Methylobacterium spp.: Emerging Opportunistic Premise Plumbing Pathogens

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Abstract

Opportunistic premise plumbing pathogens (OPPPs) are responsible for many infections linked to drinking water. The annual cost of disease caused by these waterborne pathogens is $850 million. Key characteristics of these opportunistic waterborne pathogens include: disinfectant-resistant, biofilm formation, thermal-tolerance, desiccation-resistant, growth in amoebae and growth in low oxygen conditions. *Methylobacterium* spp. have been recognized as an emerging OPPP, so the purpose of this study was to investigate these waterborne bacteria in more detail to determine whether they have all characteristics of OPPPs. Seven *Methylobacterium* spp. strains were studied to measure growth in laboratory broth medium and drinking water, measure hydrophobicity on surfaces found in household plumbing, measure adherence and biofilm formation to surfaces found in household plumbing and measure susceptibility to hot water heater temperatures. *Methylobacterium* spp. were found to aggregate in lab broth medium and drinking water, hydrophobic on different surfaces in household plumbing, adhere readily and form biofilm on different surfaces and thermal-tolerant to water heater temperatures. These results support and identify *Methylobacterium* spp. as opportunistic premise plumbing pathogens.

Methylobacterium spp.: Emerging Opportunistic Premise Plumbing Pathogens

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General Audience Abstract

Opportunistic premise plumbing pathogens (OPPPs) are microbial residents of drinking water systems and premise plumbing that cause infection. Premise plumbing includes water pipes in hospitals, houses, apartment buildings or office buildings. OPPPs share a number of characteristics that contribute to their growth and survival in drinking water systems. In this study, *Methylobacterium* spp., an emerging OPPP, were studied to see if they share all of the characteristics of OPPPs. Seven *Methylobacterium* spp. strains were studied to measure growth in laboratory broth medium and drinking water, measure hydrophobicity (ability to repel water) on surfaces found in household plumbing, measure adherence to surfaces found in household plumbing and measure susceptibility to high temperatures. *Methylobacterium* spp. were found to form clusters of cells in lab broth medium and drinking water, hydrophobic on different surfaces in household plumbing, adhere readily on different surfaces and resistant to high temperatures. These results support *Methylobacterium* spp. are opportunistic premise plumbing pathogens. This is important because there is now a better understanding of how *Methylobacterium* spp. survive in drinking water systems to prevent its growth and persistence. This study was also able to determine which pipe surfaces support the least amount of *Methylobacterium* spp. growth to be used be used by plumbers and homeowners to reduce exposure to *Methylobacterium* spp.

Table of Contents

Background

Opportunistic Premise Plumbing Pathogens (OPPPs). Opportunistic premise plumbing pathogens are normal microbial residents of drinking water distribution systems and premise plumbing. OPPPs are estimated to cause nearly 30,000 cases of human disease and cost $850 million yearly (Collier *et al*. 2012). OPPPs grow in drinking water distribution systems, unlike contaminants of drinking water, such as *Escherichia coli* and *Salmonella* spp.

A number of accepted opportunistic premise plumbing pathogens is listed in Table 1. *Legionella pneumonia*, the causative agent of Legionnaire's disease, a life-threatening pneumonia, has been isolated in samples of drinking water across the United States (Donohue *et al*. 2014). Pulmonary infections caused by *Mycobacterium avium* have been tracked by DNA fingerprinting to showerheads (Falkinham *et al*. 2008) and household plumbing (Falkinham 2011). Infections caused by *Pseudomonas aeruginosa* have been linked to bronchoscopes washed with non-sterile water (Cuttelod *et al*. 2011). Premise plumbing has unique features (Table 2) that select for distinct communities of microorganisms to grow (Falkinham *et al*. 2015). Low oxygen, low disinfection concentrations, low organic matter content, heating, periods of stagnation and pipe materials as biofilm formation surfaces all give OPPPs ideal growth conditions. The microaerobic OPPPs are able to survive low oxygen conditions created by the premise plumbing. Being disinfectant-resistant allows OPPPs to survive standard disinfectant dosages for drinking water treatment that will essentially kill off all but the opportunistic pathogens. Consequently, these premise plumbing pathogens are able to consume the available organic carbon without competition. Desiccation-resistance allows OPPPs to survive in dried conditions created by premise plumbing. Biofilm-formation provides a mechanism to prevent washing out of microorganisms in flowing systems, such as pipes. Thermal tolerance allows OPPPs to survive in high temperatures created by hot water heaters. The ability of OPPPs to survive and grow in free-living amoebae gives an extra layer of protection against disinfection and desiccation.

Recently it has been recognized that *Methylobacterium* spp. are normal drinking water

inhabitants (Gallego *et al*. 2006) and opportunistic pathogens (Kressel and Kidd 2001). Further, studies of households of patients with *Mycobacterium avium* pulmonary infections showed that when *Methylobacterium* spp. were present in biofilm samples, *M. avium* was absent (Falkinham *et al*. 2015). As *Methylobacterium* spp. have been recognized as an emerging OPPP and they demonstrate an interesting interaction with another OPPP, namely *M. avium*, we sought to study them in more detail to determine whether they have all the characteristics of OPPPs (Table 3).

***Methylobacterium* spp. Characteristics.** *Methylobacterium* spp. are Gram-negative, relatively slow growing opportunistic pathogens in premise plumbing that were originally classified as *Pseudomonas* or *Protomonas* (Green and Bousfield 1982). In agar medium they form salmon-pink colonies and aggregates in broth culture are also pink (Gallego *et al*. 2006). *Methylobacterium* spp. are able to grow on one-carbon-compounds such as formate, formaldehyde, methanol, methylamine, and a wide range of multi-carbon compounds as a sole source of carbon and energy (Green 1992). *Methylobacterium* spp. produce pink carotenoid pigments as well as phytochromes (cytokinins and auxins), which are known to stimulate plant growth (Ivanova *et al*. 2001; Koeing *et al*. 2002), and are capable of fixing atmospheric nitrogen (Sy *et al*. 2001) and help plants to fight pathogens (Holland and Polacco 1994).

***Methylobacterium* spp. Pathogenicity.** *Methylobacterium* spp. have been linked to infections in humans. Most reported *Methylobacterium* spp. infections have been nosocomial. *Methylobacterium* spp. were reported to infect immunocompromised patients and people with indwelling catheters (Poirier *et al*. 1988). *Methylobacterium mesophilica* has caused pseudo-outbreaks associated with the use of bronchoscopes and endoscopes that were contaminated by the *M. mesophilica*-containing tap water used to wash each instrument (Kressel and Kidd 2001; Flournoy *et al*. 1992). In general, *Methylobacterium* spp. cause mild clinical symptoms, such as fever, but severe infections include bloodstream infections, peritonitis, and pneumonia have also been reported (Sanders *et al*. 2000; Lai *et al*. 2011). Bloodstream infections have been reported due to *M. mesophilica* from bone marrow transport recipients caused by tap water used for oral irrigation (Gilchrist *et al*. 1986). In another case, a patient undergoing continuous ambulatory

peritoneal dialysis developed peritonitis due to *M. mesophilica*, which was isolated from stagnant water in the bathroom (Rutherford *et al*. 1988).

