

An Improved Enzyme-Linked Immunosorbent Assay for Whole-Cell Determination of Methanogens in Samples from Anaerobic Reactors

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An enzyme-linked immunosorbent assay was developed for the detection of whole cells of methanogens in samples from anaerobic continuously stirred tank digesters treating slurries of solid waste. The assay was found to allow for quantitative analysis of the most important groups of methanogens in samples from anaerobic digesters in a reproducible manner. Polyclonal antisera against eight strains of methanogens were employed in the test. The specificities of the antisera were increased by adsorption with cross-reacting cells. The reproducibility of the assay depended on the use of high-quality microtiter plates and the addition of dilute hydrochloric acid to the samples. In an experiment on different digester samples, the test demonstrated a unique pattern of different methanogenic strains present in each sample. The limited preparatory work required for the assay and the simple assay design make the test well suited for routine analysis of large numbers of samples and thus for process surveillance during operation of biogas digesters.

Optimization of the anaerobic digestion processes for treatment of waste and recovery of energy requires a refined process operation, during which the physiology of the microbes is considered. Therefore, there is a need for measurement strategies suited for monitoring of the process and characterization of the microbiota present (1, 29, 32). Investigations aimed at establishing applicable test procedures for process control have repeatedly been concentrated on the presence of selected key organisms, e.g., the methanogens (7, 22, 24, 27).

During the anaerobic digestion of complex organic matter, the methanogens are responsible for the terminal conversion of intermediate compounds to methane and carbon dioxide. Thereby, they constitute an important sink for disposal of reducing equivalents that would otherwise accumulate and disturb the overall process by inhibition of important steps (14, 25).

Immunological methods for detection of methanogens in environmental samples have been presented in a number of cases (2, 7, 20–24, 28, 31, 34, 35). Especially Conway de Macario, Macario, and coworkers have repeatedly demonstrated the usefulness of immunological methods for characterization of samples from anaerobic reactors (2, 20–24, 28, 34). The potential of the methods was highlighted by the observation that polyclonal antisera against methanogens are specific at least within families and often within genera or even species (12).

The enzyme-linked immunosorbent assay (ELISA) is a widely used immunological assay format suited for routine evaluation of large numbers of samples (9). Several attempts of adapting this procedure to the quantification of methanogens in reactor samples have been described (6, 7, 18, 19). Archer (6) and Bryniok and Trösch (7), however, concluded that the

assay was not applicable to complex environmental samples. Other approaches have concentrated on the sensitivity of the test rather than the applicability for work on complex samples (7, 18, 19).

To increase the applicability of the ELISA method for quantification of methanogens in digester samples, we have developed an improved albeit simple procedure. In this article, we describe our new method along with its use for determination of methanogens in anaerobic reactors. We include a comparison of our method with another immunological test procedure, the slide indirect immunofluorescence (IIF) test.

MATERIALS AND METHODS

Strains of methanogens and production of antisera. Table 1 lists the strains of methanogens used in this work together with their origins and growth conditions. Cells of *Methanosaeta thermophila* CALS-1 were purchased as a large batch from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). The strains Pluk and MB have been isolated in our laboratory from thermophilic sludge digesters. The isolation of strain Pluk has been described previously (3). Strains Pluk and MB are thermophilic, rod-shaped methanogens converting hydrogen to methane. Strain MB also utilizes formate. The strains have tentatively been placed in the genus *Methanobacterium*. The strains Marburg and CB12 were earlier placed in the species *Methanobacterium thermoautotrophicum* (16) and *Methanobacterium thermoformicum* (36), respectively, but their correct positions remain to be assessed (33). The four strains are, therefore, not given specific epithets, and their generic name is enclosed in quotation marks.

Polyclonal antisera were raised in rabbits against the strains listed in Table 2. Cells for immunizations were prepared as described by Conway de Macario et al. (11). Cell suspensions were emulsified in Freund's incomplete adjuvant.

Antiserum against *Methanobacterium formicum* JF-1 was kindly provided by Dieter Bryniok, Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik, Stuttgart, Germany.

