1   ANALYSIS OF MICROBIAL COMMUNITY DURING BIOFILM DEVELOPMENT IN AN

2   ANAEROBIC WASTEWATER TREATMENT REACTOR

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9   Running title: Biodiversity and structure of anaerobic biofilms

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ABSTRACT

The formation, structure and biodiversity of a multispecies anaerobic biofilm inside an Upflow Anaerobic Sludge Bed (UASB) reactor fed with brewery wastewater was examined using complementary microbial ecology methods such us Fluorescence in situ Hybridization, Denaturing Gradient Gel Electrophoresis and cloning. The biofilm development can be roughly divided into three stages: an initial attachment phase (0-36 hours) characterised by random adhesion of the cells to the surface; a consolidation phase (from 36 hours to 2 weeks) defined by the appearance of microcolonies; and maturation phase (from 2 weeks to 2 months). During the consolidation period proteobacteria with broad metabolic capabilities, mainly represented by members of alpha-*Proteobacteria* class (*Oleomonas, Azospirillum*), predominated. Beta-, gamma-, delta- (both syntrophobacteria and sulfate-reducing bacteria) and epsilon- (*Arcobacter* sp.) *Proteobacteria* were also noticeable. *Archaea* first appeared during the consolidation period. A *Methanospirillum*-like methanogen was detected after 36 hours and this was followed by the detection of *Methanosarcina,* after four days of biofilm development. The mature biofilm displayed a hill and valley topography with cells embedded in a matrix of exopolymers where the spatial distribution of the microorganisms became well-established. Compared to the earlier phases the biodiversity had greatly increased. Although alpha-*Proteobacteria* remained as predominant, members of the phyla *Firmicutes*, *Bacteroidete* and *Thermotogae* were also detected. Within the domain *Archaea*, the acetoclastic methanogen *Methanosaeta concilii* become dominant. This study provides insights on the trophic web and the shifts in population during biofilm development in an UASB reactor.

Introduction

Biofilms are structured microbial communities made up of groups of cells suspended in a self-produced hydrated polymeric matrix of variable density and permeated by channels [13, 14]. In most natural and engineered environments, a multispecies microbial community is the prevailing life form [58]. Although many species are implicated, biofilm development has been mainly studied using systems composed by one or two species which have led the formulation of a development model, in which the formation of biofilms occurs in multiple steps: (i) approach of microbes to a surface, (ii) initial attachment mainly governed by van der Waals and electrostatic forces; (iii) formation of microcolonies; and (iv) biofilm maturation in which microcolonies are stabilized by increased cell-surface adhesion due to the accumulation of extracellular polymer substance (EPS) [21, 51, 57, 58]. Mature biofilms show a complex structure (the mushroom or the tulip model) with extensive presence of extracellular polymeric substances (EPS) full of channels through which a liquid phase is free to move [34, 60].

Biofilms play an important role in wastewater treatment as they form the basis of diverse aerobic and anaerobic reactors (trickling filters, rotating biological contactors...) and are characterized by their feasibility and efficiency. Pollutants are anaerobically processed and eliminated by means of the complex food chain established within the biofilm [30]. Consequently, the process efficiency is the result of the biofilm microbial diversity. Nevertheless, studies of biofilm growth in wastewater treatment systems have focused mainly on the influence of operational parameters, physicochemical factors and the properties of the supports on biofilm development [26, 56]. On the other hand, activity, adhesion, biomass and other conventional parameters have been measured to assess the microbial community [22, 29, 52]. Reports based on microbial ecology techniques are scarce [8, 19, 49].

Conventional cultivation-dependent microbiological techniques fail to give an indication of biodiversity (about 99% of the bacterial cells in biofilms can not be cultured on standard media [60]) or the architecture of a biofilm. During the last 15 years, molecular techniques based on

66 16S rRNA/rDNA have been successfully applied to microbial ecology research. Denaturing

67 Gradient Gel Electrophoresis (DGGE) [39, 40], Fluorescence in situ Hybridization (FISH) [7, 50,

68 54], together with molecular cloning, have opened up new perspectives for the study of microbial

69 ecosystems [5]. FISH combined with Confocal Laser Scanning Microscopy (CLSM) makes it

70 possible to study biofilms in natural [34], industrial [35] and engineered [15] environments

71 without destroying their critical architecture. An excellent review of the application of molecular

72 ecology techniques to wastewater treatment systems has recently been published [42].

73 The aim of this study was to investigate in detail the formation of a multispecies

74 anaerobic biofilm inside a reactor. The Upflow Anaerobic Sludge Bed (UASB) reactor was

75 chosen as it is widely used throughout the world. Scanning electron microscopy (SEM) and

76 CLSM combined with FISH were used to monitor the development and structure of the biofilm.

77 Biodiversity was evaluated with three rRNA-based complementary methods: FISH, DGGE and

78 cloning and sequencing of 16S rDNA.

79

80   ***Methods***

81   Experimental set-up: A laboratory scale UASB reactor (0.9 L) was operated for five

82   months. Intact and crushed granular sludge (2.5 g VSS L-1) from a full-scale UASB reactor

83   treating brewery wastewater (MAHOU, Guadalajara, Spain) was used as inoculum. The reactor

84   was fed with industrial wastewater from a brewery (4000 mg L-1 COD; 2400 mg l-1 BOD; 1.3 g

85   L-1 TSS; 100 mg l-1 TNK; 15 mg L-1 NH4+, 15 mg L-1 P) pH 7.0, and operated with a hydraulic

86   retention time of 24 hours at 30ºC. The typical composition of brewery wastewater is (in mM per

87   g of COD): 1.6 acetate, 1 propionate, 6-8 ethanol, and less than 0.2 of butyrate, lactate,

88   succinate and glucose. The VFA in the effluent after UASB treatment is around 10% of their

89   content in the effluent (23, 61).

90   Once the reactor reached steady state (organic loading rate: 1 g-COD L^{-1} d^{-1}; sludge

91   loading rate 0.35 g-COD g-VSS^{-1} d^{-1}; efficiency of COD removal: 85-90%, no volatile fatty acids

92   accumulated), biofilms were allowed to develop on vertical glass slides submerged in the

93   reactor.

