RESEARCH LETTER

Assessment of the oxidant tolerance of *Methanosarcina acetivorans*

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Abstract

All methane-producing *Archaea* (methanogens) are strict anaerobes, but the majority of species are tolerant to oxidants. *Methanosarcina* species are important environmental and industrial methanogens as they are one of only two genera capable of producing methane with acetate. Importantly, *Methanosarcina* species appear to be the most oxidant-tolerant; however, the mechanisms underlying this tolerance are poorly understood. We report herein two similar methods (spot-plating and microtiter plate) developed to examine the oxidant tolerance of *Methanosarcina acetivorans* by viability assessment. Both methods revealed that *M. acetivorans* can tolerate exposure to millimolar levels of hydrogen peroxide (H_2O_2) without a complete loss of viability. The exogenous addition of catalase was also shown to protect *M. acetivorans* from H_2O_2 toxicity, indicating catalase can serve as an antioxidant enzyme in methanogens even though oxygen is a byproduct. Of the two methods, the microtiter plate method provided a simple, reliable, and inexpensive method to assess viability of *M. acetivorans*. Combined with recent advances in the genetic manipulation of methanogens, methods in assessment of methanogen oxidant tolerance will aid in the identification of components of the antioxidant defense systems.

Introduction

Methane-producing archaea (methanogens) comprise a large group of microorganisms within the domain *Archaea*. Methanogens are the only organisms capable of producing methane, serving a key role in the global cycling of carbon and in the production of methane fuel from renewable biomass (Thauer *et al.*, 2008). All methanogens are metabolic specialists, only capable of growth by methanogenesis. However, there are differences in the substrate utilization and energy conservation strategies among methanogens. With one exception, all members of the *Methanococcales*, *Methanomicrobiales*, *Methanobacteriales*, and *Methanopyrales* grow exclusively by the reduction in CO_2, and all species lack cytochromes (Thauer *et al.*, 2008). Members of the *Methanosarcinales* are more metabolically diverse, with some species capable of growth by CO_2-reduction, aceticlastic, and/or methylotrophic methanogenesis. All members of the *Methanosarcinales* contain cytochromes. It is estimated that two-thirds of biologically produced methane is derived from the methyl group of acetate (Thauer *et al.*, 2008), underscoring the importance of the *Methanosarcinales*. These fundamental differences indicate that there are likely more widespread differences in the biology of methanogens with and without cytochromes, including mechanisms for sensing and responding to environmental change.

Importantly, all methanogens are strict anaerobes, not documented to use O_2 as an electron acceptor during energy-conserving metabolism. Organisms experience oxidative stress primarily as a result of the oxidation of electron carriers by O_2, producing the reactive oxygen species (ROS) superoxide (O_2^-), H_2O_2, and hydroxyl radical ($^{\cdot}OH$) (Imlay, 2002). The production of ROS is more prevalent in anaerobes such as methanogens, due to the high number of cofactors that readily react with O_2 (Imlay, 2006). However, the majority of strict anaerobes can tolerate prolonged periods of oxygen exposure. Although it is documented that strict anaerobes possess mechanisms to combat oxidative stress, the mechanisms used by methanogens to sense and cope with oxidative stress are poorly understood. Anaerobes often do not contain superoxide dismutase (SOD) or catalase because each enzyme produces O_2 as an end product, which may

further propagate the production of ROS (Imlay, 2002). Instead, strict anaerobes typically contain superoxide reductase and rubrerythrin, which remove O_2^- and H_2O_2, respectively, without the production of O_2 (Jenney *et al.*, 1999; Lumppio *et al.*, 2001).