***Methylobacterium* spp. in the Human Environment.** Members of the *Methylobacterium* genus are found in a wide variety of natural habitats, including soil, dust, air, fresh water and aquatic sediments (Hirashi *et al*. 1995). They can be easily recognized in homes as they form pink slime in shower surfaces and on shower curtains (Yano *et al*. 2013). One species, *Methylobacterium tardum,* was even isolated from dust samples on the International Space Station (Mora *et al*. 2016). *Methylobacterium* are also commonly found in drinking water and have been isolated from water in households and hospitals (Flournoy *et al*. 1992; Kovalea *et al*. 2014). These bacteria are able to persist in drinking water due to their chlorine-resistance, surface-adherence, biofilm-formation and desiccation-tolerance (Kaneko and Hiraishi 1991; Yano *et al*. 2013). *Methylobacterium extorquens* has been isolated from amoeba from drinking water systems, makes it a member of the amoeba-resistant microorganisms (Thomas 2006).

***Methylobacterium* spp. Biofilm Formation.** Biofilms are assemblages of microbial cells attached to surfaces and enclosed in a matrix of exocellular polymeric substances (Donlan and Costerton 2002). Biofilms are difficult to remove and protect microorganisms to survive under conditions of high water flow (i.e., washout), drying (i.e., desiccation), disinfectant (e.g., chlorine) and antibiotic exposure. The extracellular matrix and layers of cells are protective by reducing the diffusion of antimicrobial compounds (Donlan and Costerton 2002). Biofilms also limit the diffusion of oxygen to cells in the biofilm matrix (Donlon and Costerton, 2002). *Methylobacterium* spp. have a strong biofilm-producing ability (Yano *et al*. 2013; Simoes *et al*. 2009). *Methylobacterium*-containing biofilms have been found on surfaces in automobile air conditioning systems (Simmons *et al*. 1999), shower curtains (Kelley *et al*. 2004), and showerheads (Feazel *et al*. 2009).

Methylobacterium spp. cells in biofilms exhibit resistance to chemical disinfectants. For example, *Methylobacterium* spp. biofilms have been found to survive one hour exposure to 100 ppm of sodium hypochlorite, a concentration that is 100-times higher than the 1ppm

concentration used for drinking water disinfection (Simoes *et al*. 2009). Regrowth of *M. extorquens* biofilms occurred following seven days after an exposure to 1% peracetic acid (Kovela 2013). *M. mesophilicum* biofilms on the tubing in an automated endoscope reprocessor were the source of a *Methylobacterium mesophilicum* infection outbreak in patients after bronchoscopy (Kressel and Kidd 2001). Further, the presence of an established *Methylobacterium* spp. biofilm has been found to substantially reduce adherence and biofilm formation by *Mycobacterium avium*, another drinking water opportunistic pathogen associated with pulmonary disease, to plumbing surfaces (Falkinham *et al*. 2016).

***Methylobacterium* spp. Aggregation and Hydrophobicity.** Aggregation of methylobacterial cells appears to be a characteristic, as it has been reported in all species characterized to date, including *Methylobacterium adhaesivum* AR27 (Gallego *et al*. 2006) and *Methylobacterium funariae* F3.2 (Schauer and Kutschera 2011). Plant growth-promoting *Methylobacterium* strains have also been found to aggregate in high C/N growth conditions (Joe *et al*. 2013). Bacterial aggregate formation may be driven by the same forces that result in biofilm formation; namely hydrophobic interactions (Kos *et al*. 2003)

***Methylobacterium* spp. Chlorine Resistance.** *Methylobacterium* spp. have been isolated from water supplies and other chlorinated environments (Furuhata and Koike 1993), suggesting that they are innately chlorine-resistant and perhaps other disinfectants. The resistance of *M. extorquens* to free residual chlorine has been found to be moderate; the CT value (the product of disinfectant concentration and time to reach 3 logs units of cell death) was 1.5 mg-min/l compared to *E. coli* whose CT value is 0.09 mg-min/l (Furuhata *et al*. 1989). *Methylobacterium aquaticum* strains isolated from tap water were all found to be mildly resistant to chlorine having average CT values of 0.89 mg-min/l (Furuhata *et al*. 2011).

***Methylobacterium* spp. Thermal Tolerance.** *Methylobacterium* spp. are recognized as resistant to elevated (>50°C) temperatures (Orphan 1999). *Methylobacterium* spp. have been isolated from hot tap water in a household (Soucie and Schuler 2006) suggesting that the laboratory-measured resistance (re: Orphan 1999) is of ecological consequence. The PhyR protein in *M.*

extorquens has been revealed to positively regulate genes involved in the resistance to heat shock and other environmental stressors (Gourion *et al*. 2007).

Hypotheses

1. *Methylobacterium* spp. are capable of growth in drinking water.
2. *Methylobacterium* spp. cells are hydrophobic.
3. *Methylobacterium* spp. are able to adhere to different surfaces found in household plumbing.
4. *Methylobacterium* spp. grow on surfaces and form biofilms.
5. *Methylobacterium* spp. can survive temperatures found in hot water heaters.

Experimental Objectives

1. Measure the growth of different *Methylobacterium* spp. strains in laboratory broth medium and drinking water.
2. Measure hydrophobicity of *Methylobacterium* spp. cells on surfaces found in household plumbing.
3. Measure adherence and biofilm formation of *Methylobacterium* spp. cells on surfaces found in household plumbing.
4. Measure susceptibility of *Methylobacterium* spp. cells to hot water heater temperatures.

Rationale of the Experimental Objectives

1. Growth of *Methylobacterium* spp. can be measured by increases in turbidity (absorbance 540 nm) or by increases in protein.
2. Hydrophobicity of *Methylobacterium* spp. cells can be measured by the contact angle of water drops on surfaces of cells.
3. Adherence of *Methylobacterium* spp. cells can be measured as colony-forming units (CFU) adhering to coupons of different pipe materials placed in water suspensions of cells for short periods of time (1-6 hr).

4. Biofilm formation of *Methylobacterium* spp. can be measured as the number of colony-forming units (CFU) on surfaces after 21 days following a short period for adherence.

Experimental Methods

***Methylobacterium* spp. Strains**. The *Methylobacterium* spp. strains used in this study are listed in Table 4 and chosen to potentially represent the range of characteristics representative of the genus. For some experiments, consortia of different species representatives were made arbitrarily to reduce the number of different species.

Growth of *Methylobacterium* spp. Strains. For all experiments, strains were grown in 25 mL of R2A broth (High Media Laboratories, India) in a 250 mL Nephalometer flask to log phase with aeration (60 rpm) at 30°C.

