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Par

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Diagnostic de Laboratoire des Infections Opportunistes à Gemmata spp. (Planctomycetes)

Thèse présentée publiquement devant les membres du Jury :

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SOMMAIRE

AVANT-PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Prof. Didier Raoult

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RESUME

Les bactéries du genre *Gemmata* sont des Planctomycetes suspectées d'être responsables d'infections opportunistes chez des patients aplasiques. Ce genre englobe non seulement un ensemble de bactéries réfractaires à la culture et dont les séquences d'ADN ont été détectées chez l'homme ; mais également deux espèces cultivées : *Gemmata obscuriglobus* et *Gemmata massiliana*. Connues pour leurs antibiorésistances et leurs associations avec l'homme, le rôle des *Gemmata* comme pathogènes opportunistes devraient donc justifier des investigations plus poussées. Au cours de notre Thèse, nous avons collecté 426 échantillons cliniques dont 34 échantillons de sang collectés chez des patients aplasiques et 392 lavages broncho-alvéolaires afin de rechercher les *Gemmata* par qPCR et par culture. Chez un patient aplasique, une PCR positive a été obtenue à partir du sang. Une PCR standard a été alors réalisée sans possibilité d'obtenir de l'ADN malgré plusieurs tentatives et aucun isolat de *Gemmata* n'a été obtenu en culture pure. Nous avons par la suite étudié les interactions entre les *Gemmata* et des phagocytes par coculture afin d'avoir un aperçu sur leur capacité à résister à la lyse phagocytaire. Trois espèces amibiennes et une lignée de cellules cancéreuses THP-1 ont été sondées et toutes se sont avérées bactéricides pour les deux souches cultivées. En raison du manque de sensibilité de nos outils actuels et surtout le caractère récalcitrant de ces bactéries qui résistent à l'isolement, nous avons effectué une revue de la littérature sur les habitats de prédilection des Planctomycetes afin de comprendre leurs exigences nutritionnelles et de concevoir de nouveaux milieux de culture. Ainsi, dans l'environnement, les Planctomycetes sont fortement associés aux éponges marines et y cohabitent par des relations symbiotiques. Sachant que ces bactéries ont besoin d'un support pour s'accrocher afin de se diviser, nous avons donc comparé un nouveau milieu de culture A, incorporant des tissus d'éponge marine de *Spongia* sp. au milieu standard ; et un milieu B incorporant le filtrat

aqueux de *Spongia* sp. dans le milieu A. Les trois milieux (standard, A et B) ont été inoculés avec les deux souches. Ainsi, nos observations ont montré qu'après 3 jours d'incubation, les colonies comptées à partir des milieux A et B ont augmenté de plus d'un log par rapport au milieu standard ($p<0.001$). Ces milieux A et B permettront donc l'isolement rapide des *Gemmata*. Aussi, ces bactéries étaient très associées aux sédiments de fer, suggérant un rôle essentiel du fer chez ces organismes. L'analyse des génomes des deux espèces a montré qu'aucune d'elles ne codait pour certains composants essentiels du métabolisme du fer. Nous avons donc forcé *Escherichia coli* à produire des sidérophores et une ferriréductase extracellulaire et recueillir le filtrat afin de supplémenter le milieu de standard. Nous avons observé qu'en présence du filtrat, la croissance des *Gemmata* était meilleure par rapport au milieu non supplémenté ($p<0.001$). Enfin, nous avons exploré l'impact des rayons X sur les bactéries du microbiote digestif, sachant que celui-ci est perturbé lors de la radiothérapie et aurait influencé la translocation des *Gemmata* du tractus digestif vers le sang des patients soumis à cette thérapie. Nos observations ont révélé une forte perturbation bactérienne après l'irradiation des selles.

Mots cléfs : *Gemmata massiliana*, *Gemmata obscuriglobus*, *Planctomycetes*, Pathogènes opportunistes, fer, siderophores, ferrireductase, éponge marines, rayon X.

ABSTRACT

Bacteria of the genus *Gemmata* are Planctomycetes suspected to be responsible for opportunistic infections in two aplastic patients. This genus *Gemmata* includes not only a set of culture-refractory (non-cultured) bacteria whose DNA sequences have been detected in various habitats and in humans; but also, two cultivated species: *Gemmata obscuriglobus* and *Gemmata massiliana*. Known for their antimicrobial resistance and their demonstrated associations in humans, the role of *Gemmata* as opportunistic pathogens should therefore warrant further investigation. During our Thesis work, we collected 426 clinical specimens including 34 blood samples collected from aplastic patients and 392 bronchoalveolar fluids to search for *Gemmata* using a specific qPCR and culture. In an aplastic patient, a positive PCR was obtained from the blood. A standard PCR was then performed without the possibility of obtaining DNA despite several attempts. Blind sequencing was then performed but sequencing was defective and no isolates of *Gemmata* spp. was obtained in pure culture. We then studied the interactions of these bacteria with phagocytic cells by co-culture to gain insight into their ability to resist phagocytic lysis. Three amoebic species and one THP-1 cancer cell line were probed, and all were shown to be bactericidal for both *Gemmata* strains. Due to the lack of sensitivity of our current tools and especially the recalcitrant nature of these bacteria resistant to isolation, we conducted a review of the literature on the predilection habitats of Planctomycetes to understand their nutritional requirements in order to design new culture media. Thus, in the marine environment, Planctomycetes are strongly associated with marine sponges and coexist with symbiotic relationships. Knowing that these bacteria need a support to hold in order to divide, we therefore compared a new culture medium A, incorporating *Spongia* sp. marine sponge tissues in standard culture medium; and medium B incorporating the aqueous filtrate of *Spongia* sp. in medium A. The three media (standard, A

and B) were inoculated with both strains. Thus, our observations showed that after 3 days of incubation, colonies counted from media A and B increased to more than one log compared to standard medium (p <0.001). These media A and B will therefore allow growth and rapid isolation of *Gemmata* spp. Also, the analysis of these different habitats allowed us to note that these bacteria were very associated with sediments of iron hydroxide, suggesting an essential role of iron in these bacteria. Genome analysis of both species showed that none of them encoded for certain essential components in the iron metabolic pathway. We therefore forced *Escherichia coli* to produce siderophores and an extracellular ferrideductase to collect the filtrate to supplement the standard medium. We observed that in the presence of *E. coli* filtrate, the growth of *Gemmata* bacteria was significantly improved compared to the unsupplemented medium (p <0.001). Finally, we explored the impact of X-rays on the digestive tract bacteria, knowing that it is disrupted during radiotherapy and has influenced the translocation of *Gemmata* from the digestive tract to the blood of patients receiving this therapy. Our observations revealed a strong disturbance of the bacteria contained in the irradiated stools.

Key words : *Gemmata massiliana*, *Gemmata obscuriglobus*, *Planctomycetes*, opportunistic pathogens, iron, siderophores, ferrireductase, marine sponges, X-ray.

Introduction

Une étude de la flore planctonique du lac Lágymányos (Budapest, Hongrie) en Septembre 1924 a permis à Nándor Gimesi (biologiste hongrois) de découvrir des microorganismes à morphologie inhabituelle, en forme de rosettes, flottant dans l'eau eutrophique du lac (Gimesi, 1924). La morphologie cryptique de cet organisme a été interprétée par Gimesi comme étant des conidies et des conidiophores. En effet, dans sa description, Gimesi a assimilé le microorganisme à un champignon sporulant avec des « thalli » sphériques formés de structures qui rappellent les couches d'aiguilles de pin rayonnant à partir d'un centre commun. Ce microorganisme a été nommé *Planctomyces bekefii* Gimesi 1924 (*Planctomyces*, champignons flottant) en hommage de Remigus Békefi (1858-1924), un prêtre et professeur hongrois décédé la même année (Gimesi, 1924). Ainsi, le premier représentant du phylum des *Planctomycetes* (dérivé du genre *Planctomyces*) a été décrit sur une simple observation microscopique comme un champignon. Une décennie plus tard (1935), Henrici et Johnson, n'ayant pas connaissance des travaux de Gimesi observèrent à partir de l'eau du Lake Alexander (Minnesota, USA), des organismes similaires attachés à des pédoncules sur lesquelles sont incrustés des dépôts d'hydroxyde ferrique qu'ils nommèrent *Blastocaulis sphaerica* en les assimilant cette fois-ci à des bactéries (Henrici and Johnson, 1935). Au fil des années, un nombre important de micro-organismes apparentés, en forme de spores, bourgeonnants, ont été signalés à plusieurs reprises dans divers habitats, notamment dans des étangs piscicoles, des ruisseaux de forêt, des sédiments marins, en eaux douces, saumâtres et marines dans tous les continents (Hirsch, 1968a, 1968b, Hirsch and Rheinheimer, 1968; Hortobágyi, 1965, 1968; Ruttner, 1952; Wawrik, 1952; Zavarzin, 1960, 1961). Toutes ces descriptions se faisaient sur la simple base des observations morphologiques. Ainsi, depuis la confusion de Gimesi et la re-description de Henrici et Johnson, les controverses se sont exacerbées pendant un demi-siècle jusqu'à ce que Peter Hirsch fournisse en 1972, une

nouvelle description avec des preuves concluantes que *Planctomyces bekefii* et *Blastocaulis sphaerica* sont indiscernables et que les deux microorganismes sont des bactéries plutôt que des champignons. Hirsch propose alors de relocaliser le phylum *Planctomycetes* parmi le monde des bactéries. Bien que *Planctomyces bekefii* ait été décrite comme un champignon sans détails physiologiques, la priorité a été donnée à ce nom et *Blastocaulis sphaerica* a été considéré comme un synonyme (Hirsch, 1972). L'étymologie déroutante de *Planctomycetes* (Signifiant "champignon flottant") a été conservée telle quelle, expliquant ainsi le nom actuel de ce phylum : *Planctomycetes*.

