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1 **Cultivating the uncultured: limits, advances and future challenges**

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5 Running title: Cultivating the uncultured.

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16
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18 high-throughput cultivation.

19
20 **Abstract**

21 **Since the invention of the Petri dish, there have been continuous efforts to**
22 **improve efficiency in microbial cultivation. These efforts were devoted to the**
23 **attainment for diverse growth conditions, simulation of *in situ* conditions and**
24 **achievement of high-throughput rates. As a result, prokaryotes catalysing novel**
25 **redox reactions as well as representatives of abundant, but not-yet cultured taxa,**

26 were isolated. Significant insights into microbial physiology have been made by
27 studying the small number of prokaryotes already cultured. However, despite
28 these numerous breakthroughs, microbial cultivation is still a low-throughput
29 process. The main hindrance to cultivation is likely due to the prevailing lack of
30 knowledge on targeted species. In this review, we focus on the limiting factors
31 surrounding cultivation. We discuss several cultivation obstacles, including the
32 loss of microbial cell–cell communication following species isolation. Future
33 research directions, including the refinement of culture media, strategies based
34 on cell-cell communication and high throughput innovations, are reviewed. We
35 further propose that a combination of these approaches is urgently required to
36 promote cultivation of uncultured species thereby dawning a new era in the field.

37

37 **Introduction**

38 Today, 1.8 million eukaryotic species are recognized, while estimates indicate that our
39 planet hosts 5 to 10-fold more species. Until now, only 7031 prokaryotic species have been
40 described (and validated by the International Committee on Systematics of Prokaryotes) in
41 the one hundred and twenty years, since the invention of the Petri dish (Achtman & Wagner,
42 2008), even though we now realize that prokaryotes represent “the unseen majority”
43 (Whitman et al., 1998). The lack of an extensive and accurate picture of the microbial
44 diversity is partly due to a lack in technical advances in the microbiology cultivation field.
45 Over the past three decades, molecular biology was an enormous driving force in
46 microbiology in uncovering the microbial diversity. Many new candidate divisions are now
47 recognized due to 16S rRNA sequence-based approaches and environmental metagenomics
48 (Curtis *et al.*, 2002). These findings exposed a gap between the known phyla and those
49 possessing cultured representatives (Fig. 1). In 1987, when much of our knowledge derived
50 from pure culture techniques, all known phyla possessed cultured representatives. Twenty
51 years later, of the 100 bacterial phyla identified only 30 possess a cultivated representative
52 (Achtman & Wagner, 2008). The number of phylum-level divisions possessing cultured
53 representatives has increased at a linear and constant rate over the last two decades. However,
54 this increase was greatly augmented with the emergence of molecular-based approaches. This
55 augmentation is seen when candidate divisions are included in these calculations (Fig. 1).

56 From a quantitative point of view, the advances made by culture-dependent
57 approaches may appear trivial, especially since only 0.1% of the existing prokaryotes have
58 been cultured so far. Yet, this quantitative approach is reductive since the past 20 years of
59 microbial cultivation have led to unprecedented advances in our knowledge of the microbial
60 world.

61 Molecular ecology and metagenomics have increased significantly our knowledge of
62 the genetic diversity and have led to interesting hypotheses (Hugenholtz & Tyson, 2008). The
63 advanced techniques have also revealed how far we are from measuring the full extent of
64 genetic diversity encoded by microbial life (Hugenholtz & Tyson, 2008; Pignatelli *et al.*,
65 2008). Considering that many of the genes stored in the databases have unknown functions or
66 are incorrectly annotated, it is probable that metagenomes alone will not offer sufficient
67 knowledge to cultivate all organisms. There is evidence that many of the candidate divisions
68 revealed by molecular approaches, and known only from molecular signatures, represent a
69 significant fraction of the microbial diversity. Some members of these ‘not-yet-cultured’ taxa
70 are probably key ecological players. Today, one of the main challenges for microbiologists is
71 to develop strategies to cultivate this uncultured majority. A comprehensive understanding of
72 biology and ecology of prokaryotes will require cultivation. Therefore, it is not surprising to
73 observe an increasing interest for the field of microbial cultivation (Leadbetter, 2003).

74 The aim of this review is to provide an overview of the new cultivation-based
75 approaches while documenting their limitations and outcomes. Further, we highlight how
76 cultivation has led to valuable advances in our understanding of microbial physiology and
77 identify the future challenges for microbiologists in the microbial cultivation field. Lastly, a
78 discussion is provided on the technical developments that may drive innovative research in
79 the near future.

80

81 **I- Review on some cultivation successes**

82 Prokaryotic growth necessitates an energy source (light or chemical compounds),
83 nutrients and proper physicochemical conditions. Challenges for the microbiologist are to
84 identify required nutrients, to provide them in the growth medium in the appropriate
85 concentrations to sustain the microbial growth, and to avoid the co-precipitation of the

86 introduced chemicals. Since different organisms require a different set of nutrients in varying
87 concentrations and forms, the design of growth media remains a difficult task. Conversely,
88 the intrinsic selectivity of any growth medium imposes limitations on the type, diversity and
89 number of prokaryotes recovered from the natural environment. This phenomenon is known
90 as the “great plate count anomaly” (Staley & Konopka, 1985). Indeed, there is a difference of
91 several orders of magnitude between colony counts on laboratory medium and total numbers
92 of prokaryotic cells present in natural environments. Only a minor fraction of the naturally
93 occurring microbial community is recovered by conventional selective media (Skinner *et al.*,
94 1952; Amann *et al.*, 1995). Depending on the nature of the samples, the cultivation efficiency
95 of active cells by standard plating techniques is estimated between 0.001% and 1% (Kogure
96 *et al.*, 1979; Staley & Konopka, 1985; Amman *et al.*, 1995). Thus, cultured microorganisms
97 do not reflect the functional and phylogenetic diversity present within any natural habitat.

98 Two main strategies are used for the isolation of pure cultures in microbiology. In
99 both strategies, enrichment culture is performed as a first step. The first strategy aims to
100 isolate colonies by repetitive streaking on solid medium (or alternatively by performing pour
101 plates or agar shake tubes), while the second strategy aims to isolate cells following repeated
102 series of dilutions in liquid medium. These classical approaches have led to the isolation of a
103 large number of strains belonging to taxa with few or no representatives in pure culture
104 (Janssen *et al.*, 1997; Joseph *et al.*, 2003). Nevertheless, the enrichment and pure culture
105 isolation strategies often select for opportunistic fast-growing organisms also called lab
106 weeds. In nutrient-rich artificial media, the community members with ‘r’-strategy, or fast-
107 growers, often overgrow and out-compete the naturally abundant ‘K’-strategists (Watve *et al.*,
108 2000). Consequently, these conventional culture-dependent approaches do not reflect the
109 actual microbial communities (Amann *et al.*, 1995). Certain taxa are still severely under-
110 represented in pure cultures (Hugenholtz *et al.*, 1998). As direct consequence, most of our

111 current knowledge of the nutrition, physiology and biochemistry of prokaryotes is based on
112 easily cultivable organisms. In recent years, novel cultivation strategies were developed to
113 overcome these limitations. Schematically, they are classified into four categories (Fig. 2).