Reactor samples. The anaerobic reactor samples used for the different experiments were sampled from thermophilic (55°C) bench-scale continuously stirred tank reactors fed with cow or swine manure and operated at a retention time of 15 days (4). In addition, homogenized granular sludge from a mesophilic bench-scale upflow anaerobic sludge bed reactor treating wastewater from celery processing was used. The biomass samples were fixed by the addition of 3.7% (wt/vol) formaldehyde and stored at 4°C. Before being analyzed, the samples were diluted 10-fold in saline (0.85% [wt/vol] NaCl) with 1.6% (wt/vol) formaldehyde (formalin salt water [FSW]) and homogenized with a tissue grinder (Teflon pestle tissue homogenizer; Thomas Scientific, Swedesboro, N.J.) and passages through successively smaller needles. Subsequent dilutions were done in FSW.

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TABLE 1. Methanogenic strains used in this work

Strain	Medium (reference)	Substrate	Temp (°C)	Source	DSM strain no.
<i>Methanobacterium thermoautotrophicum</i> ΔH	BA medium (5)	H ₂ -CO ₂	60	DSM	1053
" <i>Methanobacterium</i> " sp. strain PLUK ^a	BA medium	H ₂ -CO ₂	60	Our lab	
" <i>Methanobacterium</i> " sp. strain MB	BA medium	H ₂ -CO ₂	60	Our lab	
" <i>Methanobacterium</i> " sp. strain Marburg	BA medium	H ₂ -CO ₂	60	DSM	2133
" <i>Methanobacterium</i> " sp. strain CB12	BA medium	H ₂ -CO ₂	60	DSM	3664
<i>Methanobacterium formicicum</i> MF	Mod. BA medium ^b	H ₂ -CO ₂	37	DSM	1535
<i>Methanobacterium bryantii</i> M.o.H.	Mod. BA medium	H ₂ -CO ₂	37	DSM	863
<i>Methanobrevibacter arborophilicus</i> AZ	Mod. BA medium	H ₂ -CO ₂	37	DSM	744
<i>Methanococcus vannielii</i> SB	DSM medium 141 (13)	H ₂ -CO ₂	37	DSM	1224
<i>Methanosarcina mazei</i> S-6	Mod. DSM medium 120 (13) ^c	TMA ^f	37	Wadsworth ^d	2053
<i>Methanosarcina thermophila</i> TM-1	BA TM-1 medium ^e	TMA	50	DSM	1825
<i>Methanosaeta concilii</i> GP6	DSM medium 334 (13)	Acetate	37	DSM	3671
<i>Methanosaeta concilii</i> Opfikon	DSM medium 334 (13)	Acetate	37	DSM	2139
<i>Methanosaeta thermophila</i> CALS-1	DSM medium 387 (13)	Acetate	60	DSM	3870

^a The binary names of strains with genus names in quotation marks have not been assessed (see text for details).

^b Mod. BA medium, BA medium (5) modified by Hendriksen and Ahring (17) supplemented with 1 g of yeast extract per liter, 0.5 g of cysteine hydrochloride per liter, and 2 mM sodium acetate.

^c Mod. DSM medium, DSM medium supplemented with MgCl₂ to 10.5 mM and CaCl₂ to 5 mM and with methanol replaced by 50 mM trimethylamine. This medium supported growth of *Methanosarcina mazei* S-6 as single cells.

^d *Methanosarcina mazei* S-6 was a gift from E. Conway de Macario, Wadsworth Centre for Laboratories and Research, Albany, N.Y.

^e BA TM-1 medium, BA medium (5) supplemented with 100 mM trimethylamine, 1 g of yeast extract per liter, MgCl₂ to 150 mM, and CaCl₂ to 5 mM. This medium supported growth of *Methanosarcina thermophila* TM-1 as single cells.

^f TMA, trimethylamine.

The ELISA method. A 100-μl amount of cell suspension or diluted biomass sample was added to each well of a polystyrene microtitration plate (Immuno Plate MaxiSorp F96; Nunc A/S, Roskilde, Denmark) together with 100 μl of 0.1 M HCl. The plates were incubated at 37°C for 2 h and at 4°C overnight. The plates were emptied and washed four times with washing buffer containing, per liter of MilliQ water, 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH₂PO₄, 1.15 g of Na₂HPO₄, and 1 cm³ of Tween 20 (pH 7.4).