Of methanogens, *Methanosarcina* species appear to be the most aerotolerant. For example, *Methanosarcina* and the related genus *Methanocella* were identified as the dominant methanogens in arid soils and produced methane even under aerobic conditions (Angel *et al.*, 2011). The aerotolerance of *Methanosarcina* is likely related to its metabolic diversity and the higher prevalence of antioxidant enzymes encoded in the genomes of sequenced species (Erkel *et al.*, 2006). *Methanosarcina* species typically contain the aerobic-type enzymes, SOD, and catalase, as well as superoxide reductase and rubrerythrin. Catalase and SOD activity has been documented in *Methanosarcina barkeri* (Brioukhanov *et al.*, 2006). $F_{420}H_2$ oxidase is an antioxidant enzyme catalyzing a reaction unique to methanogens, whereby $F_{420}H_2$ donates electrons to reduce O_2 to water (Seedorf *et al.*, 2004). However, the importance of these enzymes in protecting cells from O_2/ROS and the mechanisms used to repair damage due to oxidative stress is poorly understood.

Methanosarcina acetivorans serves as an excellent model methanogen to elucidate the mechanisms used by *Methanosarcina* to sense and respond to oxidants. Not only have the methanogenesis pathways within *M. acetivorans* been well-characterized (Li *et al.*, 2005a, b, 2006, 2007; Lessner *et al.*, 2006), the genome of *M. acetivorans* encodes homologs of SOD, catalase, superoxide reductase, rubrerythrin, $F_{420}H_2$ oxidase, and peroxidases (http://img.jgi.doe.gov). For comparison, *M. acetivorans* encodes two putative catalase/peroxidases, one of which is similar to the single characterized catalase (Mbar_a0814) found in *M. barkeri* (Shima *et al.*, 1999). The genome also encodes MdrA, a novel Fe-S cluster containing disulfide reductase, proposed to function in repair of oxidatively damaged proteins (Lessner & Ferry, 2007), and a homolog of MsvR, a putative redox-sensing transcription factor originally identified in *Methanothermobacter thermautotrophicus*, which lacks cytochromes (Karr, 2010). What makes *M. acetivorans* a particularly attractive model to explore the oxidative stress response of methanogens is the availability of a robust genetic system allowing for the *in vivo* role of putative genes to be tested (Guss *et al.*, 2008). A prerequisite to the use of *M. acetivorans* as an oxidative stress model is the development of methods to reliably quantitate the tolerance to specific oxidants. We report herein two methods developed to quantitatively assess the tolerance of *M. acetivorans* to H_2O_2 by measuring viability.

Materials and methods

Growth of *Methanosarcina acetivorans*

Methanosarcina acetivorans WWM73 was grown in high-salt (HS) medium supplemented with 125 mM methanol as a carbon and energy source and 0.025% Na_2S as a reductant as described (Sowers *et al.*, 1984). Growth was monitored spectrophotometrically as optical density at 600 nm (OD_{600}).

Oxidant challenge of *M. acetivorans*

All manipulations were performed within an anaerobic chamber (Coy laboratories) containing 75% N_2, 20% CO_2 and 5% H_2 atmosphere. Once an OD_{600} of 0.4 to 0.75 was reached, cultures of *M. acetivorans* strain WWM73 were pelleted, washed twice with assay buffer (HS medium devoid of methanol, resazurin, cysteine, and sulfide), and normalized to an OD_{600} of 0.5 with assay buffer. The cells were dispensed into 0.5 mL aliquots. Fresh stock solutions of H_2O_2 (30% solution, EMD chemicals) were made in water and brought into the anaerobic chamber 1 h prior to use. 10 μL of H_2O_2 stock solution or water (control) was added to the 0.5 mL aliquots (final H_2O_2 concentrations: 0–10 mM). 68 units of catalase from *Corynebacterium glutamicum* (Sigma-Aldrich) were added to some aliquots prior to and after the addition of H_2O_2. The cells were incubated at 25 °C for 1 h. Viability was then assessed by either the spot-plating or microtiter plate method.