94

95   *Growth curve:* Once the reactor reached steady state, a growth curve during the 10 first

96   days of biofilm development was obtained to provide insights on the initial period of biofilm

97   development. At the beginning of the experiment, several Falcon 3911 MicroTest III assay plates

98   were vertically placed inside the reactor. Subsequently three of them were removed at each

99   sampling time. The biofilm formation was determined by optical density at 560 nm as previously

100   described [25]

101

102   *Fluorescent in situ hybridization (FISH):* Supports consisting glass slides with 10-wells

103   (75X25 mm, with wells of 5 mm diameter) (Superior, Marienfeld, Germany) were hung vertically

104   by a nylon line at a distance of a few centimetres over the sludge bed. All the slides were

105   introduced at the start of the biofilm formation experiment and they were removed at different

106 times (1, 2, 4, 12, 24 and 36 hours, and 2, 4, 8, 18, 25, 30 and 60 days) for *in situ* hybridization.

107 Samples were rinsed with filtered water to remove loosely attached planktonic forms and

108 immediately fixed with ethanol for Gram-positive bacteria detection or with 4% paraformaldehyde

109 in phosphate buffered saline solution (PBS) during 4 hours at 4 ºC for Gram-negative bacteria.

110 The samples were then washed in PBS, and stored in PBS: Ethanol (1:1) solution at –20ºC. All

111 samples were further dehydrated by immersion in 50%, 80% and 100% ethanol solutions for

112 three minutes each time. Hybridization was performed following the protocol described

113 elsewhere [6, 32]. The probes used in this work are listed in Table 1. The NON338 probe was

114 used as negative control. The total cells present in the samples were determined by direct

115 counting of 4',6'-diamin phenylindol (DAPI, 1mg/ml) stained cells when possible. Samples were

116 examined under a Zeiss Axiovert 200 microscope. Structural and morphological studies on intact

117 biofilms were carried out with a Confocal Radiance 2000 scanning system coupled to a Zeiss

118 Axiovert S100 TV confocal laser-scanning microscope.

119

120 *Scanning electron microscopy (SEM):* Samples for scanning electron microscopy were

121 prepared as follows: biofilms were grown on glass slides (Φ 0.5 cm, FEDELCO, ER-308). The

122 location of the slides and sampling protocol were similar to FISH procedure. Once a sample was

123 taken out from the reactor it was fixed with glutaraldehyde (2.5% v/v) in 0.2 M sodium cacodylate

124 buffer (pH 7.1) and dehydrated with graded ethanol solutions (10%, 30%, 50%, 70%, 90% and

125 100% ethanol). The samples were dehydrated by the critical point drying method and coated

126 with gold. Micrographs were taken with a Phillips XL30 EDAX DX4i SEM.

127

128 *DNA extraction and 16S rRNA amplification:* Two- and 60-day old biofilms were detached

129 from the slides using Triton X-100 (0.25%) solution. DNA was extracted using FastDNA kit for

130 soils BIO101 according to the manufacturer's protocol. The 16S rRNA genes from mixed

131 microbial DNA were amplified by PCR. To obtain almost complete 16S rRNA gene, two

132 oligonucleotide primer pairs were used: 27F and 1492R (annealing T: 56ºC) for the domain

133 *Bacteria* [28] and 25F and 1492R (annealing T: 52 ºC) for the domain *Archaea* [28]. For

134 subsequent DGGE analysis, a fragment of DNA was amplified with two primer pairs: 341F-GC

135 and 907R (annealing T: 52 ºC) for the domain *Bacteria* [11] and 622F-GC and 1492R (annealing

136 T: 42ºC) for the domain *Archaea* [12] (GC clamp: 5'-CGC CCG CCG CGC CCC GCG CCC GTC

137 CCG CCC CCG CCC-3'). PCR reactions were performed with the following thermocycler

138 program: pre-denaturation at 94 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 1 min,

139 annealing at corresponding temperature for 1 min, and elongation at 72 ºC for 3 min (1 min for

140 DGGE use); and post-elongation at 72 ºC for 10 min.

141

142   *Denaturing Gradient Gel Electrophoresis (DGGE):* The PCR products of the same length

143 were separated by DGGE [36], which was performed according to the Dcode-System (BioRad,

144 Germany). Polyacrylamide gels 6% (wt/vol, acrylamide-bisacrylamide 37.5:1) were prepared

145 with denaturing gradients ranging from 30% to 60% (in which 100% denaturant contained 7M

146 urea and 40% v/v formamide) and were run at 60ºC and 80V for 15 h. Bands detected by

147 fluorescence using a UV transilluminator were excised and re-amplified for sequencing.

148

149   *Clone libraries, ARDRA analysis and sequencing***:** For a further comparison between

150 young and mature biofilm communities, two- and 60-day old biofilms were analyzed by clone

151 libraries. The 16S rRNA gene amplificates (length 1465 and1467 bp for *Bacteria* and *Archaea*,

152 respectively) were cloned using TOPO Cloning Kit (Invitrogen Corporation, San Diego,

153 California) and then transformed into competent *E. coli* cells. Plasmid inserts were screened by

154 Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the enzyme *Sau*3AI (BioLabs

155 Inc., New England). Fragments were separated by 2% (w/v) agarose (Pronadisa, Madrid) gel

156 electrophoresis and visualized by ethidium bromide staining. Clones were grouped according to

157 their restriction patterns defining different Operational Taxonomic Units (OTUs). Subsequently,

158   two clones of each OTU were amplified by PCR using the M13 primer set (Invitrogen).

159   Automated DNA sequencing was performed with an ABI model 377 sequencer (Applied

160   Biosystems).

161

162   *Sequence analysis:* All sequences obtained in this work were compared with the

163   databases by using Basic Local Alignment Search Tool (BLAST) [1] to identify the closest

164   sequence. Sequence data were aligned and analyzed with the ARB program package [31].

165   Parsimony was used to construct phylogenetic trees.

166

167   *Nucleotide sequence accession numbers:* The sequences obtained in this study have

168   been deposited in the GenBank database under accession numbers AY692039 to AY692074.

169

170   **Results**

171   Three complementary approaches were used to monitor the kinetics of biofilm formation,

172   their phylogenetic diversity, and the spatial distribution of the populations: SEM, FISH and DNA

173   sequencing after direct cloning of the 16S rRNA gene as well as resolution by DGGE of

174   amplified fragments of this gene.