The antibodies were diluted in phosphate-buffered saline (PBS; pH 7.6) with 1% (wt/vol) bovine albumin (Sigma A-4503) and 1% (vol/vol) Triton X-100. An amount of 100 μl of diluted rabbit antiserum or control preimmune serum was added to each well, and the plates were incubated overnight at 4°C, emptied, and washed as described above. Peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) was diluted 1,000-fold, and 100 μl was added to each well. After 3 h at 37°C, the plates were washed; addition of 100 μl of substrate per well followed. The substrate was a solution of *ortho*-phenylenediamine and hydrogen peroxide, prepared as described in the manufacturer's instructions (KEM-EN-TECH, Copenhagen, Denmark). The reaction was stopped after exactly 30 min by the addition of 100 μl of 1 M H₂SO₄ to each well, and the A₄₉₀ was measured with a Biotec EL311SX plate reader against air. The test was in all cases performed with at least four parallel wells.

Standard curves were established in all experiments by use of defined suspensions of cells in FSW. Cell density in suspensions of the filamentous strains *Methanosaeta concilii* GP6 and *Methanosaeta concilii* Opfikon could, however, not be counted directly. The A_{578s} of the suspensions of the two strains were, therefore, adjusted to approximately 0.2, the absorbance from which the suspensions were diluted for use in standard curves. The results (absorbance values) obtained in the ELISA with the reference cell suspensions were plotted versus the logarithm of the cell concentrations. The standard curves were sigmoidal as also shown by Bryniok and Trösch (8). Curve fits were performed by use of the SigmaPlot scientific graphing system software version 4.10 (Jandel Corporation) with the following formula for a sigmoid curve (8): $y = A + B \cdot [\exp(C + D \cdot \ln x)] / [1 + \exp(C + D \cdot \ln x)]$, where y is A₄₉₀, x is the log(concentration of cells), A is the lower asymptotic A₄₉₀ value, B is the upper asymptotic A₄₉₀ value, and C and D are constants. For the curve fits, the following approximate values were set as the starting point: $A = 0$; $B = 6$; $C = -20$; $D = 11$. Standard curves of *Methanosaeta concilii* GP6 were related to dilution of the reference antigen suspension instead of cell numbers. A₄₉₀ values from different tests were compared by the two-sided *t* test, with each sample consisting of the values from four parallel wells.

Adhesion of samples. The effect of the addition of HCl or methylglyoxal (10) on sample adhesion was tested. The antigen was cells of "*Methanobacterium*" sp. strain Pluk, 3.7 × 10⁶ cells/cm³. The addition consisted of 18 μl of 2% (wt/vol) methylglyoxal, 100 μl of 0.1 M HCl, 36 μl of methylglyoxal plus 100 μl of HCl, or nothing. Twelve wells were prepared with each addition.

Likewise, results with a standard microtitration plate (Nunc 2-69620, polystyrene, flat bottom) were compared with those with the MaxiSorp plate. With the standard plate, 100 μl of water was added to the sample instead of 0.1 M HCl. Two MaxiSorp plates were tested in the same trial, one with 0.1 M HCl and one with an equal amount of water added to the sample. The antigen was cells of "*Methanobacterium*" sp. strain Marburg or digested and diluted cow manure.

Removal of cross-reactions. In cases of extensive cross-reactions of the rabbit antisera with strains of methanogens other than the immunizing strain (listed in Table 1), the cross-reacting antibodies were removed by an adsorption procedure adopted from Conway de Macario et al. (12). Each serum was diluted 100 times and mixed with a pellet of the cross-reacting cells. The mixture was agitated overnight at 4°C and then filtered through a 0.2-μm-pore-size filter. Diluted and adsorbed antiserum was supplemented with 0.1% NaN₃ and stored at 4°C.

Use of the ELISA method on reactor samples. A number of experiments were performed for testing the method for use with reactor samples. The recovery of cells added to a biomass sample was tested with cells of "*Methanobacterium*" sp. strain Pluk added to homogenized granular sludge. A cell number corresponding to 3.3 × 10⁶ cells/cm³ was added to each well. In addition, the recoveries of "*Methanobacterium*" sp. strain CB12 suspended in homogenized granular sludge and in FSW were compared. Finally, the applicability of the ELISA to establish the difference between biomass samples was demonstrated by comparing samples from reactors fed with swine or cow manure.