114

115 **1- Refinement of standard cultivation strategies**

116 Different studies have demonstrated that a fraction of the “not-yet-cultured” groups of
117 prokaryotes can be grown by the refinement of classical approaches. Changes in the media
118 formulations, including the use of non-traditional electron donors, electron acceptors and
119 carbon sources have proven efficient in recovery of uncultured taxa (Köpke *et al.*, 2005).
120 Diversification of the media and multiplication of culture conditions are simple methods to
121 by-pass approaches that are selective by nature. For instance, the cultivable fraction from
122 coastal sub-surface sediments was shown to yield a higher number and diversity of isolates
123 when culture collections were performed with diverse electron acceptors and carbon sources
124 (Köpke *et al.*, 2005). The qualitative composition of carbon sources is also a determinant
125 factor for cultivation efficiency. As shown with seawater samples from the North Sea, media
126 prepared using several different carbon sources and complex compounds yield higher number
127 and more diverse isolates than similar media with only one carbon source. These isolates
128 obtained with a single substrate belonged almost exclusively to the *Gammaproteobacteria*
129 while representatives of four other classes grew on complex media (Uphoff *et al.*, 2001). In
130 recent years, it was shown that novel redox reactions, catalysed by specific ecological
131 communities of prokaryotes, can be identified within enrichment cultures onto non-
132 conventional media. Many novel physiotypes can be isolated using this method. Significant
133 advances were made in the field of the anaerobic degradation of hydrocarbons. For example,
134 different microbial consortia of Archaea and Bacteria, which couple the anaerobic oxidation
135 of methane to sulphate or nitrate reduction, were enriched from anoxic marine or freshwater

136 sediments. These findings enhanced our understanding of the global biological cycles
137 (Nauhaus *et al.*, 2002; Raghoebarsing *et al.*, 2006). Moreover, hydrocarbon-degrading
138 bacteria were identified using refined media containing only hydrocarbon/carbon energy
139 sources and nitrate, iron or sulphate as electron acceptors. These concurrent studies led to a
140 better understanding of the biochemistry and energetics of anaerobes. Several biochemical
141 mechanisms involved in the activation of some of these chemically non-reactive compounds
142 have been identified (Lovley and Lonergan, 1990; Rabus *et al.*, 1993; Galushko *et al.*, 1999;
143 Coates *et al.*, 2001; Widdel *et al.*, 2007). However, hydrocarbon-degrading capacities,
144 activation mechanisms and species or ecological guilds, which are involved in hydrocarbon
145 degradation, remain to be discovered. For example, a microbiological anaerobic oxidation of
146 methane with iron (III) or manganese (IV) as a terminal electron acceptor is unknown, yet this
147 reaction is thermodynamically possible (Raghoebarsing *et al.*, 2006). The enrichment culture
148 with anoxic ditch sediment, discovered in 1999 by Zengler and co-workers, was another
149 growth-supporting reaction of relevance. This team demonstrated that the conversion of long-
150 chain alkanes to methane, under strictly anoxic conditions, is biologically performed by an
151 ecological guild assumed to be acetogenic syntrophic bacteria associated to acetoclastic and
152 hydrogenotrophic methanogenic archaea (Zengler *et al.*, 1999). The discovery of this process
153 in nature might help to understand the terminal degradation of organic matter in areas of deep
154 and old marine sediments where sulphate is depleted. Another novel physiotype recently
155 identified due to advances in cultivation and isolation was an autotrophic anaerobe, which
156 couples the oxidation of phosphite (III) to sulphate reduction (Schink and Friedrich, 2000).
157 These novel physiotypes along with the newly recognized biological redox reactions are only
158 a few of the several examples of the significant advances made in uncovering the microbial
159 diversity through the enhancement of cultivating techniques.

160 Some recent successes in improving traditional cultivation methods include the
161 following. The use of relatively low concentration of nutrients to increase the cultivability and
162 to improve the recovery of prokaryotes from different types of natural samples (Button *et al.*,
163 1993; Janssen *et al.*, 1997; Watve *et al.*, 2000; Connon and Giovannoni, 2002; Rappé *et al.*,
164 2002; Sangwan *et al.*, 2005). The use of increased incubation periods to allow for the
165 development of strains from rarely isolated taxa (Sait *et al.*, 2002; Stevenson *et al.*, 2004;
166 Davis *et al.*, 2005; Sangwan *et al.*, 2005; Stott *et al.*, 2008) and, the addition of signalling
167 compounds known to mediate communication between bacteria (Bruns *et al.*, 2002; Bruns *et*
168 *al.*, 2003). Moreover, other less-documented approaches also yielded new isolates. These
169 included: the use of gellan gum (phytagel) as a gelling reagent instead of agar (Tamaki *et al.*,
170 2005; Stott *et al.*, 2008); the decrease in inoculum size (Davis *et al.*, 2005); the addition of
171 electron transporters to the culture media (Stevenson *et al.*, 2004); the addition of enzymes to
172 cope with reactive oxygen species (Stevenson *et al.*, 2004); the addition of inhibitors of
173 undesired organisms (Leadbetter *et al.*, 1999); and, the combination of an unusual energy
174 source with antibiotics to exclude Bacteria (Könneke *et al.*, 2005). Finally, sophisticated
175 single-cell isolation tools allowing for the manipulation of a targeted cell from a mixed
176 community (with a micro-capillary tube or with ‘optical tweezers’) have been developed
177 (Huber *et al.*, 1995; Fröhlich and König, 2000; Huber *et al.*, 2000). The principal limitations
178 of these single-cell isolation strategies include their labour intensive requirements and the
179 determination of the suitable growth conditions to cultivate prokaryotic cells of unknown
180 metabolism and systematic affiliation. The identification of a cell of interest among a complex
181 community in absence of clear distinctive morphological features is also challenging.
182 Altogether, these limitations account for the lack in isolating numerous undiscovered strains.