Curieusement, aucun des auteurs cités plus haut n'a réussi à isoler un *Planctomycetes* en culture pure malgré les tentatives d'enrichissement. Ce n'est qu'en 1973 que James Staley a réussi ce pari grâce à l'utilisation de milieux nutritifs dilués oligotrophes (Staley, 1973). En 1976, la première espèce de *Planctomycetes* (*Planctomyces mavis*) a été formellement décrite sur la base des caractères morphologiques, phénotypiques, génotypiques et culturaux (Bauld and Stanley, 1976). En 2006, un superphylum PVC a été désigné pour incorporer les phyla *Planctomycetes, Verrucomicrobia* et *Chlamydiae* sur la base de données phylogénétiques (Wagner and Horn, 2006). Récemment, cette taxonomie a été élargie et le superphylum PVC regroupe actuellement sept phyla bactériens (*Planctomycetes, Verrucomicrobiae, Chlamydia, Lentisphaerae, Poribactéries, OP3, WWE2*) (Gupta et al., 2012; Pinos et al., 2016; Siegl et al., 2011; Ward et al., 2006a)

A l'interface de la cellule procaryote et eucaryote, les *Planctomycetes* apparaissent comme un phylum ayant brouillé la ligne de démarcation entre ces deux entités. Leur morphologie et leur position phylogénétique font d'elles aujourd'hui, des organismes modèles particulièrement fascinant qui alimentent les débats sur la théorie l'évolution (Fuerst, 2012, 1995, 2004; Pinos et al., 2016b). En effet, elles présentent de nombreuses caractéristiques qui rappellent celles

de la cellule eucaryote telles que la compartimentalisation intracellulaire, très caractéristique des planctomycetes en général et en particulier chez le genre *Gemmata* où le nucléoïde est entouré d'une double membrane (Acehan et al., 2014; Fuerst, 2005; Fuerst and Webb, 1991), bien que des études aient contesté cela en l'assimilant plutôt à une invagination de la membrane externe (Santarella-Mellwig et al., 2013). Plus récemment, des structures proches des pores nucléaires des eucaryotes ont été rapportées au sein de ces systèmes endomembranaires (Sagulenko et al., 2017). Aussi, ces bactéries sont capables d'endocytose (Fuerst and Sagulenko, 2010; Lonhienne et al., 2010), de synthèse de stérols (Gudde et al., 2019; Pearson et al., 2003), d'oxydation de l'ammoniac dans une double membrane interne (anammoxosome) en anaérobie (anammox), propriété mise à profit dans le traitement des eaux polluées (Jetten et al., 2001; Niftrik and Jetten, 2012). Elles se distinguent également des bactéries ordinaires Gram positif et Gram négatif par une paroi bactérienne protéique, dépourvue de peptidoglycane (Cayrou et al., 2012; König et al., 1984; Liesack et al., 1986) bien que la présence de peptidoglycane soit controversée (Jeske et al., 2015). Cependant, elles résistent aux antibiotiques ciblant le peptidoglycane (Betalactamines et Glycopeptides) (Cayrou et al., 2010). La présence de lipopolysaccharide, dévolue aux bactéries Gram négatif, vient d'être rapportée (Mahat et al., 2016). Elles sont très fastidieuses, à croissance très lente, se divisent de manière indépendante de FtsZ via une fission polaire par bourgeonnement. Ceci contraste avec les bactéries ordinaires pour lesquelles FtsZ est la protéine de division cellulaire centrale (Bernander and Ettema, 2010; Fuerst, 1995; Pilhofer et al., 2008).

Certains membres du superphylum *PVC* sont des agents pathogènes classiques aussi bien connus chez les animaux que chez l'homme (*Chlamydiaceae*) (AbdelRahman and Belland, 2005; Belland et al., 2004). La bactérie *Akkermansia muciniphila*, membre du phylum des *Verrucomicrobia*, a été isolée des selles et dans le sang (septicémie) de patients suite une forte

colonisation du microbiote intestinal par celle-ci dans un contexte d'antibiothérapie à large spectre et pourrait donc être un pathogène opportuniste. Par ailleurs, certains auteurs rapportent que cette bactérie dégradant la mucine, est inversement associée à l'obésité, au diabète, et semble être un bon biomarqueur reflétant l'état de santé (3-5 % de la diversité totale du microbiote intestinal) de l'intestin et l'équilibre métabolique (propriétés probiotiques) (Derrien et al., 2008; Devos and Ward, 2014; Dubourg et al., 2013, 2017). Quant aux bactéries classées au sein du phylum des *Planctomycetes*, le genre *Gemmata* est le seul ayant suscité un grand intérêt en microbiologie clinique ces dernières années (Aghnatios and Drancourt, 2016; Cayrou et al., 2010, 2013a; Drancourt et al., 2014). Ce genre englobe non seulement un vaste ensemble de bactéries non-cultivées dont les séquences nucléotidiques ont été détectées dans divers habitats et chez l'homme, mais également deux espèces cultivées : *Gemmata obscuriglobus* et *Gemmata massiliana* (Aghnatios and Drancourt, 2015, 2016; Aghnatios et al., 2015; Franzmann and Skerman, 1984a). Des séquences d'ADN de ces dernières ont été détectées dans les selles de patients atteints d'endocardite et d'individus sains, indiquant qu'elles sont membres du microbiote digestif humain (Cayrou et al., 2013a). En outre, en utilisant une approche de PCR suivie de séquençage, des séquences d'ADN proches du genre *Gemmata* ont été détectées dans le sang de deux patients fébriles (2/100 testés) atteints de leucémie et d'une neutropénie aplasique. Il était peu probable que ceci corresponde à une contamination puisqu'aucun ADN de *Planctomycetes* n'a été détecté chez les témoins (Drancourt et al., 2014). Cependant, malgré plusieurs tentatives d'isolement, aucun isolat de *Planctomycetes* n'a été obtenu en culture en raison de défaillance des systèmes de cultures automatisés en vigueur dans notre laboratoire pour détecter de telles bactéries (Christen et al., 2018). L'isolement de *G. massiliana* (Aghnatios and Drancourt, 2015, 2015) dans l'eau du réseau hospitalier à proximité de ces patients a conforté l'hypothèse

d'une porte d'entrée digestive par ingestion d'eau contaminée suivie d'une translocation dans le sang de ces patients immunodéprimés, chez qui un tel mécanisme est décrit pour d'autres bactéries du tractus digestif (Tancrède and Andremont, 1985).

Connues pour leur multirésistance à la plupart des antibiotiques couramment utilisés dans les traitements empiriques aux posologies usuelles (Cayrou et al., 2010), leur proximité phylogénétique avec les pathogènes *Chlamydia* et les *Verrucomicrobia* (Wagner and Horn, 2006), et leur association récemment démontrée chez l'homme (Cayrou et al., 2013a; Drancourt et al., 2014), le rôle des *Gemmata* comme pathogènes opportunistes (Aghnatios and Drancourt, 2016) devrait donc justifier des investigations plus poussées avec une mise en avant de la culture bactérienne comme la technique de référence (gold standard) pour la confirmation du diagnostic d'une infection bactérienne.

Ainsi, nos travaux de Thèse ont porté sur la recherche de *Gemmata* spp. dans les échantillons cliniques (Sang et Lavages Bronchiolo-Alvéolaires), à l'étude des interactions des *Gemmata* avec des phagocytes environnementaux et humains *in vitro* dans un premier temps. Ensuite, le manque de performances des outils diagnostiques actuels (PCR et culture) (Christen et al., 2018) nous a conduit à mettre au point des milieux et des méthodes de cultures innovantes sur la base d'une revue de la littérature concernant les différents écosystèmes des *Planctomycetes* ; ceci afin d'explorer plus en avant la relation éventuelle existant entre l'homme et les *Gemmata*. Enfin, nous avons exploré l'impact des rayons X sur les bactéries du microbiote digestif, sachant que celui-ci est perturbé lors de la radiothérapie et pourrait favoriser la translocation des *Gemmata* du tractus intestinal vers le sang chez les patients leucémiques soumis à cette thérapie.

PARTIE I : REVUE

Les planctomycetes dans leurs ecosystemes : une perspective pour ameliorer leur isolement dans les laboratoires de microbiologie clinique.

L'absence de certaines conditions complexes de culture dans les laboratoires de microbiologie clinique contribue souvent à des échecs d'isolement d'espèces bactériennes fastidieuses et non-cultivées (Kaeberlein et al., 2002; Vartoukian et al., 2010; Winkelmann and Harder, 2009). A cet égard, fournir des conditions environnementales et/ou nutritionnelles semblables à celles qui existent dans l'habitat naturel de ces bactéries pourraient être une option intéressante pour favoriser la croissance et le succès de l'isolement des *Gemmata* spp. non-cultivées, dont une petite portion de leur génome a été séquencée à partir de nombreuses niches écologiques.

L'objet de cette revue a consisté donc à suggérer un protocole qui soutienne le mieux la croissance des Planctomycetes en général en étudiant leur distribution, leur abondance et les facteurs physicochimiques de leurs niches écologiques tout en se focalisant sur le genre *Gemmata*. Cette approche qui a pour objectif de reproduire en laboratoire, les conditions naturelles du biotope dans lequel vit un microorganisme, a déjà montré son efficacité dans l'isolement des deux souches cultivables en ajoutant au milieu de base utilisé pour leur culture, une partie de l'eau naturelle d'où elles avaient été isolées (Aghnatios et al., 2015; Franzmann and Skerman, 1984b). Ainsi, nous avons analysé les caractéristiques de ces niches environnementales pour préciser leur distribution, leur abondance et leurs associations à tel ou tel habitats de prédilection comme les éponges, algues, lichens, eaux douces, saumâtres marines, sédiments, animaux et hommes. En outre les facteurs physicochimiques tels que les variations saisonnières (les planctomycetes sont plus abondantes dans leurs niches écologiques en été qu'en hiver), le pH, l'humidité et la température de ces niches ont été discutés. La littérature sur les données de métagénomiques et des séquences des différentes espèces de *Gemmata* non-cultivées détectées dans divers environnements, nous a permis de décrire ces niches environnementales. Également, nous avons mis en exergue la richesse des

Planctomycetes en général dans certaines niches environnementales qui pourraient constituer de nouvelles sources de nutriments naturels pour la préparation de nouveaux milieux de cultures. L'ensemble de ces données expliquent les mécanismes d'association de ces bactéries à croissance lente et leur abondance relative dans des environnements polymicrobiens, pollués, riches en oligo-éléments et leur persistance périodique dans certains habitats. Par ailleurs, un des buts de cette revue était de comprendre les relations écosystémiques des Planctomycetes afin d'utiliser ces données pour jeter les bases d'une nouvelle approche de culture et d'isolement de nouvelles espèces *Gemmata* au laboratoire de microbiologie clinique. Ce travail a fait l'objet d'un article

Article 1

REVIEW ARTICLE

Planctomycetes in their ecosystems: a perspective to improve their isolation in clinical microbiology laboratories

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REVIEW ARTICLE

Planctomycetes in their ecosystems: a perspective to improve their isolation in clinical microbiology laboratories.

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Abstract

Within the Planctomycetes phylum, the genus *Gemmata* represents the only particular group of organisms that has aroused a great interest in clinical microbiology**.** It consists of both a vast world of nucleic acid sequences of yet-uncultured bacteria and only two cultured representatives *Gemmata obscuriglobus* and *Gemmata massiliana*. They are very slow growing and fastidious bacteria. Several Planctomycetes have no cultivable members and are recognized only by detection of their DNAs by molecular methods. Possible explanations for the resistance of certain Planctomycetes to cultivation *in vitro* include unmet fastidious growth requirements; inhibition by environmental conditions (pH, temperature, attachment) or chemical factors produced by neighboring organisms (bacteria, sponge, algae) in mixed cultures; or conversely, dependence on interactions with other organism in the natural environment, without which they cannot survive in isolation. Some bacteria, with metabolic pathways lacking in the necessary genetic material to encode for essential nutrients, frequently rely on close symbiotic relationships with other bacteria for survival and may therefore be recalcitrant to cultivation in purity. Then, the absence of some complex conditions of cultivation in clinical microbiology laboratories has contributed to numerous isolation failures of fastidious species of bacteria that were considered "yet-unculturable". To this end, providing environmental and/or nutritional conditions like those in the natural habitat of these bacteria could be an interesting option for the improvement of culture and the success of isolation.

For decades, most of the plankton studies have focused on the molecular detection of the 16S rRNA gene, and only describe their diversity, distribution and relative temporal or permanent abundance in such or such habitat. Unfortunately, research and analysis on the physicochemical compositions of these habitats of predilections of Planctomycetes remains

scarce. Known for long time as environmental bacteria, Planctomycetes recently faced shift toward bacteria of clinical microbiology interest. To address the needed of new culture media to isolate *Gemmata* species, this review covers the perspective of new culture approaches as well as the current understanding of this conspicuous environmentally and medical important bacterial phylum. Then, we systematically review, the various ecological niches of the natural habitats of the Planctomycetes in general and the *Gemmata* in particular, to have a good apprehension of their nutritional requirements, the physicochemical factors of these natural ecological niches, the current methods of cultivation of the Planctomycetes and their gaps, from a perspective of collecting data in order to optimize conditions and the protocols of cultivation of these fastidious, slow growing and recalcitrant to isolation.