Assessment of viability: spot-plating method

To assess viability, the challenged *M. acetivorans* cells were serially diluted up to 10^{-5} in assay buffer using a sterile 96-well polycarbonate microtiter plate. Aliquots (3 μL) of each serial dilution were spotted on HS medium agar plates (60 × 15 mm) containing 125 mM methanol and cysteine, but lacking Na_2S. The spots were allowed to dry and the plates were placed in canning jars (Apolinario & Sowers, 1996). A vial containing 2.5 mL of 2.5% Na_2S was placed in the jar and the jar was sealed. The anaerobic canning jars were incubated at 35 °C and growth was monitored visually. After 10–14 days, the jars were opened outside of the anaerobic chamber, the plates were imaged, and growth scored. Spots of growth on the plates were scored based on the presence of colonies, where a spot was deemed positive for the presence of viable cells at a particular dilution regardless of the amount of colonies or confluent growth.

Assessment of viability: microtiter plate method

To assess viability, the challenged *M. acetivorans* cells were serially diluted up to 10^{-5} in assay buffer using a sterile 96-well polycarbonate microtiter plate. Aliquots (3 μL) of each serial dilution were transferred to a microtiter well containing 100 μL of HS medium supplemented with 125 mM methanol and 0.025% Na_2S. The microtiter plate was covered with a sterile polycarbonate lid. The inoculated microtiter plates were placed within glass storage jars (Walmart® 2qt cracker jar #857587MN) and sealed with an accompanying aluminum lid. However, to ensure an airtight seal, the lid was fitted with a rubber gasket (Korky® Flush valve seal, no. 7301111-0070A), which was modified by removing the inner lip. Sealed anaerobic jars containing microtiter plates were incubated at 35 °C, and growth within the microtiter wells was monitored visually. After 8–10 days, the jars were opened outside of the anaerobic chamber and the plates were removed. Growth was visually scored. Growth within a well was deemed positive for the presence of viable cells at a particular dilution regardless of the amount of turbidity.

Results and discussion

Development of a spot-plating method to assess the effect of H_2O_2 on the viability of *M. acetivorans*

Methanogens can withstand exposure to millimolar levels of H_2O_2. Sublethal levels of H_2O_2 induce changes in methanogen gene expression, including an increase in the expression of antioxidant enzymes; however, the mechanisms of sensing are unknown. For example, methanol-grown *M. barkeri* can overcome the exogenous addition of up to 0.8 mM H_2O_2 to growth medium (Brioukhanov *et al.*, 2006). However, it is important to note that methanogen medium typically contains sulfide and/or cysteine as a reductant and resazurin as a redox indicator. Therefore, the cellular stress induced by the addition of H_2O_2 to methanogen growth medium is influenced by the levels of sulfide, cysteine, and resazurin in the medium (see results below) and may not reflect the true tolerance of a particular strain. Moreover, this approach does not quantitatively assess the effect of H_2O_2 on viability. Our aim was to develop a reliable and simple method to enumerate the loss of methanogen viability due to the exposure to oxidants. We first set out to develop a modified spot-plating method to assess the effect of H_2O_2 on *M. acetivorans* viability.

To allow comparison to previous oxidant challenge studies performed with *M. barkeri* (Brioukhanov *et al.*, 2006), the oxidant tolerance was determined with mid-exponential phase cells of *M. acetivorans* grown with methanol as a carbon and energy source. Because a long-term goal is to use genetics to elucidate the mechanisms important in the oxidative stress response of *M. acetivorans*, we chose to determine the basal H_2O_2 tolerance of *M. acetivorans* strain WWM73. Strain WWM73 has been engineered to serve as a parent strain for deletion, expression, and complementation studies, but is otherwise identical to the wild-type strain C2A (Guss *et al.*, 2008).