175

176   *SEM:* Microscopic examination showed an erratic colonization sequence during the first

177   few hours of biofilm formation. The cell density on the surface changed continuously without a

178   trend and the microorganisms were widely spaced on the surface of the support (Fig. 1, 3h-24h).

179   After two days, the growth tended to stabilize, the production of a matrix of exopolymers began

180   and the first microcolonies could be observed (Fig 1, 4d). The microcolonies then progressively

181   spread to form a biofilm (Fig. 1, 8d-14d). SEM images showed that the mature biofilm consisted

182   of both densely populated and less dense areas during the growth period (Fig. 1, 60d), results

183   that were similar to those previously reported [52].

184   These data were corroborated by monitoring the adhered cells during the first 10 days

185   (Fig. 2). The growth curve showed that adhesion behaviour was random during the first 36

186   hours, corresponding to the period before growth of microcolonies was observed by SEM.

187   Subsequently, a constant rate of adhesion was observed which tended to stabilize in the final

188   stage of the studied period.

189   This microscopic analysis also revealed extensive morphological diversity including

190   different kinds of spirillum, straight and curved rods and small coccoid cells (Fig. 1, 4d detail).

191   Two methanogens could be identified due to their particular shape: *Methanospirillum*-like cells

192   appeared after one day of growth whereas *Methanosaeta*-like cells were clearly identified after

193   four days.

194

195   *FISH*. Based on SEM and growth curve results, to determine the spatial distribution of

196   the microorganisms the development of the biofilm was divided roughly into three stages: initial

197   attachment (0-36 hours), consolidation (from 36 hours to 2 weeks) and maturation (from 2 weeks

198   to 2 months).

199   Different groups belonging to the domain *Bacteria* (α, β, γ-*Proteobacteria*,

200   *Syntrophobacter*, sulfate-reducing bacteria, *Bacteroides* and Gram-positive bacteria) were

201   analyzed by FISH and quantified for each stage. During initial attachment, the number of

202   microorganisms reached 10^6 cells/cm^2 (total DAPI stained cells), with a percentage of hybridized

203   cells between 50-70% (probes EUB338 plus ARC915 versus DAPI stained cells). Of those, 85-

204   95% corresponded to the domain *Bacteria*. During this period, α–*Proteobacteria* (35-55% of

205   detected bacterial cells, Fig. 3A) was the most representative group. β- and γ-*Proteobacteria* (5-

206   15% each) were also detected, always associated to colonies of α–*Proteobacteria* (Fig. 3B and

207   3C). The presence of *Syntrophobacter* (5-10%) and sulfate-reducing bacteria (5-8% each) was

208   also noticeable (Fig. 3D and 3E). The presence of archaea could be detected after only 36

209   hours, although these microorganisms were always scarce in comparison to bacteria. These

cells had the form of long bowed rods that formed small *Methanospirillum*-like filaments, which hybridized with the MG1200 (*Methanomicrobiales*) probe (Fig. 3F), confirming the results of SEM.

The consolidation stage of the biofilm was marked by the formation of colonies that began to interconnect but still appeared as independent entities (Fig. 3H). β-*Proteobacteria* were specifically located on the edges of the colonies. These colonies were mainly made up of α–*Proteobacteria* together with the other groups detected: γ-*Proteobacteria*, *Syntrophobacter* and sulfate-reducing bacteria which had not a tendency to occupy specific positions within the colonies. Archaea were basically represented by *Methanospirillum*-like cells (probe MG1200: *Methanomicrobiales*), as large rods and small filaments scattered throughout the bacterial colonies forming a network. *Methanosarcina* genus (probe MS1414) were observed after four days of growth, making up dense packed within the bacterial colonies (Fig. 3H and 3I), although in smaller amounts than *Methanospirillum*-like cells. In addition to this *Methanobacteriales* group (probe MEB859, Fig. 3G) was found in very small quantities. The presence of *Methanosaeta* was not detected.

Mature biofilm covered almost the entire surface of the support, with cells embedded in an exopolymeric matrix. 3D reconstructions based on biofilm sections obtained using confocal microscopy revealed their spatial distribution. The groups detected were not very different from previous stages. The main body was formed mostly of bacteria, with the archaeal cells clearly defined within it (Fig. 3J). They were basically α–*Proteobacteria*, with β-*Proteobacteria* always located on the edge of the biofilm and γ- and δ-*Proteobacteria* scattered within it. *Bacteroides* and Gram-positives were also detected at this stage. Archaea appeared as individual cells or short filaments that spread forming a network throughout the biofilm; however, it was mainly composed of *Methanosaeta* filaments. It appears that *Methanospirillum* was displaced by *Methanosaeta*. As noted before, densely packed groups of very bright *Methanosarcina* were normally located inside the colonies (Fig. 3J).

236

237   *DGGE profiles:* Changes in microbial diversity during biofilm formation were studied

238   using DGGE profiling. Figure 4 shows the band patterns resolved with DGGE after partial 16S

239   rRNA gene amplification using specific primers for the domains *Archaea* and *Bacteria.* Archaea

240   were not detected during the first 24 hours of development. Since then and during the rest of the

241   studied period, a stable pattern was maintained for this domain (Fig. 4A). The bacterial patterns

242   were highly variable during the first two days reflecting large changes in diversity in this domain

243   at the beginning of the growth (Fig. 4B). After that, the bacterial diversity remained fairly

244   constant.

245   All visible bands were excised from the DGGE fingerprints, re-amplified, purified and

246   sequenced. A total of eleven bacterial bands and three archaeal bands yielded sequences that

247   were analyzed using the BLAST program. The taxonomic affiliations of the 16S rRNA partial

248   sequences are shown in Table 2. All sequenced bacterial bands belonged to *Proteobacteria*,

249   *Firmicutes*, *Actinobacteria* and *Bacteroidetes* phyla. Some of them were present only during the

250   initial period: B5 (*Flavobacteriaceae*), B7 (*Hydrogenophilaceae*) and B11 (*Nocardiaceae*). Bands

251   B2 (*Acetobacteraceae*), B3 (*Rhodocyclaceae*), B9 (*Syntrophomonadaceae*) and B10

252   (*Campylobacteraceae*) were also detected in the consolidation period. The remaining bands

253   were found in the consolidation and mature stage: B4 (*Comamonadaceae*), B1

254   (*Syntrophobacteraceae*), B6 and B8 (order *Clostridiales*). In the case of the domain *Archaea*,

255   the bands belonged to *Methanosarcinales* (A1 and A2) and *Methanomicrobiales* orders from the

256   *Methanomicrobia* class.

