An Anaerobic, Intrachamber Incubator for Growth of *Methanosarcina* spp. on Methanol-Containing Solid Media

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To simplify the incubation of *Methanosarcina* spp. on solid agar medium, a two-port, manual, rectangular air lock was modified to serve as an anaerobic incubator. In one operation, it is possible to incubate 153 petri plates, the equivalent of 11 standard anaerobic jars, with plating efficiencies identical to those of traditional protocols.

Genetic analysis of methanoarchaea has lagged far behind that of other organisms in part due to the inconvenience of anaerobically plating cells on agar medium when the reducing potential must be -330 mV or lower. Recently, a summary of techniques has been compiled (7) in which individual researchers have contributed protocols for growth of these organisms on solid media; these protocols are a convenient, in-depth source of information to the original literature and cover a wide variety of techniques including genetic and molecular ones. The procedures for incubation of methanoarchaea on solid media, protocol 1 by Sowers and Noll (7), are limited by the capacity of an anaerobic jar; a standard anaerobic jar such as Oxoid USA (HP 11) holds 14 dishes (100 by 15 mm). Some investigators prefer to use smaller petri dishes (60 by 15 mm or 35 by 10 mm) and a modified pressure tank for incubation, protocol 2 by Tumbula et al. (7), but handling small dishes with the heavy rubber gloves of an anaerobic chamber is a demanding procedure.

Despite recent developments in the genetics of methanoarchaea (2, 3, 4, 5, 7), genetic analysis of methanogenic pathways has not been reported. We have chosen to examine *Methanosarcina* because members of this genus are the most metabolically diverse of the methanoarchaea and offer different options for mutagenesis of the metabolic pathways. Genetic study of these organisms is now possible because of their growth as single cells, protocol 4 by Sowers (7). To simplify the examination of many hundreds of *Methanosarcina* colonies, we have developed an anaerobic incubator that is placed inside an anaerobic chamber so that inoculated standard petri dishes (100 by 15 mm) may be placed directly into the incubator.

Development of the incubator. A two-port, manual, rectangular air lock body was obtained from Coy Laboratory Products (Grass Lake, Mich.) with valves and a vacuum gauge in place. For the front port only, a metal door housing (with an attached acrylic door that is not screwed to the main air lock body) was obtained. The other port was closed with a (19-mm) thick clear acrylic panel (28 by 38 cm) with stainless steel bolts aligned to the 10 holes in the air lock body and sealed with a silicone sealer between the acrylic panel and the metal air lock body (Fig. 1). Because the door to the Coy air lock chamber is designed to function with a negative pressure within the air lock and may release gases when a positive pressure occurs, a

mechanism for pressing the acrylic door securely against the heavy rubber seal was developed. After the metal door housing (Fig. 2A, part a) had been bolted to the incubator body and the silicone sealer had cured, six screws were removed from the metal housing of the incubator door: two at the top, two at the bottom, and one on each side. To each of these six holes, a U-shaped metal bracket (18 by 18 by 18 mm) fabricated from metal, 16 mm wide and 1 mm thick, was bolted through the door frame to the incubator housing (silicone sealer used) (Fig. 2A, part c). Stainless steel (316) was used to fabricate all metal parts described in this report. On each side of the U-shaped bracket, a 2.3-mm-diameter hole had been drilled previously to accommodate a metal pin (2 by 15 mm), which was secured in place by a clip at each end. The pin was used to attach a metal bolt to the U-shaped bracket (Fig. 2A, part c). Each bolt was fabricated from a metal rod (1 by 5 cm); one end was turned on a lathe so that the last 1.5 cm could be threaded with standard threads and the other end was rounded. A 2.3-mm-diameter hole was drilled 5 mm from the rounded end so as to accept the metal pin in the U-shaped bracket, forming a swivel joint (Fig. 2A, part c). On the acrylic door exactly opposite each swivel bolt, a slotted bracket of 4-mm-thick metal (38 by 75 mm) with a slot (8 by 20 mm) at one end and with three drilled holes at the other end (Fig. 2A, part b) was screwed in place. A threaded knob was placed on the end of each swivel bolt (Fig. 2A, part c).

