-like chromosomal protein. The construction, pMipuid, was based on pMip1, and carried apart from one of the reporter cassettes also a *hisA* fragment allowing integration [134]. In order to be able to design experiments for studying coordinate regulation of methanogenic genes, another reporter gene was tested in *M. voltae: treA*, encoding trehalase activity. The pMiptre constructions, pWay1 and pWay2, contained the reporter gene was tested in *M. voltae* allowing coordinate regulation of methanogenic genes, another construction series of integration and shuttle vectors for *M. acetivorans* as mentioned above. It was fused with the *pac* cassette to pBluescript. The shuttle vectors, pWM series, were fusions between the natural *M. acetivorans* plasmid pC2A (see Section 3.3.1) and fragments from pR6K (an *E. coli*-based plasmid) and pJK3, providing the required genetic elements such as replication origins, a polyclinker sequence and selective markers for both *E. coli* and *Methanosarcina*. The resulting plasmids were able to replicate in *E. coli* and in the majority of the tested *Methanosarcina* strains.

The series of integration and shuttle vectors for *M. acetivorans* is becoming more extensive. A resistance marker for pseudomonic acid has been developed as an alternative to *pac* as mentioned above. It was fused with the pC2A as well as an *E. coli* origin of replication and a selectable marker to give the shuttle vectors pPB31 through pPB35 [97]. A transposon-based approach has also been used successfully by the group of Dr. Metcalf where a modified version of the transposable element *Himar1* originating from the insect *Hematobia irritans*, which has the advantage of being independent of host factors [96], was used to construct pWM368 through pWM370.

We used the *Methanosarcina* plasmid pJK3 and the shuttle vector pWM321 as basis for a reporter gene system for *M. mazei* S-6. A *lacZ* gene, encoding β-galactosidase,
under the control of a promoter fragment from the universal stress genes dnaK or grpE of *M. mazei* was inserted in the *Nor*I site of pWM629 or pJK3, see Table 6 [111].

### 3.4.7. Other shuttle vectors

A shuttle vector was constructed for *M. maripaludis*, by partially digesting pURB500 and pMEB.2 with *EcoRI*. After ligation the mixture was transformed into *M. maripaludis* and the vector pDLT44, which proved able to replicate in both *E. coli* and *M. maripaludis*, was isolated. The vector was not able to replicate in *M. voltae* [119].

Shuttle vectors for hyper-thermophilic and halophilic Archaea were constructed according to the same general idea as that of Metcalf et al. [104] and Tumbula et al. [119]. The vectors were hybrids between regions of natural cryptic halobacterial plasmids and commercial *E. coli* plasmids, e.g. pUC19 [135,136]. However, for thermophilic methanogens no such work has yet been published.

### 3.5. Transformation methods for methanogens

Different approaches for transformation of methanogens have been used. An overview is given in Table 7. Below, the most important progress within the field of transformation will be described.

#### 3.5.1. Total DNA-mediated transformations

The earliest report on genetic transformation of methanogens was by Bertani and Baresi in 1987 [38]. They performed a so-called DNA-mediated transformation of mutants requiring histidine, purine, or vitamin B₁₂. The mutants were isolated after UV treatment. The transformation consisted of a preincubation for 30 min at 4–12°C in buffer with high CaCl₂ concentration. The purified wild-type DNA was added and the incubation with the mutant cells continued for 60 min, a heat-shock at 43°C for 2–3 min and incubation at 30°C. Cells in suspension were transferred to agar medium lacking the specific growth requirements, and poured into petri-dishes [38]. The number of transformants was maximally 133 transformants μg⁻¹ DNA, but varied significantly. Although the number of transformants was low, it was proved that genetic transformation of the methanogenic Archaea is indeed possible.

In the same way Worrell et al. and Micheletti et al. transformed mutant strains of *M. thermoautotrophicum* ΔH and *M. voltae*, respectively, with genomically wild-type DNA [89,113]. The plating strategy, consisting of simply applying DNA to a plate, and inoculating this with *M. thermoautotrophicum* ΔH, used by Worrell and co-workers gave few transformed colonies. A liquid incubation did not result in transformants [113].