183

184 **2- Cultures *in situ* or cultures in simulated natural conditions**

185 Often, the laboratory conditions poorly mimic the natural environmental conditions.
186 Therefore, strategies aimed at simulating natural conditions or culturing *in situ* have been
187 proven efficient. Schematically, two types of “*in situ* colonization devices” have been
188 developed: the diffusion chambers and the carriers (of organic or inorganic nature)
189 (Kaeberlein *et al.*, 2002; Ferrari *et al.*, 2005; Yasumoto-Hirose *et al.*, 2006; Bollmann *et al.*,
190 2007). Diffusion chambers are apparatuses equipped with filter membranes, which restrict the
191 movement of cells in the chamber. They allow for the removal of low-molecular weight
192 inhibitory end-products, as well as the exchange of chemicals between the chamber and the
193 environment, thereby making high density cultivation possible (Pörtner and Märkl, 1998;
194 Kaeberlein *et al.*, 2002). Different types of membrane-based systems have been developed to
195 grow microbial communities directly in the natural habitats (Kaeberlein *et al.*, 2002; Plugge
196 and Stams, 2002; Ferrari *et al.*, 2005; Bollmann *et al.*, 2007; Ferrari *et al.*, 2008). Uncultured
197 bacteria from soil, marine or activated sludge were grown in diffusion chambers. This led to
198 the hypothesis that *in situ* cultivation of environmental prokaryotes in diffusion chambers
199 either enriches sufficiently the strains for their subsequent isolation onto classical solid media,
200 or conditions them for growth under otherwise prohibitive *in vitro* conditions (Bollmann *et*
201 *al.*, 2007). Interestingly, slow-growing organisms were cultivated using this method.

202 In natural ecosystems, many prokaryotes live attached to surfaces. This is well known
203 for microbes living in sediments, soils, or in association with eukaryotes. It is less recognized
204 for microbes living in aquatic habitats, where free-living forms were supposed to be
205 dominant, but other associations, with various interfaces, exist. The attached existence
206 provides several advantages for the prokaryotes. Attached cells escape grazing better than
207 their free-living neighbours. Attachment also allows cells to develop metabolic inter-relations,
208 resistance to different stresses and better access to adsorbed substrates (Schink, 1999). *In situ*
209 colonization carriers are useful tools to overcome cultivation limits induced by attachment of

210 prokaryotes to solid surfaces. Several publications describe the deposition in natural
211 ecosystems of different carriers such as glass, ceramic, titanium devices, porous inorganic
212 substrates or polyurethane foams (Araki *et al.*, 1999; Alain *et al.*, 2004; Yasumoto-Hirose *et*
213 *al.*, 2006). *In situ* collectors coated with selective substrates, are effective for the selective
214 enrichment of targeted prokaryotes (Yasumoto-Hirose *et al.*, 2006). In addition, specialized
215 techniques simulating one or several important spatial or physical parameters allow the
216 cultivation of novel physiotypes. For instance, gradient systems (Nelson and Jannasch, 1983;
217 Emerson and Moyer, 1997), high pressure reactors (Marteinsson *et al.*, 1999; Alain *et al.*,
218 2002), flow-through devices (Houghton *et al.*, 2007) and gas-lift reactors (Postec *et al.*, 2005;
219 Postec *et al.*, 2007) were successfully used. Pressure is also an essential parameter given that
220 high-pressure environments occupy the largest fraction of the known biosphere. This
221 parameter was often neglected in microbial cultivation. However, its effects on microbial life
222 are as important as those of temperature or salinity. Pressure acts upon physiology and upon
223 biochemical reactions. Thus, these types of reactors are effective to grow microbial strains
224 from high-pressure habitats. However, only few piezophilic prokaryotes have been enriched
225 or isolated under elevated pressures (i.e. Yayanos *et al.*, 1979, 1981; Marteinson *et al.*, 1999;
226 Alain *et al.*, 2002; Houghton *et al.*, 2007). Recently, the first obligate piezophilic and
227 hyperthermophilic archaeon (*Pyrococcus* sp. strain CH1) was isolated from the deepest
228 hydrothermal vent field explored so far, using a high-pressure reactor (Zeng *et al.*, in press).
229 Finally, different types of bio-reactors are used to reproduce diverse environmental
230 conditions. The physical and chemical conditions of seafloor hydrothermal systems are
231 among the most difficult to reproduce in laboratories, considering the multiplicity of *in situ*
232 physical and chemical gradients. Gas-lift reactors and flow-through devices are powerful
233 tools to simulate hydrothermal vent *in situ* conditions (Postec *et al.*, 2005; Postec *et al.*, 2007;
234 Houghton *et al.*, 2007). Flow-through devices allow for continuous cultures under *in situ*

235 temperature, pressure and fluid flow. They make possible the simulation of the deep-sea vent
236 changes of fluid chemistry (Houghton *et al.*, 2007). Both systems allow for continuous
237 enrichment culturing under controlled conditions. Significant fractions of microbial
238 communities were grown using these systems (Postec *et al.*, 2007).

239

240 **3- Cultures of microbial communities**

241 The cultivation methods allowing for the growth of mixed populations offer great
242 potential to cultivate not-yet cultivated organisms. Indeed, in natural environments, most
243 organisms live as a part of a community in which distinct cells work in concert and
244 communicate either by trading metabolites, by exchanging dedicated signalling molecules, or
245 by competition for limited resources (West *et al.*, 2007; Nadell *et al.*, 2009). These
246 relationships, in addition to complex cell-cell communications, are hardly reproducible in
247 monocultures. This “in group” lifestyle, in biofilm or multi-cellular assemblage, is thought to
248 generate robustness for nutritional, biotic and abiotic changes by providing a capacity of
249 adaptation to environmental fluctuations. It is critical for microbial ecology and evolution. A
250 striking example is biofilm, in which cell-cell communication determines biofilm structure,
251 maturation and microbial niche construction thereby optimizing microbial survival and
252 reproduction. Mixed microbial assemblages have also the capacity to perform multiple-step
253 functions that are often not possible for individual strains or species. Examples are the
254 degradation of cellulose or the methanogenic conversion of complex organic matter (Brenner
255 *et al.*, 2008). In some cases, the cooperation within the microbial community is based on
256 sharing metabolic intermediates, micronutrients (e.g. vitamins) or chelating agents that either
257 assist or compromise the growth of other community members. When identified, such
258 facultative dependencies can be reproduced experimentally by supplying the medium with
259 these micronutrients or co-substrates. Besides facultative associations, syntrophic associations

260 are often compulsory and this interdependence cannot easily be by-passed or suppressed by
261 the addition of factors to the media (Schink, 1999). Many syntrophic associations are
262 explained by unfavourable energetic conditions. Both facultative and syntrophic associations
263 are widespread in natural habitats.

264 Consequently, approaches based on community cultures are effective methods to grow
265 facultative associations and syntrophic organisms. Using community culture approaches in
266 addition to dialysis membrane reactors, thermophilic syntrophic anaerobic glutamate-
267 degrading consortia from anaerobic sludge have been successfully enriched (Plugge and
268 Stams, 2002). Similarly, batch reactors operating under anaerobic-aerobic cycling conditions
269 have allowed the enrichment of mixed microbial sludge communities (Crocetti *et al.*, 2002).

