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Development of a chemically-defined minimal medium for studies on growth and protein uptake of *Gemmata obscuriglobus*

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ABSTRACT

We experimentally determined minimal media requirements for *Gemmata obscuriglobus*, a Gram-negative Planctomycete bacteria with several unusual physiological features. We find that supplementing media with the usual vitamins solution does not improve viability, but does result in an increased growth rate in liquid cultures and a larger colony size on agar plates. By systematically including individual vitamins, or omitting individual vitamins, from media we find that the addition of only two vitamins, biotin and cyanocobalamin, are sufficient to restore colony growth to comparable rates as other commonly used media. Overall, our findings define minimal media requirements for the culturing of this low-nutrient organism. One of *G. obscuriglobus* unusual physiological features is the ability to internalize fully-folded proteins. Using fluorescence microscopy and flow cytometery we show that this physiological behavior is dependent on media state and composition. The percentage of cells exhibiting internalization of GFP when grown on a particular, solid minimal medium is far greater than cells grown in liquid medium of similar composition or other solid media with different compositions.

1. Introduction

Gemmata obscuriglobus is a member of the deeply divergent Planctomycetes-Verrucomicrobia-Chlamydiae (PVC) superphylum. Similar to other members of the PVC superphylum, *G. obscuriglobus* has a number of evolutionary interesting features distinct from typical Gram-negative bacteria which make it an emerging model for bacterial and evolutionary cell biology (van Niftrik and Devos, 2017). For *G. obscuriglobus*, these include the presence of a highly invaginated inner membrane (Sagulenko et al., 2014; Santarella-Mellwig et al., 2013), the ability to synthesize sterols (Pearson et al., 2003), a highly condensed nucleoid (Yee et al., 2012), the absence of common bacterial cytoskeletal proteins (Rivas-Marin et al., 2016b), and the ability to internalize fully-folded proteins (Lonhienne et al., 2010) and dextrans (Boedeker et al., 2017). Additionally, the *G. obscuriglobus* genome contains several predicted eukaryotic-like membrane-coat proteins (Santarella-Mellwig et al., 2010). Recently, membrane-associated protein complexes with several features similar to eukaryotic nuclear pore complexes were reported (Sagulenko et al., 2017). While much of what we know of the organism is based on direct interrogation of a wild type isolate, recent studies have demonstrated that certain genetic manipulations are possible and pave the way for more focused studies of the unique features of this organism (Rivas-Marin et al., 2016a) and other Planctomycetes (Jogler et al., 2011; Schreier et al., 2012).

Gemmata species are present in diverse environments throughout the world; including, freshwater and soil in Australia (Franzmann and Skerman, 1984; Wang et al., 2002), peat bogs in Russia (Kulichevskaya et al., 2017) and leakage water from a compost heap in Northern Germany (Ward et al., 1995). *Gemmata* species were also recently found in hospital and clinical settings (Aghnatios and Drancourt, 2015; Aghnatios and Drancourt, 2016). Further, based on 16S rDNA gene analysis of microbiomes, natural habitats of *Gemmata* also include a water spring in South Africa (Tekere et al., 2011), a geothermal steam vent in Hawaii (Benson et al., 2011) and surface sea water from the Pacific Ocean (Shu and Jiao, 2008). Analysis of 16S rDNA has additionally shown the presence of *Gemmata* in the gastrointestinal tract of carp (van Kessel et al., 2011) and humans (Cayrou et al., 2013). Given these diverse habitats, defining a standardized *G. obscuriglobus* medium is an essential step for comparative studies.

When culturing microorganisms, defining appropriate growth media is critical. Firstly, many organisms, including *G. obscuriglobus*, are sensitive to the concentration of nutrients in the media. *G. obscuriglobus* and many other Planctomycetes require very low concentrations of nutrients for culturing. If nutrient conditions are not consistent when culturing microorganisms, continued culturing can result in the accumulation of genetic mutations as the organism adapts

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to the growth conditions of a particular medium (Dragosits and Mattanovich, 2013; Winkler et al., 2014). This can become problematic when different research groups attempt to replicate work. Secondly, media state (liquid/planktonic growth versus solid/colonial growth) and media composition can affect microbial morphology and physiology (Azadmanesh et al., 2017). Further, the type and composition of the solid matrix can affect cells within the colony during colonial growth, as the colony microenvironment changes significantly as colony size increases (Jeanson and Thierry, 2015; Skandamis and Jeanson, 2015). Given the unique cell-biological features associated with *G. obscuriglobus*, understanding how media state and composition may affect these processes is important to our overall understanding of the organism. Lastly, the culturing conditions of an organism can affect the success rate of certain experimental manipulations (e.g., transformation efficiency of foreign DNA). Recent studies in *G. obscuriglobus* showed that genetic manipulations are possible (Rivas-Marin et al., 2016a) and optimizing growth conditions may significantly improve the success rate of these and enable the possibility of other genetic approaches. To date, little is known about which commonly used media components are essential for *G. obscuriglobus* viability and growth and how these affect its physiology.

Here, we define a *G. obscuriglobus* minimal medium by assessing the requirements of commonly used additives in *G. obscuriglobus* media. We compared viability and growth rates of G. *obscuriglobus* in liquid and solid versions of different media. We show that while a vitamin mixture is routinely added to media, these vitamins are not essential for viability but do aid in growth. Interestingly, supplementing with only two of the vitamins in the mixture is sufficient for growth comparable to the full vitamin mixture. We determine the relationship between optical density and cell density and show that the internalization of native proteins has a preference on media state and composition.