Measurement of Growth. Samples of 1 mL of cultures from each strain were used to inoculate 25 mL of R2A broth in a 250 Nephalometer flask and grown at 30°C with aeration (60 rpm). Immediately and every six hours for two days the turbidity was measured as Abs_{540} nm.

Measurement of Growth as Protein Content. Due to aggregation of all, but one, of the *Methylobacterium* spp. cells (i.e., *Methylobacterium hispanicum* strain JMI-5/SS-2), the total protein content of cells was measured in 1 mL samples of the cultures at different times. Comparison of measurements of absorbance at 540 nm and protein content of the non-aggregating strain *Methylobacterium hispanicum* strain JMI-5/SS-2 demonstrated a strong correlation (R=0.9841) (Figure 3). Protein measurements were performed using the method of Lowry et al. (1956) using 1 mL of the undiluted cultures at each time point. Absorbance was measured A_{500} and protein content determined by comparison to a standard curve of bovine serum albumin, fraction V.

Water Adaptation of *Methylobacterium* spp.. For adherence and biofilm experiments, cells grown to log phase in R2A broth medium were collected by centrifugation (5000 x g) for 20 min. The supernatant medium was discarded and the cells suspended in 25 mL of sterile Blacksburg tap water. Cells were pelleted a second time by centrifugation (5000 x g for 20 min) and suspended in the same volume of autoclaved Blacksburg tap water (total organic carbon= 2·5 $\pm$ 2·5 mg l^{-1}). The twice-washed cells were incubated for 1 week at room temperature in sterile Blacksburg tap water to acclimate the cells to low nutrient, low ionic strength conditions (Falkinham 2003). Following water adaptation, colony counts were measured using serial dilutions in sterile Blacksburg tap water on R2A agar.

Measurement of Temperature Susceptibility of *Methylobacterium* spp. Strains. For the temperature susceptibility measurements, cells previously grown to log phase in R2A broth medium were collected by collected by centrifugation (5000 x g) for 20 min. The supernatant medium was discarded and the cells suspended in 10 mL of sterile Blacksburg tap water. Cells were pelleted again by centrifugation and suspended in 10 mL of sterile Standard Hardness Water (17.5 mL of 10% (wt/vol) $CaCl_2 \bullet 6H_2O$ and 5 mL of 10% (wt/vol) $MgSO_4 \bullet 7H_2O$ in 3,300 mL MilliQ water). The washed cells were incubated for one week at room temperature.

Biofilm Device. The CDC biofilm reactor (Figure 1, BioSurfaces Technologies Corporation, Bozeman, MT) was used with glass, steel, copper, PVC and galvanized coupons. Each coupon is 0.3 cm thick with a 1.27 cm diameter, presenting a total surface area of 2.54 cm^2. The glass vessel allows for approximately 500 ml of operational fluid capacity and contains eight, removable, polypropylene rods, each capable of holding three coupons, which allows for 24 sampling opportunities. The use of the CDC biofilm reactor allows for easy manipulation of biofilm growth surfaces, temperature and shear (Donlon et al., 2002).

Measurement of Cell Hydrophobicity. Hydrophobicity, as reflected in contact angle provides good estimate of bacterial cell surface hydrophobicity (van Oss et al., 1975) and have a predictive value for adhesion (Van Loosdrecht et al. 1987). Coupons in the CDC biofilm reactor (CBR) were incubated at room temperature for 6 hours in the presence of 10^5 CFU mL^{-1} of a *Methylobacterium* spp. strain for adherence. Rods with the coupons were removed from the paddle support system and rinsed by dipping three times in sterile Blacksburg tap water. Rods were allowed to air dry before recording measurements. 10µL drops of 1% saline were placed on cell biofilms on coupons in triplicate. The measurement of the contact angle was done by turning the rim of the goniometer until one of the hairs of the cross-hair was tangential to the drop at the place where it is in contact with the surface (van Oss et al., 1975).

Measurement of Adherence of *Methylobacterium* spp. Cells to Surfaces. The CBR was filled with sterile Blacksburg tap water to fully cover the coupons (500 mL) and the liquid was inoculated with a sufficient volume of a water acclimated suspension of methylobacterial cells to reach a final density of 10^5 CFU mL^{-1}. Immediately and after 1, 2, 3 and 6 hours incubation at room temperature, a paddle was removed and washed by dipping three times in the same 35 mL of sterile Blacksburg tap water. Individual coupons were removed aseptically and placed in separate sterile 50 mL-screw cap tubes containing 2 mL of sterile Blacksburg tap water. Each tube was vortexed for 1 minute at the highest setting and 0.1 mL of the resulting undiluted and diluted suspension was spread on R2A agar and incubated at 30°C for 5 days. After incubation, colonies were counted and CFU cm^{-2} calculated for each coupon. To estimate the number of cells still adhering to the coupons after vortexing, the coupons were placed on R2A agar medium for 1 hour and removed. The plate incubated at 30°C for 5 days and the number of colonies were counted.

Measurement of Biofilm Formation of Adherent *Methylobacterium* spp. Cells. Coupons in the CDC biofilm device were incubated at room temperature for 6 hours in the presence of 10^5

CFU mL^{-1} of a *Methylobacterium* spp. strain for adherence as described above. The entire paddle support system (eight paddles) was removed and rinsed by dipping sequentially in three separate 500 mL volumes of autoclaved Blacksburg tap water. A second and autoclaved CDC biofilm device was filled with autoclaved Blacksburg tap water and the paddle support with coupons and adherent methylobacterial cells placed in the device. One coupon of each plumbing surface paddle was immediately removed, washed by dipping three times in autoclaved Blacksburg tap water and processed for measurement of methylobacterial CFU cm^{-2} as described above. After 21 days incubation at room temperature, each paddle was removed, washed by dipping in autoclaved Blacksburg tap water and processed for measurement of methylobacterial CFU cm^{-2} as described above.

Measurement of Temperature Susceptibility of *Methylobacterium* spp. Strains. The temperature of a water bath was set to 50°C, 55°C or 60°C. Water-adapted suspensions were diluted 1,000-fold into Standard Hardness Water (Schulze-Röbbecke and Buchholtz 1992). Two mL of the diluted suspension was transferred to a sterile 13 x 100 mm glass tube and 10 µL immediately spread on R2A agar in triplicate. At 1, 2 and 3 minutes of exposure to each temperature, 10µL of suspension was spread on R2A agar in triplicate. Plates were incubated at 30°C for 5 days and colonies counted to calculate survival as a percent of the non-heat exposed control.

Results

Methylobacterium spp. growth in R2A broth and water.