IIF test on glass slides. The IIF procedure was modified from the method described by Conway de Macario et al. (11). To enhance sample attachment, the wells were precoated with poly-L-lysine (Sigma Diagnostics P 8920) as described in the manufacturer's instructions. The samples were flame fixed to the glass surface, and the salt was gently washed away with MilliQ water. The antiserum was diluted in PBS supplemented with 1.5% bovine albumin. To the secondary antibody mixture, 1 μg of 4',6-diamidino-2-phenylindole (DAPI) per cm³ was added. The slides were mounted with Citifluor AF1 (UKC Chemical Laboratory, Canterbury, United Kingdom). The labelling was evaluated with an epifluorescence microscope (Olympus BH2-RFL) equipped with an exciter filter B, a

TABLE 2. Antisera used in this work

Immunizing strain ^a	Dilution (fold)	Adsorbed with cells of strain(s) ^b :
ΔH and Pluk ^c	40,000 each	Marburg, CB12, M.o.H., TM-1
Marburg	20,000	TM-1
CB12	20,000	ΔH, Pluk, MB, M.o.H.
JF-1	20,000	Marburg
M.o.H.	20,000	CB12
S-6	20,000	SB
GP6	20,000	
CALS-1	20,000	GP6, Opfikon

^a See Table 1 for full binary names.

^b Diluted antiserum was incubated with cells of the strains listed to eliminate cross-reactions (see text for details).

^c A mixture of antisera against the two strains. The total concentration of rabbit serum was 1/20,000.

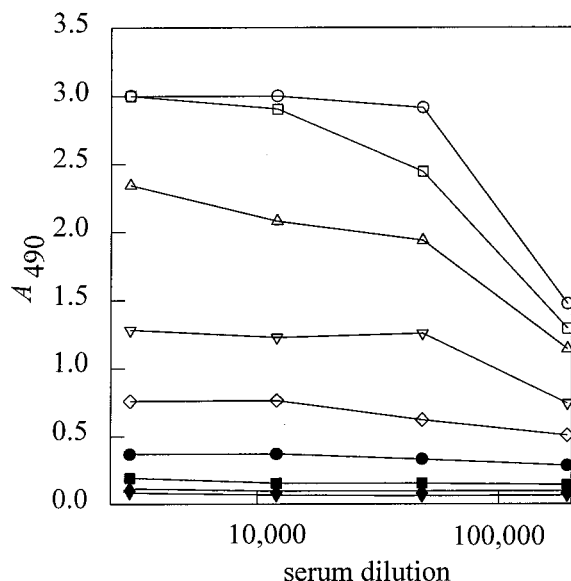


FIG. 1. Results from an ELISA with different dilutions of antiserum against *Methanoseta thermophila* CALS-1 applied to different concentrations of *Methanoseta thermophila* CALS-1 cells. Symbols: \circ , 3.3×10^7 ; \square , 1.1×10^7 ; \triangle , 3.7×10^6 ; ∇ , 1.2×10^6 ; \diamond , 4.1×10^5 ; \bullet , 1.4×10^5 ; \blacksquare , 4.6×10^4 ; \blacktriangle , 1.5×10^4 ; \blacktriangledown , 5.1×10^3 cells/cm³.

dichroic mirror B, and a HBO100W/2 mercury burner. The DAPI staining was visualized with exciter filter U and dichroic mirror U.

Analytical procedures. The A_{578} of the cell suspensions was measured in glass test tubes (light path, 10 mm) against FSW in a Spectronic 301 spectrophotometer (Milton Roy).

RESULTS

Antisera. Strains used for elimination of cross-reactions are presented in Table 2. Antiserum against *Methanobacterium formicicum* MF was used in preliminary experiments but was found to cross-react strongly with cells of "*Methanobacterium*" sp. strain CB12. The antiserum against *Methanobacterium formicicum* JF-1 was specific for *Methanobacterium formicicum* MF after adsorption with cells of "*Methanobacterium*" sp. strain Marburg (data not shown). The antiserum against *Methanobacterium formicicum* JF-1 was, therefore, used for assays with cells of *Methanobacterium formicicum* MF throughout this work.

Preparatory work of the ELISA method. The optimum dilutions of the antisera for the ELISA were determined by use of a number of cell concentrations and serum dilutions. Figure 1 shows an example with the antiserum against *Methanoseta thermophila* CALS-1. To optimize the specificity as well as the detection limit, the optimum concentration of the serum is the lowest concentration still giving a maximum reaction (corresponds to the S probe described by Conway de Macario et al. [11] for immunofluorescence). For the present work, however, the specificity was increased considerably by adsorption of the antisera, and a convenient, common serum dilution of 20,000 times could be chosen. This value was close to the optimum concentration as defined above found for all the antisera tested.