2. Materials and methods

2.1. Organism and media components

G. obscuriglobus was obtained from the Leibbniz-Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; DSM No. 5831). Media components were either purchased from Research Products International (agar, peptone, yeast extract, D-glucose and calcium carbonate) or Sigma (*N*-acetylglucosamine, individual vitamins and individual minerals). A standard 100 × vitamin solution (V) was prepared as follows: 4 mg biotin, 4 mg folic acid, 20 mg pyridoxine hydrochloride, 10 mg riboflavin, 10 mg thiamine HCl, 10 mg nicotinamide, 10 mg calcium pantothenate, 0.2 mg cyanocobalamin and 10 mg of p-aminobenzoic acid were dissolved in 1 L of water and then filter sterilized with a 0.22 μm filter. A standard 50 × mineral salt solution (Hutner's salts) was prepared as follows: 10 g nitriloacetic acid, 29.7 g $MgSO_4$ heptahydrate, 3.34 g $CaCl_2$ dihydrate, 12.7 mg Na_2MoO_4 dihydrate, 99 mg $FeSO_4$ heptahydrate and 50 mL of Metals 44 were brought up in 1 L of water, adjusted to pH 7.2 and filter sterilized. To control for potential pH fluctuations, a 50 × Buffer solution was prepared as follows: 64 g Na_2HPO_4, 15 g KH_2PO_4 and 5 g NH_4SO_4 were dissolved in 0.5 L of water, the pH was adjusted to 7.5 (if necessary) and the 50 × Buffer solution was autoclaved. The working concentration of each component in this Buffer solution, after appropriate dilution in media, is: 1 mM sodium phosphate, 4 mM potassium phosphate and 1.5 mM ammonium sulfate.

In addition to the above components, different media contained additional components as follows: (i) PYGV medium contained yeast extract (0.25 g/L), peptone (0.25 g/L) and glucose (0.2% final concentration), (ii) M1 contained $CaCO_3$ (5 g/L) and *N*-acetylglucosamine (0.2% final concentration) and (iii) minimal media (MM) contained either glucose (G) or *N*-acetylglucosamine (N) at a final concentration of 0.2%. Both glucose and *N*-acetylglucosamine were prepared as 10% stock solutions and filter sterilized.

During media preparation, water, mineral salts, yeast extract (for PYGV medium), peptone (for PYGV medium) and agar (for solid/colonial growth; 15 g/L) were combined and autoclaved. The 50 × Buffer solution, 100 × vitamin solution and the carbon source (10% glucose or 10% *N*-acetylglucosamine) were added aseptically in a biological cabinet after autoclaved media had cooled to 55 °C.

To examine vitamin requirements, separate 100 × vitamin solutions were prepared for each vitamin routinely added to *G. obscuriglobus* media. These solutions were then individually pipetted into minimal media with glucose (MM-G) to prepare: (i) single vitamin add-back, (ii) multiple vitamin add-back and (iii) drop-out vitamin agar plates. For select vitamin composition plates colony diameters were determined by selecting a region of the imaged agar plate that contained at least 20 colonies. The diameter of each colony was then determined using Image J.

Viability tests on solid media. Log-phase cells grown in liquid PYGV were collected, washed three times in sterile water and sonicated (Branson Digital Sonifier 250) at 10% maximum intensity for 10 s to break clumps of cells into individual cells. Cells were then diluted using serial 10-fold dilutions in sterile water and a similar volume of the dilution was plated on each different media. Four plates of each type were assayed.

2.2. Growth curves, doubling times and maximum density

Log-phase cells were collected and washed twice in sterile water to remove any trace of previous growth media. Cells were resuspended in 1 mL of sterile water, sonicated briefly to disperse, and then an equal volume of the cell suspension was pipetted into individual 250 mL erlenmeyer flasks containing 50 mL of media. An initial OD_{600} measurement was taken to ensure each initially inoculated culture had an OD_{600} of < 0.025 (Spectronic 200). During growth, flasks were incubated at 28 °C and shaken at 150 rpm (Excella E24, New Brunswick). For any individual optical density measurement, the flasks were opened in a biological cabinet and any adhered clumps of cells were displaced with a pipet tip. The culture media was transferred to a 50 mL conical tube and then sonicated at 15% of maximum intensity for up to 25 s until visible clumps were dispersed. The culture was then transferred back to its original 250 mL erlenmeyer flask and a 1 mL sample was removed. Each 1 mL sample was then sonicated at 10% for 10 s to fully disperse any nonvisible clumps prior to the optical density measurement.

2.3. Determining the relationship between optical density and cell density

Log-phase cells were harvested, washed twice in water and then sonicated at 10% for 10 s to disperse. Cell suspensions were diluted in water to an OD_{600} of 0.1 and 1.0. Four 10-fold serial dilutions were performed for each OD_{600} solution and then 100 μl of each diluted cell suspension was plated on four minimal media plates containing the vitamins solution and *N*-acetyl glucosamine (MM-N-V). After 12 days colonies were counted to determine the cell density of the original culture.

2.4. Flow cytometry and microscopy

Log-phase cells were harvested, washed twice in water and then sonicated at 10% for 10 s to disperse. The cell density was determined based on OD_{600} as described above. Dilutions in water were performed and a volume containing ~200 cells was spread on four different agar plates. Plates were incubated at 28 °C for 13 days and then 2 mL of incubation buffer (10 mM Tris-Cl pH 7.5) was added to each plate. A cell scraper was used to dislodge colonies which were then transferred to a 1.5 mL microfuge tube. Each sample was sonicated at 10% for 10 s to disperse cells. A volume containing a comparable amount of cells was transferred to a fresh microfuge tube. The cells were pelleted and washed in 1 mL of incubation buffer. After pelleting the cells again, the