257

258   *Clone libraries.* Two specific periods during the biofilm formation were studied by cloning: after

259   two days, at the beginning of the consolidation stage when the microorganisms can be

260   considered to be specifically attached to the support, and after two months, when the biofilm is

261   mature. Almost complete 16S rRNA sequences were amplified from total DNA extracted from

262   the biofilm using universal primers for the bacterial and archaeal domains. ARDRA analysis of

263   the 90 (bacterial Domain) and 85 (archaeal Domain) clones for the two day-old biofilm and 77

264   (bacterial Domain) and 96 (archaeal Domain) clones for the sixty days-old biofilm allowed us to

265   group them and to define different Operational Taxonomic Units (OTUs) which were formed from

266   clones with the same restriction band pattern (Table 2).

267   After two days of biofilm formation, 5 different OTUs were detected for *Bacteria* domain

268   and 1 for *Archaea* domain, while after 60 days, 6 and 3 OTUs were found, respectively. The 16S

269   rRNA gene sequences were affiliated with mainly uncultured bacteria from different habitats and

270   only remotely related to known bacterial species (Table 3).

271   Taxonomic similarities and phylogenetic relationships showed that most of the bacteria

272   belonged to the phylum *Proteobacteria* (Table 3, Fig. 6): two OTUs could be included in the α

273   class, one was related to *Acetobacteraceae* (Cb1) and the other to *Rhodospirillaceae* (Cb2);

274   another one in the β class was related to *Rhodocyclaceae* (Cb3); one OTU in the δ class was

275   related to sulfate-reducing bacteria (Cb4); and one OTU in the ε class was related to the

276   *Arcobacter* genus (Cb5). Many of the remaining sequences were members of Gram-positive

277   bacteria: three OTUs belonging to the *Clostridiales* (Cb6, Cb7 and Cb8). Members of the

278   *Flavobacterium-Cytophaga* group (Cb9) and *Thermotogae phylum* (Cb10) were also identified.

279   Three methanobacteria were identified inside the domain *Archaea* (Table 3, Fig. 6). Two

280   could be included in the *Methanosarcinales* order: *Methanosaeta* (Ca1 and Ca2) and

281   *Methanosarcina* genera (Ca3). The other sequences belonged to the *Methanomicrobiales* order,

282   probably to the *Methanospirillum* genera (Ca4).

283

284   ***Discussion***

285   The main phases described for single-species biofilm formation were also observed

286   during the development of a multispecies anaerobic biofilm. It is known that microbial cells might

287   be affected by the adsorption-desorption processes caused by the electrostatic and shearing

288   forces that take place between a surface charges and charges on the bacterial surface [30]. This

289   could explain the behavior observed during the initial stage of development in which the

290   microbial adhesion to the support was a random process, as indicated by SEM (Fig. 1) and

291   supported by optical density measurements (Fig. 2), FISH (Fig. 3 A, to F) and the band patterns

292   generated with DGGE (Fig. 4). In this study, the influence of physicochemical and operational

293   conditions on the growth trend was minimized since the reactor was operated at steady-state

294   throughout the entire experiment and the environmental conditions were expected to be fairly

295   constant. It should be noted that neither archaea nor Gram-positive bacteria appeared during the

296   first 36 hours. It is plausible that the chemical characteristics of their external envelopes, and the

297   lower number of fimbriae of Gram-positive with respect to the Gram-negative bacteria could be

298   critical factors during the initial colonization and could be implicated in their delay in colonizing

299   the surface.

300   According to the accepted model, once beyond this initial stage, the influence of the

301   stochastic processes and physicochemical conditions waned and the nature of the bacteria-

302   surface interaction could be determined by the attachment of bacterial fimbriae [18] and by the

303   excretion of an exopolymeric matrix [18, 53]. Such a matrix was observed after 1-2 days and

304   marked the beginning of the consolidation stage. From this point on the physical structure of

305   biofilm evolved, changing from isolated microcolonies to a mature stage in which the cells were

306   embedded in the matrix, adopting rounded shapes stabilized by EPS (Fig. 1, 60th day). In

307   addition, the bacterial diversity of the biofilm, confirmed by FISH and DGGE band patterns,

308   remained fairly constant in comparison with the initial stage. This implies that the basis of the

309   microbial community and therefore the main pathways of the trophic web were formed during the

310   consolidation stage.

311   Once irreversible adhesions to the surface had occurred and the microcolonies started to

312   form, the first community mainly comprised α-*Proteobacteria* according to FISH. *Oleomonas*

313   *sagaranensis* (Cb1, B2) is able to form aggregates [27] producing some EPS, so facilitating its

adhesion to the surface. This characteristic and its high metabolic versatility [27]—are advantages that make this microorganism a pioneer in the colonization of the surface. At the same time *Azospirillum* (Cb2), which also has a broad metabolic capability, appeared. These two α-*Proteobacteria* could participate in the breakdown of organic polymers.

In general, the microorganisms that initiated the formation of the biofilm have a broad metabolic flexibility that facilitated invasion of the surface. The intermediates produced after the lysis of macromolecules could be degraded by proteobacteria: *Oleomonas* (Cb1, B2), *Azonexus* (B3), *Azospirillum* (Cb2), a γ-*Proteobacteria* (enterobacteria were detected by FISH), *Arcobacter* (Cb5, B10), or sulfate reducers (FISH data) [10], or by the firmicutes *Thermovirga* (B9). *Syntrophobacteria* (B1) was a expected member of the microbial community because it is implicated in the degradation of fatty acids and others intermediate products during fermentation to acetate and hydrogen. However *Syntrophobacteria* appeared in low proportions (FISH data) in the first stages. We must emphasize that *Azonexus* is able to grow under microaerophilic conditions, in line with the observation that the β-*Proteobacteria* were always located in the exterior zones of the microcolonies where they had access to traces of oxygen present in the reactor. The presence of a microorganism related to the class *Flavobacteria* (Cb9, B5) seems odd because they are typical of aerobes environments. Nonetheless, sequences similar to *Cytophaga* have been found in anaerobic environments such as sulfate-reducing enrichment cultures [41] and in granular sludge [12]. It has been suggested that the *Cytophaga-Flavobacterium* cluster play an important role in the degradation of complex organic matter in anaerobic marine sediments [45]. This role might be extended to all anaerobic environments.