270

271 **4- High-throughput automatable microbial culture formats**

272 The past decade was marked by the emergence of high-throughput cultivation methods
273 (Connon and Giovannoni, 2002; Zengler *et al.*, 2002; Zengler *et al.*, 2005; Ingham *et al.*,
274 2007). Several advances in high-throughput culture formats have originated from industrial
275 endeavours. Indeed, the myriad of organisms inhabiting our planet represents a tremendous
276 reservoir of bio-molecules for pharmaceutical, agricultural, industrial and chemical
277 applications. While culture-independent recombinant approaches are used to screen novel
278 molecules and enzymes from natural samples, cultivation of organisms greatly simplifies such
279 studies and allows the use of a strategy biased for the desired phenotype.

280 A very efficient high-throughput cultivation method resulted from modifying the
281 pioneering work, called extinction cultures, of Button and colleagues (Button *et al.*, 1993).
282 Briefly, extinction culturing requires dilutions of natural communities to a low number of
283 cells (1 to 5 cells per ml) in natural environmental water followed by their incubation in
284 defined conditions. Following incubation, growth is measured. The initial procedure was

285 refined by Giovannoni and colleagues to increase high-throughput rates by using microtiter
286 plates and by fluorescence microscopy screening (Connon and Giovannoni, 2002; Stingl *et*
287 *al.*, 2007). This improved technique resulted in better sensitivity and cultivation efficiency.
288 This technique allows notably for the growth of slow-growers. It also allowed for the
289 isolation of the first representatives of two bacterial clades: the SAR11 clade, a ubiquitous
290 alpha-proteobacterial lineage found abundantly in marine pelagic environment (Rappé *et al.*,
291 2002) and the OM43 clade (Connon and Giovannoni, 2002).

292 Another technology for massive cultivation of prokaryotes was developed recently.
293 This high-throughput approach consists in the encapsulation of cells in gel micro-droplets
294 (GMDs) incubated in a single column for long intervals of time under low nutrient flux
295 conditions. Micro-capsules are subsequently sorted by flow cytometry. This technique is
296 applied to samples from different habitats and provides more than 10 000 bacterial and fungal
297 isolates per natural sample (Zengler *et al.*, 2005). To our knowledge, this technology, which
298 allows the development and detection of micro-colonies (20 to 100 cells), has never been used
299 for anaerobes. It is advantageous as cell-containing micro-droplets are grown together in
300 capsules allowing for the exchange of signal compounds and metabolites between cells from
301 different micro-colonies but originating from the same natural community. Using the micro-
302 encapsulation approach, members of numerically abundant clades were isolated. Although
303 very promising, this technique is not yet easily implemented in microbiology laboratories
304 (Zengler *et al.*, 2002; Zengler *et al.*, 2005).

305 Finally, a multiwell microbial culture-chip was recently developed by a team of
306 microbiologists, nanotechnologists and micro-engineering experts (Ingham *et al.*, 2007). This
307 micro-Petri dish is composed of a unique porous ceramic subdivided into millions of
308 compartments in which cultures can be separately grown. The growth of microorganisms is
309 sustained by the nutrients diffused through a porous membrane. The innovation lies in the

310 conception of the chip that is composed of micron-scale wells, is readily manufactured,
311 affordable and easy-to-use in microbiology laboratories not equipped with micromanipulator.
312 This system combines automation and miniaturisation, prerequisites for modern
313 microbiology.

314

315 All approaches described in this chapter aimed to optimize prokaryote cultivation
316 efficiency (Fig. 2). Overall, these studies allow for the isolation of numerous novel species
317 and permit major breakthroughs in cultivation. Despite continuous efforts, culture dependent
318 approaches undergo changes characterized by gradual improvements rather than by a radical
319 paradigm shift. It is noteworthy that the so-called “Moore Law” in microbiology (Gefen and
320 Balaban, 2008) applies to the volume used in culture and not to the output in number of novel
321 species described.

322

323 **II- Why do so many microbes resist cultivation?**

324 Despite extensive studies on ecosystems where sampling is conducted, the conditions
325 used in classical cultivating conditions are often far from endogenous abiotic and biotic
326 conditions required for microbial growth. Thus, it is not surprising that only a small number
327 of the microbial repertoire has been cultured so far.

328 The lack of efficient cultivation techniques stems from many factors that largely
329 remain unknown. These factors include limited knowledge of (i) the diverse organisms, (ii)
330 the chemistry of the natural habitats, (iii) the natural biotic and abiotic interactions and, (iv)
331 the global functioning of the ecosystems at microbial level. Cultivation aims to create an
332 artificial system mimicking *in situ* conditions. However, we do not have sufficient knowledge
333 to reproduce the natural conditions in the laboratory or to create viable synthetic conditions
334 for all organisms. This lack of knowledge has led to regrettable mistakes. For example, traces

335 of tungsten element, which inactivates nitrogenase, have been included for a long time in the
336 culture media for *Spirochaetes* (Leadbetter, 2003). It was recently discovered that
337 *Spirochaetes* are able to grow *via* nitrogen fixation (Lilburn *et al.*, 2001), a property that can
338 be expressed only in tungsten-depleted media. Another remarkable example is the unexpected
339 discovery of nanoarchaea in enrichment cultures (Huber *et al.*, 2002). This discovery was
340 unattainable by current PCR-based approaches since the universal primers commonly used for
341 diversity studies could not target this group.

342 Another reason that can explain the failure to cultivate many prokaryotes is our lack of
343 patience and sensitive detection methods for low cell yields. As previously discussed, in some
344 cases, cultivation efficiency was significantly increased by long periods of incubation. This is
345 true for organisms originating from oligotrophic habitats and potentially in a non-growing or
346 dormancy state. It is important to note that transition from a non-growing to a growing state in
347 a synthetic medium is a critical and stressful event. Thus, adaptations of cells to laboratory
348 growth conditions may require increased cultivation times. For instance, the duration of
349 growth log phase can depend on the cell status (i.e. healthy, stressed or sub-lethally damaged
350 cells) and the gap between *in situ* and *in vitro* conditions (*de novo* synthesis of an enzymatic
351 set to grow in the synthetic medium, etc). Without permitting adequate time intervals for
352 growth, many organisms and many redox reactions would never have been discovered. For
353 example, without lengthy incubation periods, the observation that methane is formed from
354 long-chain alkanes under anaerobic conditions would not have been documented. Under
355 tested conditions, gas formation in the presence of hexadecane started only after four-month
356 incubation of the culture (Zengler *et al.*, 1999). While bioenergetics calculations can predict
357 thermodynamical feasibility of a reaction, our current knowledge can not predict kinetics for
358 ‘resuscitation’ and for the growth of *de novo* enrichment cultures *via* a given reaction under a
359 defined experimental conditions. Indeed, the characteristics of natural uncultured organism(s)

360 (physico-chemical optima for growth, transport systems, etc) and of their enzymes (catalytic
361 rates, substrate affinities, regulation mechanisms) are unknown. At best, we can suppose that
362 enrichment cultures performed with highly stable substrates, weak oxidants and calculated to
363 have low net free energy gain, will require long-term incubations and patience. Otherwise,
364 extremely long growth kinetics or low cell densities are sometimes observed in enrichment or
365 pure culture. Inappropriate or non-optimal growth conditions might be responsible for these
366 low yields. Thus, once parameters that inhibit growth are identified, “normal” growth kinetics
367 and high yields can be restored (i.e. Flagan *et al.*, 2003). For the organisms characterized by
368 meagre yields, highly sensitive detection technologies such as tangential flow filtration and
369 concentration are critical (Giovannoni and Stingl, 2007).

