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Taxonomy of methanogens by ELISA techniques

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Summary. Polyclonal antisera have been used for the development of a novel serotaxonomic system for methanogenic bacteria by quantifying immunological cross-reactions of the antisera with heterologous strains. This system corresponds well to phylogenetic relationships among methanogenic bacteria as analysed by other authors, and is in good agreement with the existing immunological fingerprint for immunotyping of methanogenic bacteria.

Introduction

A recent paper (Bryniok and Trösch 1989) describes the development of enzyme-linked immunosorbent assay (ELISA) techniques for quantitative determination of methanogenic species from pure cultures as well as from complex environments. For these tests polyclonal antisera were used against 29 methanogenic strains.

Identification of methanogenic bacteria from complex populations with unknown microbial composition occurs via immunological cross-reactions. Therefore relationship-correlated cross-reactions are necessary for the application of immunological methods in such populations. Cross-reactions between different strains of the same species should be significantly high, and high cross-reactions between different families should not occur, as shown in previous serological analyses of the relationships among methanogenic bacteria (Conway de Macario and Macario 1986; Conway de Macario et al. 1982b; Macario and Conway de Macario 1983). These conditions were verified using a semiquantitative slide assay (Conway de Macario et al. 1982a, 1983) and resulted in an immunological fingerprint system for the taxonomy of methanogenic bacteria.

With respect to the main purpose of our investigation, the quantitative determination of methanogens, it was necessary to evaluate the cross-reactions between the strains used and to quantify them. This means that the portion of antibodies reacting with each of the heterologous strains had to be quantified. Such a portion can be measured with an indirect ELISA by building up calibration curves for the antisera with their homologous antigens and comparing the absorbance values from a second ELISA performed with the heterologous antigen with those of the calibration curve.

Materials and methods

Bacteria. Methanogenic bacteria used as antigens for the production of polyclonal antisera are listed in Table 1, which also includes media and incubation temperatures. Eight of the strains used in this investigation were isolated in our own laboratory; the others were obtained from the German type culture collection (Dt. Slg. für Mikroorganismen, DSM, Braunschweig, FRG). At least one representative strain of each genus of methanogenic bacteria was selected, except for *Methanothrix* and some other genera that are not as relevant to technological applications. More than two strains have been selected from *Methanobacterium* and *Methanosarcina* as these are the most commonly occurring methanogens in biogas reactors.

Culture media. The methanogens were cultured using the serum bottle modification (Miller and Wolin 1974) of the Hungate technique (Hungate 1969) in the media of Balch et al. (1979): Methanobacteriales and *Methanospirillum* in medium 1; Methosarcinaceae in medium 3 supplemented with 5 ml methanol/l (MeOH 3); all other methanogens in medium 3 without supplement.

Determination of working dilutions and cross-reactions. The working dilutions for the indirect ELISA were fixed by adsorbing 1:2 dilution series of the antisera to microtitration

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Table 1. Methanogenic bacteria and culture conditions as described in Materials and methods

Species	Strain	Source[1]	Medium	Temperature
Methanobacterium formicicum	MF	DSM 1535	Balch 1	37 °C
Methanobacterium formicicum	JF-1	DSM 2639	Balch 1	37 °C
Methanobacterium formicicum	IVA	IGB[2]	Balch 1	37 °C
Methanobacterium bryantii	M.o.H.	DSM 863	Balch 1	37 °C
Methanobacterium thermoautotrophicum	ΔH	DSM 1053	Balch 1	55 °C
Methanobacterium thermoautotrophicum	IIA	IGB[2]	Balch 1	55 °C
Methanobacterium thermoautotrophicum	IIB	IGB[2]	Balch 1	55 °C
Methanobacterium thermoautotrophicum	IIC	IGB[2]	Balch 1	55 °C
Methanobrevibacter ruminantium	M1	DSM 1093	Balch 1	37 °C
Methanococcus vannielii	SB	DSM 1224	Balch 3	37 °C
Methanococcus voltae	PS	DSM 1537	Balch 3	37 °C
Methanogenium marisnigri	JR1	DSM 1498	Balch 3	25 °C
Methanogenium sp.	I1	IGB[2]	Balch 3	55 °C
Methanogenium sp.	II1	IGB[2]	Balch 3	55 °C
Methanospirillum hungatei	JF1	DSM 864	Balch 1	37 °C
Methanoplanus limicola	M3	DSM 2279	Balch 3	37 °C
Methanoplanus sp.	VIII2	IGB[2]	Balch 3	37 °C
Methanosarcina barkeri	MS	DSM 800	MeOH 3	37 °C
Methanosarcina sp.	KS	IGB[2]	MeOH 3	37 °C
Methanosarcina sp.	G1	DSM 3338	MeOH 3	37 °C
Methanosarcina mazei	MC_3	DSM 2907	MeOH 3	37 °C
Methanosarcina mazei	S-6	DSM 2053	MeOH 3	37 °C
Methanosarcina acetivorans	C2A	DSM 2834	MeOH 3	37 °C
Methanosarcina sp.	MST-A1	DSM 2905	MeOH 3	55 °C
Methanosarcina thermophila	TM-1	DSM 1825	MeOH 3	55 °C
Methanosarcina sp.	CHTI-55	DSM 2906	MeOH 3	55 °C
Methanosarcina sp.	MP	DSM 2980	MeOH 3	55 °C
Methanolobus tindarius	Tindari 3	DSM 2278	MeOH 3	25 °C
Methanolobus vulcani	PL-12/M	DSM 3029	MeOH 3	37 °C