The fitting for addition of gases in the top of the air lock was replaced with a three-way stainless steel valve with standard Swagelok connectors (Peoria Valve and Fitting Co., Morton, Ill.) for 1/4-in. (6.2-mm)-thick copper tubing (Fig. 1). After the modified air lock (incubator) was placed in a Coy type B anaerobic chamber (Fig. 2C), one port of the three-way valve was attached with metal tubing through the chamber wall to an external cylinder of compressed gases (79.9% N₂, 20% CO₂, and 0.1% H₂S). The other port was similarly attached to a cylinder of compressed gases (5% H₂, 75% N₂, and 20% CO₂); a heated copper scrubber (1) was placed in the line of this gas mixture to ensure reduction of any free oxygen in the gas mixture. A vacuum line of rigid polyvinyl chloride plumbing pipe (12-mm inner diameter) was constructed from the vacuum pump through the chamber wall and connected to the vacuum port of the incubator. A similar line was constructed from the vacuum pump exhaust port to a fume hood. By use of a 23 Series oilless vacuum pump (Gast Mfg. Corp., Benton Harbor, Mich.) evacuation of the atmosphere in the incubator is rapid. For convenience in accessing incubated plates, a circular turntable (a lazy Susan) of acrylic plastic mounted on ball

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FIG. 1. Line drawing of the air lock body, showing the acrylic panel in place and the three-way valve with gas mixture supply lines.

bearings was placed inside the incubator. This turntable would accommodate 119 petri plates (seven metal racks of 17 petri plates each). Without the turntable, nine metal racks (153 petri plates) may be placed on the floor of the incubator.

Operation of the incubator. After the inoculated petri dishes are placed in the incubator and the door is sealed, the incubator is evacuated to a negative pressure of 0.5 atm (−50 kPa); a negative pressure greater than this may collapse agar overlays. With the door firmly compressed against the rubber seal, each of six swivel bolts is placed in its stainless steel bracket on the door and its knob is tightened by hand firmly (Fig. 2B). The incubator is then filled with the H₂S-N₂-CO₂ mixture to about zero pressure. This cycle of evacuation and filling should be repeated seven times to ensure that the final atmosphere approximates that of the cylinder. On the last filling with H₂S-N₂-CO₂, we recommend that a slight negative pressure (−5 kPa) should remain in the incubator. Prior to removal of plates from the incubator after incubation, the atmosphere of the incubator is evacuated through the fume hood to −50 kPa and the incubator is filled with H₂-N₂-CO₂, a gas mixture which approximates that of the chamber, until the pressure gauge reads about zero. This cycle is repeated 13 times to ensure that the gas atmosphere inside the incubator is free of H₂S. On the last vacuum cycle at −50 kPa, the knob on each of the six swivel bolts is released so that the bolt is quite loose before the final addition of H₂-N₂-CO₂. When the pressure from the addition of the gas mixture within the incubator equals that of the chamber, the swivel bolts are moved aside, and the door should open easily.

Because all methanococci studied so far require the addition of H₂S to the atmosphere when grown on solid media in petri plates, we have chosen to use a gas mixture that contains H₂S. This simplifies the addition of precise amounts of H₂S, and importantly, the removal of H₂S after incubation. To absorb traces of H₂S in the anaerobic chamber, we use trays of activated charcoal mounted on the catalytic boxes and on a separate circulation fan to absorb H₂S, which can easily destroy the catalyst. Charcoal is replaced weekly. We also have found it efficient to have outlets for gases and vacuum within the chamber for use with anaerobic jars. On the incubator shown in Fig. 2B (part d), the vacuum and gas lines from external sources have been tapped and connected through additional three-way valves to a rubber pressure tubing that can be attached to an anaerobic jar. Jars may be incubated within the chamber at 37°C. To conserve the gases used with

the incubator, space fillers (Fig. 2D) may be placed inside the chamber when it was not completely filled with plates. These space fillers were constructed from rigid standard polyvinyl chloride pipe (77-mm inner diameter; 5-mm-thick wall) with a solid cap at the top (the base was a sleeve with female threads and the insert cap had male threads). The total height was 30.5 cm.