370 Another rational put forward for prokaryotic resistance to cultivation is inherent to the
371 *in vitro* cultivation techniques widely used. More specifically, the *in vitro* cultivation
372 techniques used paradoxically aim to isolate strains in pure culture, while most organisms in
373 nature live in community and establish complex relationships (see previous chapter). The
374 main consequence of this general practice is a disruption of inter- and intra-species
375 communication during the very first stage of isolation. The impact of this perturbation on
376 cultivability is unknown for most prokaryotic species and might vary depending of the
377 species. Cell-cell communication (CCC) has gained considerable attention in recent years, in
378 particular density-dependent cell-signalling mechanisms known as quorum sensing (QS).
379 Cell-cell interactions have been investigated mainly in Bacteria due to their tremendous
380 importance in health, environmental and industrial applications. Bacteria respond to a wide
381 range of signalling molecules at intra-species level (species-specific compounds) and/or at
382 inter-species level (Camilli and Bassler, 2006). Inter-species interactions appear to be
383 ubiquitous among prokaryotes and are not limited to signalling molecules such as
384 autoinducer-2 (AI-2) and *N*-acyl-homoserine lactones (*N*-AHLs) but extend to antibiotics at

385 sub-inhibitory concentrations (Ryan and Dow, 2008). Globally, prokaryotes and eukaryotes
386 have co-existed for millions of years and frequently have co-evolved in the same
387 environments. Therefore, it is not surprising that interactions between them range from
388 mutually beneficial to virulent. QS compounds like AHLs found initially in Bacteria are
389 involved in virulence, biofilm formation, motility, antibiotic production and are recognized by
390 eukaryotes with effects on immuno-modulation, intracellular calcium signalling and apoptosis
391 (Hughes and Sperandio, 2008). QS mechanisms have been extensively investigated during
392 the past decade. Many Bacteria have one QS circuit while some display two or three circuits
393 to coordinate their population density. Yet, these circuits are themselves under the control of a
394 master QS regulator (Hooshangi and Bentley 2008). Recently, Kolodkin-Gal *et al.* (2007,
395 2008) showed that the mode of action of antibiotics in *E. coli* is determined by the ability to
396 communicate through the Extra-cellular Death Factor (EDF) as a function of cell density.
397 CCC was initially considered as an exception limited to a few specialized bacteria, but has
398 recently emerged as the norm in the bacterial world. Several indications strengthen this trend:
399 (i) the high frequency of QS among genome-sequenced bacteria (40% of the 800 sequenced
400 bacterial genomes contain the *luxS* gene suggesting that the AI-2 precursor functions as a
401 universal signal (Pereira *et al.*, 2008)) and, (ii) the discovery of signalling molecules in
402 microbial metagenomic data. In addition, there is growing evidence that QS signals provide to
403 bacteria more information than cell density. Surprisingly, the abundance of results on QS,
404 biofilms and cell-cell communication, at intra-species or inter-species levels have been
405 analysed in the perspective of pathology (virulence, biofilm formation and control),
406 biotechnology, synthetic biology (Hooshangi and Bentley, 2008), evolution (Keller and
407 Surette, 2006; West *et al.*, 2007), ecology (Nadell *et al.*, 2008) but seldom for cultivation
408 purposes.

409 To account for CCC mechanisms in cultivation procedures remains a challenge due to
410 our lack of knowledge on the cell-cell interaction requirements of targeted species.
411 Considering the small fraction of prokaryotes cultivated, it seems likely that most cell-cell
412 communication mechanisms are unknown. CCC mechanisms described up to now, like those
413 classically observed with *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Escherichia coli*,
414 operate at high cell densities. We cannot exclude that some CCC mechanisms could also act
415 at low cell densities (10^2 to 10^4 cells/ml) to regulate cell growth of some species. In that case,
416 it may be necessary to reconsider isolation procedures accordingly.

417 Finally, one last reason for which prokaryotes remain uncultured is due to enrichment-
418 isolation process whereby the abiotic interactions are broken down. This disruption of the bio-
419 geochemical factors, that collectively represent the environment, is a source of stress for the
420 organisms from natural habitats. The depreciation of these bio-geochemical factors from the
421 native extra-cellular habitats leads the experimentalists to design synthetic conditions, which
422 might introduce a stressful parameter or a main change in resource type or concentration. For
423 example, a phenomenon similar to substrate-accelerated death might occur. Growth inhibition
424 is observed when cells are suddenly grown in the presence of concentrations greater than 1-10
425 μM of a given substrate (i.e. ammonia, phosphate or glucose) whereas they were previously
426 starved of this substrate (Overmann, 2006).

427

428 **III- Cultivating the uncultured: future directions**

429 There is current growing consensus among microbiologists that improvements are
430 needed in microbial cultivation. A comprehensive understanding of the microbial world will
431 undoubtedly require cultivation. It is clear that the keys to cultivation will not come from a
432 single technological breakthrough but will depend on our knowledge of the natural microbial
433 systems. The numerous examples of successes in cultivation that we have mentioned in this

434 review demonstrate that through patience and advances in technology, cultivation is a
435 surmountable obstacle for many organisms. We still have a lot to learn from pure culture
436 microbiology, even if these isolates might display unnatural behaviours in the synthetic
437 laboratory. Pure culture microbiology still represents the best method to study microbial
438 physiology including detailed investigations on the role of genes, proteins, and metabolic
439 pathways. This is true since several unexpected physiological discoveries were made after
440 isolation of novel species belonging to taxa presumed to be already well documented. In turn,
441 these data provide important guidance for the optimization of cultivation media.

442 While several difficulties remain in cultivating microorganisms, future directions can
443 be summarized as follows: refining culture medium, mimicking nature through *in situ*
444 cultivation systems or designing devices supporting CCC, and developing automated
445 procedures through robotics. Undoubtedly, combinations of these diverse approaches will
446 yield successes in cultivation.

447

448 **1- Refinement of culture media**

449 Culture medium optimization will require the use of various complementary tools
450 including: (i) the ability to define the range of electron donors, acceptors and key elements
451 sources based on a better knowledge of the environment, (ii) molecular probes for screening
452 novel species and, (iii) high-sensitive methods with low detection thresholds to uncover rare
453 and slow-growing species in culture. In the future, results from single cell genomics and
454 metagenomics analyses will contribute to better isolation strategies for prokaryotes.