#### 3.5.2. Transduction

Only one other report on genetic modification of a *Methanobacterium* exists [126]. The *M. thermoautotrophicum* Marburg virus-like particles were demonstrated to transduce certain genomic marker traits. Mutant strains of Marburg with the requirement for amino acids were reverted by transduction with phages: Y⁺M1 and Y⁺M2. Phages originating from a pseudomonadic acid A-resistant strain were able to transfer the resistance to a wild-type strain [126]. Cells and phage lysate were incubated for 10 min at room temperature before spreading on selective plates. The transduction frequency was between 5 × 10⁻⁶ and 6 × 10⁻⁵ per plaque forming unit [126]. A polyethylene glycol (PEG)-based method was used for transduction of *M. maripaludis* [95].

#### 3.5.3. Progressing transformation

Micheletti et al. [89] used the natural transformation described by Bertani and Baresi [38] on *M. voltae*, but further introduced an electroporation step on whole cells, which increased the number of transformants up to 740 transformants μg⁻¹ of genomic DNA as opposed to between 2 and 100 transformants μg⁻¹ of genomic DNA.

The next improvement came with the pac-containing vector for *M. voltae*, see Section 3.4.2 [81]. Transformation with the vectors, pMip1 and pMip2, was performed by the method of Bertani and Baresi [38] omitting the CaCl₂ incubation and cold treatment, and vector DNA was used instead of genomic DNA. The number of transformants obtained was in the same range [81]. Sandbeck and Leigh...
achieved approximately a 10-fold higher transformation rate following approximately the same method, but using the integration vectors pKAS100 and pKAS102 and *M. maripaludis*.

In the first report on transformation of a member of *Methanosarcina* [105], the integration vector was transformed into single cells of *M. mazei* S-6 simply by resuspending the cultures with the plasmid DNA on ice for 2 h using the procedure of Bertani and Baresi [38]. Cells were previously made competent to DNA by incubation in high CaCl$_2$- and MgCl$_2$-containing medium for 48–72 h. In this study there is no report on the efficiency of the transformations and the transformed cultures all originated from liquid cultures, i.e. no attempt was made to isolate single colonies [105].

### 3.5.4. Protoplasting and PEG transformation of *Methanococcus*

In 1994 two new transformation strategies were reported for the use in *M. voltae* and *M. maripaludis*, respectively. The ability of *M. voltae* to form protoplasts in an anaerobic suspension of Tris–HCl buffer, containing NaCl and sucrose, was used. At least 50% of the cells lysed during the treatment, but protoplasts also formed [137]. These protoplasts turned out to be competent. 705 transformants µg$^{-1}$ plasmid DNA were obtained using the method of Bertani and Baresi on the protoplasts, and up to 3417 transformants µg$^{-1}$ DNA when the culture was electroporated and the plasmid linearized [93]. In a later report on transformation of *M. voltae*, protoplast transformation was also used. The electroporation procedure, which is somewhat tricky to perform under anaerobic conditions, may be left out [94,132,138].

An optimized PEG-mediated transformation of *M. maripaludis* was presented by Tumbula et al. [80] and has been used in all later studies with this organism [82,95,106]. The main components of the transformation buffer were 50 mM Tris–HCl, 0.38 M NaCl, 0.35 M sucrose. The cells were washed in buffer and incubated under shaking with plasmid DNA and PEG8000. The optimal concentration of PEG8000 in the transformation solution was 13%, but the molecular mass of PEG or the incubation time of cells and plasmid in the solution did not affect the efficiency. With the integration plasmid pKAS102, frequencies of 1.8×10$^5$ transformants µg$^{-1}$ were obtained [80]. It is not clear at which puromycin concentration the selection was performed.