Due to the characteristic clumping of *Methylobacterium* spp. cells during growth in broth medium (Gallego *et al*. 2006; Joe *et al*. 2013), it was not possible to measure growth as increases in turbidity (absorbance at 540 nm). However, it was possible to measure the increase in turbidity of *M. hispanicum* strain JM1-5, the one isolate (Table 4) that failed to form clumps in R2A broth culture. Exponential growth was seen for *M. hispanicum* strain JM1-5 within the first 6 hours at 30° C with stationary phase reached by 24 hours (Figure 2). Although it was evident from the increase in the number and size of aggregates in R2A broth cultures of the other *Methylobacterium* spp. strains that they did grow in R2A broth, it was not possible to follow growth as increases in turbidity. The ability of the aggregating *Methylobacterium* spp. strains to adhere to the flask, forming a pink ring, could have caused ineffective sampling due to smaller amounts of cells in the R2A broth.

Alternatively, growth was monitored by increases in total protein of cells. To determine if protein measurements would be suitable surrogates for turbidity, both turbidity (abs 540 nm) and protein content of the non-aggregating *M. hispanicum* strain JM1-5 were measured. The data (Figure 3) demonstrate a high correlation (R=0.9841) existed between protein concentration and turbidity. Thus, for the remaining aggregating *Methylobacterium* spp. strains, protein content was measured as a substitute for turbidity. The protein content, expressed as mg protein/mL of culture, was measured to follow growth for the other *Methylobacterium* spp. strains (Figure 4). Measuring the protein content of *Methylobacterium* spp. in water showed that the strains grew modestly within the first 7 days, exhibiting a doubling in most strains (Figure 5). However, the protein content of those aggregating strains decreased after 21 days (Figure 5). Quite possibly that decrease in protein reflected difficulty sampling and the degradation of the cellular protein. Interestingly, *E. coli* was able to grow and persist in water for 21 days. *E. coli* is not a water inhabitant and is commonly found in water as a contaminant.

Measurement of hydrophobicity of *Methylobacterium* spp. cells on different plumbing surfaces

Hydrophobicity, as reflected in contact angle provides good estimate of bacterial cell surface hydrophobicity (van Oss et al., 1975). All *Methylobacterium* spp. strains were grown in R2A broth at 30°C with aeration (120 rpm). Coupons for each plumbing in the biofilm reactor were incubated at room temperature for 6 hours in the presence of 10^5 CFU mL^{-1} of each strain for adherence before being processed for measurement. All *Methylobacterium* spp. strains (triplicate replicates) produced higher contact angles than the control stainless steel coupon with no methylobacterial cells (Table 5). The *Methylobacterium* spp. strains grown on plumbing surfaces produced high contact angles when grown on PVC and galvanized coupons, but low contact angles on glass (Table 6). Significantly, *M. hispanicum* strain JM1-5, the strain that did not aggregate in R2A broth or water, exhibited the lowest contact angles compared to all the rest of the *Methylobacterium* spp. strains (Table 6). The *M. extorquens* strain produced higher contact angles than the JM1-5 strain, which can be related to *M. extorquens* ability to aggregate compared to JM1-5. The *E. coli* strain grown on the coupons produced contact angles smaller than the control contact angles.

Measurement of adherence of *Methylobacterium* spp. cells to plumbing surfaces

Plumbing surface coupons (i.e., glass, copper, galvanized, PVC, and stainless steel) were exposed to water-adapted *Methylobacterium* spp. cells at 10^5 CFU mL^{-1} for 1-6 hours in the CDC Biofilm Reactor to measure adherence. Cells of the *Methylobacterium* spp. strains rapidly adhered and reached high numbers, reaching between 350 and 4,800 CFU cm^{-2} within 6 hours (Tables 7-9). The values for the adherent *Methylobacterium spp*. are likely underestimates as cells remained on the coupons despite vortexing. Approximately 1-10% of the cell numbers in suspension were detected by placing the vortexed coupons on R2A agar medium (independent experiment). The standard deviations of the triplicate measurements were high for some samples

(Tables 7-9). There could be several reasons for this: (1) if both single and aggregated cells adhered, there would be a wide range of adherent cells and, (2) if aggregates adhered, the suspensions derived would consist of aggregates and single cells that would yield widely different colony counts depending on the ability of spreading to separate aggregates into single cells.

PVC and galvanized surfaces supported the greatest number of adherent cells for both *M. hispanicum* strains JM1-5 and JM1-8. The order of the JM1-5 adherence was as follows: PVC, galvanized, steel, glass and copper (Table 7). The order of the JM1-8 adherence was as follows: PVC, galvanized, copper, steel and glass (Table 8). The aggregated JM1-8 strain had a higher number of adherent cells to surfaces compared to the non-aggregated JM1-5 strain possibly due to its higher hydrophobicity from aggregating. The order of the *M. extorquens* strain adherence is as follows: PVC, glass, galvanized, copper and steel (Table 9). Based on these results, PVC and galvanized plumbing surfaces can be assumed to cause the most adherence of all *Methylobacterium* spp. strains.

Measurement of biofilm formation of *Methylobacterium* spp. on plumbing surfaces

Following a 6 hour period for attachment, paddles and coupons were rinsed in autoclaved Blacksburg tap water and replaced in the CDC biofilm device containing autoclaved Blacksburg tap water. The paddles and coupons were removed after 21 days incubation and the number of adherent CFU were counted. The results show, like adherence, plumbing material influenced biofilm formation. Both the aggregating *M. hispanicum* JM1-8 and *M. extorquens* strains had similar numbers of adherent cells per surface area (i.e., CFU/cm^2) and preferred plumbing surfaces (Tables 10 and 11). The order of biofilm numbers for *M. hispanicum* strain JM1-8 was as follows: PVC, galvanized, copper, steel and glass (Table 10). The order of biofilm numbers for *M. extorquens* was as follows: PVC, galvanized, copper, glass and steel (Table 11).

Measurement of the thermal tolerance of *Methylobacterium* spp. cells

To assess the thermal-tolerance *Methylobacterium* spp. at hot water heater temperatures, survival measurements were done at 50, 55 and 60°C. Water-adapted *Methylobacterium* spp. suspensions were diluted and exposed to each temperature for 1, 2 and 3 minutes. At 50°C, all *Methylobacterium* spp. isolates had a survival of at least 80% after three minutes of exposure (Figure 5). *M. adhaesivum* strain 14625, *M. variable* strain 14628, *M. hispanicum* strain JM1-8 and *M. aquaticum* strain 14006 survival values exceeded 100% after three minutes of exposure. This could be due to aggregates of each of these strains breaking up at the high temperature. In contrast, only 40% of *E. coli* cells survived after three minutes of exposure. At 55°C, most *Methylobacterium* spp. isolates had less than 60% survival after three minutes of exposure (Figure 6). *M. adhaesivum* strain 14625, *M. hispanicum* strain JM1-8 and *M. isbiliense* strain 14626 had a survival of 80% or greater after three minutes of exposure. *E. coli* did not survive at this temperature, as its survival dropped to undetectable numbers (<1% survival) after three minutes of exposure. At 60°C, almost all *Methylobacterium* spp. isolates had a survival at less than 20% after three minutes of exposure (Figure 7). *M. adhaesivum* strain 14625 had over 80% survival after three minutes of exposure and less than 20% survival after six minutes of exposure.