455

456 **2- Design of isolation strategies based on cell-cell interactions**

457 As seen previously, microbial landscapes are dominated by biofilms or aggregates.
458 This isolation is not the preferred state of most Bacteria and Archaea. Despite the growing

459 knowledge on cell-cell signalling molecules, the use of antibiotics and auto-inducers in
460 culture is limited. Indeed, the vast majority of these compounds were characterized from
461 microorganisms in pure culture, some of which were subsequently retrieved in metagenomic
462 analyses. Metagenomics will yield numerous genes coding for novel auto-inducers or their
463 precursors, but these will be classified as unknown genes until discovered in culture.
464 Therefore, it is not surprising that our previous analytical knowledge is hardly applicable to
465 isolation procedures of novel species. For isolation of a strain, one way to preserve the
466 endogenous CCC mechanisms is to grow it in microbial community conditions, using
467 bioreactors, either under planktonic stage, or incorporated in gel micro-droplets (GMDs)
468 (Zengler *et al.*, 2002), or attached to a surface. So far, the community culture step is more
469 often followed by an isolation step through various means: streaking, dilution-to-extinction,
470 sorting (mechanically or by FACS). For the last method, single cells are distributed in wells
471 or micro-wells filled with appropriate medium and incubated for further culture. The major
472 drawback is that the appropriate medium used to grow the cells after the isolation step is
473 different from the medium that supported growth in the community culture, and that all
474 chemical CCC are suddenly disrupted. What is the impact on the cultivability of some strains?
475 In order to address this question it seems necessary to design novel culture equipments. The
476 easiest method would be to substitute culture micro-plates by micro-bioreactors in the
477 Zengler's method (Fig. 3). In such a system, the flow from the community culture would
478 nourish each well of the micro-bioreactor with all the metabolites produced by the community
479 culture. This system would combine community culture and isolation. Further, it would allow
480 for the circulation of chemical compounds in the micro-bioreactor, partly preserving cell-cell
481 communication, even though physical cell-cell contacts would be disrupted. Since the
482 community culture would operate as a black box for signalling compounds, we can not
483 exclude that some metabolites could have inhibitory effects. The benefits of this combination

484 of enrichment culture and isolation remain to be assessed. By dissociating and, in a following
485 step, combining community culture and isolation, this system could address the culture of
486 consortia components and symbionts, which are almost impossible to solve with current
487 practices.

488

489 **3- High throughput issues**

490 A quiet revolution occurred during the past decade in liquid handling applied to life
491 sciences with the development of pipetting workstations. The main line of action was the
492 reproduction at high throughput of manual procedures by pipetting robots. Culture medium
493 distribution in micro-plate wells and inoculation of a single cell or GMD per well is now
494 performed routinely by robots. The combination of GMD encapsulation and flow cytometry
495 sorting is effective for aerobes (Akselband *et al.*, 2006). Further, these studies show that some
496 cells remain viable after staining and sorting, however these findings await confirmation. This
497 remains a challenge for obligate anaerobes especially due to the sorting step with flow
498 cytometers. FACS equipment could be adapted to operate in anaerobic chambers, but might
499 prove inconvenient and costly. The development of direct sorting of GMDs on liquid
500 handling workstations would be an excellent alternative to avoid FACS analysis, to limit the
501 cost of equipments and, to allow dissemination of these approaches in microbiology
502 laboratories. What seems also to be certain is that the widespread use of high-throughput
503 cultivation procedures implies a simultaneous development of affordable high-throughput
504 identification procedures.

505

506 **4- Combination principle**

507 Microbiologists have long recognized that our understanding of the microbial world
508 critically depends on the technological advances that broaden the knowledge-base for

509 chemical, biological and physical processes. Today, it is established that an integration of the
510 knowledge from multiple hierarchical levels of organization and from the molecule to the
511 ecosystem are also required. Despite these facts, most efforts in cultivation focused only in
512 the optimization of a limited set of parameters. We are convinced that this situation will
513 change in the near future for several reasons. First, a consensus is gradually emerging: culture
514 is not only needed to describe randomly novel species as a function of researcher main field
515 of interest. Culture appears more and more as the *sine qua non* condition for understanding
516 how the microbial world functions and is a prerequisite to predict changes in the frame of
517 global change. Secondly, it seems that the complexity of the microbial world emerging from
518 the growing knowledge of genomic and proteomic advances can not be analyzed only by
519 combining these approaches and relying on systems biology. Finally, the advent of
520 automation in culture and the combination of innovative methods lead to a new era in
521 cultivation, assuming that some specific equipments are developed to handle the cell-cell
522 interaction mechanisms during the clonal culture phase. Combinations theoretically possible
523 are detailed in Fig. 3.

524 Ecosystems encompass abiotic conditions, living species and all their interactions
525 (resource limitations, competition, predation, parasitism, among others). It is obvious that any
526 cultivation attempt is by nature highly reductive and cannot reproduce the conditions
527 observed in nature. The combination of existing methods and the development of novel
528 approaches will help to come close to conditions where a significant fraction of microbes is
529 amenable to culture (Fig. 2).

530

531 **Concluding remarks**

532 The extent of microbial diversity is still unknown and prokaryotes have undoubtedly
533 many secrets to reveal. The success of microbial life on Earth stems from its great

534 physiological diversity that collectively allows prokaryotes to derive energy from a wide
535 range of redox reactions and to colonize several types of habitats; including the extreme ones.
536 It is remarkable how much we have learned about microbial life by studying only a small
537 fraction of its diversity. New examples of microbial ingenuity were recently discovered due to
538 cultivation, either independently or in combination with metagenomics. There are still
539 important essentials hidden in the 99.9% of the microbial world that are not yet cultured.
540 They might change our understanding of biochemical processes, redox reactions,
541 physiological adaptations and microbial behaviours. We postulate that in the future major
542 advances in the understanding of microbial life will be achieved by innovative approaches in
543 cultivation, but not cultivation alone. Coordinated efforts of researchers studying microbial
544 systems at different levels should guide us to rethink culture strategies and to design growth
545 conditions as close as possible to the natural interactions and conditions. In conclusion, the
546 different cultivation successes discussed in this review demonstrate that contrary to widely
547 held beliefs, many prokaryotes can be cultivated. In view of the gap between the number of
548 phylotypes with or without representative cultured species, an additional question arises: is
549 there a need to define relative priorities between phylotypes? The division amongst
550 microbiologists at the international realm suggests that a consensus is not possible. The only
551 consensus that could emerge, and successfully funded, is the need to investigate novel
552 cultivation approaches, to develop new equipments and bring microbial cultivation in the 21st
553 century as a technologically advanced and a data rich discipline.