Thus, we found that most Planctomycetes grew in a nutrient-poor, oligotrophic environments with few exceptions, pH ranges from 3.4 to 11. Also, a seasonality variation of abundance was observed, and bloom occurs in summer-early autumn than in winter and spring, correlated with the strong growth of algae in the marine environments. According to temperature, most planctomycetes are mesophilic, but there are some rare thermophilic planctomycetes (50°C to 60°C). Planctomycetes are widespread in freshwater, seawater and terrestrial environments. In some habitats, they are strongly associated with macroalgae, marine sponges, moss and lichens depending on the species and metabolizable polysaccharides by their sulfatases. In many studies, the commonly added nutrients are N-acetyl-glucosamine, yeast-extracts, peptone and some oligo and macro-elements. Some studies found that vitamin B12 and vitamin B6 promoted growth. Low concentration of glucose can be added. For the culture technique, Petri dish method, host-associated extract (macroalgae, sponge extract) conjugated with a diluted basal medium provide favorable results for the success of isolation in pure culture.

Introduction

The history of Planctomycetes dates back to 1924 with a study of the September plankton of Lake Langymanyos (Budapest, Hungary). This study led Nador Gimesi (an Hungarian biologist) to discover for the first time, an unusual planktonic microscopic organism consisting of threadlike forms which bore spherical structures floated in the eutrophic waters (Gimesi, 1924). The cryptic morphology of this organism was interpreted by Gimesi as fungal conidia and conidiophores respectively. Therefore, this organism was described as a fungus, and named *Planctomyces bekefii* Gimesi 1924 (Gimesi, 1924; Jenkins and Staley, 2013). In 1935, Henrici and Johnson found morphologically similar stalked, budding microorganisms in Lake Alexander, Minnesota, USA, but as these authors were unaware of Gimesi's previous report, they named this microorganism which they interpreted as bacteria *Blastocaulis sphaerica* (Henrici and Johnson, 1935). The authors also found spore-forming, drop-shaped, budding bacteria lacking a stalk, which they considered to be identical to *Pasteuria ramosa* Metchnikoff 1888, a spore-forming bacterium that infects *Daphnia* species (Metchnikoff, 1888). These bacteria were later given the name *Blastobacter henricii* (Zavarzin, 1961). Since these early studies numerous authors have reported the presence of the same or similar organisms (*Planctomyes stranskae*, *P. subulatus*, *P. ferrimorula*, *P. condensatus*, *P. guttaeformis*, *P. crassus*) from diverse habitats worldwide, including freshwater lakes, fish ponds, brackish water, aquarium water, marine sediments, forest brook and seawater (Hirsch, 1968, 1974; Hirsch and Rheinheimer, 1968; Hortobágyi, 1965, 1968; J, 1971; Kahan, 1961; OLAH et al., 1972; Razumov, 1949; Ruttner, 1952; Skuja, 1964; Wawrik, 1952, 1956; Zavarzin, 1960). All these organisms were described based on morphological observations with stalks that are heavily encrusted with iron. Peter Hirsch provided a new description and conclusive evidence that *Planctomyces bekefii* and *Blastocaulis sphaerica* were

indistinguishable, being both bacteria rather than fungi. Indeed, Hirsch reviewed properties of both organisms, pointed to their identity and proposed to relocate the phylum *Planctomycetes* among bacteria. Although *Planctomyces bekefii* has been described as a fungus without physiological details, priority was given to this name, of which *Blastocaulis sphaerica* must be considered a later subjective synonym (Hirsch, 1972). The initial misidentification of *Planctomyces* as a fungus, and the confusing etymology of *Planctomycetes* (Gr. adj. planktos wandering, floating ; Gr. masc. n. mukês fungus ; N.L. masc. n. Planctomyces floating fungus.), meaning "floating fungus" (Gimesi, 1924) has been conserved as such and thus explaining the current name of this phylum (*Planctomycetes*). It was not until 1973, that the first isolation of these budding, rosette-forming bacteria in a pure culture was achieved by Staley, who introduced dilute nutrient media into the practice of oligotrophic bacteria cultivation (Staley, 1973). In 1976, the first species of *Planctomycetes* phylum (*Planctomyces mavis* sp. nov.) was formally described on the basis of phenotypic, genetic and cultural characters (Bauld and Staley, 1976). In 1987, Carl Woese stated that *Planctomycetes* had a distant relationship to Chlamydiae (Woese, 1987) and Strous et *al*. later verified this by comparison of 49 concatenated protein sequences (Strous et al., 2006). In the same year (2006), a superphylum was designated to incorporate the phyla *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* on the basis of comprehensive analysis of the 16S rRNAs gene (Wagner and Horn, 2006) and later by 23S rRNA gene sequences (Glöckner et al., 2010). The current taxonomy includes the Planctomycetes within the PVC superphylum, which encompass seven bacterial phyla, and comports 37 species of bacteria whose genome was entirely sequenced. (*Planctomycetes*, *Verrucomicrobiae*, *Chlamydiae*, *Lentisphaerae*, *Poribacteria*, *OP3*, *WWE2*)(Cho et al., 2004; Gupta et al., 2012; Pinos et al., 2016; Siegl et al., 2010; Wagner and Horn, 2006). Some members of PVC superphylum are well-known

pathogens of animals and humans (*Chlamydia*), others are probable opportunistic pathogens for humans (*Planctomycetes* and *Verrucomicrobiae*) (AbdelRahman and Belland, 2005; Belland et al., 2004; Derrien et al., 2008; Devos and Ward, 2014; Drancourt et al., 2014; Dubourg et al., 2013, 2017; Subtil and Dautry-Varsat, 2004). Amongst *Planctomycetes*, the genus *Gemmata* represents the only particular group of organisms that has aroused a great interest in medical and clinical microbiology (Aghnatios and Drancourt, 2016; Cayrou et al., 2013; Christen et al., 2018; Drancourt et al., 2014)**.** It consists of both a vast world of nucleotidic sequences of yet-uncultured organisms and only two cultured representatives, *Gemmata obscuriglobus* and *Gemmata massiliana* (Aghnatios et al., 2015; Franzmann and Skerman, 1984). *Gemmata* organisms colonize the soil (Ivanova et al., 2016b; Stackebrandt et al., 1993; Wang et al., 2002), tap water and hospital chlorinated water systems in proximity with patients (Aghnatios and Drancourt, 2015). Analysis of the 16S rDNA has additionally shown the presence of *Gemmata* in the gastrointestinal tract of patients with endocarditis and healthy individuals, suggesting they were members of the human digestive microbiota (Cayrou et al., 2013). More recently, using a PCR approach followed by sequencing, DNAs sequences close related to the genus *Gemmata* were detected in the blood of two febrile patients with leukemia and aplastic neutropenia (Drancourt et al., 2014). The isolation of *G. massiliana* in the water from hospital network in close proximity to these patients might supported the hypothesis of a digestive tract entry pathway by ingestion of contaminated water followed by translocation in the blood of immunocompromised patients, in whom this mechanism has been described for other bacteria belonging to the digestive tract (Tancrède and Andremont, 1985). Known as bacteria testing most commonly used antibiotics in clinical practice with usual dosages (Cayrou et al., 2010), and for their association recently demonstrated in humans, the great probability about the *Gemmata* to behave as potential

opportunistic pathogens should therefore justify further investigations (Aghnatios and Drancourt, 2016; Cayrou et al., 2013; Drancourt et al., 2014).

Thus, in the practice of clinical microbiology, bacterial culture remains the gold standard for infectious disease confirmations. However, despite several attempts to isolate *Gemmata* from clinical specimens, we failed to isolate any Planctomycetes in pure culture due to their recalcitrance to "traditional" culture. They require a specific culture medium and long incubation times (Aghnatios et al., 2015; Franzmann and Skerman, 1984; Lee et al., 2009). Accordingly, most knowledge regarding the environmental and host-associated microbiota niches of Planctomycetes organisms is derived from DNA-based studies including PCR-sequencing-based analyses and metagenomics studies (Bondoso et al., 2014; Delmont et al., 2018; Vega-Avila et al., 2015). To date, although there have been considerable advances that have made it possible to isolate many species of this phylum, enormous work remains to be done to improve their culture and their isolation, in view of the enormity of sequenced genomes deposited in GenBank but have never yet been isolated in pure culture (uncultured planctomycetes) up to the present day (see review Wiegand et al., 2018 for more details).

For decades, most of the plankton studies have focused on the molecular detection of the 16S rRNA genes and only describe their diversity, distribution and relative temporal or permanent abundance in such or such habitat (Buckley et al., 2006; Nacke et al., 2011; Stackebrandt et al., 1993; Steven et al., 2013). However, research and analysis on the physicochemical compositions of the habitats of predilections of Planctomycetes remains poorly described. In this respect, we systematically review the various ecological niches of the natural habitats of the Planctomycetes in general with a focus in *Gemmata* species, to have a good apprehension of their nutritional requirements, the physical and chemical factors characteristics of these natural ecological niches. Furthermore, we will emphasize the current strategies and methods

of cultivation of Planctomycetes and their gaps, in the perspective to identify issues and/or opportunities in order to optimize the current cultivation conditions and protocols of these slow growing bacteria, recalcitrant and refractory to isolation. We hope that this review could help researchers choose appropriate methods to isolate new species by applying our recommendations.

I. *Planctomycetes* and key characteristics

Planctomycetes (*Planctomycetes* / *Planctomycetia* / *Planctomycetales* / *Planctomycetaceae*) form a fascinating distinct phylum of the domain Bacteria on the basis of the 16s rRNA gene sequence analysis (Schlesner and Stackebrandt, 1986; Ward et al., 1995; Woese, 1987). The phylogenetic relations between PVC superphylum and other bacteria are still a controversial topic (Jun et al., 2010; Kamneva et al., 2012; van Niftrik and Devos, 2017) and the authors argue that they are related most closely to bacterial phyla *Verrucomicrobia* and *Chlamydia* (Pilhofer et al., 2008; Ward et al., 2006, 2015). Some members of this superphylum are classical pathogens of humans and animals (Chlamydia). The authors suggest that members of the mucus-degrading genus *Akkermansia* (Verrucomicrobia) may be potential biomarkers of a healthy gut status as it growth is favored by low availability of enteral nutrients in patients with long-term fasting, malnutrition and long-term betalactamin-antibiotherapy (peptidoglycan-less, and then no-susceptible bacteria) (AbdelRahman and Belland, 2005; Belland et al., 2004; Belzer and De Vos, 2012; Dubourg et al., 2013, 2017; Fujio-Vejar et al., 2017). Currently, the bacteria of the planctomycetes group are considered to be of increasing relevance to at least four major areas of research in microbiology: emerging models for microbial evolution, cell biology, ecology and medical interest (genus *Gemmata*) as