Discussion

The experimental approaches were devised to determine whether representative *Methylobacterium* spp. strains had characteristics found in OPPPs. The findings indicate *Methylobacterium* spp. form aggregates, hydrophobic, adhere to many plumbing surfaces, form biofilms and are thermal-tolerant. While *Methylobacterium spp*. have been identified as slow growers, the non-aggregating *M. hispanicum* strain JM1-5 was a fast growing strain, with its doubling time being six hours. The ability of the other *Methylobacterium* spp. strains to form aggregates made it difficult to record their growth accurately. In attempt to solve this problem, protein content was found to have a correlation (R=0.9841) to turbidity by absorbance in the *M. hispanicum* strain JM1-5. Unfortunately, the growth curve of the protein content of the aggregating *Methylobacterium* spp. strains in R2A broth could not give a consistent result (Figure 4). The aggregating strains formed clumps of cells when measurements were taken so sampling error could have caused the inconsistency of the data recorded.

Methylobacterium spp. was not found to grow longer than seven days in sterile water, but could persist in this environment for 21 days (Figure 5). The growth and persistence of *E.coli* (non-aggregating) in sterile water could be due to favorable conditions, as it has been found to survive in autoclaved filtered river water for up to 260 days (Flint 1987). New methods need to be used to accurately measure *Methylobacterium* spp. in its aggregated form without disturbing its natural growth form. One way of doing this could be to add detergents to the aggregating samples to break up the aggregates.

Both coupon composition and strain were major factors influencing adherence and biofilm formation. Hydrophobicity has been found to be a factor in surface adherence (van Loosdrecht *et al*. 1987). Therefore, it is not surprising the more hydrophobic *M. extorquens* has more adherent cells than the non-aggregating *M. hispanicum* strain JM1-5. The ability of each *Methylobacterium* spp. strain to aggregate has been listed in Table 4. While *Methylobacterium* spp. has been found to be hydrophobic, in comparison, *Mycobacterium intracellulare* biofilms have formed a 54.0° angle on the surface of a glass slide (Steed and Falkinham 2006). The

materials in each plumbing surface also influenced the number of adherent cells and biofilm formation. Zinc-galvanized steel and PVC materials are generally hydrophobic surfaces, which leads to higher adherence and biofilm formation, while glass and steel are more hydrophilic surfaces, leading to less adherence and biofilm formation. Although speculation, high zinc concentrations in soil and water have also been associated with high mycobacterial numbers (Kirschner *et al*. 1992) so this could be related to the high number of adherent *Methylobacterium* spp. cells.

Methylobacterium spp. exhibited relative thermal tolerance at hot water temperatures with survival at 50°C (Figure 6). Killing was not observed until the temperatures were raised to 55°C (Figure 7) and all *Methylobacterium* spp. strains were killed at 60°C (Figure 8). These results suggest that one way to reduce *Methylobacterium* spp. exposure is to elevate water heater temperatures to 55°C. In a study of the numbers of nontuberculous mycobacteria (NTM) in household plumbing samples, it was found that NTM were less frequently recovered from household samples whose water heater temperatures were $\geq$ 130°F ($\geq$55°C) compared to $\leq$ 125°F ($\leq$50°C) (Falkinham 2011). Other ways to reduce *Methylobacterium* spp. exposure in hot water heaters would be to drain and refill the water heater periodically.

In summary, *Methylobacterium* spp. share characteristics of other OPPPs including being hydrophobic, ability to aggregate, adhere and form biofilms on different plumbing surfaces and its thermal tolerance. Knowledge of pipe surfaces to support the lowest amount of *Methylobacterium* spp. can be used by plumbers and homeowners to reduce exposure to *Methylobacterium* spp. in household plumbing.

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List of Tables

Table 1. Opportunistic Premise Plumbing Pathogens

Species
Legionella pneumophila
Mycobacterium avium complex
Pseudomonas aeruginosa
Stenotrophomonas maltophila
Acinetobacter baumanii
Sphingomonas paucimobilis
Acanthamoeba polyphaga

Table 2. Common Characteristics of Premise Plumbing

Common Characteristics of Premise Plumbing
Unique Pipe Materials
Low Oxygen and Carbon Levels
Periods of Stagnation
Heating
Low disinfection concentrations

Table 3. Common Characteristics of OPPPs

Common Characteristics of OPPPs
Infection Linked to Drinking Water Exposure
Persistence and Growth in Drinking Water Systems
Microaerobic
Disinfectant (Chlorine, Chloramine-)-Resistance
Biofilm-Formation
Thermal-Tolerance
Growth in Amoebae and Protozoa
Desiccation-Resistant

Table 4. *Methylobacterium* spp. strains

Consortium	Species	Strain	Source	Aggregates
Consortium 1	*M. extorquens*	ATCC 43645	soil, Japan	+
	M. aquaticum	NCIMB 14006	drinking water, Seville	+
	M. adhaesivum	NCIMB 14625	drinking water, Seville	+
	M. isbiliense	NCIMB 14626	drinking water, Seville	+
	M. variable	NCIMB 14628	drinking water, Seville	+
Consortium 2	*M. hispanicum*	JM1-5/SS-2	Summer's Shower	-
	M. hispanicum	JM8/PPESSw-2	PA Patient Shower	+

+, positive; -, negative

Table 5. *Methylobacterium* spp. contact angles on stainless steel

Species	Strain	Mean Contact Angle	Standard Deviation
Control		43.2°	1.0
M. hispanicum	JM1-5	45.4°	3.0
M. adhaesivum	14625	57.3°	2.9
M. extorquens	MEXT	53.8°	1.8
M. variable	14628	51.3°	0.9
M. aquaticum	14006	48.5°	1.0
M. isbiliense	14626	52.7°	1.1
M. hispanicum	JM1-8	57.3°	1.4

*Average contact angle and standard deviation of adherent cells to stainless steel coupons in triplicate measurements

Table 6. Contact angles of *Methylobacterium* spp. and *Escherichia coli* cells grown on different plumbing surfaces

Strain	Steel	PVC	Galvanized	Copper	Glass
Control	58.0±5.3	97.0±5.9	85.4±5.0	66.5±9.8	35.3±2.7
JM1-5	63.9±3.7	83.7±3.1	86.5±1.9	70.4±2.6	48.2±2.7
E. coli	58.6±3.3	66.1±1.5	74.5±3.6	49.4±11.0	47.8±3.5
MEXT	65.6±3.1	97.3±5.3	93.3±4.6	71.0±2.7	50.8±4.6

*Average contact angle ± standard deviation of adhering cells to each coupon type of triplicate measurements

Table 7. Adherence of *Methylobacterium hispanicum* strain JM1-5 cells to plumbing surfaces

	CFU cm^{-2} surface				
Duration of Exposure	Copper	Glass	PVC	Galvanized	Steel
0 h	80 ± 7.5	8 ± 1.0	110 ± 20	200 ± 10	160 ± 5.5
1 h	40 ± 4.0	120 ± 4.0	1000 ± 80	1400 ± 20	560 ± 50
2 h	100 ± 2.0	210 ± 4.0	430 ± 20	2400 ± 30	480 ± 30
3 h	200 ± 10	220 ± 10	580 ± 7.1	1100 ± 70	390 ± 5.6
6 h	350 ± 30	450 ± 20	1600 ± 110	1400 ± 50	870 ± 30

*Average number of CFU/cm^2 ± standard deviation adhering to each coupon type of triplicate measurements

Table 8. Adherence of *Methylobacterium hispanicum* strain JM1-8 cells to plumbing surfaces.