[1] Strains obtained from the German type culture collection (Deutsche Sammlung für Mikroorganismen, DSM)
[2] Strains isolated in the Fraunhoferinstitut für Grenzflächen- und Bioverfahrenstechnik

plates, which were precoated with the homologous antigens, and performing the indirect ELISA as described in Bryniok and Trösch (1989). When the resulting absorbance values were plotted against the dilution steps, sigmoid calibration curves appeared.

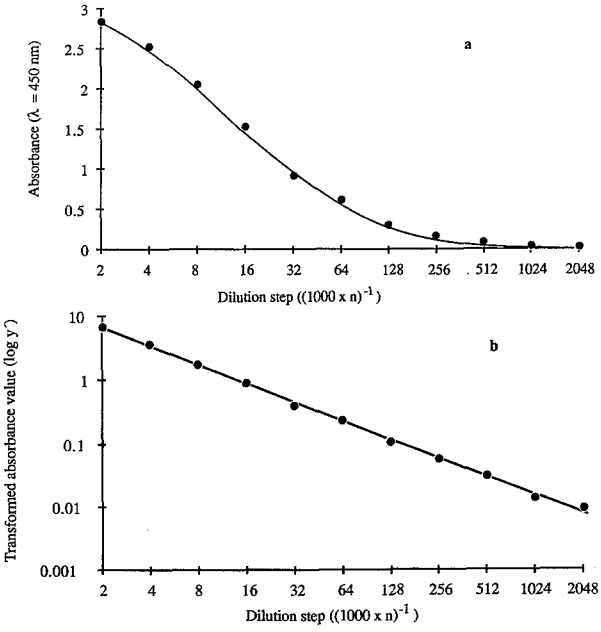


Fig. 1. Calibration curve for anti-*Methanosarcina thermophila* strain TM-1-serum **(a)**, and linearized form, calculated parameters: A = −0.0346, B = 2.961, C = 4.256, D = 1.727 **(b)**; for A–D see Materials and methods

For the determination of cross-reactions the antisera were serially diluted 1:2. The wells of a microtitration plate, precoated with the heterologous antigen, were filled with the serum dilutions, as was another plate precoated with the homologous antigen. The indirect ELISA was performed in both microtitration plates. The quasi-calibration curve obtained from the heterologous test cold be compared to the calibration curve from the homologous control experiment and cross-reactions could thus be calculated.

Evaluation of calibration curves. The most exact mathematical procedure for the evaluation of the sigmoid ELISA calibration curves is the linearization by a log-logistic function and subsequent statistical analysis (Dudley et al. 1985; Winkler et al. 1986). The function with four unknown parameters to be determined is therefore called a four-component logit:

$$y = A + B \cdot \frac{\exp(C + D \cdot \ln x)}{1 + \exp(C + D \cdot \ln x)} \tag{1}$$

or in an algebraic transformation:

$$\ln\left(\frac{y - A}{B - y - A}\right) = C \cdot D \ln x \tag{2}$$

or:

$$\bar{y} = C + D\bar{x} \tag{2.1}$$

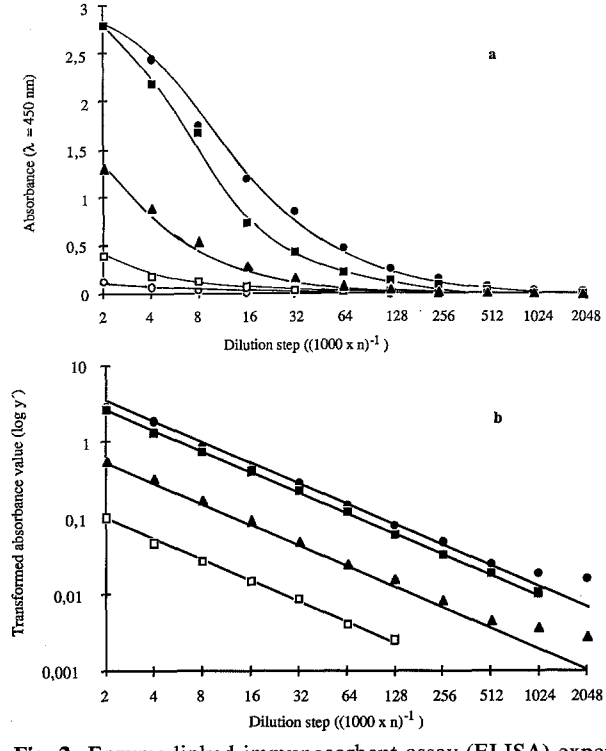
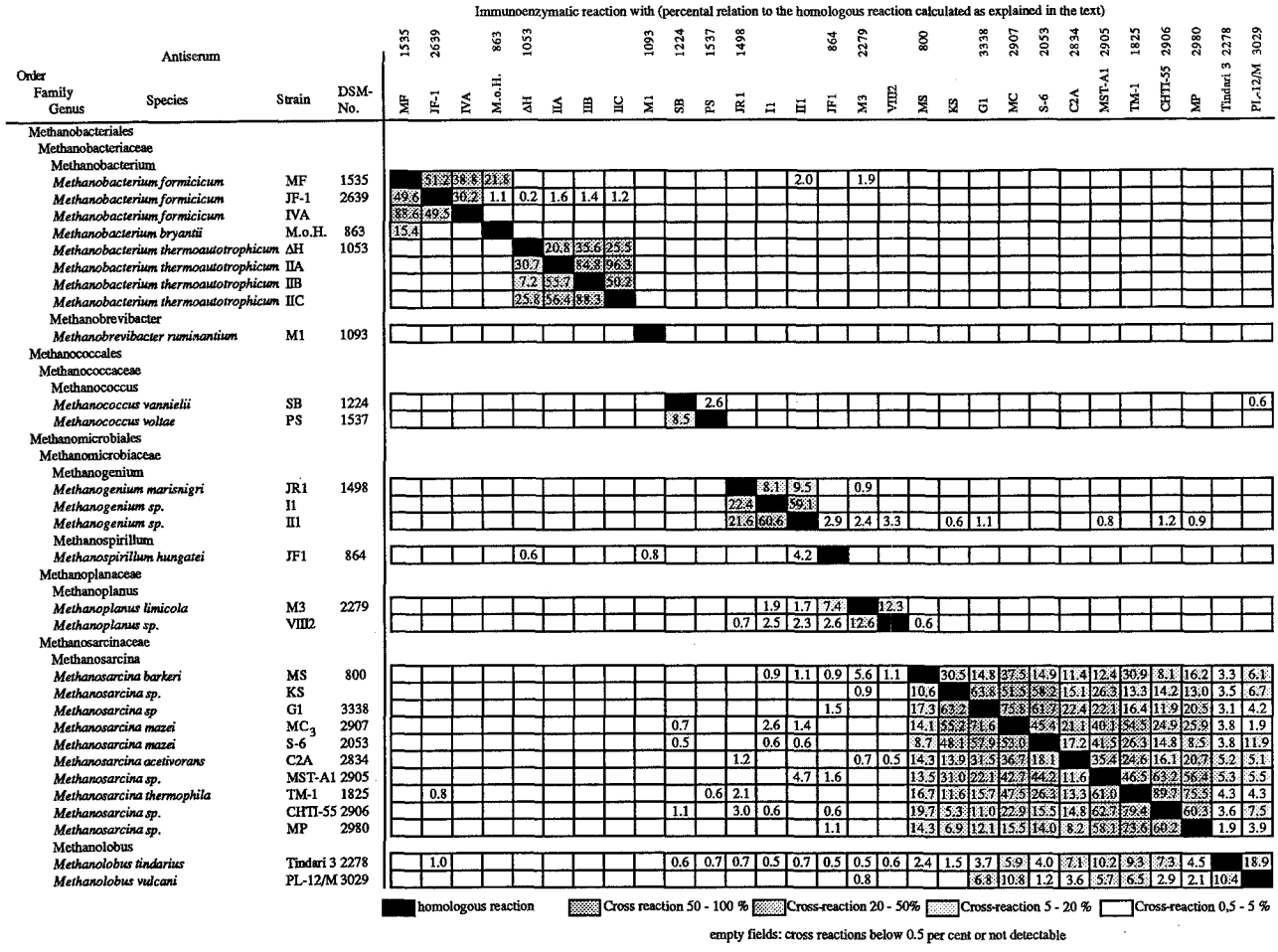