To avoid the influence of sulfide, cysteine, or resazurin, mid-exponential cells were pelleted, washed and the cell density normalized in medium devoid of sulfide, cysteine, or resazurin. Using this single approach, the tolerance of multiple strains, growth conditions, and growth phases with multiple replicates can be assessed. Moreover, the assay buffer can be modified to include additives (e.g. energy source, antioxidants, etc.) to test the influence on viability. Specifically, strain WWM73 cell suspensions were challenged with increasing concentrations of H_2O_2 (0.25–2.0 mM). After 1 h, the cells were serially diluted by 10-fold (up to 10^{-5}), and an aliquot of each dilution was spotted onto an agar plate. The plates were then transferred to canning jars for cultivation (Apolinario & Sowers, 1996). Due to the limitation of the number of plates that fit in a single jar, replicate plates were placed in different jars and incubated for 10–14 days at 35 °C. After this time, the plates were removed and growth within each spot was scored. An example of the amount of growth typically observed within the spots on a plate is depicted in Fig. 1. A summary of the results obtained from five separate experiments, each with multiple replicates (> 3), is shown in Fig. 1b. A complete loss of viability was observed at H_2O_2 concentrations of > 1.5 mM and a concentration of 0.5 mM resulted in approximately 50% loss of viability. The limit of tolerance to H_2O_2 was within the range of that observed for *M. barkeri* (Brioukhanov *et al.*, 2006); however, standardizing conditions in the future will allow further comparisons between these and other methanogens.

Similar to other methanogens, we have observed that *M. acetivorans* can survive exposure to atmospheric levels of O_2 (20%) in growth medium for extended periods. Although growth and methanogenesis are inhibited, once oxygen is removed, methanogenesis and growth resume (data not shown). Consistent with this observation, exposure of methanol-grown, mid-exponential phase WWM73 cells to 20% O_2 for 1 h did not result in a detectable loss of viability using the spot-plate method (data not shown), indicating that short-term O_2 exposure does not significantly damage *M. acetivorans*.

Results obtained from multiple independent experiments were reproducible (Fig. 1b). However, reliability of growth on agar plates within the canning jars was of

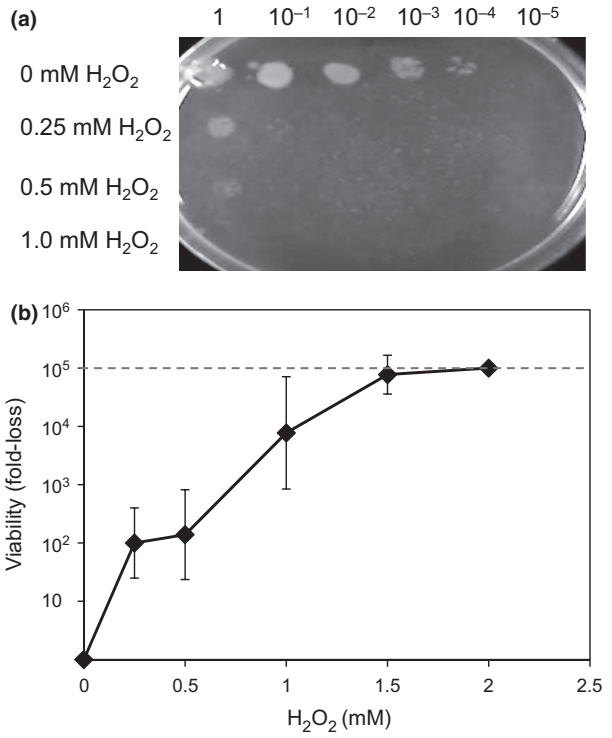

Fig. 1. Effect of H_2O_2 on the viability of *Methanosarcina acetivorans* WWM73 as analyzed by the spot-plating method. (a) Image of plate depicting typical results seen for viability of cells challenged with 0.25–1.0 mM H_2O_2. (b) Fold loss of viability of cells challenged with increasing concentrations of H_2O_2. Fold loss is defined as the lowest 10-fold serial dilution that resulted in the absence of growth, relative to the control without the addition of H_2O_2 (growth observed up to a 10^{-5}-fold dilution). The dashed line represents the highest fold loss that results in a complete absence of viable cells.