554

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560

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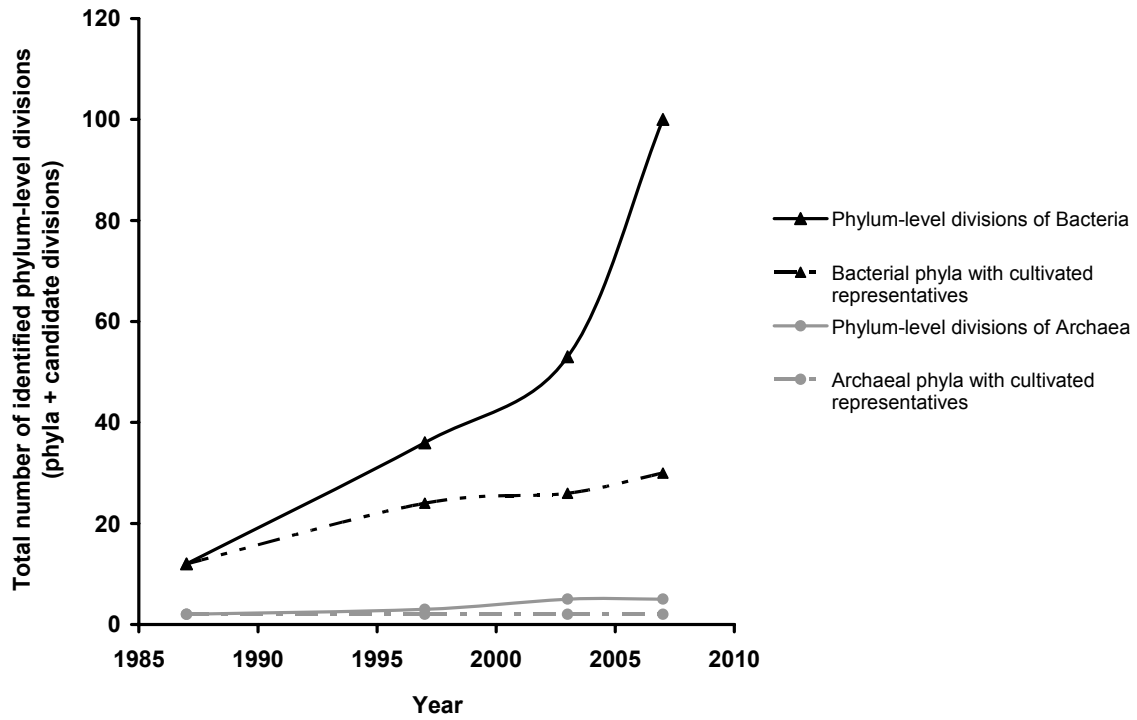
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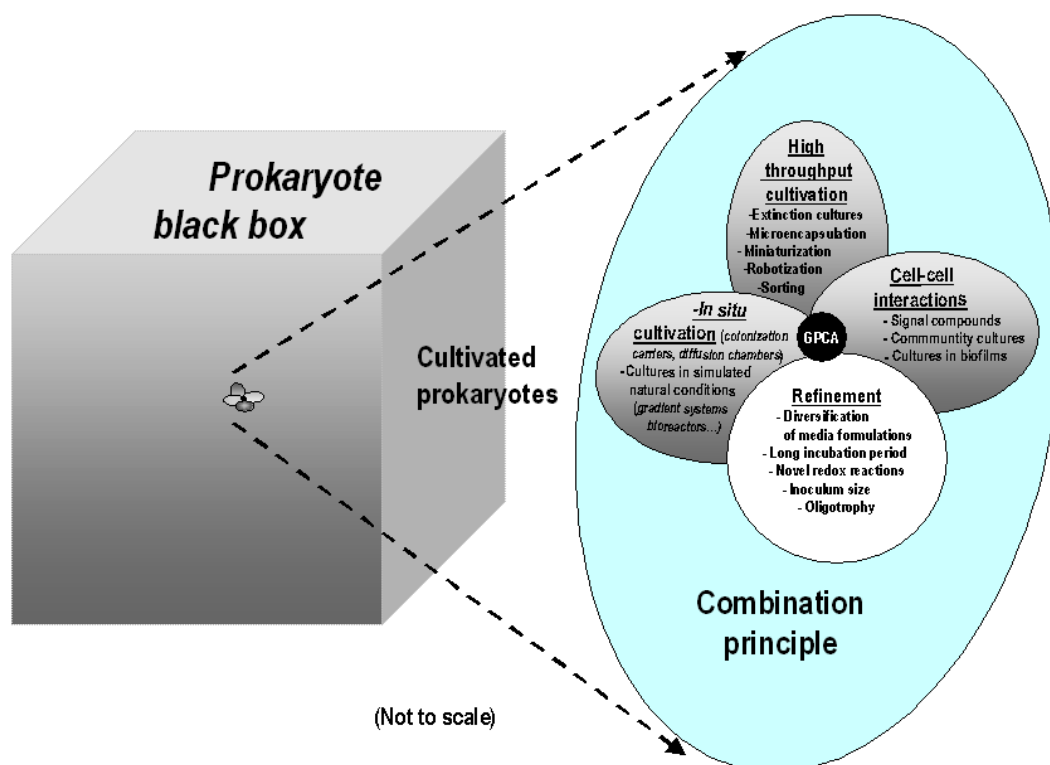
782 **FIGURES and FIGURE LEGENDS**



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784 Fig. 1. Numbers of phylum-level divisions (phyla with cultivated members + candidate
785 divisions with no cultivated representative) identified since 1987 among Bacteria (black line)
786 and Archaea (grey line), and numbers of phyla with cultivated representatives (dotted lines).