### 3.5.5. Liposome-mediated transformation of *Methanosarcina*

Liposome-mediated transformation was first used on *Methanosarcina* in 1997 [104] and has proven very efficient. Exponentially growing cells were harvested and resuspended in a sucrose solution. Plasmid DNA was allowed to form liposome complexes in a DOTAP/HEPES solution for 15 min before applying this mixture to the cell suspension, which was then incubated for 4 h at room temperature. HEPES was later exchanged with a 0.85 M sucrose buffer [96]. After incubation the sample was transferred to growth medium and incubated at the optimal growth temperature for 12–16 h before transferring to medium containing 1 µg ml$^{-1}$ puromycin [104]. Up to 2×10$^8$ transformants µg$^{-1}$ DNA was achieved when the method was optimized with *M. acetivorans* [104].

The method was also used to introduce the so-called mini-mariner transposons into *M. acetivorans* [96]. We have successfully used the liposome method as described by Metcalf et al. [104] for single cells of *M. mazei* S-6 [111] and the method was recently also used on *M. voltae* [134].

### 3.5.6. The challenge of morphology

One of the challenges faced with some of the *Methanosarcina* species is their morphological characteristics, which are also found in members of *Haloarcula* [136]. Ideally, a transformation/permeabilization method should be applicable in every morphological form of the strain. It is, however, unlikely that a straightforward transformation method will soon be developed for the aggregate or clump form of *Methanosarcina*, unless a novel enzyme product, e.g. disaggregatase (see Section 1.2.6), is developed or discovered. We transformed single cells of *M. mazei* S-6 but had difficulties in keeping the single-cell morphology; the puromycin somehow triggered aggregate formation [111]. We tested the puromycin tolerance of a wild-type aggregate culture of *M. mazei* (the only difference in growth conditions from single cells was lower CaCl$_2$ and MgCl$_2$ concentrations). The puromycin tolerance in single cells and aggregates was similar, indicating that aggregate formation does not protect the cell [111]. A similar feature was observed in experiments of the halo-philic archaean *Haloarcula* [136], which is able to undergo a cell cycle similar to that of *Methanosarcina* including conversion between single cells and clusters. The frequency of the cluster formation was much higher under selective pressure of mevinolin than in non-selective media for two transformant strains [136]. As previously mentioned, a change in cell permeability is the cause of antibiotic resistance in some organisms and we speculate that aggregate formation encountered under selective pressure is a protection response by the cells. However, since the aggregates and single cells of *M. mazei* are equally sensitive to the antibiotic the shut-out strategy does not work. After 10–15 transfers of the aggregated transformants reversion to the single-cell form in the presence of puromycin was observed in some cultures [111].

### 3.5.7. Special considerations for thermophilic methanogens

Most of the above mentioned experiments were performed with mesophilic strains, except for *M. thermoautotrophicum*, which grow optimally between 65 and 70°C. Reports are lacking completely on transformation of hyper-thermophilic methanogens, possibly because several
challenges are faced in order to adapt methods to hyperthermophilic conditions, e.g. that no thermostable vectors are known and that the stability of many antibiotics, for example, is greatly dependent on the temperature. Also the functionality of the resistance enzymes is affected. The puromycin transacetylase (from the pac cassette) is naturally stable for only 12 min at 70°C [139]. The gene for a thermostable hygromycin phosphotransferase from E. coli was used to transform Sulfolobus solfataricus, which is a thermophilic Archaea [140]. Alternative genetic markers, to those that are degraded under thermophilic conditions, must be found, and selection markers for hyper-thermophiles are under development [114].

Thermostable reporter proteins are also rare, but as an example of a promising candidate, a β-galactosidase, lacS, originating from the thermophilic archaeon S. solfataricus, has been isolated [141]. This β-galactosidase has optimal activity at 90°C. The gene has been sequenced and expressed under control of a galactosidase inducible upstream activating factor in Saccharomyces cerevisiae [141, 142]. The enzyme retained the properties of the native protein, but the translational level was judged to be relatively low, which was assumed to be caused by codon usage biases [143].