Fig. 2. Enzyme-linked immunosorbent assay (ELISA) experiments with anti-*Methanosarcina* sp. strain CHTI-55 **(a)**; ●, homologous reaction, cross-reaction with ■ *Methanosarcina thermophila* strain TM-1; ▲, *Methanosarcina acetivorans* strain C2A; □, *Methanogenium marisnigri* strain JRI; and ○, *Methanobacterium formicium* strain JF-1. **b** Linearized form, cross-reactions with *Methanobacterium formicium* JF-I were too low to obtain a reasonable linearization of the measured values

Table 2. Immunological cross reactions



$$\bar{y}=\ln\left(\frac{y-A}{B-y-A}\right) \text{ and } \bar{x}=\ln x \tag{2.2}$$

where y = absorbance in the ELISA; x = concentration of antiserum or antigen; A = lower asymptotic absorbance value; B = upper asymptotic absorbance value; C = intersection of the linear form; D = slope of the linear form.

Winkler et al. (1986) have written a program for the calculation of the four parameters, identification and possible elimination of outliners, and for the calculation of the concentrations of unknown samples with the evaluated calibration curve. This program was slightly modified and completed with input and output routines to be suitable here.

Calculation of working dilutions. The sigmoid curves resulting from the ELISA described above were evaluated with this computer program and the dilution producing an absorbance value of 2.0 in the ELISA was calculated. To ascertain a sufficient excess of antibodies in the test, a fivefold concentration was used for routine application of the indirect ELISA. To avoid high values of unspecific reactions, higher concentrations were not used.

Calculation of cross-reactions. The quasi-calibration curves obtained from ELISA experiments with heterologous antigens were linearized in the same way as calibration curves obtained with homologous antigens. For each dilution step the transformed absorbance value:

$$y'=\frac{y-A}{B-y-A} \tag{3}$$

of the quasi-calibration curve of the heterologous ELISA was divided by the transformed homologous value of the same dilution step and multiplied by 100 to give the percentage of the cross-reaction.

Results and discussion

Absorbance values of ELISAs performed with dilution series of polyclonal antisera and homologous antigens gave sigmoid calibration curves when plotted versus dilution steps. Calibration curves as well as cross-reaction determinations were performed with triple values for each dilution step. Standard deviations generally did not exceed 5% and certain exceptions reached maxi-