According to the FISH results, major microbial groups within the biofilm maintained the same architecture during biofilm maturation. Nevertheless, with time, new members appeared thus increasing the complexity of the ecosystem. In the maturation stage, some new sequences were retrieved (Cb6, Cb7, Cb8, B6, B8). Based on the closest genus described (*Aminobacterium*, *Catabacter* and *Clostridium*), these sequences belong to the Gram-positive

340   class *Clostridia*, which together with *Bacteroidetes* members (FISH data) could be involved not

341   only in hydrolysis and but also in fermentation steps. Some closely related sequences have

342   been found in methanogenic sludge (AF323770, not published) and granular sludge [16],

343   suggesting that these microorganisms are extremely widespread in anaerobic systems and that

344   they must have an important role in anaerobic digestion Even thought, an interesting observation

345   was that microorganisms from the phylum *Thermotogae* were detected in the mature biofilm.

346   Mesophilic *Thermotogae* have been isolated from anaerobic ecosystems [37, 59] and also

347   several similar sequences have been reported in an anaerobic reactor (AB195923, not

348   published).

349   With regard to the domain *Archaea*, all microorganisms identified in the biofilm

350   corresponded to methanogenic archaea. They were absent until 36 hours of biofilm

351   development. During the consolidation stage, the number of methanogens grew in parallel with

352   increase in microcolony size, mainly represented by *Methanospirillum* sp., a hydrogenotrophic

353   methanogen that began to form an increasingly complex network within the bacterial colonies

354   (FISH data, A3). Because of the energy released, the biomass yield is lower for acetate-

355   consuming methanobacteria than for hydrogen-consumers favoring initial predominance

356   of.*Methanospirillum* over *Methanosarcina* and *Methanosaeta*. The increased number of

357   fermentative microorganisms implied a higher availability of acetate for acetoclastic

358   methanogens, which appeared later than hydrogenotrophic ones. The first acetoclastic

359   methanogen to appear was the genus *Methanosarcina* (Ca3,A1), which was found in the biofilm

360   as dense, bright packs. In the case of granular sludge, it has been reported [20] that substrates

361   that are easily fermented—such as the brewery wastewater used to feed our reactor—are

362   degraded mainly on the surface of granular sludge, while the intermediate products are

363   converted into acetate in the middle layers. This would explain the position in the biofilm of

364   *Methanosarcina*, which has a high Ks (4.02 mmoles L^{-1}) for acetate. DGGE and clone libraries

365   showed the presence of *Methanosaeta* genus (Ca1, Ca2, A2) in the former steps but it was

366 impossible to detect by FISH. This implies that few of these microorganisms were present (less

367 than 1%) but their metabolic role was negligible.

368 In the evolution to a mature biofilm, *Methanospirillum hungateii*, was displaced over time

369 by *Methanosaeta concilii*. This archaea is the dominant methanogen in the anaerobic granular

370 sludge reactors [2, 47, 48]. which was used to inoculate our reactor. *M. concilii* is an exclusively

371 acetoclastic organism with a slow growth rate (Vmax = 0.11 d^{-1}). This could explain its early

372 detection by means of PCR-based technologies whereas with FISH it only appeared in

373 abundance in the mature stages of the biofilm. It is well known [33] that the complexity of the

374 trophic web of an engineered ecosystem leads to the prevalence of acetate over hydrogen as

375 the final product of the fermentation, resulting in overgrowth of *Methanosaeta* compared to

376 *Methanospirillum*.

377 The techniques based on the 16S rRNA gene allowed us to describe the development

378 and diversity in the microbial community of an anaerobic biofilm. The insight gained on the

379 formation of biofilms during anaerobic wastewater treatment can be applied to control anaerobic

380 bioreactor technology for a wide range of applications.

381

386

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Table 1.
rDNA oligonucleotide probes used in this study

Probe	Specificity	Probe sequence (5'-3')	Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	3
ARC915	*Achaea*	GTGCTCCCCCGCCAATTCCT	50
NON338	Negative control	ACTCCTACGGGAGGCAGC	55
ALF968	α-*Proteobacteria*	GGTAAGGTTCTGCGCGTT	42
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	32
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	32
SRB385	Sulfate-reducing bacteria	CGGCGTCGCTGCGTCAGG	3
DSS658	*Desulfosarcina, Desulfococcus*	TCCACTTCCCTCTCCCAT	33
DSV698	*Desulfovibrio* sp.	GTTCCTCCAGATATCTACGG	33
SYN835	*Syntrophobacter*	GCAGGAATGAGTACCCGC	24
BAC1080	*Bacteroides*	GCACTTAAGCCGACACCT	17
LGC354	Gram-positive bacteria with low G+C content	TGGAAGATTCCCTACTGC	36
HGC69A	Gram-positive bacteria with high G+C content	TATAGTTACCACCGCGT	44
MEB859	*Methanobacteriales* (except M*ethanothermaceae*)	GGACTTAACAGCTTCCCT	9
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	43
MG1200	*Methanomicrobiales*	CGGATAATTCGGGGCATGCTG	43
MSSH859	*Methanosarcinales*	CTCACCCATACCTCACTCGGG	9
MS1414	*Methanosarcinales* (except *Methanosaeta*)	CTCACCCATACCTCACTCGGG	43
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	43

Table 2.
Sequences from NCBI database with the highest similarity to each band. For uncultured microorganisms a brief description of the environment of origin is given.