opportunistic pathogens and as sources of novel bioactive compounds (antibiotics and anticancer drugs) (Aghnatios and Drancourt, 2016; Calisto et al., 2019; Drancourt et al., 2014; Fuerst, 1995; Graça et al., 2016; Jetten et al., 2001; Kuypers et al., 2003; Lindsay et al., 2001; Niftrik and Jetten, 2012; Sagulenko et al., 2014; Schmid et al., 2003; van Teeseling et al., 2015). The Planctomycetes comprise 3 orders (*Planctomycetales*, *Phycisphaerales*, and "*Candidatus Brocadiales*") but to date, the class *Planctomycetia* consists of one validly described order (*Planctomycetales*), which encompass three families with standing in nomenclature: *Planctomycetaceae* (Schlesner and Stackebrandt, 1986), *Isosphaeraceae* (Kulichevskaya et al., 2016) and *Gemmataceae* (Kulichevskaya et al., 2017a). At the interface of the prokaryotic and eukaryotic cell, *Planctomycetes* appear as a phylum having blurred the lines of demarcation between these two cellular entities. Their morphology and phylogenetic position make them currently a fascinating model that provokes debates on evolutionary theory with their complex cellular architectures, previously considered to be traits exclusive to eukaryotes (Fuerst, 2012, 1995, 2004; Jun et al., 2010; Kamneva et al., 2012; van Niftrik and Devos, 2017; Pinos et al., 2016). Indeed, they have many characteristics that recall those of the eukaryotic cell such as intracellular compartmentalization, very characteristic of planctomycetes in general and in particular in the genus *Gemmata* where the nucleoid is surrounded by a double membrane (Acehan et al., 2014; Fuerst, 2005; Fuerst and Webb, 1991), although studies have challenged this by likening it to external membrane invagination (Santarella-Mellwig et al., 2013). Some genes were found to match genomic genes found in the domain Eukarya such as the integrin alpha-V and inter-alpha-trypsin inhibitor protein (Jenkins et al., 2002). More recently, nuclear pore-like structures of eukaryotes have been reported in these endomembrane systems (Sagulenko et al., 2017). Also, these bacteria are capable of endocytosis process (Lonhienne et al., 2010), sterol biosynthesis (Gudde et al.,

2019; Pearson et al., 2003), ammonia oxidation in an anaerobic double membrane (anammoxosome) which is an advantage in the treatment of polluted water (Jetten et al., 2001; Niftrik and Jetten, 2012; Park et al., 2017a). They are also distinguished from ordinary Gram-positive and Gram negative bacteria with cell wall stabilized by a proteinaceous layer rather than a peptidoglycan layer; a characteristic shared only with the chlamydiae and mycoplasmas among the Bacteria (Cayrou et al., 2012; König et al., 1984; Liesack et al., 1986) although this is a controversial topic (Jeske et al., 2015). Accordingly, they are resistant to antibiotics targeting peptidoglycan (β-lactams, glycopeptides) but they are susceptible to antibiotics targeting protein synthesis (chloramphenicol, tetracyclin, doxycyclin, minocyclin, erythromycin, clindamycin) or DNA replication as Fluoroquinolone (Cayrou et al., 2010; Godinho et al., 2019). The presence of lipopolysaccharide, devolving to Gram-negative bacteria has also been reported (Mahat et al., 2016). The *Planctomycetales* is an order, which accommodates budding, fastidious bacteria, slow growing and divided independently of FtsZ via a polar fission by budding. This contrasts with ordinary bacteria for which FtsZ is the central cell division protein (Bernander and Ettema, 2010; Fuerst, 1995; Pilhofer et al., 2008). Indeed, *Gemmata*, like other *Planctomycetale* bacteria, have a polar, yeast-like cell division. During division, the cells attach their vegetative poles to surfaces with stalks or excreted substances. In budding reproduction, the mother cell is preserved and a new daughter cell is formed, unlike in binary fission where the parent cell splits into two equal daughter cells. In budding, the cell wall extends from one point instead of growing evenly throughout the cell which allows polar division. From this aggregate a motile cell is formed. Members of Planctomycetes are also characterized by a non-cellular stalk terminating in a holdfast opposite the reproductive cell pole (Fuerst, 1995). They are very slow growing fastidious bacteria and several Planctomycetes have no cultivable members and are recognized only by

detection of their DNA by molecular methods (see review Wiegand et al., 2018). They represent an ubiquitously distributed bacterial group in diverse habitats.

II. *Planctomycetes* and their habitats

The application of cultural en culture-independent approaches had already revealed and is still revealing a wide distribution of planctomycetes of a wide variety geographically and ecologically from various habitats (Bergmann et al., 2011; Buckley et al., 2006; Kamneva et al., 2012). Since the observations of *Planctomyces bekefii* in a pond in Budapest (Gimesi, 1924), numerous reports of similar morphotypes and other types of budding bacteria in Africa (Woebken et al., 2007), Europe (Aghnatios et al., 2015; Chouari et al., 2003; Slobodkina et al., 2015), South East Asia (Bolhuis et al., 2014), and America (Kuske et al., 1997) have been followed and widely spread across all continents. Planctomycetes are widely distributed in terrestrial (Buckley et al., 2006; Ivanova et al., 2016b; Slobodkina et al., 2015; Stackebrandt et al., 1993; Wang et al., 2002) and aquatic environments (Aghnatios and Drancourt, 2015; Franzmann and Skerman, 1984; Gimesi, 1924; Glöckner et al., 2003; Pimentel-Elardo et al., 2003; Schlesner, 1994; Sipkema et al., 2011; Webster and Taylor, 2012, 2012; Woebken et al., 2007). These organisms have been found to be abundant in brackish and_marine water (Hempel et al., 2008; Wang et al., 2002; Woebken et al., 2007), freshwater (Andrei et al., 2019; Bondoso et al., 2011; Franzmann and Skerman, 1984; Wang et al., 2002), wastewater (Chouari et al., 2003; Lage et al., 2012). Both terrestrial and aquatic habitats differing in salinity (from hypersaline to freshwater), oxygen availability (from the oxic water-column to anoxic sediments), trophic level (from oligotrophic lakes to eutrophic wastewater) and temperature (from cold-water marine snow to hot springs) (DeLong et al., 1993; Giovannoni

et al., 1987; Kahan, 1961; Kerger et al., 1988; Kirkpatrick et al., 2006; Miskin et al., 1999; Schlesner, 1994; Slobodkina et al., 2015, 2016; Vergin et al., 1998)

III. Cultured *Planctomycetes*

The first report of the isolation of a planctomycete in axenic cultures is due to the work of James T. Staley (Staley, 1973). Recent study found that 44 planctomycetal species are validly described, with the vast majority (28) belonging to the family *Planctomycetaceae* (Wiegand et al., 2018). Although still small, last decade several members of planctomycetes were isolated in pure cultures and described as representing the new genera, namely *Schlesneria*, *Singulisphaera*, *Zavarzinella*, *Telmatocola*, *Paludisphaera*, *Fimbriiglobus*, and *Tundrisphaera* (Kulichevskaya et al., 2007a, 2008, 2009, 2012a, 2012b, 2016, 2017a, 2017b). The cultured strains continue to increase due to various methods of isolation (Gade et al., 2004; Lage and Bondoso, 2012; Pimentel-Elardo et al., 2003; Schlesner, 1994; Wang et al., 2002). However, the cultured strains are not at all representative of the great diversity and ubiquity that has been revealed by molecular microbial ecology techniques (Fuchsman et al., 2012; Hamersley et al., 2007; Ivanova and Dedysh, 2012; Kirkpatrick et al., 2006; Penton et al., 2006; Pizzetti et al., 2011a; Pollet et al., 2011a; Schmid et al., 2007). Recent study revealed that on the 8.312 operational taxonomic units (OTUs) defined by a 99% identity threshold (full length 16S rRNA (SILVA SSU Ref NR99 database (release 128 from 07-09-2016) (Quast et al., 2013), only 0.6% of the known planctomycetal diversity on OTU level is covered by axenic cultures (Wiegand et al., 2018). There are very likely even more diverse linages of Planctomycetes out there that have so far escaped detection.

IV. Yet Uncultured *Planctomycetes*

Owing to difficulties to obtain planctomycetes in pure culture, the number of characterized Planctomycetes is quite limited and therefore, most studies frequently are focused on the 16S rRNA gene-based detection from various habitats (Cayrou et al., 2013; Chouari et al., 2003; Ivanova et al., 2016, 2018; Kulichevskaya et al., 2006; Shu and Jiao, 2008; Yang et al., 2016). Furthermore, the low number of Planctomycetes must be interpreted with caution, because the 16S rRNA genes of these bacteria do have mismatches to some PCR primers that are widely used in environmental diversity surveys (Pollet et al., 2011a). This characteristic might lead therefore to underrepresentation of the Planctomycetes in environmental 16S rRNA libraries. (see above). Less than 4% of the existing planctomycetal OTUs are partly sequenced and 99.4% still await cultivation. Metagenomics studies contribute about 250 potential planctomycetal bins which would correspond to 3% of the known diversity, (Anantharaman et al., 2016; Baker et al., 2015; Dudek et al., 2017; Kim et al., 2016; Park et al., 2017b; Tully et al., 2017; Vollmers et al., 2017) while only 4 (0.05%) of the known planctomycetal genomes were obtained via single cell approaches clone sequences belonging to Planctomycetes have been isolated in pure culture (Quast et al., 2013; Wiegand et al., 2018). Within the classes of Planctomycetes, *i.e Planctomycetia* and *Phycisphaerae*, the anammox Planctomycetes form the class Brocadiaceae, which has Candidatus status due to the lack of an axenic culture (for review see Kartal et al., 2013), although sequences that are phylogenetically affiliated with cultured heterotrophic Planctomycetes were identified, the majority of the sequences belonged to several globally distributed, as-yet-uncultured Planctomycetes lineages. (Elshahed et al., 2007). Taken together, the phylum Planctomycetes is heavily under sampled and most planctomycetes remain uncultivated although their partial genome have been detected in various environment. The current challenges of this group, together represent the need to

isolate new strains in pure cultures to extend our knowledge of their physiological role in microbial communities, and medical interests

V. Opportunistic *Planctomycetes*

Some members of PVC (*Planctomycetes-Verrucomicrobia-Chlamydia*) superphylum are among the most successful human pathogens. Indeed, the pathogenicity of *Chlamydia* is no longer to be demonstrated (AbdelRahman and Belland, 2005; Belland et al., 2004). The members of the phylum Verrucomicrobia were also found in the stool (Dubourg et al., 2013) and blood (Dubourg et al., 2017) of patients. The balance of the genus *Akkermancia* in the human gut microbiota is considered today as a potential biomarker of a healthy gut status (see above for more details)(Belzer and De Vos, 2012) and Crohn disease (Tedjo et al., 2016). More recently, the genus *Gemmata* have been detected also in stool and blood samples of immunocompromised patients, making this particular bacteria a genus of medical interests among the members of *Planctomycetes* (Aghnatios and Drancourt, 2016; Cayrou et al., 2013; Drancourt et al., 2014). The genus *Gemmata* forms a large group of so-called "uncultivable" and "cultivable" bacteria among which only two species (*Gemmata obscuriglobus*, *Gemmata massiliana* have been isolated, cultivated and taxonomically described today. Also, there are most likely new *Gemmata* species whose partial DNA sequences have been detected in different habitats but have hitherto escaped isolation in pure culture.