concern because approximately 40% of the jars failed to yield any growth. For each experiment, two or more jars were used to incubate replicate plates derived from the same medium and reagents. Surprisingly, a jar would fail to yield any growth, while the other(s) would yield growth similar to that observed in previous experiments. The lack of growth was unexplained, because in no instances did the plates turn pink to indicate O_2 contamination and that a jar was improperly sealed. Due to the unexplained lack of growth in some jars and the time frame of data acquisition of the spot-plating method, we sought to develop a method that was as simple, but more reliable and high-throughput.

Development of a microtiter plate method to assess the effect of H_2O_2 on the viability of *M. acetivorans*

Cultivation of methanogens in liquid medium is typically less fastidious than cultivation on solid medium. Therefore, we sought to combine the benefits of growing in liquid medium with the use of inexpensive glass jars. Analysis of numerous samples in liquid medium necessitates a high-throughput method to cultivate *M. acetivorans*. Thus, we sought to develop a method using microtiter plates, which have recently been used to cultivate methanogens (Anderson *et al.*, 2012; Bang *et al.*, 2012).

The developed method was identical to the spot-plating method in cell normalization and serial dilutions, except aliquots of the diluted cells were transferred to a microtiter plate containing HS medium. A microtiter plate will not fit within a wide-mouth canning jar. Therefore, a two-quart glass cracker jar fitted with an aluminum screw-cap lid was used. However, to generate an airtight seal, the lid was fitted with a rubber gasket that is the exact internal diameter of the aluminum lid (Fig. 2), despite being designed for use as a toilet flush valve seal. Each jar can contain up to four microtiter plates. The lids were not fitted with a pressure gauge or a septum because the small volume of medium (9.6 mL per plate) and large headspace within the jar led to a minimal increase in pressure. Importantly, the use of the modified two-quart jar provided a reliable method (100% success rate) to cultivate *M. acetivorans* strain WWM73 within the wells of the microtiter plates. Growth was also complete

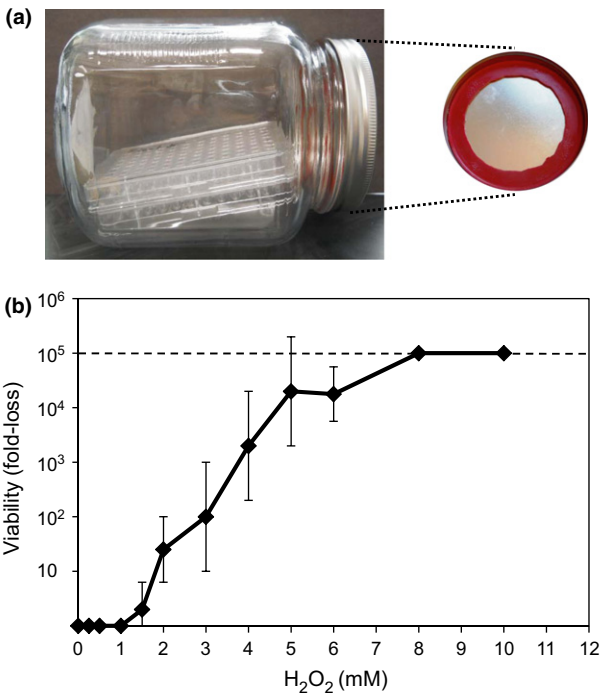

Fig. 2. (a) Glass jar and lid modified with a red rubber gasket used for cultivation of *Methanosarcina acetivorans* in microtiter plates. (b) Effect of H_2O_2 on the viability of *M. acetivorans* WWM73 as analyzed by the microtiter plate method. The dashed line represents the highest fold loss that results in a complete absence of viable cells.