	CFU cm^{-2} surface				
Duration of Exposure	Steel	Copper	PVC	Galvanized	Glass
0 h	850 ± 30	1800 ± 9.6	350 ± 20	440 ± 10	120 ± 9.0
1 h	1500 ± 110	2500 ± 7.8	2200 ± 4.0	1900 ± 20	420 ± 40
2 h	1500 ± 40	2600 ± 9.9	2300 ± 10	2500 ± 20	580 ± 20
3 h	1900 ± 70	3000 ± 10	3700 ± 20	3700 ± 10	880 ± 40
6 h	2000 ± 20	3400 ± 10	4800 ± 10	4800 ± 20	1300 ± 30

*Average number of CFU/cm^2 ± standard deviation adhering to each coupon type of triplicate measurements

Table 9. Adherence of *Methylobacterium extorquens* strain ATCC 43645 cells to plumbing surfaces.

	CFU cm^{-2} surface				
Duration of Exposure	Steel	Copper	PVC	Galvanized	Glass
0 h	180 ± 10	250 ± 2.6	1100 ± 90	640 ± 40	270 ± 3.5
1 h	460 ± 20	650 ± 30	1900 ± 70	1300 ± 30	1500 ± 90
2 h	680 ± 20	1000 ± 20	2200 ± 40	870 ± 30	1900 ± 30
3 h	870 ± 40	1300 ± 10	2100 ± 30	2000 ± 50	1900 ± 30
6 h	1100 ± 50	2000 ± 20	3800 ± 30	3000 ± 50	3800 ± 20

*Average number of CFU/cm^2 ± standard deviation adhering to each coupon type of triplicate measurements

Table 10. Biofilm formation by *Methylobacterium hispanicum* strain JM1-8 to plumbing surfaces.

	Duration of Exposure	
CFU cm^{-2} surface	6 h	21 d
Steel	$2.9 \pm 0.67 \times 10^3$	$3.0 \pm 3.8 \times 10^4$
Copper	$4.1 \pm 0.44 \times 10^3$	$2.9 \pm 4.7 \times 10^5$
PVC	$6.6 \pm 0.32 \times 10^4$	$7.1 \pm 9.6 \times 10^6$
Galvanized	$7.6 \pm 2.2 \times 10^4$	$3.4 \pm 3.9 \times 10^6$
Glass	$2.7 \pm 1.3 \times 10^3$	$2.0 \pm 5.4 \times 10^4$

*Average number of CFU/cm^2 $\pm$ standard deviation adhering to each coupon type of triplicate measurements

Table 11. Biofilm formation by *Methylobacterium extorquens* strain ATCC 43645 to plumbing surfaces.

	Duration of Exposure	
CFU cm^{-2} surface	6 h	21 d
Steel	$1.1 \pm 0.98 \times 10^2$	$3.1 \pm 6.7 \times 10^2$
Copper	$1.0 \pm 2.1 \times 10^3$	$9.2 \pm 2.3 \times 10^3$
PVC	$4.5 \pm 3.4 \times 10^3$	$6.2 \pm 9.7 \times 10^5$
Galvanized	$6.7 \pm 1.2 \times 10^3$	$4.5 \pm 9.8 \times 10^5$
Glass	$9.4 \pm 0.56 \times 10^2$	$2.2 \pm 5.4 \times 10^3$

*Average number of CFU/cm^2 $\pm$ standard deviation adhering to each coupon type of triplicate measurements

List of Figures

Fig. 1. CDC Biofilm Reactor

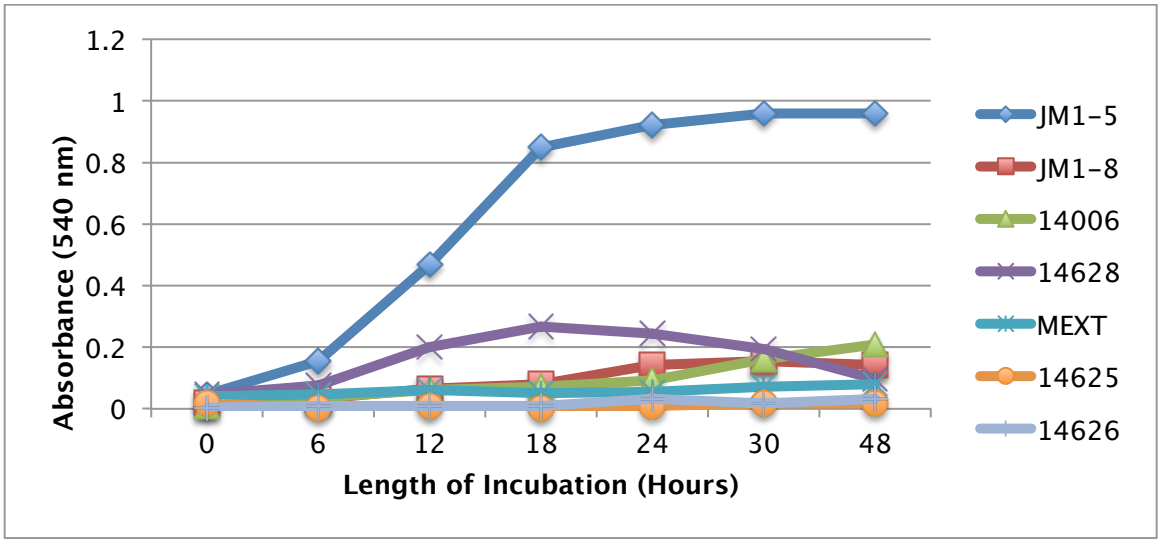

Fig. 2. Representative growth curve of *Methylobacterium* spp. strains in R2A broth at 30° C with aeration (120 rpm).