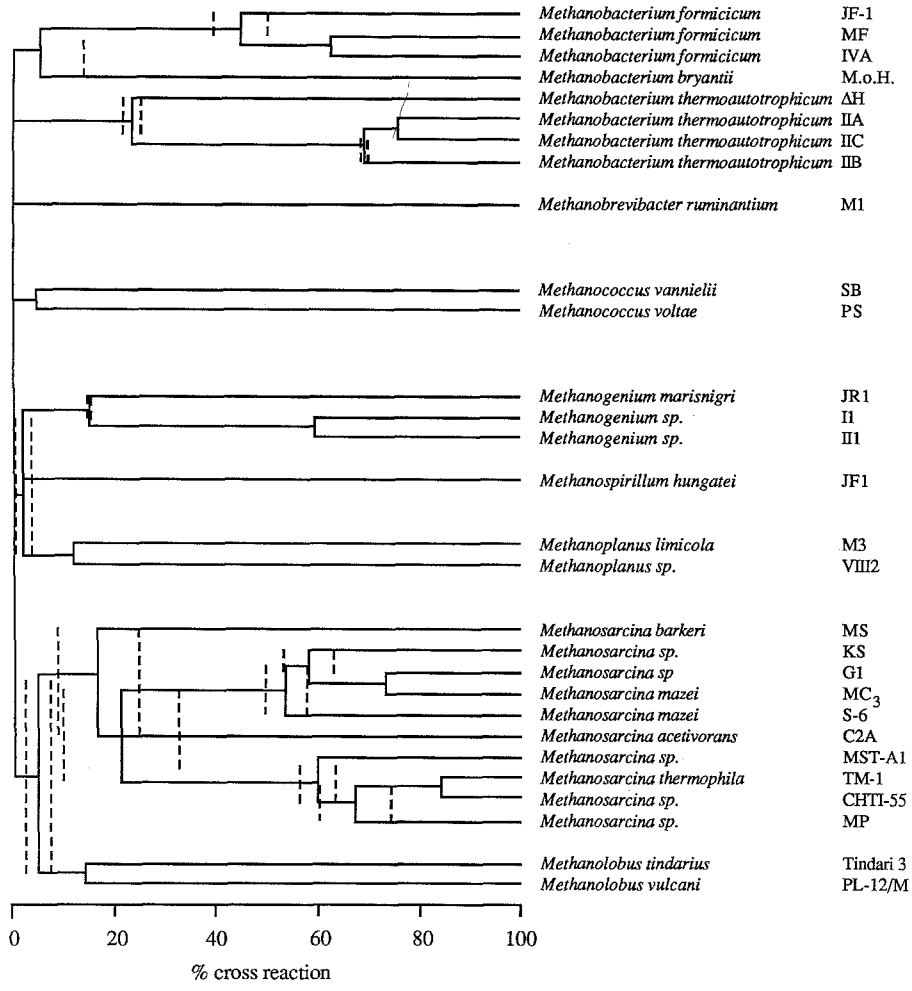


Fig. 3. Dendrogram of the relationships among methanogenic bacteria based on the values in Table 2. The cross-reaction between two strains are calculated as average between all cross-reactions performed with these strains. The dendrogram is based on averages of all binary cross reaction values. SD indicated by the *dotted lines*

mally 10%. Figure 1a shows a calibration curve for anti-*Methanosarcina thermophila* TM-1 serum as a typical example for calibration curves for antisera, as used for the determination of working dilutions of the antisera. Figure 1b shows the linearized form as evaluated with the computer program basing on the four-component logit (Eq. 1).

If cross-reactions were high enough to result in a complete sigmoid curve, the curves obtained with heterologous antigens in sigmoid curves shifted leftwards parallel to the X-axis. Poor cross-reactions showed only the lower part of the sigmoid plots (Fig. 2). All cross-reactions between methanogenic strains were determined at least twice, and if they were higher than 20% at least three times. The mean values exceeding 0.5% are listed completely in Table 2.

Cross-reactions between the methanogenic bacteria were considerable, according to their phylogenetic relationships as indicated by 16 s rRNA sequences, DNA homology or morphological and physiological properties (Balch et al. 1979; Tu et al. 1982; Sowers et al. 1984). Cross-reactions were high between strains of the same species and low or not detectable between strains of different species or genera. The only cross-reactions between different families exceeding 1% occurred between Methanoplanaceae and Methanomicrobiaceae. In fact, the relationship of Methanoplanaceae to Methanomicrobiaceae is shown to be much higher than any other relationship between methanogenic families (Tu et al. 1982). In other cases *Methanoplanus* is even seen as a member of Methanomicrobiaceae (Jones et al. 1987). Moreover, the results shown were in good agreement with other investigations (Conway de Macario and Macario 1986; Macario and Conway de Macario 1983). Significant cross-reactions were found here in the same clusters as in their investigations.

Isolates from the Fraunhofer-Institut für

Grenzflächen- und Bioverfahrenstechnik were also immunotyped in Table 2, showing perfect agreement with the physiological characterization performed in parallel, as will be shown elsewhere (Bryniok et al. in preparation).

By registration of iummunoenzymatic cross-reactions in dilution series of antisera and calculation by linearizing and division of transformed values by those of homologous reactions, it has been demonstrated for the first time, to our knowledge, that it is possible to obtain dilution-independent immunological values comparable to the S_{AB} values of rRNA sequencing. As with DNA or RNA sequences, amino acid sequences of unique enzymes or other molecular fine structures of biological macromolecular, antigenic determinants of cell surfaces are subject to statistically detectable evolutionary changes.

Phylogenetic investigations which are based on immunological data might otherwise be uncertain in respect to individual properties of the immune system of the specific immunized animal species. Moreover, antibodies in polyclonal antisera should react with all surface determinants of the immunizing antigen, but varying immunogenity due to the different chemical compositions of surface components could then lead to cross-reactions which do not correspond to the phylogenetic relationship of the antigens. Nevertheless, a dendrogram (Fig. 3) constructed with the data of Table 2 was in very good agreement with dendograms constructed by rRNA or DNA sequence analysis (Balch et al. 1979; Sowers et al. 1984) or cross-hybridization (Tu et al. 1982).

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