V. 1. Cultured *Gemmata* spp.

V.1.1 *Gemmata obscuriglobus*

By analyzing a sample of water collected from the surface waters of Maroon Dam in southeastern Queensland, Australia in 1984, Franzman and skerman isolated for the first time *Gemmata obscuriglobus (*L.v. gemmare: buds, L. adj. obscurus. dark and L.n. globus meaning a sphere*)* which remained, for three decades, the sole taxonomically characterized member of the lineage of the genus *Gemmata* (Franzmann and Skerman, 1984). *Gemmata obscuriglobus* is represented by single, spherical and stalk-less cells and this planctomycetes is a distinctive member of the deeply divergent PVC superphylum. Similar to other members of the phylum *Planctomycetes*, *G. obscuriglobus* has a number of evolutionary interesting features distinct from typical Gram-negative bacteria which make it an emerging model for bacterial and evolutionary cell biology (Fuerst, 1995; Fuerst and Sagulenko, 2011; Lieber et al., 2009a; Lonhienne et al., 2010; Sagulenko et al., 2017). This budding bacterium had a life cycle, with a multitrichous swarm stage, and produced a phase-dark inclusion of packaged ribosomes and nuclear materials. *Gemmataceae* in general have the largest genome (the mol % G + C of DNA was 64.4 ± 1.0). This bacterium has become today the key bacterium of the study of evolution with many debates around its unusual morphology. Indeed, the nucleus is surrounded by a double membrane that reminds the morphology of the eukaryotic cell, the bacterial cytoplasm is compartmentalized, it is capable of endocyting macromolecules (Fuerst, 1995; Fuerst and Sagulenko, 2010), a phenomenon hitherto devolted to the eukaryotic cell, sterol synthesis (Gudde et al., 2019; Pearson et al., 2003), resists high dose of radiation and environments polluted by heavy metals (Aghnatios and Drancourt, 2016; Lieber et al.,

2009) ; The absence of the protein Fstz, divide by a budding process, the long doubling time (13 hours) (Lee et al., 2009) make it a very fastious and slow-growing bacteria.

V.1.2 *Gemmata massiliana*

The detection of *Gemmata*-like DNAs in stool (Cayrou et al., 2013) and blood (Drancourt et al., 2014) of aplastic patients with neutropenia has raised doubts about their opportunistic pathogenicity. In this respect, Rita agnanthos (Aghnatios et al., 2015) undertook a study on the sources of contamination of these germs in the hospital environment of these patients. Thus, by analyzing the hospital water network close to these patients, she isolated the second member of this genus, ' *Gemmata massiliana* ' of the genus *Gemmata* almost 30 years after the isolation of the first (due to their recalcitrance traits to resist isolation), using a new culture medium that she has developed. *Gemmata massiliana* is fastidious and slow growing bacteria when grow on axenix culture compared to *G. obscuriglobus* (personal data), resistant to β-lactam antibiotics (peptidoglycan-less) and shares a 97 % 16S rRNA gene sequence similarity with, *Gemmata obscuriglobus.* It is an aerobic oligotrophic microorganism with optimal growth at temperature range (mesophile, an optimum at 30°C). the optimal pH ranges at 6–8; and the salinity ≤1.25 % NaCl. As *Gemmata obscuriglobus*, its 9,24 bp genome consists in one chromosome and 64.07 % G + C content (Aghnatios et al., 2015).

V.2. Uncultured *Gemmata* spp.

The two cultured strains of the genus *Gemmata* described above (Aghnatios et al., 2015; Franzmann and Skerman, 1984) are not at all representative of the great diversity and ubiquity of these bacteria. Indeed, techniques of direct molecular tools to estimating microbial community diversity in environmental habitats approaches have broadened and deepened our

knowledge, revealing the distribution of *Gemmata* species not only in habitats where they have been commonly cultured but also many where culture has not yet been attempted or failed to isolate news species. In this respect, *Gemmata* -related 16S rRNA gene sequences have been retrieved from diverse habitats including, municipal wastewater treatment plants (Chouari et al., 2003), freshwater in polluted rivers in Germany (Brummer et al., 2004), non-sulfur, sulfur and iron geothermal steam vents (Benson et al., 2011), sulfidic water, tailing ponds (Ramos-Padrón et al., 2011), lakes (Pollet et al., 2011b)., sphagnum peat bogs (Kulichevskaya et al., 2006), glacial soil (Yang et al., 2016), soil (Buckley et al., 2006), clean rooms where spacecraft are assembled (Moissl et al., 2007), a water specimen of the Western Pacific Ocean and sediments (Shu and Jiao, 2008), a South African water spring (Tekere et al., 2011), from the freshwater macrophyte *Nuphar lutea* (Ivanova et al., 2018), Northern wetlands environments (Dedysh et al., 2006; Ivanova and Dedysh, 2012; Serkebaeva et al., 2013; Wilhelm et al., 2011), the gastrointestinal tract of fish (van Kessel et al., 2011), and recently from human stool (Cayrou et al., 2013) and blood specimens (Drancourt et al., 2014). All these bacteria are waiting to be cultivated. The great challenges today remain the invention of new approaches and culture strategies to isolate them.

V.3. *Gemmata* - related *Planctomycetes*

Gemmata-like planctomycetes bacteria have been also cultivated from the leakage water of a compost heap (Ward et al., 1995), an Australian soil and ornamental fountain freshwater (Wang et al., 2002), and an acidic boreal sphagnum Peat bogs (Kulichevskaya et al., 2006). Futhermore, two other *Gemmata*-related planctomycetes, *Zavarzinella formosa* (Kulichevskaya et al., 2009) and *Telmatocola sphagniphila* (Kulichevskaya et al., 2012b) have been isolated from northern Sphagnum-dominated wetlands. Recently, a peat-inhabiting planctomycetes, *Fimbriiglobus ruber*, which is related to *G. obscuriglobus* but represents a

novel genus and specie has been isolated (Kulichevskaya et al., 2017a). In contrast to stalk-less *Gemmata*, some cells of these planctomycetes form thick stalks and can be assembled in large rosette-like clusters (*Zavarzinella*) or dendriform-like structures (*Telmatocola*). All these planctomycetes belong from *Gemmataceae* family (Kulichevskaya et al., 2009, 2012b, 2017a).

VI. Composition of media existing to date for their isolation and gaps

James T. Staley is the one who laid the first foundation of Planctomycetes in axenic cultures using a chemoheterotrophs low nutrient-media (Staley, 1973). Since then, many species have been isolated by the utilization of media that have a relatively low content by other authors. A great technological accomplishment using several culture methods and media formulations leading to the isolation of a great number of planctomycetes isolates from various habitats were subsequently performed in 1994. These breakthrough were especially due to Dr. Heinz Schlesner's work (Schlesner, 1994). Currently, the formulation and development of many planctomycetes isolation media existing varied with respect to the planctomycetes species targated from diverse habitats with a wide range of salinity (fresh to hypersaline water), pH (4.2-11.6), pollution (oligotrophic vs eutrophic) and few differences in the composition and concentrations used. The axenic cultures using a chemoheterotrophs low nutrient-media containing yeast extract and peptone with sometimes the addition of glucose as a carbon source is useful for the isolation of current planctomycetes cultured organisms. Most authors commonly use the addition of complex vitamins named Staley vitamin solution (see medium 600 from DSMZ), required by some members of the Planctomycetes to the isolation media. Also, some micronutrients (Hutner's salts (see medium 590) and macronutrients as Metals 44"

(see medium 600) are used. In the literature, most culture media used are Staley's maintenance medium (see medium 629) as described from https://www.dsmz.de/microorganisms), the M1N, M31 (Kulichevskaya et al., 2009), PYGV (Peptone, Yeast extract, Glucose supplemented with 20 ml Hutner's basal salts and Vitamin solution) (Staley, 1968) and Caulobacter medium (Christen et al., 2018; Kaboré et al., 2018). The major components of all media are concentration of peptone (0.05% or less), yeast extract (0.1 g/L or less) and glucose (0.025 %) plus trace elements ($MgSO_4$, $CaCO_3$, $FeSO_4$). The introduction of N-acetylglucosamine (1g / L or less), the monomere of chitin, which constitutes both a carbonaceous and nitrogenous source for planctomycetes metabolism has allowed Schlesner to the isolation of numerous strains (Schlesner, 1994). In all these previously described media, in general, planctomycetes are comparatively slow growing organisms with low demand for carbon and nitrogen sources. This makes them difficult to isolate in common media because they are easily outgrown by bacteria with faster growth rates (such as *E. coli*, doubling time: 20 min). Indeed, in a non-selective medium, competition between planctomycetes and fast-growing microorganisms hampers their isolation by agar invasion and depletion of nutrients. However, antibiotics targeting the petidoglycan biosynthesis, such as β-lactam antibiotics (1 mg/L penicillin, 200 mg·mL−1 ampicillin and, 100 mg/L imipenem), aminoglycosides (1000 mg·mL−1 streptomycin), glycopeptides (40 mg/L vancomycin) are commonly added to the growth media and solve this problem with the selective isolation of Planctomycetes. This allowed the authors to obtain a large collection of culturable Planctomycetes (Aghnatios et al., 2015; Lage and Bondoso, 2011; Schlesner, 1994; Wang et al., 2002). Besides the overgrowth of rapid growing bacteria, another problem, commonly faced when isolating bacteria from environmental samples, is the rapid and invasive growth of fungi. The bottleneck in these cases are usually solved by addition to the growth media of cycloheximide or amphotericin B

(Cayrou et al., 2010; Schlesner, 1994; Wang et al., 2002; Winkelmann and Harder, 2009). However, these antifungal compounds have not always proven to be effective and fungicides like pevaryl (econazole nitrate; 1%) and benlate (benomyl, or methyl l-(butylcarbamoyl)-2-benzimidazole carbamate;4 mg·mL−1) appear to be more adequate in inhibiting fungal growth (Lage and Bondoso, 2011). Futhermore, when the targated strains belong from marine sample, the authors commonly use filtired marine water or artificial seawater in order to complement the medium and to reach the bacterial natural environment (Schlesner, 1994). This was especially relevant for marine aquatic planctomycetes. In contrast, when the targated strains is a freshwater planctomycetes, tap filtired waters are used or soil extracts for soil strains (Aghnatios et al., 2015; Wang et al., 2002). For exemple, the culture medium used to isolate *G. obscuriglobus* (freshwater bacterium) consisted of 2.0 µm filtered lake water solidified with 1.5% agar. Micro-colonies were then subcultured using a microloop and a microforge (device and technique described by Skerman) to a new plate to boost their growth (Franzmann and Skerman, 1984; Skerman, 1968). Similar strategy has been used to isolate and culture *G. massiliana* from Hospital network water supplemented with the basic culture medium with the appropriate nutrients (Aghnatios et al., 2015). According to the salinity, temperature, and pH growth, for *Gemmata* spp., freshwater bacteria, salinity must be <0.6%, glucose concentration ≤0.1% and pH growth range at (7.8-8.8). For *Gemmata*-like planctomycetes, salinity must be <0.6% (*Zavarzinella formosa*), glucose concentration ≤0.05% (*Zavarzinella formosa*), ≤0.025% and pH growth range (4.0–7.0), temperature range, 6–30 °C with an optimum at 20-26 °C. Both *Gemmata* spp and *Gemmata*-like should be required a long time of incubation to be isolated. The difficulties related to the culture of planctomycetes are mainly due to their lack of Fstz, their particular mode of reproduction by budding and their generation time relatively very long (e.g., *G. obscuriglobus* with a 13-h

generation time, *Gimesia maris* 13–100 h depending on medium, and the anammox planctomycetes with typical generation times of more 2 weeks) (Fuerst, 1995, 2017; Lee et al., 2009). As an example of recalcitrant bacteria, so far and curiously, no isolate of *Planctomyces bekefii* Gimesi in 1924 was obtained in pure culture although it represents the first morphologically observation of Planctomycetes (Gimesi, 1924). In addition, the need for attachment support to produce a mobile bud in a liquid medium would make the task difficult on ordinary solid agar. Also, the premature drying of agar plates in petri dishes is a one limit for long time incubation. Although still small, last decade several representatives of planctomycetes were isolated in pure cultures and described as representing the new genera, including *Schlesneria* (Kulichevskaya et al., 2007a), *Singulisphaera* (Kulichevskaya et al., 2012a), *Zavarzinella* (Kulichevskaya et al., 2009), *Telmatocola* (Kulichevskaya et al., 2012b), *Paludisphaera* (Kulichevskaya et al., 2016), *Fimbriiglobus* (Kulichevskaya et al., 2017a), and *Tundrisphaera* (Kulichevskaya et al., 2017b). However, altogether, the current cultured strains (Aghnatios et al., 2015; Elshahed et al., 2007; Franzmann and Skerman, 1984; Lage and Bondoso, 2011; Pimentel-Elardo et al., 2003; Schlesner, 1994; Slobodkina et al., 2015; Wang et al., 2002) are not at all representative of the great diversity and ubiquity that has been revealed by molecular microbial ecology techniques (Cayrou et al., 2013; Fuchsman et al., 2012; Ivanova and Dedysh, 2012; Kirkpatrick et al., 2006; Lachnit et al., 2011; Pizzetti et al., 2011a; Pollet et al., 2011a; Woebken et al., 2007) and many news strains are waiting to be cultivated but some bottlenecks will have to be elucidated using their natural environment physicochemical studies. Hence, to circumvent these isolation bottlenecks, news culture media formulation, novel approaches and technical manipulations are needed to recovery news novel strains from various habitats, including human blood which are very important for us.