after 10 days, decreasing the time frame for data collection. However, growth of *M. acetivorans* on agar plates within the modified two-quart jars proved as unreliable as the canning jars (data not shown) negating the use of the larger jars for incubation of agar plates. Cultivation of H_2O_2-challenged cells in microtiter plates produced similar results to those obtained by the spot-plating method; however, the observed tolerance was higher (Fig. 2). Complete loss of viability was not obtained until the addition of 6 mM H_2O_2, approximately three times higher than that observed with the spot-plating method. The difference in viability likely stems from the more fastidious growth on solid medium versus liquid medium. In addition, after 1 h of oxidant exposure, there may have been residual H_2O_2 that was more rapidly diluted and reduced in the liquid medium compared with the solid medium. The microtiter plate method was also used to experimentally verify that the presence of sulfide and cysteine in methanogen medium increases the tolerance of cells to oxidants. WWM73 cells challenged with three different concentrations of H_2O_2 exhibited a 10- to 100-fold increase in viability when assayed in buffer containing sulfide and cysteine, compared with assay buffer alone (Fig. 3). Overall, the microtiter plate method provided a simple, reliable, and inexpensive method to assess the viability of *M. acetivorans*.

Protection of *M. acetivorans* from H_2O_2 by catalase

Methanogens have been documented to possess catalases (Leadbetter & Breznak, 1996; Shima *et al.*, 1999; Brioukhanov *et al.*, 2006). However, the presence of catalase is not universal among methanogens. *Methanosarcina barkeri* contains a catalase and activity was modestly upregulated after exposure to sublethal levels of H_2O_2 (Brioukhanov *et al.*, 2006). To demonstrate that the loss of viability was specific to the addition of H_2O_2 and to test whether extrinsic catalase activity could protect *M. acetivorans* from H_2O_2, assays were performed in which catalase was added to the assay buffer before or after the addition of H_2O_2. Using the spot-plating method, the cells were challenged with 1 mM H_2O_2, a concentration that does not result in a complete loss of viability (Fig. 1). When catalase was added prior to the addition of 1 mM H_2O_2, there was a substantially higher viability compared with cells that were not pretreated with catalase or when catalase was added after the 1 h incubation (Fig. 4). These results confirm that the loss of viability is specific to H_2O_2 and reveal that catalase can protect *M. acetivorans* by decreasing H_2O_2 to a level that is nontoxic. Similar results were seen with the microtiter plate method; in this case, there was no detectable loss of viability when cells were pretreated with catalase and exposed to 3 mM H_2O_2 or even a lethal dose of 10 mM H_2O_2 (Fig. 5). The O_2 produced by the reaction of catalase with the concentrations of H_2O_2 tested does not appear to significantly damage the cells.

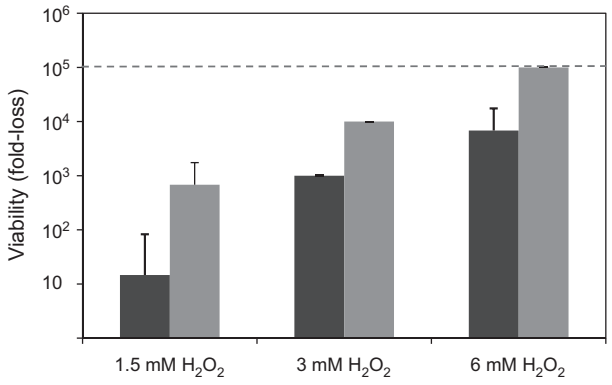

Fig. 3. Protection of *Methanosarcina acetivorans* WWM73 from H_2O_2 by the presence of Na_2S and cysteine in methanogen growth medium: microtiter plate method. Fold loss of viability of cells challenged with the indicated concentrations of H_2O_2 normalized in assay buffer (gray) or assay buffer supplemented with 0.025% Na_2S and 4 mM cysteine (black). The dashed line represents the highest fold loss that results in a complete absence of viable cells.