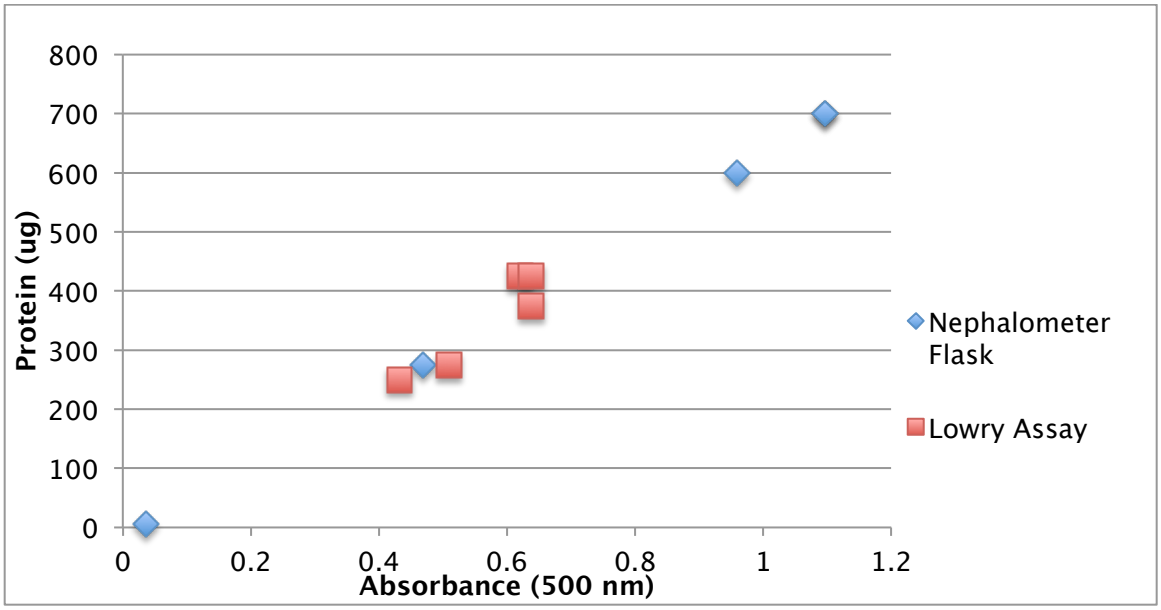

Fig. 3. Representative growth curve of *Methylobacterium hispanicum* strain JMI-5/SS-2 turbidity vs. protein content (Lowry Assay) at 30° C with aeration (120 rpm). R=0.9841

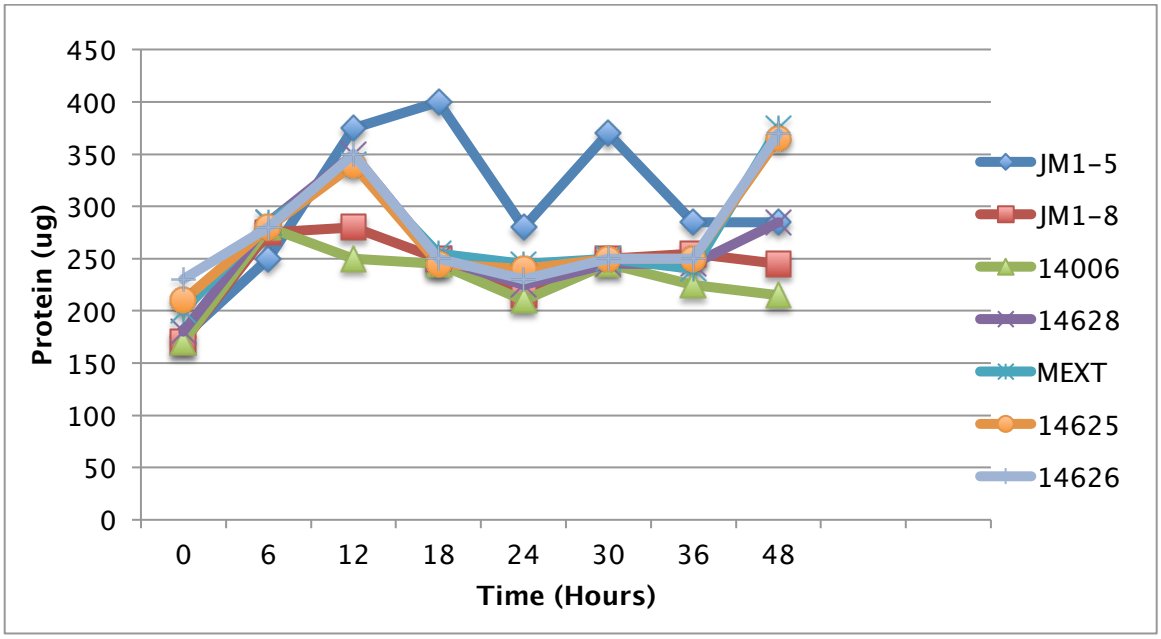

Fig. 4. Representative growth curve of protein content of *Methylobacterium* spp. in R2A broth (Lowry Assay) at 30° C with aeration (120 rpm).

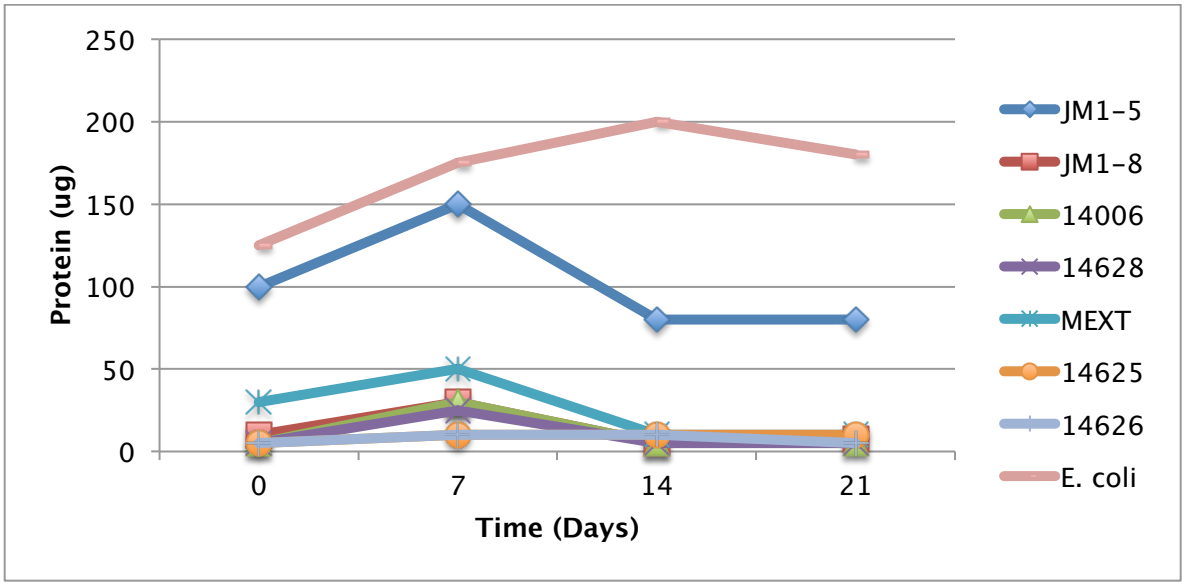

Fig. 5. Representative growth curve of protein content of *Methylobacterium* spp. and *E. coli* in water (Lowry Assay) at 30° C with aeration (120 rpm).