Figure 2 shows an example of a standard curve obtained with pure cells of *Methanobacterium bryantii* M.o.H. and homologous antiserum. The detection limit was approximately 10^5 cells/cm³ (10^4 cells/well). The A_{490} found with preimmune rabbit serum was never above 0.08. The antisera slowly deterio-

rated at 4°C, and the standard curve was therefore always compared to that of earlier experiments. Freezing injured the antisera in some cases.

Specificities of the sera. The specificities of the antisera were tested against the methanogens listed in Table 1. A number of cross-reactions were found, but after adsorption of the sera with cross-reacting cells, these never gave A_{490} values above 0.1. This value was chosen as the cutoff value. Reaction between homologous cells (3.7×10^6 cells/cm³) and antibodies gave A_{490} values between 1.19 and 2.70 (this value could not be comparably determined for the filamentous *Methanoseta concilii* strains Opfikon and GP6). Cross-reactions between the strains *Methanobacterium thermoautotrophicum* Δ H, "*Methanobacterium*" sp. strain MB, and "*Methanobacterium*" sp. strain Pluk were pronounced, and a mixture of antiserum against *M. thermoautotrophicum* Δ H and "*Methanobacterium*" sp. strain Pluk was applied to ensure an equal reaction with all three strains. Likewise, the two strains of *Methanoseta concilii*, GP6 and Opfikon, could be targeted comparably with antiserum against strain GP6.

Adhesion of cellular antigen to the microtitration plates. In the test with "*Methanobacterium*" sp. strain Pluk as the antigen, the average A_{490} obtained in the ELISA was 1.51 with no addition (coefficient of variance, 22.3%). The same figures for wells with methylglyoxal, HCl, or both added were 1.72 (9.3%), 2.37 (4.6%), and 1.72 (5.5%), respectively.

Figure 3 shows the influence of hydrochloric acid added to cell suspensions or biomass samples and the results obtained when two different microtitration plates were used. The standard curve had a smooth, sigmoid shape only in the MaxiSorp plates with addition of HCl. Likewise, the highest A_{490} values were obtained with the biomass sample by use of MaxiSorp plates and the addition of HCl.

Verification of the ELISA method for use on biomass samples. Table 3 shows cell numbers recovered by ELISA after addition of a known cell number to a biomass sample. The table further shows the cell number determined in the biomass

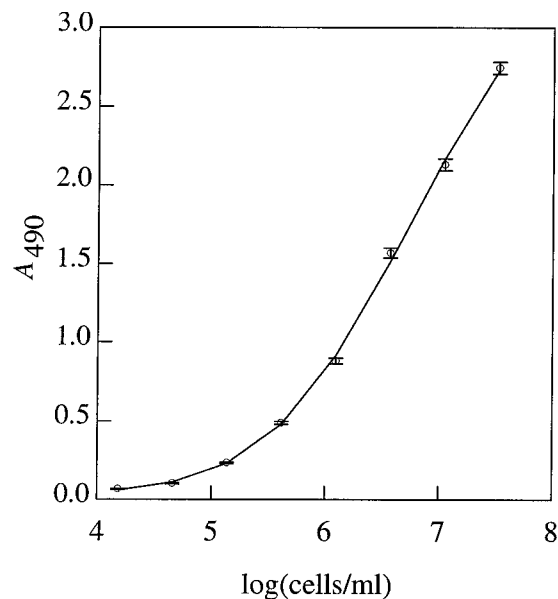


FIG. 2. Example of a standard curve of *Methanobacterium bryantii* M.o.H. Both the data points (\circ) and the fitted curve (—) are shown. The error bars show the standard deviations of the data ($n = 4$).