Comparison of incubator and anaerobic jars. To test the anaerobic incubator, we have compared the plating efficiency of *M. acetivorans* C2A on agar medium grown in anaerobic jars to that obtained in the incubator. Petri plates of methanol-agar (HS-MA) medium were prepared inside the anaerobic chamber as previously described (6). At each step in the serial dilution of a stationary-phase broth culture, identical sample volumes were plated in triplicate on HS-MA. All plates were prepared in the same time period, with one series of plates incubated by each method (Table 1). For this experiment, we compared the following: (i) the anaerobic incubator as described above; (ii) our traditional method for incubation of plates, in which the anaerobic jars are removed from the incubator for the addition of H₂S and returned to the anaerobic chamber for incubation (6); and (iii) a simpler method in which the atmosphere in the jar was replaced inside the anaerobic chamber utilizing the premixed H₂S-N₂-CO₂ gas via the gas lines attached to the incubator as described above. For the last method, the same cycles of filling and evacuation were used as described above for the incubator. The data clearly show that there are no significant differences in plating efficiency be-

FIG. 2. (A) Modified air lock-incubator with the door partially open before installation inside an anaerobic chamber. The metal housing of the door frame (a), slotted bracket (b), and U-shaped bracket and pin holding a swivel threaded-bolt with a threaded knob on the free end (c) are indicated. (B) Incubator with door locked and tightened against black rubber seal by knobs on threaded swivel bolts. The rubber vacuum hose for attachment to anaerobic jar (d) is indicated. (C) Incubator installed inside an anaerobic chamber. (D) Space fillers for use inside incubator.

TABLE 1. Relative plating efficiencies of *M. acetivorans* C2A with different incubation methods

Trial no.	Plating efficiency (10 ⁸ CFU/ml) by the following method ^a :		
	Anaerobic jar with H ₂ S		Anaerobic incubator ^d
	Additions outside chamber ^b	Additions inside chamber ^c	
1	1.66	1.45	1.68
2	1.58	1.45	1.46
3	1.25	1.48	1.64

^a Serial dilutions of a stationary-phase broth culture were plated in triplicate on HS-MA medium, as previously described (6). Each dilution series was then incubated by one of the three methods.

^b Plates were placed inside an anaerobic jar and removed from the anaerobic chamber. The gas inside the jar was replaced with N₂-CO₂ (80 and 20%, respectively) by three cycles of evacuation and filling, with a final pressure of ca. 5 kPa inside the jar. H₂S was then added to a final concentration of 0.1%, and the jars were returned to the anaerobic chamber for incubation.

^c Plates were placed inside an anaerobic jar, and the gas inside the jar was replaced with N₂-CO₂-H₂S (79.9, 20, and 0.1%, respectively) by seven cycles of evacuation and filling, with a final pressure of ca. -5 kPa inside the jar.

^d The anaerobic incubator was used as described in the text.

tween the three methods employed here (Table 1). Further, because there is no need for time-consuming transfers to and from the air lock, the methods using the anaerobic incubator and the anaerobic jars with additions inside the chamber are considerably simpler and faster to perform.

The system described herein has been developed to plate mesophilic species of *Methanosarcina* which grow well at 37°C, the temperature at which the chamber is maintained, so no

additional heating element for the incubator is required. We have not attempted to grow plated organisms on H₂ and CO₂ within the incubator.

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