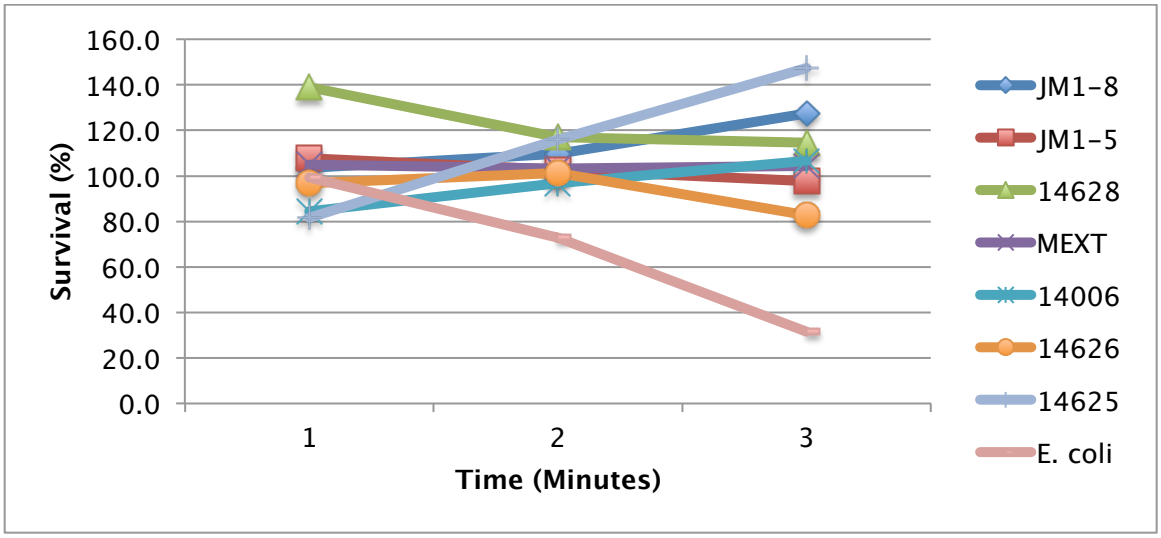

Fig. 6. Survival of *Methylobacterium* spp. and *E. coli* at 50°C.

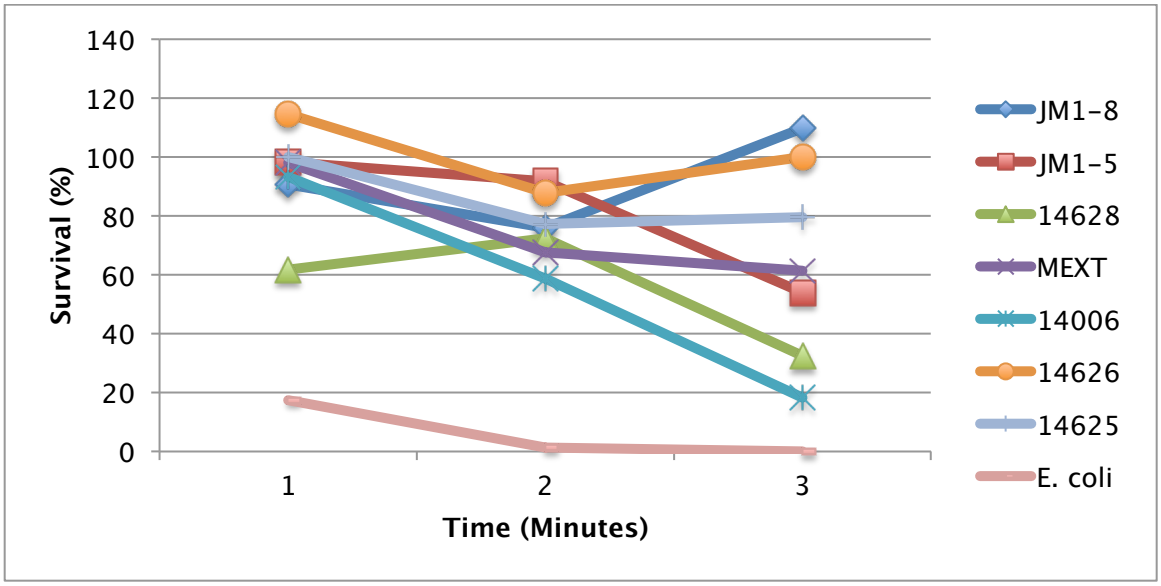

Fig. 7. Survival of *Methylobacterium* spp. and *E. coli* at 55°C.

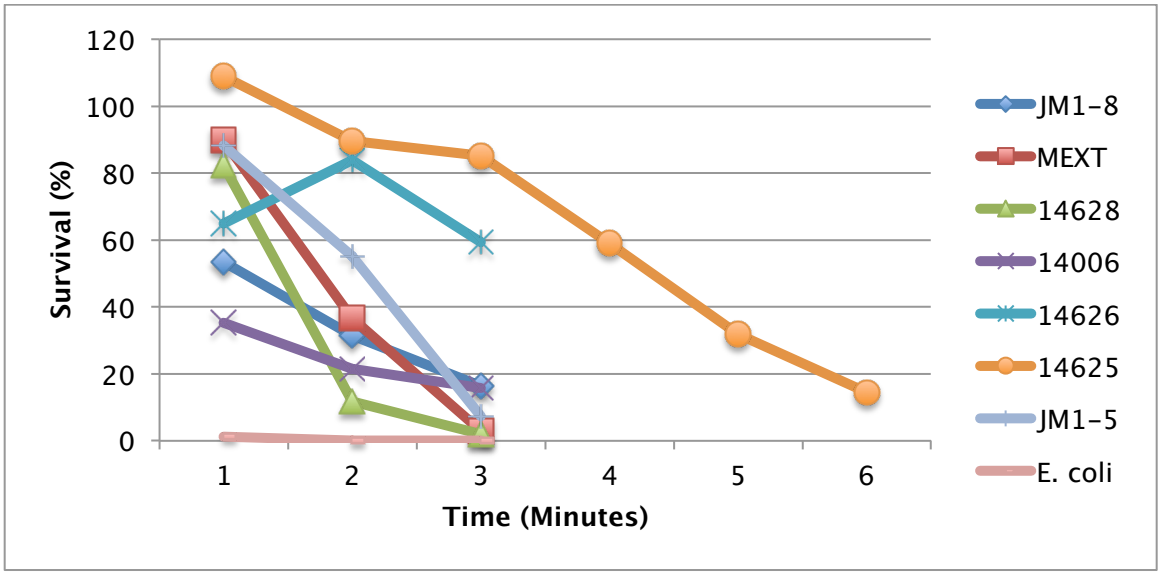

Fig. 8. Survival of *Methylobacterium* spp. and *E. coli* at 60°C.