VII. Physicochemical and environmental factors affecting Planctomycetes geographical distribution

Members of the bacterial phylum Planctomycetes inhabit a wide range of aquatic and terrestrial environments with diverse environmental and physicochemical conditions. However, the relationship between environmental factors, host-associated organisms and Planctomycetes diversity is remain vague.

VII.1. Laltitude

The diversity and abundance of planctomycetes have been studied along a higher and low latitudes in surface seawater of the open sea and in sediment. Planctomycetes diversity in seawater at low latitudes appeared to have higher diversity than mid-latitudes (Shu and Jiao, 2008). Indeed, authors reported that the diversity of planctomycetes in the surface seawater in latitude of the western Pacific Ocean showed that the *Pirellula-Rhodopirellula-Blastopirellula* clade dominated (between 83.3% and 94.1%) the Planctomycetes community at all surface seawater sites, while the minority genera *Gemmata* and *Planctomyces* were present only on sedimentary sites. Moreover, integral-LIBSHUFF software analysis revealed significantly different diversity patterns between in latitudinal surface seawater and in the sediment of one station in the South China Sea (Shu and Jiao, 2008). Another study has showed a sharp decline of planctomycete abundance in most peatland sites in relation to depth while in other particular sites this decline was followed by a type of population in an anoxic part of the bog profile (Ivanova and Dedysh, 2012). Thus,

the fact that certain types of planctomycetes prefer to be abundant on the surface area compared to sediments may involves a tropism for oxygen or nitrogen present in the environmental air and / or attached lifestyle in the sediments. In this respect, recently, some others authors have reported that nitrogen-fixing populations of Planctomycetes and Proteobacteria are abundant in the surface ocean metagenomes (Delmont et al., 2018). However, these parameters remain confused because their distributions could be related to the flowering of lichens, marine sponges, macroalgae and mosses which would provide them with heteropolysaccharides for their metabolisms (Kulichevskaya et al., 2006; Lage and Bondoso, 2011; Webster and Taylor, 2012). In fact, the proportion of sphagnum-dominated wetlands decrease in high latitude regions, where mosses are replaced by lichens (Kulichevskaya et al., 2007b), hence this provokes the moving of planctomycetes via a symbiotic relations. Finally, this statement should be counterbalanced by the fact that the different hydrological and geographic features would contributed to the shift of Planctomycetes diversity in marine environment (Shu and Jiao, 2008)

VII.2. Humidity

The bacterial community richness and diversity are significantly positively correlated with environment with relative humidity. Indeed, a study conducted to evaluate fields in drylands worldwide using DNA-sequencing approaches has found that increases in aridity reduce the diversity and abundance of soil bacteria (Maestre et al., 2015). Strongest and most significant correlations (Spearman's rank correlation [rs] = >0.81; false-discovery rate [q] = ≤0.005) between water rate in soil and phylum relative abundance have been observed for Acidobacteria, Proteobacteria, Planctomycetes (r^2= 0.76), Verrucomicrobia, and Euryarchaeota (Neilson et al., 2017). This makes sense because humidity and water

temperature have the greatest impact on bacterial metabolism (Scofield et al., 2015). Hence, the high rate of humidity is a strong parameter that influences the diversity and abundance of planctomycetes. For example, in the wetlands, Sphagnum-dominated boreal represent one of the most extensive terrestrial environments where Planctomycetes are widespread and abundant. Northern peatlands represent a major global carbon store harboring approximately one-third of the global reserves of soil organic carbon. A large proportion of these peatlands consists of acidic *Sphagnum*-dominated ombrotrophic bogs, which are characterized by extremely low rates of plant debris decomposition. (Bragina et al., 2012; Dedysh and Ivanova, 2019; Dedysh et al., 2006; Moore et al., 2015; Serkebaeva et al., 2013a). Bacterial populations and environmental factors controlling polysaccharids degradation in an acidic Sphagnum peat in the wetland have been described in details in Obukhovskoye, Yaroslavl region, European North Russia. (Pankratov et al., 2011) The predominant populations of planctomycetes are represented by members of the phylogenetic group of the *Isosphaera* and *Singulisphaera* (Ivanova and Dedysh, 2012; Ivanova et al., 2016a; Serkebaeva et al., 2013). However, some authors have repoted that, in some desert soils habitats, planctomycetes may be significantly more abundant and they comprise up to 20% of total bacterial diversity in biological soil crusts inhabiting polar desert soils at the northern land limit of the arctic polar region (Steven et al., 2013). In summay changes in humidity and aridity affect planctomycetes microbial communities.

VII.3. Oxygen requirement

Oxygen requirements of bacteria reflect the mechanism used by them, to satisfy their energy needs. Most members of planctomycetes bacteria, such as *Pirellula, Blastopirellula, Rhodopirellula, Planctopirus, Gimesia*, and *Gemmata* species are chemoheterotrophic

aerobes. However, some oligotrophic (*Isosphaera pallida*) and slow-growing strains have the ability to reduce elemental sulfur to sulfide under anaerobic conditions.(Fuerst, 2017). In Acidic Northern Wetlands, Planctomycetes are most abundant in peatland sites and oxic peat layers are dominated by representatives of the *Isosphaera–Singulisphaera* group, while anoxic peat was inhabited mostly by *Zavarzinella (Gemmata*-like*)* and *Pirellula*-like planctomycetes. However, Planctomycetes related bacteria of the candidate division OP3 are mainly detected in both oxic and anoxic peat layers (Ivanova and Dedysh, 2012). Among Planctomycetes, a particular group of obligately anaerobic, lithoautotrophic, the so-called 'anammox' planctomycetes, have the ability to oxidize ammonium under aerobic conditions. This process is dependent on the anammoxosome (a membrane-bound cell compartment), which might be a functional analogue of the eukaryotic mitochondrion. Anammox planctomycetes have been found in wastewater plants, coastal marine sediments, and oceanic and freshwater anoxic zones (Fuerst and Sagulenko, 2011). They have very slow growth rate (two weeks) and their activity is inhibited by exposure to molecular oxygen even at sub ppm levels, thus it is believed that anammox bacteria are difficult to cultivate (van de Graaf et al., 1996). Futhermore, *Thermostilla marina*, a marine thermophilic anaerobic and microaerobic planctomycete from a submarine hydrothermal vent environment can definitely use elemental sulfur as an electron acceptor generating sulfide as well as being able to respire with nitrate, using mono-, di-, or polysaccharides as electron donors (Slobodkina et al., 2016). Anaerobic metabolic abilities are widely distributed among all major Planctomycetes, and both cultured heterotrophic strains and yet-uncultured Planctomycetes may have the ability to use carbohydrate fermentation and sulfur reduction as possible mechanisms employed for growth and survival under anaerobic conditions (Elshahed et al., 2007; Spring et al., 2018). As an example, a Zodletone organism in the *Blastopirellula* group grows under anaerobic conditions

with sulfur, probably using carbohydrate fermentation (Elshahed et al., 2007). In summary, Planctomycetes have many capabilities to adapt under aerobic and anaerobic conditions. Under acidic conditions and in low phosphate level as described in ombrotrophic wetlands, Planctomycetes use a sequential methylation to produce larger fractional abundance of mono-di-and Trimethylornithine membrane lipids to ensure their membrane stability under at micro-oxic and acidic stress conditions (Moore et al., 2013).

VII.4 Temperature

Temperature governs the kinds of organisms that can live in rivers and lakes. It is also important because of its influence on water chemistry. Planctomycetes can inhabit numerous environment differing in temperature from cold-water marine snow to hot springs)(Giovannoni et al., 1987; Miskin et al., 1999; Schlesner, 1994; Vergin et al., 1998; Wang et al., 2002; Ward et al., 1995). Most planctomycetes described in pure culture are mesophiles (Aghnatios et al., 2015; Franzmann and Skerman, 1984; Giovannoni et al., 1987; Kulichevskaya et al., 2015), but some moderately thermophilic and thermophilic members exist, such as *Thermostilla marina* (optimum at 55°C), *Tepidisphaera mucosa* (optimal growth at 47–50°C), *Thermogutta terrifontis* (optimal at 60°C), *Thermogutta hypogea* (optimal at 52°C) and they are capable to grow at temperature ranges at 30–68°C (Kovaleva et al., 2015; Slobodkina et al., 2015, 2016). Psychrotolerant bacteria that are capable to grow at low temperatures, down to 4–6°C, such as *Singulisphaera acidiphila*, *Singulisphaera rosea*, *Paludisphaera borealis* and *Tundrisphaera lichenicola* that have been isolated from northern peatlands, and lichen-dominated tundra soils (Kulichevskaya et al., 2008, 2012a, 2016, 2017b). However, Psychrophilic members of this phylum are not yet known. Planctomycetes inhabit various low-temperature ecosystems and they are commonly detected by molecular

surveys. In permafrost-affected soils of arctic and sub-arctic tundra, they comprise one of the minor groups (several percent of total diversity) of the bacterial community (Kim et al., 2014; Wagner et al., 2009). Authors reported that planctomycetes were more abundant in summer than in winter in biofilms of two polluted rivers, suggesting the importance of temperature of planctomycetes growth and abundance (Brummer et al., 2004). This aspect is very important for sampling period for isolation.