The cell envelope of hyper-thermophilic organisms has evolved to resist the strain of high temperatures. Electroporation, although only used in few cases with methanogens, is usually performed quickly and at low temperatures. At low temperatures, the cell membranes of thermophiles are lacking flexibility hindering the penetration of the DNA, but at higher temperatures the increased oxygen sensitivity of the organism will cause a decrease in transformation efficiency [114].

4. Perspectives

4.1. Present tools

At present the tools for addressing several issues of methanogenic genetics are becoming available. The past few years have been very fruitful in the terms of development of transformation systems, cloning vehicles, and identification techniques, which are also appropriate for in situ investigations.

At this point the use of integration vectors for M. maripaludis and M. voltae is quite advanced. Reporter genes are available; two for M. voltae and one for M. maripaludis, but it is likely that all three genes are functional in both organisms as well as in other methanogens. Two antibiotic markers are also available, the frequently used puromycin, and neomycin, which has only been applied in one study. The availability of more reporter gene and selective antibiotics from now on enables more sophisticated experimental set-ups. In Methanosarcina important advances have also been made within the last 3 years. For some species of Methanosarcina the challenge of developing transformation systems for all morphological forms still persists.

For some genera transformation studies have not yet been reported, e.g. Methanoseta and Methanobrevibacter. Thermophilic and hyper-thermophilic organisms bring other challenges, e.g. in the special nature of their cell envelope, in suitable thermostable molecular markers and in transformation systems. Much effort is presently put into research in extremophiles, since this group of organisms is believed to be of great potential for the biotechnological industry [144]. The encouraging results with mesophilic methanogens and the large interest in thermophilic organisms in general (e.g. in the genome sequencing projects [43, 44]) will undoubtedly result in rapid progress within this field.

4.2. Questions to be answered

In basic research, a group as versatile, e.g. with respect to cell envelope and natural habitats, and yet so uniform, e.g. with respect to end-product of metabolism, as the methanogens will always trigger the curiosity of scientists. For comparative studies of genetic regulation between the three domains, Archaea, Bacteria and Eukarya, the methanogens are important. For studies of adaptation to extreme conditions the methanogens occupy a range of habitats with different temperatures and osmolarity, and anaerobiosis is also an intriguing feature. Methanogens have very limited substrate spectra reflecting a very limited metabolic capability. The reason why so little metabolic development seems to have occurred in the course of evolution is further interesting to study. Much more knowledge exists today on the genetics of Bacteria and Eukarya compared to Archaea. The origin of replication of DNA in Archaea is unknown, and under the genome sequencing projects it was found that a large fraction of the genes were Archaea-specific [43, 44]. The areas in need of investigations are vast.

More specifically, since the methanogens constitute an important fraction of the active biomass in biogas reactors for waste and wastewater treatment and fuel production, issues of biotechnological importance should be addressed. The reactor performance, where Methanosarcina is involved, is dependent on the morphology of the methanogens, which is previously studied [145, 146]. Not only is the morphology important for reactor performance but it also provides an ideal system for studying gene expression and regulation in methanogens. The enzyme disaggregatase from Methanosarcina is known and could be used as an ideal starting point for analysis of the regulation.

In Methanosarcina several heat-shock or stress-induced genes are known and their transcription has been analyzed (e.g. [72, 147–149]). The stress-induced genes provide another excellent system for studying genetic regulation in methanogens.
Tools for identification of methanogens, for genetic manipulation, and for studying molecular regulation are available. Particularly in the latter two areas the practical applications are still limited. Consequently, the scientific possibilities within molecular biology research in methanogens are almost endless.

Acknowledgements

We thank Anders Hay Sørensen for critical reading of the manuscript. This work was supported by the Danish National Research Council, Grant no. 9502848.

References


M. Lange, B.K. Ahring | FEMS Microbiology Reviews 25 (2001) 553–571


M. Lange, B.K. Ahring / FEMS Microbiology Reviews 25 (2001) 553–571