Band[a]	P[b]	Closest relative (Accession number)	S[c] (%)
B1	c,m	*Syntrophobacter fumaroxidans* (X82874)	92
B2	i,c	Clone I79 (AY692039), this work	99
		Oleomonas sagaranensis (D45202)	97
B3	i,c	Uncultured IMCC1716 (DQ664239), freshwater bacteria	99
		Azonexus fungiphilum (AJ630292)	97
B4	c,m	Uncultured clone SsB12 (AB291302), UASB reactor	92
		Hydrogenophaga defluvii (AJ585993)	90
B5	i	Clone I73 (AY692051), this work	99
		Flavobacterium frigoris (AJ557887)	93
B6	c,m	Clone M77 (AY692049), this work	99
		Catabacter hongkongensis (AY574991)	91
B7	i	*Dechloromonas aromatica* (CP000089)	98
B8	c,m	Clone M78 (AY692048), this work	99
		Aminobacterium colombiense (AF069287)	90
B9	i,c	Clone BA128 (AF323770), methanogenic consortium	99
		Thermovirga lienii (DQ071273)	96
B10	i,c	Clon I92 (AY692047) this work	99
		Arcobacter sp. R-28314 (AM084114)	98
B11	i	*Rhodococcus opacus* (AY027583)	99
A1	c,m	Uncultured CLONG74 (DQ478747), anaerobic sludge	98
		Metanosarcina mazei (AY196685)	94
A2	c,m	Clone M1 (AY692055), this work	98
		Methanosaeta concilli (X51423)	96
A3	c,m	Uncultured methanospirillum (DQ478753), UASB reactor	100
		Methanoculeus palmolei (Y16382)	95

563   [a] A: *Archaea*, B: *Bacteria*
564   [b] Period, i: initial, c: consolidation, m: mature
565   [c] Similarity
566

567

Table 3.

Abundance of operational taxonomic units (OTUs) determined with each clone library and sequences from NCBI database with the highest similarity to each OTU. For uncultured microorganisms, a brief description of the environment of origin is given.

OTU[a]	P[b]	Abundance (%)	Closest relative (accession number)	S[c] (%)
			α-*Proteobacteria*	
Cb1	c	16.7	*Oleomonas sagaranensis* (D45202)	97
Cb2	c	5.6	*Azospirillum brasilense* (X79733)	96
			β-Proteobacteria	
Cb3	c	27.8	*Dechloromonas* sp. LT-1 (AY124797)	98
			δ-Proteobacteria	
Cb4	m	36.3	Uncultured bacterium PL-37B10 (AY570628), oil reservoir.	99
			ε-Proteobacteria	
Cb5	c	38.9	Uncultured *Arcobacter* sp. DS081 (DQ234164), mangrove bacterioplankton.	99
			Clostridia	
Cb6	m	27.3	Uncultured bacterium CLONG96 (DQ478749), UASB reactor.	95
Cb7	m	18.2	Uncultured bacterium SJA-136 (AJ009493), anaerobic trichlorobenzene-transforming microbial consortium	96
Cb8	m	9.1	Uncultured bacterium SHA-74 (AJ306755), dechlorinate consortium	94
			Flavobacteria	
Cb9	c	11.1	*Flavobacterium frigidarium* (AY771722)	93
			Thermotogae	
Cb10	m	9.1	Uncultured bacterium (AB195923), anaerobic reactor.	99
			Methanomicrobia	
Ca1	c	100	*Methanosaeta concilii* (X51423)	99
Ca2	m	91.7	*Methanosaeta concilii* (X51423)	98
Ca3	m	6.2	*Methanosarcina mazei* (AE008384)	98
Ca4	m	2.1	*Methanospirillum hungatei* (M60880)	96

568   [a] Ca: *Archaea*, Cb: *Bacteria*

569   [b] Period, c: consolidation, m: mature

570   [c] Similarity

FIGURE LEGENDS

572   Figure 1. Micrographs of a biofilm over time using scanning electron microscopy. 1000x

573   magnification for all the pictures except for 60 days (200x). Details shown in the inner square

574   are 4000x. (B)

575

576   Figure 2. Growth curve of a biofilm during the first 10 days of development.

577

578   Figure 3. FISH of different development stages of a biofilm viewed by epifluorescence and CLS

579   microscopies. A, B, C, D, E and F: 36 hours. G, H and I: 4 days. J: 60 days. The samples A, B,

580   D. E, F, I were simultaneously stained with DAPI (blue) and specific-group Cy3-labeled probes

581   (red). (A) *α-Proteobacteria* (ALF338-Cy3). (B) *β-Proteobacteria* (BET42a-Cy3). (D) Sulfate-

582   reducing bacteria (SRB385-Cy3). (E) *Syntrophobacter* (SYN835-Cy3). (F) *Methanomicrobiales*

583   (MG1200-Cy3), with *Methanospirillum*-like red rods. (G) Overlay of *Methanobacteriales*

584   (MEB859-Cy3, red) and *Archaea* (ARC915-fluos, green). The samples C and H were

585   simultaneously hybridized with a bacterial probe (EUB338-fluos, green) and other probes: (C) γ-

586   *Proteobacteria* (GAM42a-Cy3, yellow) and (H) *Archaea* (ARC915-Cy3, red). (I) Detail of

587   *Methanosarcina* group (ARC915-Cy3). (J) Orthogonal CLMS section of the XZ plane of a mature

588   biofilm hybridized with specific probes for *Archaea* and *Bacteria* (ARC915-Cy3, red and

589   EUB338-fluos, green). All the micrographs have a 630x magnification with the exception of I

590   which has a 1000x magnification.

591

592   Figure 4. DGGE fingerprint for *Archaea* (A) and *Bacteria* (B) throughout the experiment.

593

594   Figure 5. Archaeal phylogenetic tree based on almost complete 16S rRNA gene sequences

595   retrieved from the cloning analysis. The bar scale represents 10 nucleotide substitutions per 100

596   nucleotides. The tree was constructed using parsimony.

597

598   Figure 6. Bacterial phylogenetic tree based on complete 16S rRNA gene sequences retrieved

599   from the cloning analysis. The bar scale represents 10 nucleotide substitutions per 100

600   nucleotides. The tree was constructed using parsimony.