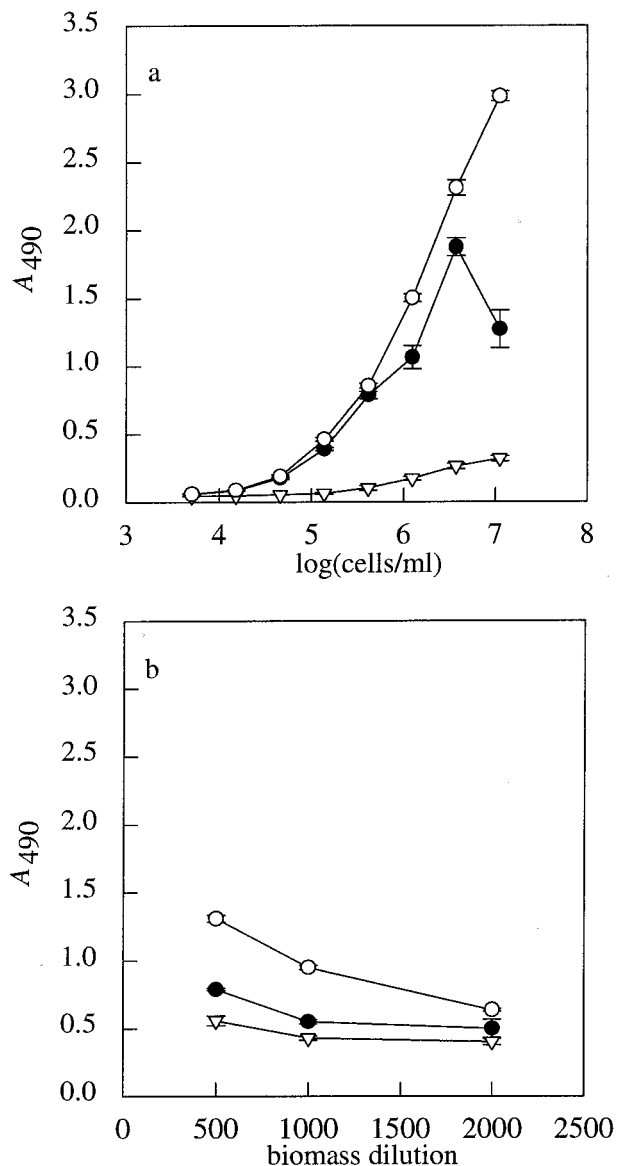


FIG. 3. Comparison of the MaxiSorp plate with (○) and without (●) HCl added and a standard polystyrene plate without HCl (▽). (a) A_{490} values obtained with antiserum against “*Methanobacterium*” sp. strain Marburg and suspensions of “*Methanobacterium*” sp. strain Marburg cells; (b) A_{490} values obtained with a biomass sample diluted 500, 1,000, and 2,000 times and antiserum against “*Methanobacterium*” sp. strain Marburg. The error bars show the standard deviations ($n = 8$).

sample without additions. The added cells were recovered at all the dilutions of biomass tested.

The recovery of cells of “*Methanobacterium*” sp. strain CB12 added to biomass was also tested. Measurements obtained with cells added to a biomass sample diluted 1,000 or 2,000 times in total were compared with a standard curve obtained with cells suspended in FSW. The biomass itself did not contain cells of “*Methanobacterium*” sp. strain CB12. Figure 4 shows that the values obtained from cells suspended in dilute biomass were indistinguishable from the values obtained from cells suspended in FSW if the biomass was diluted 2,000 times. If the biomass was diluted only 1,000 times, there was a clear influence on the values obtained.

The cell numbers determined in two different biomass sam-

TABLE 3. Recovery of cells of “*Methanobacterium*” sp. strain Pluk added to a biomass sample

Biomass dilution	Estimated cell density without added cells (no. of cells [10^8]/ cm^3 of undiluted biomass)	Surplus of cells found with 3.3×10^6 cells/ cm^3 of mixture added (no. of cells [10^6]/ cm^3 of mixture)
1:1,000	6.8, 7.0 ^a	5.0, 4.8
1:3,000	8.7, 8.3	3.6, 4.1
1:9,000	9.4, 8.7	3.5, 4.2

^a Values from two parallel tests (four wells each) are given.

ples are presented in Table 4. The samples differed by the content of the different strains. This difference was more pronounced for some strains than for others. The methane production of the reactors was 0.4 and 0.7 $\text{m}^3 \text{m}^{-3}$ of reactor day^{-1} for the swine and cow manure reactors, respectively. Table 4 further shows that cell numbers as low as 0.39×10^8 cells/ cm^3 in undiluted biomass, corresponding to 1,950 cells/well, could be reliably determined.

The biomass samples presented in Table 4 were also characterized by the IIF test. In the samples from the digester fed with cow manure, a relatively high amount of rod-shaped cells reacted with the antiserum mixture against *Methanobacterium thermoautotrophicum* ΔH and “*Methanobacterium*” sp. strain Pluk or with antiserum against “*Methanobacterium*” sp. strain Marburg. Only very few positive cells were seen in specimens with antisera against “*Methanobacterium*” sp. strain CB12, *Methanobacterium formicicum* JF-1, or *Methanosarcina mazei* S-6, and no positive reaction was found with antiserum against *Methanosaeta thermophila* CALS-1, *Methanobacterium bryantii* M.o.H., or *Methanosaeta concilii* GP6. In samples from the swine manure digester, some rods reacted with an antiserum mixture against *Methanobacterium thermoautotrophicum* ΔH and “*Methanobacterium*” sp. strain Pluk, while only very few or no cells with a positive reaction were seen with the other antisera.