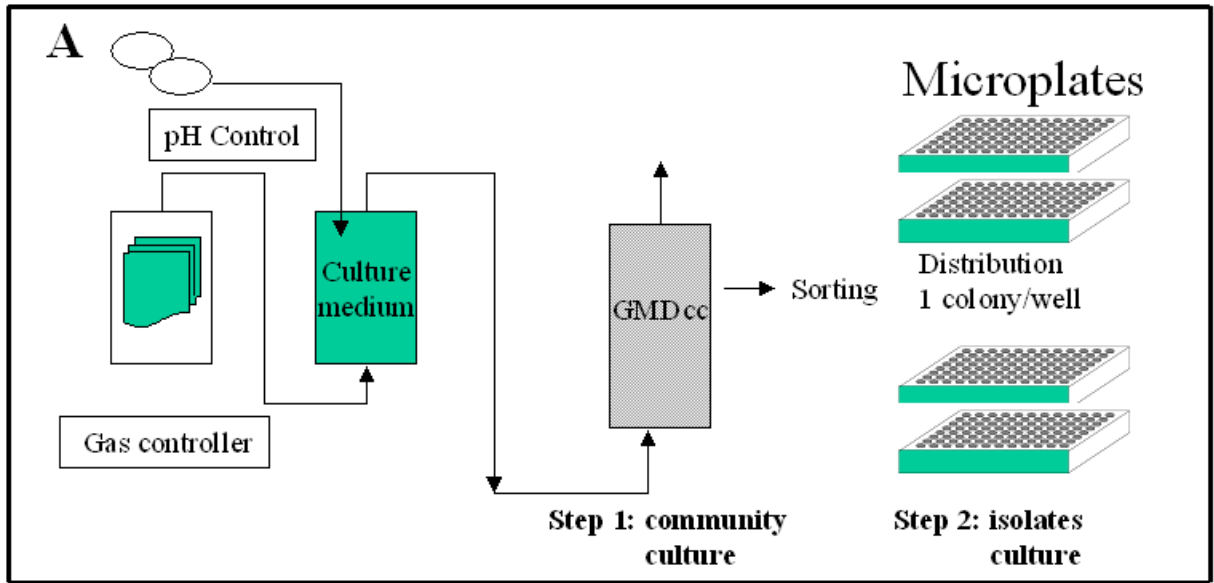
787 Adapted from Achtman & Wagner (2008).



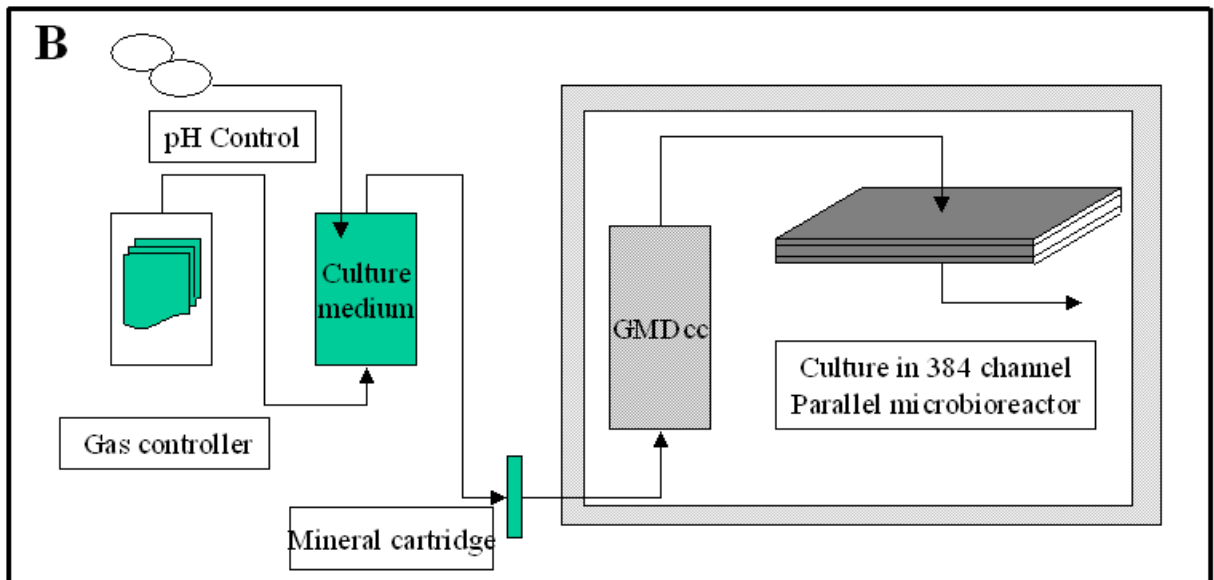
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789 Fig. 2. Schematic representation of the four main categories of cultivation strategies. The
 790 small black circle schematizes the minor fraction of a naturally occurring microbial
 791 community that are recovered onto conventional selective media, the so-called “Great Plate
 792 Count Anomaly”. Improved cultivation strategies developed so far aim to enhance one or few
 793 aspects of growth conditions among four main categories (represented by four bullets) and
 794 each allowed individually recovering more numerous and/or more diverse isolates than
 795 traditional approaches. None of these improved approaches is universal. We postulate that the
 796 combination (“the combination principle”) of already existing methods belonging to these
 797 four categories will help to come close to conditions where a significant fraction of microbes
 798 is amenable to culture.

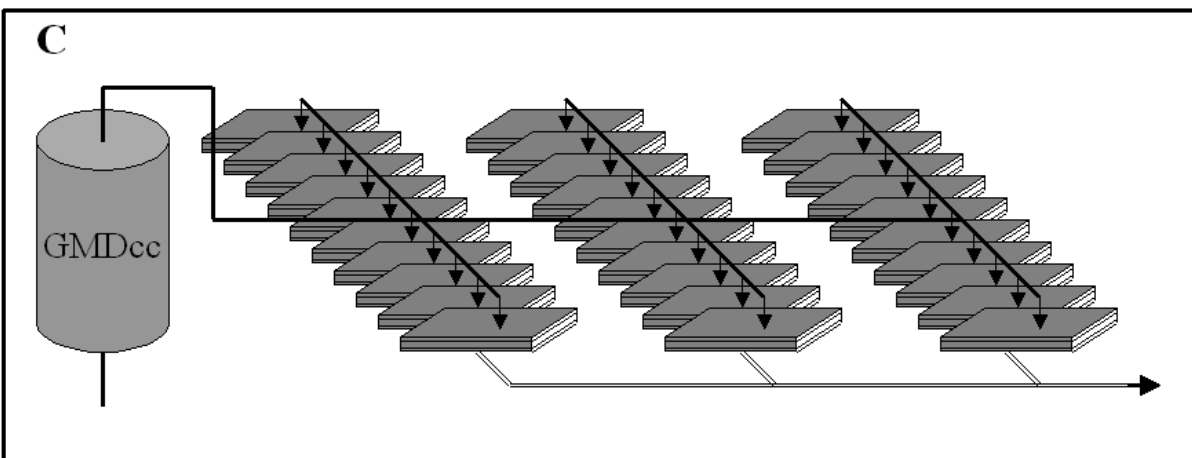
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802 Fig. 3. Schematic diagram of cultures and isolation procedures based on the combination
803 principle.

804 Abbreviation: GMDcc: community culture of microbial cells incorporated in gel micro-
805 droplets

806 A: GMD community culture followed by sorting and microplate cultivation (From Zengler *et*
807 *al.*, 2002, modified).

808 B: Flow-through culture in parallel micro-bioreactors nourished by community culture
809 medium and metabolite products. Micro-plates are replaced by micro-bioreactors directly
810 connected to the GMD community culture. This system can be operated in aerobic or
811 anaerobic conditions. Micro-bioreactors (either 96 or 384 parallel channels SBS format) and
812 flow-through equipments await development.

813 C: GMD and micro-bioreactors (384 channels) coupled in a nearly theoretical 10 000 parallel
814 channels configuration.