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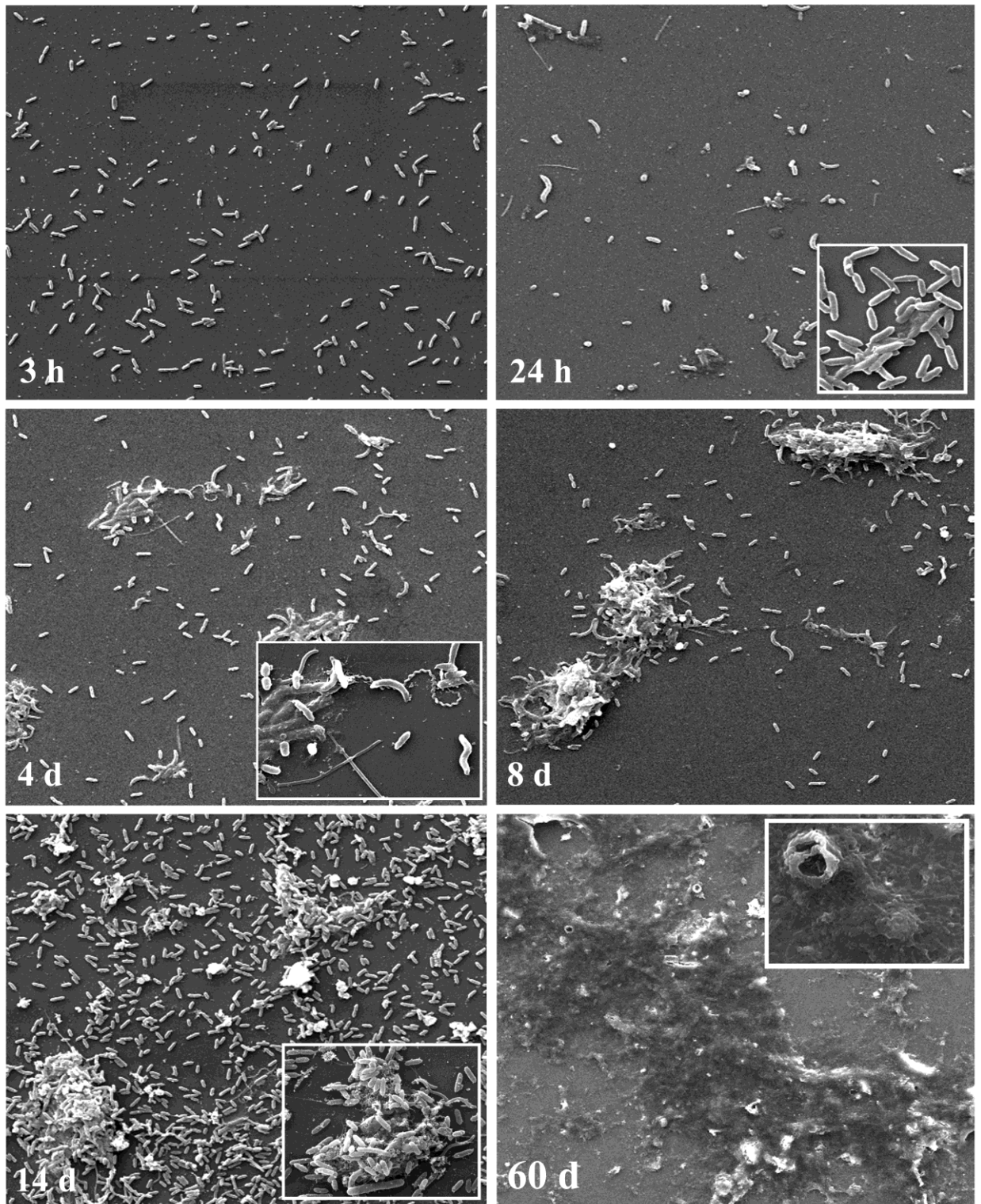609

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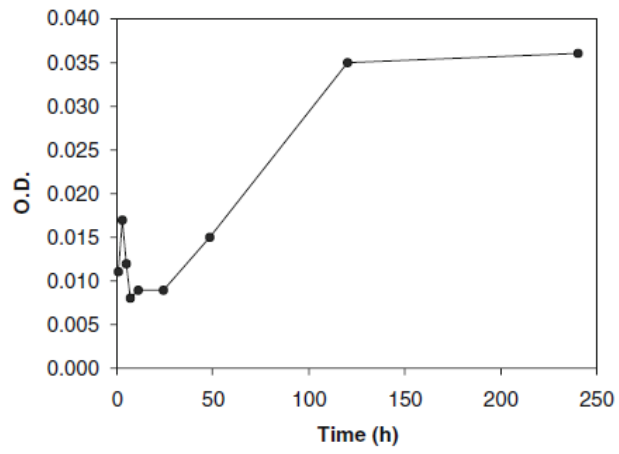612