VII.5 pH

Planctomycetes can inhabit in different large level of pH growth ranged from 4.2 to 11.6 (Schlesner, 1994). However, most strains are mildly acidophilic and mesophilic organisms capable of growth at pH values between pH 4.2 and 7.1 (with an optimum at pH 6.0–6.5). Some planctomyctes, such has *Zavarzinella formosa*, (Kulichevskaya et al., 2009), *Telmatocola sphagniphila* (Kulichevskaya et al., 2012b) has been isolated at the pH range at 3.8–7.2 from the acidic peat soil of the Sphagnum-dominated ombrotrophic peat bog (Staroselsky moss, Tver region, European North Russia). According to the *Gemmata* species, Both are mesophilic organisms capable of growth at pH values between 6- 8.8 (Aghnatios et al., 2015; Franzmann and Skerman, 1984). The pH is a parameter than exerts an influence on baterial community. Accordingly, two studies have reported that the pH emerges as a filter exhibiting a most important correlation with the distribution of certain soil phyla, and thus has a strong influence on the community composition as a whole a-Proteobacteria, d-Proteobacteria, Planctomycetes and Verrucomicrobia which were strongly correlated with soil pH (both positively and negatively correlation). The acidophilic attributes of some genera belonging to a-Proteobacteria and Verrucomicrobia and the basophilic attributes of some genera belonging to Planctomycetes and d-Proteobacteria were coherent with the correlation

between these taxa and pH levels between these studies (Constancias et al., 2015; Nacke et al., 2011).

VII.6 Seasonal variation associated with the diversity and abundance of planctomycetes

Diversity and seasonal changes of Planctomycetes in river biofilms have been reported. In this study , cell counts of planctomycetes showed that there were high levels of these organisms in the summer and low levels in the winter in biofilms grown in situ in two polluted rivers (Brummer et al., 2003). Indeed, seasonal abundance peaks were observed using fluorescent in situ hybridization methods of the Planctomycetales phylum with a proportion of 7 to 10 % in summer, compared to 2% of the number of DAPIs in winter in these polluted rivers. These seasonal changes appeared to be more related to the relative higher temperature and higher abundance of summer algae (bloom) than the differences in xenobiotic compounds in the two rivers. High levels of Planctomycetales during the production period have also been found in Elbe snow and in a eutrophic lake. Also, the relationship between Planctomycetes and macroalgae had been unveiled by Bengtsson et *al*. 2010, who found that Planctomycetes account for 51–53% of the bacterial biofilm cells in July and September and 24% in February at the surface of the kelp *Laminaria hyperborea* from 2 sites on the southwestern coast of Norway, while Verrucomicrobia, Cyanobacteria, Gamma proteobacteria, Betaproteobacteria, and Bacteroidetes were more sporadically detected. Indeed, the microbial cell density of the biofilm in the surface of the kelp *Laminaria hyperborea* appeared to be highly affected by the seasonal growth cycle of the kelp and was found to be lowest on growing kelp in March (minimum 8.3 $\times 10^2$cells cm^{-2}), while on non-growing kelp in July to February, it was around 1.0 $\times 10^7$cells cm^{-2} with large fluctuations. These observation of the composition of the

bacterial community of the biofilm with a continuous seasonal succession may be explained by the influence of both biotic factors such as seasonal changes in the kelp substrate and abiotic factors such as seawater temperature. The role of planctomycetes as degraders of sulfated polymeric carbon in the marine environment as kelps produce such substance would explained this seasonal fluctuation (Bengtsson and Øvreås, 2010; Bengtsson et al., 2010). Lachnit et *al*. (2011) also found a temporal fluctuation of Planctomycetes in association with *Fucus vesiculosus*, *Gracilaria vermiculophylla* and *Ulva intestinalis* (Lachnit et al., 2011). Another comparative study has shown that seasonal variations in temperature, nutrient concentration (related to temperature), and solar radiation (high temperature in summer) have the potential to change microbial community composition and to promote the occurrence of species that are well adapted to the glacial snow constraints. Indeed, authors reported that Actinobacteria and Proteobacteria were dominant in both glacial snow and glacial soil (ubiquitous) with extensive metabolic versatility whereas, the other phyla, including Planctomycetes and Verrucomicrobia were only dominant in glacial soil (Yang et al., 2016). In summary, generally Planctomycetes are more abundant in samples collected in summer and earlier-autumn than in samples collected in winter and spring. Some authors found that these fluctuations seemed related to the algae or diatom bloom of, which constitute a nutrient source for planctomycetes. In addition, authors state that the successive blooms of , Dinoflagellates and Pennales might provide different types of substrates which support the growth of specialized clades of Planctomycetes (Lage and Bondoso, 2014; Pizzetti et al., 2011b). In this regard, many strains of planctomycetes have been isolated when sampling takes place in summer.

VIII. Ecosystem and chemical composition of planctomycetes habitats.

The planctomycetes are widely distributed in terrestrial (Buckley et al., 2006; Ivanova et al., 2016; Slobodkina et al., 2015) and aquatic environments (Aghnatios and Drancourt, 2015; Franzmann and Skerman, 1984; Gimesi, 1924; Glöckner et al., 2003; Pimentel-Elardo et al., 2003; Schlesner, 1994; Sipkema et al., 2011; Webster and Taylor, 2012; Woebken et al., 2007). These habitats differed in many respects as salinity, pH, temperature and seasonality and the importance of nutrient availability throughout the entire growing season. Indeed, both terrestrial and aquatic habitats differing in salinity (from hypersaline to freshwater), oxygen availability (from the oxic water-column to anoxic sediments), trophic level (from oligotrophic lakes to eutrophic wastewater) and temperature (from cold-water marine snow to hot springs). Since the observations of *Planctomyces bekefii* in a pond in Budapest (Gimesi, 1924), numerous reports of similar morphotypes and other types of budding bacteria in Africa (Slobodkina et al., 2015), Europe (Aghnatios et al., 2015; Chouari et al., 2003; Slobodkina et al., 2015), South East Asia (Bolhuis et al., 2014), and America (Kuske et al., 1997) have been reported. These organisms have been found to be abundant in various aquatic environments including, brackish and marine water (Hempel et al., 2008; Woebken et al., 2007), freshwater (Andrei et al., 2019; Bondoso et al., 2011; Franzmann and Skerman, 1984; Wang et al., 2002) and wastewater (Chouari et al., 2003; Lage et al., 2012).

VIII.1 Planctomycetes associated with aquatic environments

The cosmopolitan distribution of Planctomycetes suggests a wide capacity to adapt distinct habitats with diverse environmental conditions. They are widely distributed in many aquatic environments such as seawater, freshwater, and wastewater. In aquatic ecosystems, they

represent between 0 and 11% of planktonic prokaryotic communities (Bouvier and Del Giorgio, 2007; Gade et al., 2004; Neef et al., 1998; Pizzetti et al., 2011b; Tadonléké, 2007). These observations should be taken with caution because the molecular probes are not very sensitive and specific in view of many mismatches possessed by 16S planctomycetes genes. Despite this low abundance, the role of *Planctomycetes* in aquatic habitat functioning is increasingly recognized, given their involvement in nitrogen and carbon cycling (Kuypers et al., 2003; Schubert et al., 2006; Strous et al., 1999; Tadonléké, 2007).

VIII.1.a Planctomycetes associated with sea water

Members of the bacterial phylum Planctomycetes are reported in marine environment samples worldwide (Bauld and Staley, 1976; Schlesner, 1994, 2015; Winkelmann and Harder, 2009). The diversity and distribution of Planctomycetes in the suboxic zone of the Black Sea have been reported and a gradient in phylotype diversity was found. The distributions of known anaerobic ammonium oxidation (anammox) bacteria, many unknown Planctomycetes, and other phylotypes have been reported in marine habitats. This cosmopolitan distribution in marine water suggests a wide capacity to adapt marine salt tolerance. They have been suggested to play a role in carbon recycling in marine ecosystem. The high numbers of sulfatases in marine planctomycetes characterizes them as specialists for the initial breakdown of sulfated heteropolysaccharides and indicate their importance for recycling carbon from these compounds. (Schlesner, 1994; Shu and Jiao, 2008; Vergin et al., 1998). Some authors reported that in the lightless habitats of the deep-sea cold seeps the most abundant OTUs (21.1%) on chitin-tube forming worms (*Escarpia* sp.) are Planctomycetes, an extreme natural enrichment as they represented no more than 0.1% of the total bacterial detection from the surrounding water or sediments. Also, in association with the deep-sea octocoral *Paramuricea placomus*,10% of the bacterial community were Planctomycetes (Kellogg et al., 2016). Also,

Planctomycetes have been found to dominate deep sea sediments in the gulf of Mexico (28 ± 3%), where they seem to be involved in the nitrogen cycle and breakdown of detrital organic matter delivered to the sediment as marine snow (Vigneron et al., 2017). Moreover, the massive losses of fixed nitrogen in the environment are mainly caused by planctomycetes living via anammox- the comproportionation of nitrite and ammonia to dinitrogen gas. Planctomycetes are also part of the microbial communities that are attached to macroscopic detrital aggregates, where they are likely involved in the breakdown of complex heteropolysaccharides of host-associated eukaryotes such as macroalgae (Bondoso et al., 2014; Lage and Bondoso, 2011, 2014; Crump et al., 1999; DeLong et al., 1993; Kuypers et al., 2005; van Teeseling et al., 2015).

VIII.1.b Planctomycetes associated with Freshwater

Lakes, ponds, rivers, streams and wetlands that have a low salt concentration (usually below 1%) and serve as habitats are called freshwater ecosystems. Plants and animals in freshwater regions are adapted to the low salt content and would not be able to survive in areas of high salt concentration (*i.e.*, ocean) as *Gemmata* species, taxonomically described , which do not tolerate salt (NaCl) at high level (Aghnatios et al., 2015; Franzmann and Skerman, 1984). Others members of Planctomycetes (*Pirellula*, and *Planctomyces*) clade are usually detected and isolated found freshwaters (Bondoso et al., 2011; Gimesi, 1924; Henrici and Johnson, 1935; Hirsch, 1972; Hirsch and Müller, 1985; Schmidt and Starr, 1978; Wang et al., 2002; Zhang et al., 2015). In a study of bacterioplankton composition using FISH method, some authors have found Planctomycetes in small numbers in all freshwater samples (1 to 3%; 2.8 $\times 10^4$ to 9.0 $\times 10^4$ cells/ml) compared to other clades but there was frequently an uneven distribution of the fluorescent signal in the cell (Glöckner et al., 1999), due to the lack of

probe hybridization to the nucleoid (Fuerst, 1995) and by the fact that the 16SrRNA gene clone libraries of great members have many mismatches to commonly used universal primers, and therefore, they are underrepresented in freshwater environments (Vergin et al., 1998). Another point of view have been reported and argue that sulfate concentrations are low in most freshwater systems (only 100-200 µM) compared to 20-30 mM in seawater (Capone and Kiene, 1988), and sulfate is rapidly depleted with sediment depth, allowing certain Planctomycetes to be specific in freshwater environments near the sediment surface. Also, some authors state that Planctomycetes might be a poor competitor for phosphorus and suggest that the generally well-accepted statement that bacteria (as a whole) are superior to phytoplankton in the ability to obtain phosphorus under phosphorus limitation might not hold for Planctomycetes. Hence, Planctomycetes might be poor competitors for phosphorus that do not respond quickly to the nutrient supply, which may help explain why their abundance is low in aquatic systems (Pollet et al., 2014). However, these observations should be counterbalanced by the Schlesner's statement who found that the highest number of budding aggregate bacteria are mainly found in aquatic habitats in attached state to biofilm surfaces and therefore, the cells are not sampled in the free water column (Schlesner, 1994). Accordingly, from the point of view of the theory of evolution of freshwater species, a recent study reports that the evolutionary history inferences showed that sediment/soil Planctomycetes transitioned to aquatic environments, where they gave rise to new freshwater-specific clades. Indeed, most abundant lineage founded in the freshwater habitats has the most specialized lifestyle (increased regulatory genetic circuits, metabolism tuned for mineralization of proteinaceous sinking aggregates, psychrotrophic behavior) and harbor the smallest freshwater Planctomycetes genomes. This highlighting a genomic architecture

shaped by niche-directed evolution (through loss of functions and pathways not needed in the newly acquired freshwater niche) (Andrei et al., 2019).