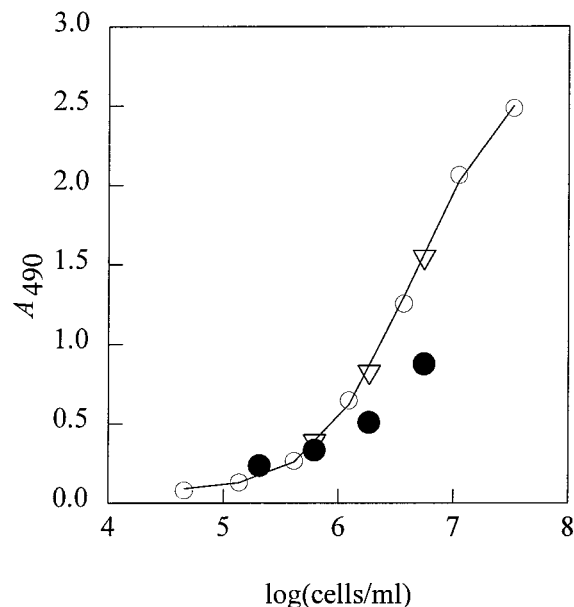


FIG. 4. Standard curve of “*Methanobacterium*” sp. strain CB12 obtained with cells suspended in FSW (○) compared to values obtained with cells suspended in biomass diluted 1,000 (●) and 2,000 (▽) times. The solid line is the standard curve obtained by fitting the data from FSW to the formula of a sigmoid curve.

TABLE 4. Cell numbers estimated in biomass samples from digesters fed swine manure or cow manure^a

Sample	Dilution (fold)	Estimated no. of cells (10 ⁸)/cm ³ of undiluted biomass							
		Pluk + ΔH	Marburg	CB12	MF	M.o.H.	S-6	GP6 ^b	CALS-1
Swine	1,000	1.0 (0.11) ^c	0.55 (0.06)	— ^d	5.1 (0.29)	0.24 (0.07)	1.7 (0.08)	7.5 (2.4)	—
	2,000	0.47 (0.13)	0.39 (0.04)	—	6.3 (0.30)	—	2.0 (0.11)	8.4 (0.82)	—
Cow	1,000	3.2 (0.05)	0.65 (0.03)	6.4 (0.39)	8.8 (0.29)	1.2 (0.27)	3.4 (0.15)	8.8 (0.80)	0.93 (0.06)
	2,000	3.8 (0.40)	0.72 (0.13)	8.5 (0.54)	15 (0.26)	1.7 (0.29)	4.4 (0.28)	13 (1.46)	1.2 (0.18)

^a See Table 1 for full binary names.

^b The values for strain GP6 are the responses from the test, calculated back to undiluted biomass, relative to the responses of the most concentrated reference suspension of GP6 cells.

^c Values in parentheses are the standard deviations between the results for four parallel wells. The standard deviation of the subtracted value obtained with preimmune serum is included, while the standard deviation of the standard curve is not.

^d —, value (gross cell number) not different from, or slightly below, the value obtained with the preimmune serum (background cell number).

Preimmune serum included in parallel to the antisera never showed any reaction.

Cells found to react with an antiserum also showed a reaction with DAPI, indicating that they were not only dead and partially degraded cells present in the sample at the time of fixation.

DISCUSSION

The results demonstrate that the ELISA procedure is applicable for quantification of whole cells of methanogens in environmental samples.

After a simple adsorption procedure, the polyclonal antibodies used were found to distinguish between the methanogenic strains tested except for closely related strains that gave comparable reaction intensities. Cross-reactions between closely related cells can, however, never be excluded with polyclonal antibodies, but these cross-reactions allow detection of yet-unknown cells related to the immunizing strain (24, 34). Cross-reactions are, therefore, of great value if limited to closely related strains of similar physiology.

Results of the ELISA described in this work are expressed in cell numbers. It is important to note that it is not necessarily the number of cells in the samples that is measured but rather a quantitative response that is subsequently compared to the cell numbers in defined reference suspensions.