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623   Figure 1

3 h

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8 d

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60 d

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626   Figure 2

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644   Figure 3

A

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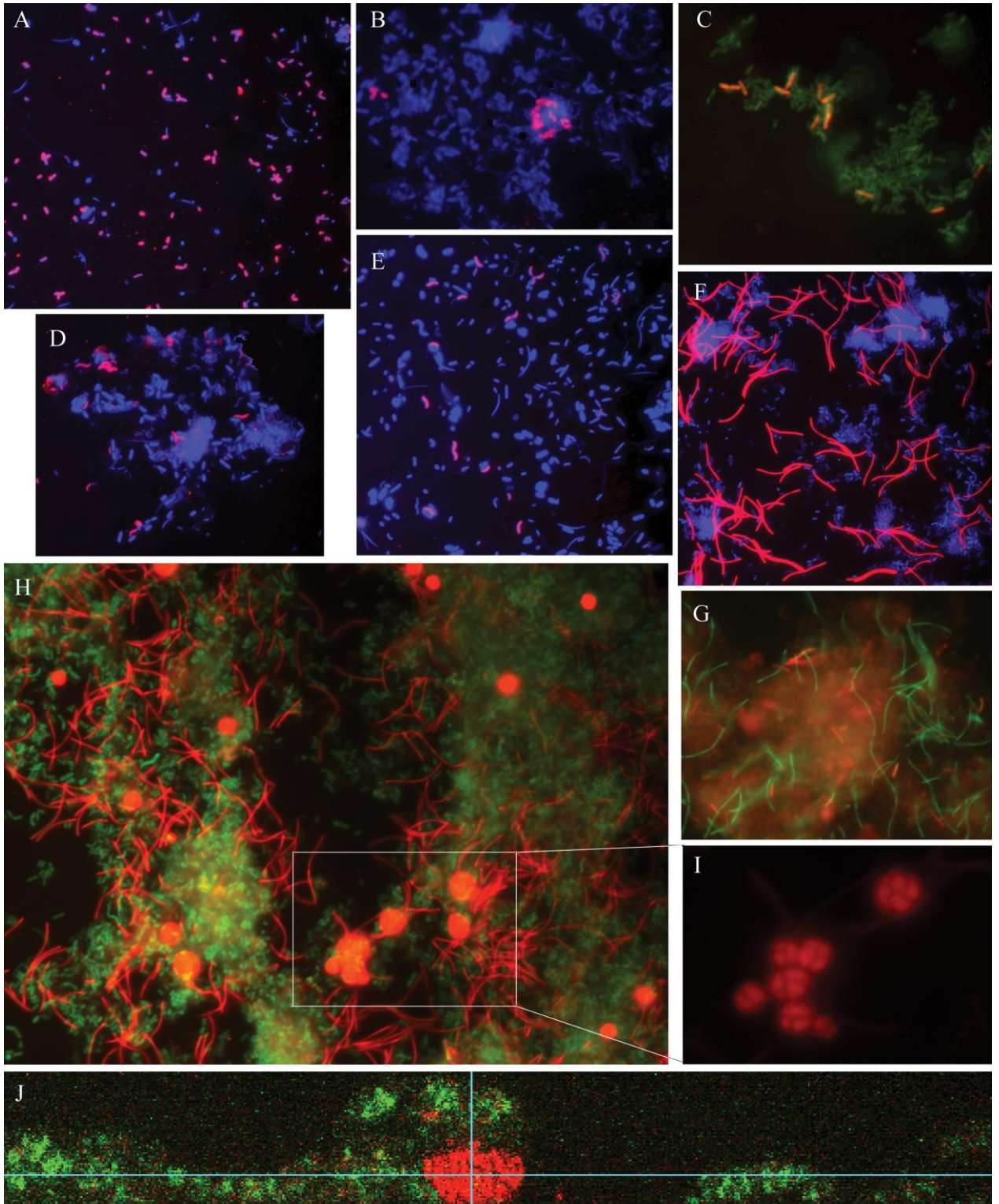
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647   Figure 4
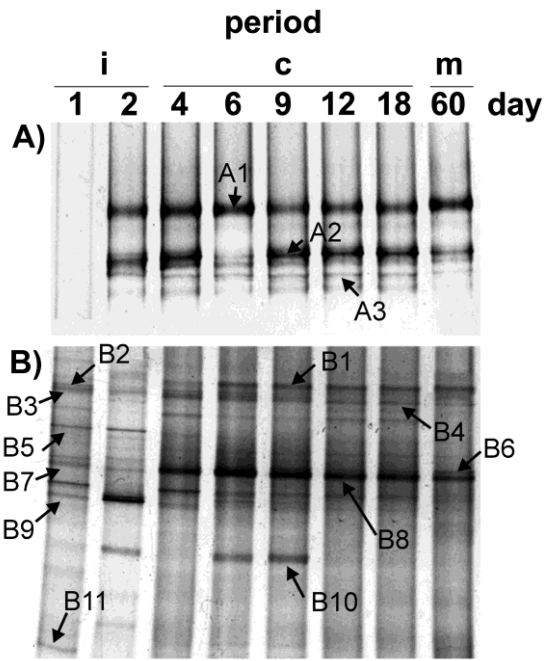
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663   Figure 5

Ca2 (AY692055)
Ca1 (AY692053)
Methanosaeta concillii (M59146)
Methanosaeta thermoacetophila (AF334401)
Methanosarcina mazei (AF028691)
Ca3 (AY692059)
Methanosarcina barkeri (AF028692)
Methanospirillum hungatei (M60880)
Ca4 (AY692060)
Methanomicrobium mobile (M59142)
Methanobacterium formicicum (AF028689)
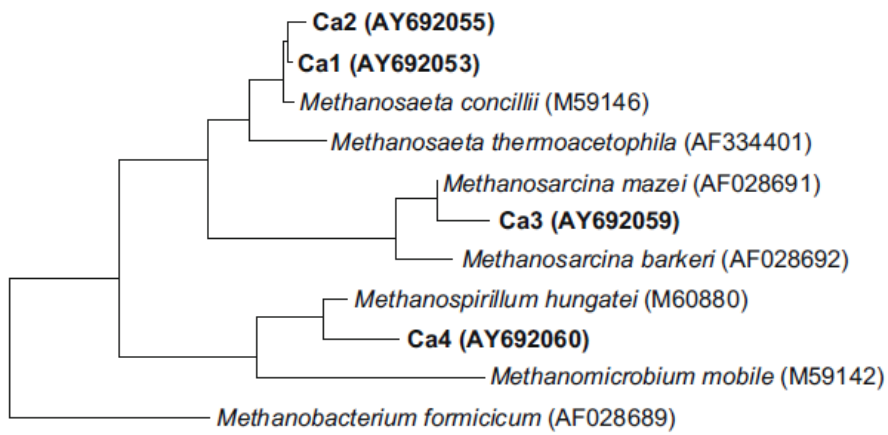
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681   Figure 6

Azospirillum brasilense (X79733)

Cb2 (AY692040)

Rhodospirillum rubrum (M55597)

Oleomonas sagaranensis (D45202)

Cb1 (AY692039)

Rhodocylus tenuis (D16209)

Dechloromonas sp. LT-1 (AY124797)

Cb3 (AY692041)

Arcobacter butzleri (AF314538)

Cb5 (AY692046)

Desulfuromonas michiganensis (AF357915)

Cb4 (AY692042)

Flavobacterium frigidarium (AF162266)

Cb9 (AY692051)

Bacteroides fragilis (X83959)

Aminobacterium colombiense (AF069287)

Cb6 (AY692048)

Ruminococcus hansenii (M59114)

Clostridium aminobutyricum (X76161)

Cb8 (AY692050)

Catabacter hongkongensis (AY574991)

Cb7 (AY692049)

Cb10 (AY692052)

Fervidobacterium islandicum (AY434670)

Thermotoga maritima (M21774)

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