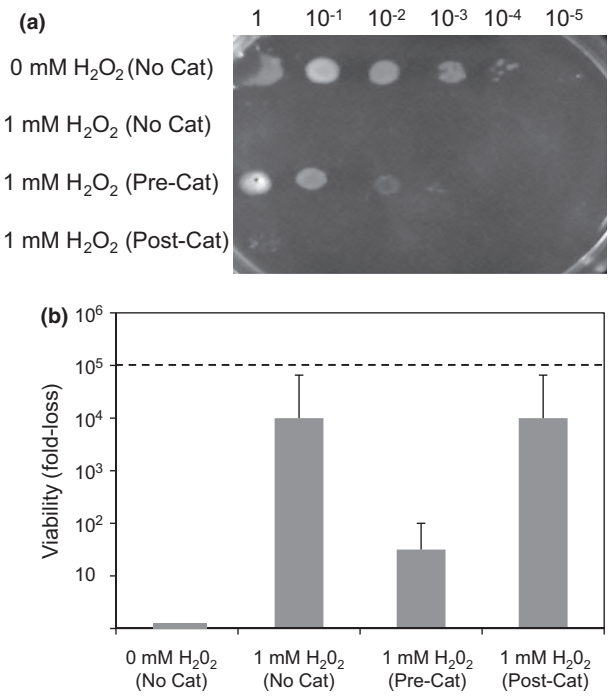

Fig. 4. Protection of *Methanosarcina acetivorans* WWM73 from H_2O_2 by the exogenous addition of catalase: spot-plating method. (a) Image of plate depicting typical results seen for viability of cells challenged with 1.0 mM H_2O_2. Pre-Cat: catalase was added to cells prior to the addition of H_2O_2. Post-Cat: catalase was added to cells after 1 h incubation with H_2O_2. (b) Fold loss of viability of cells challenged with 1.0 mM H_2O_2 and the effect of the addition of catalase. The dashed line represents the highest fold loss that results in a complete absence of viable cells.

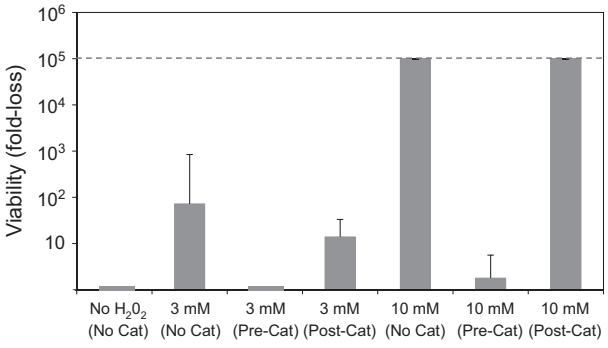

Fig. 5. Protection of *Methanosarcina acetivorans* WWM73 from H_2O_2 by the exogenous addition of catalase: microtiter plate method. Fold loss of viability of cells challenged with 3.0 mM or 10.0 mM H_2O_2 and the effect of the pre- and postaddition of catalase. The dashed line represents the highest fold loss that results in a complete absence of viable cells.

Conclusions

Two similar methods were developed to assess the viability of *M. acetivorans* after exposure to H_2O_2. Each method revealed that *M. acetivorans* can tolerate millimolar levels of H_2O_2 and that catalase provides substantial protection from H_2O_2 toxicity. The microtiter plate method was the preferred method, offering a simple, reliable, inexpensive and high-throughput method to assess the viability of *M. acetivorans*. This method, or with modification, could be used to assess the effect of additional oxidants, other stressors, growth conditions, and abiotic/biotic antioxidants on the viability of *M. acetivorans* and other methanogens. Overall, the use of the *M. acetivorans* genetic system combined with robust methods to assess viability will lead to a better understanding of the antioxidant mechanisms in methanogens.

Acknowledgments

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