Challacombe (10) described a procedure for increasing the adhesion of cells to polystyrene microtitration plates by the addition of methylglyoxal. We found no positive effect of this treatment but observed enhanced signal intensity and reproducibility after the addition of dilute hydrochloric acid. A 1:1 dilution of a cell suspension with 0.1 M HCl is used by other authors to enhance cell attachment to glass surfaces in a bacterium-counting chamber (26). The attachment of cells to the microtitration plate wells was improved further by application of the MaxiSorp plates. The MaxiSorp plates have a hydrophilic surface intended to attach hydrophilic molecules by hydrogen bonds (15).

The recovery of cells of "*Methanobacterium*" sp. strain Pluk added to biomass samples demonstrated that the quantification of cells was not influenced by the presence of biomass in the concentrations tested. In general, a biomass sample must be diluted to remove any interference exerted by the sample. A possible influence is the competition between methanogens and other particles for available space for adhesion in the well (7). This is illustrated by the test on recovery of "*Methanobacterium*" sp. strain CB12 in another biomass sample, where the biomass should be diluted 2,000 times so as not to influence the measurement. A detection limit of 10⁵ cells/cm³ (see above)

would in this case correspond to a cell density of 2 × 10⁸ cells/cm³ in the sample.

Preimmune serum was found to react slightly but significantly with biomass samples. Due to the nonlinear shape of the standard curve, the A_{490} value obtained with preimmune serum must be converted to a background cell number before it is subtracted from the gross cell number determined with the antiserum itself. Hence, the detection limit of the ELISA is dictated by measurement variance, since this determines how great the differences between the gross cell number determined with the antiserum and the background cell number found with the preimmune serum must be for these figures to be significantly different, i.e., for the actual cell number to be above zero.

The comparison of different biomass samples illustrates the feasibility of the ELISA. The higher cell numbers found in the cow manure reactor were paralleled by a higher methane production of this reactor.

In most cases, no or only very few positive cells were found by the IIF test in the biomass samples, even in cases where the ELISA indicated cell numbers around 10⁸ cells/cm³ (Table 4). With 10⁸ cells of a certain strain per cm³ of undiluted biomass sample, approximately 2 cells per microscope field should be found with the actual setup. The low number of cells found in the IIF test is probably due to a loss of cells attached to the glass slide during the washing steps. Cell detachment is demonstrated by the frequent observation that distinct cell morphotypes are transferred between separate wells of the immunoslide during the procedure (30). The IIF test used here is thus not suited for quantitative analyses. For this purpose, a filter technique (31) should have been applied. This method is, however, much more laborious and requires a skilled eye to separate stained cells from the autofluorescence of inert material and for determination of reaction intensities.

The fact that no cells at all of *Methanosaeta concilii* GP6 were found in the IIF test despite the high response found in the ELISA, however, indicates that this high number of cells could be an artifact and should be regarded with precaution. The immunofluorescence test can thus be a valuable control of the ELISA. Besides, the DAPI staining included in the IIF test reveals whether a significant fraction of the positive cells do not contain DNA and thus do not contribute to the performance of the reactor. In anaerobic wastewater reactors with high sludge retention times, a large fraction of the cells found lack DNA (30).

In a similar ELISA, Bryniok and Trösch (7) found that samples from anaerobic reactors should be diluted 1,000 times. This is confirmed by our results, but in contrast to these authors, we have shown that the results can be converted to cell

numbers in samples from biogas digesters. Likewise, the sensitive competitive assay developed by Kemp and coworkers (19) required a 1,000-fold dilution of the biomass, i.e., approximately the same dilution as that required in our ELISA. In contrast to the complex and sensitive assays presented by others (7, 18, 19), we have focused on the feasibility of the test for routine detection of the most prevalent methanogens in active methanogenic ecosystems like anaerobic reactors with high cell numbers.

None of the ELISAs described in the literature for detection of methanogens has demonstrated reliable detection of inherent strains in samples from continuously stirred tank reactors treating a complex slurry of solid waste. The work on reactor samples actually presented (7, 18) has in all cases been done on defined mixed cultures or granular sludge and other samples from wastewater treatment reactors, where the amount of inert organic matter will be much below the amount in samples from continuously stirred tank reactors. In contrast to this, our ELISA method has been shown to be well suited for quantitative detection of the main groups of methanogens in complex samples. Moreover, the concept is amenable to automatization and routine testing of multiple samples without any need of highly specialized manpower.

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