VIII.1.c Planctomycetes associated with Wastewater

Pollution is an important driver of biodiversity and ecosystem change throughout all biomes, with particularly devastating direct effects on freshwater and marine habitats (WHO 2018). Planctomycetes, including *Gemmata* species inhabit polluted wastewater habitats and they are well equipped to adapt a large range of such environment (Aghnatios and Drancourt, 2016; Fuerst, 2004). The genera, *Pirellula* (32%), *Planctomyces* (18.4%), *Gemmata* (3.8%), and *Isosphaera* (0.4%) have been detected in an anoxic and aerobic basins and an anaerobic digestor of a municipal wastewater treatment plant (Chouari et al., 2003). The anaerobic so-called anammox Planctomycetes represents the main biotechnological application of Planctomycetes for the removal of ammonium from wastewater (Strous et al., 1999). All known anammox bacteria ("Candidatus" genera "Brocadia," "Kuenenia," and "Scalindua) belong to the bacterial phylum Planctomycetes and their interests has been well known to occur in wastewater treatment facilities (Chouari et al., 2003; Fuerst, 2004; Jetten et al., 2001).

VIII.2 Planctomycetes associated with terrestrial environments

Members of the *Planctomycetes*, which were once thought to occur primarily in aquatic habitats, have been discovered in soils on five continents, revealing that these Bacteria are a widespread and numerically abundant component of terrestrial microbial ecosystems (Buckley et al., 2006; Kuske et al., 1997; Wang et al., 2002). Planctomycetes comprise up to 20% of total bacterial diversity in biological soil crusts inhabiting polar desert soils at the northern land limit of the arctic polar region (Steven et al., 2013). Also, they have been

detected in and the potato rhizosphere (da Rocha et al., 2010), and many strains have been detected and isolated from soil samples (Kulichevskaya et al., 2017b; Kuske et al., 1997; Neilson et al., 2017; Wang et al., 2002; Yang et al., 2016).

VIII.3. Planctomycetes associated with living organisms and their nutritional and intimate relationship.

In the naturel environments, *Planctomycetes* have been found to be associated with specific eukaryotes in close relationship which enable them to expand their physiological capacities. Indeed, they have been isolated from the giant tiger prawn *Penaeus monodon* (Fuerst et al., 1997), macroalga (Lage and Bondoso, 2012, 2014, 2014), marine sponges (Pimentel-Elardo et al., 2003), Sphagnum peat bog (Kulichevskaya et al., 2007a, 2009), lichens (Ivanova et al., 2016b). Their sequences have been also detected in a termite *Coptotermes formosanus* gut (Shinzato et al., 2005), in an acidic Sphagnum peat bog (Dedysh et al., 2006), human oral microbiota (Takeshita et al., 2015), human gut microbiota (Cayrou et al., 2013) and from immunocompromised patient's blood (Drancourt et al., 2014). Some of these host-associated planctomycetes would promote the proliferation of planctomycetes and may be act as potential sources for the isolation of new species or enriched substrata for formulation of new culture media.

VIII.3. a Planctomycetes associated with algae.

A great number of published data has reported that Planctomycetes are frequently associated with the epibacterial community of several macroalgae (Bengtsson and Øvreås, 2010; Burke et al., 2011; Hollants et al., 2013, 2013; Lachnit et al., 2011; Lage and Bondoso, 2011, 2012, 2014; Longford et al., 2007). Indeed, Planctomycetes represent the dominant group in the kelp

Laminaria hyperborea of the total bacterial community with 51-53% of the bacterial biofilm cells in July-September and 24% in February on the basis of FISH observation (Bengtsson and Øvreås, 2010). Lachnit et *al*. also have reported a temporal fluctuation of Planctomycetes in association with *Fucus vesiculosus*, *Gracilaria vermiculophylla*, and *Ulva intestinalis* (Lachnit et al., 2011). The abundant planctomycete populations on kelp surfaces and in association with other macroalga suggest that coexistence with these eukaryotes may be a key feature of many planctomycete lifestyles. The mainly genus of Planctomycetes are represented by *Rhodopirellula, Planctomyces,* and *Blastopirellula* (Lage and Bondoso, 2012) . Some authors speculate that the existence of a specific Planctomycetes communities associated with the algal host is likely independent of geographical variation, suggesting an symbiotic relationship (Bondoso et al., 2014). Furthermore, a specific order of Phycisphaerales, was proposed to accommodate the genus isolated from algae Porphyra sp., Phycisphaera (Fukunaga et al., 2009). Biofilms composed of complex communities at the surfaces of macroalga comprise a large number of bacteria, fungi and other eukaryotes embedded in extracellular polymeric organic colloids such as agarases, carrageenases, alginate lyases, dehalogenases, antimicrobial compounds and they constitute an interesting source of nutrients for Planctomycetes (Martin et al., 2014) as planctomycetes are well known to contain an high number of sulfatase genes (Wegner et al., 2013), which are involved in the degradation of the sulphated polymers produced by the algae. A great number of Planctomycetes strains have been recovered from the surface of macroalgae (Lage and Bondoso, 2011) and this, make evidence that the growth of *Planctomycetes* can be supported by macroalgae compounds, which provide a suitable source of nutrients that would support the growth of specific Planctomycetes. This hypothesis have been supported by growth experiments carried out with water-soluble extracts of *Ulva* sp. and *F. spiralis* (Lage and

Bondoso, 2011). A specific group of OTUs were specifically associated with the type of macroalgae (Bondoso et al., 2014) and suggest the specificity of the sulfatase to metabolize such or such algal sulfated heteropolysaccharides. In summary, marine macroalga have emerged as significant habitats for planctomycetes and sources of inoculum for isolation (Bengtsson and Øvreås, 2010; Lage and Bondoso, 2014). More than 140 planctomycetes from the biofilm community of macroalgae have been isolated by (Lage and Bondoso, 2012). Despite increasing knowledge of the successful association of planctomycetes and macroalgae, considerable effort is required to fully understand this interaction (bacterial attachment via planctomycetes holdfast or stalk and / or via chemotaxis or a symbiotic relationship). An identification and purification of these polysaccharides secreted by algae could be a research track to test them individually and serve as growth factors to enrich new culture media of planctomycetes.

VIII.3. b Planctomycetes associated with sphagnum and lichens

Planctomycetes can also be found in association with lichens (Ivanova et al., 2016a) and Sphagnum (*Sphagnaceae*) peat bogs (Dedysh et al., 2006; Kulichevskaya et al., 2006, 2007a; Pankratov et al., 2011). Authors reported that *Planctomycetes* make up an important part of the bacterial population responsible for *Sphagnum* decomposition, accounting for up to 14% of total bacterial cells (Ivanova and Dedysh, 2012; Kulichevskaya et al., 2006, 2007b). Most *Gemmata* like-related planctomycetes isolated (*Zavarzinella formosa, Telmatocola sphagniphila, Fimbriiglobus ruber*) and detected (DNAs sequences) have been recovery from *Sphagnum* moss-dominated wetlands (Ivanova and Dedysh, 2012; Kulichevskaya et al., 2007a, 2008, 2012b, 2015). Northern peatlands represent a significant global carbon store and commonly originate from *Sphagnum* moss-dominated wetlands. These ombrotrophic

ecosystems are rain fed, resulting in nutrient-poor, acidic conditions. Members of the bacterial phylum *Planctomycetes* have been found to be very highly abundant and appear to play an important role in the decomposition of *Sphagnum*-derived litter in these ecosystems.(Gorham, 1991; Kivinen and Pakarinen, 1981). Growth experiments showed that peat-inhabiting planctomycetes have the ability to catabolize a great number of heteropolysaccharides belonging to Sphagnum peat, as the addition of Sphagnum peat resulted in the relative abundance of planctomycetes compared to the total microbial community (Kulichevskaya et al., 2007a, 2012b; Pankratov et al., 2011). During the last decade, several peat-inhabiting planctomycetes representatives, including the new genera, *Schlesneria*, *Singulisphaera*, *Zavarzinella* (*Gemmataceae*), *Telmatocola*, *Paludisphaera*, *Fimbriiglobus* (*Gemmataceae*), and *Tundrisphaera* (Kulichevskaya et al., 2007a, 2008, 2009, 2012a, 2012b, 2017b, 2017) have isolated in pure cultures. Also, in a recent experiment, performed to compare the microbial communities of two lichen-dominated ecosystems of the sub-arctic zone of northwestern Siberia, that is a forested tundra soil and a shallow acidic peatland, authors have shown that soil and peat layers just beneath the lichen cover were abundantly colonized by Planctomycetes, ranged from 2.2 to 2.7 × 10^7 cells/gram of wet weight, using molecular tools. In addition, authors noticed that lichen-associated assemblages of planctomycetes displayed unexpectedly high diversity, with a total of 1723 OTUs determined at 97% sequence identity. Uncultivated members of the Planctomycetaceae (53–71% of total Planctomycetes-like reads), were the most abundant populations of forested tundra soil while sequences affiliated with the Phycisphaera-related group (order Tepidisphaerales) were most abundant in peat (28–51% of total reads). From both habitats, representatives of the Isosphaera–Singulisphaera group (14–28% of total reads), *Gemmata* (1–4%) and Planctopirus–Rubinisphaera (1–3%) were represented (Ivanova et al., 2016b). Finally, this observation suggests that both

Sphagnum and lichen are a potential sources of growth factors that would revolutionize the culture of yet-uncultured planctomycetes. Despite the increasing knowledge of planctomycetes-lichens-mosses interactions, considerable effort is required to better understand these relationships in order to identify and purify these presumably growth factors secreted by the Sphagnum/lichens to enrich new planctomycetes culture media, as media improvement represent the current challenge for furthur isolation of new strains to enlarge our knowledge.

VIII.3.c. Planctomycetes associated with other bacterial communities

In nature, no organism exists in isolation and biological interactions are inevitable. Most DNAs of the Planctomycetes, including those of the genus *Gemmata* are constantly detected is association with some other bacterial phyla. In general, the bacterial community structures in the most studies, revealed a great association between *Alphaproteobacteria* and *Planctomycetes*, followed by others such as Bacteroidetes Gemmatimonadetes, Verrucomicrobia (Delmont et al., 2018; Kim et al., 2014; Miyashita, 2015; Wang et al., 2011), suggesting symbiotic co-operation with these bacteria. In ecology, these interactions can either be intraspecific, involving only members of the same species, or interspecific, involving one or more different species. Thus, in both aquatic, terrestrial environments and human gut, Planctomycetes are commonly associated with other bacteria-eukaryote where they form multispecies assemblages. While these assemblages can form on basically with all bacteria, Planctomycetes associated with other bacterial clades were found to play important roles in supporting the growth of planctomycetes by providing them with nutrients. Major bacterial groups found in these associations are for example Proteobacteria, Bacteroidetes,

Verrucomicrobia which are dependent on each other. Thus, possible explanations for the resistance of yet uncultured planctomycetes in purity in vitro include: unmet fastidious growth requirements; inhibition by environmental conditions (pH, temperature, attachment) or chemical factors produced by neighboring organisms bacteria in mixed cultures; or conversely, dependence on interactions with other organism in the natural environment, without which they cannot survive in isolation. Some Planctomycetes, with metabolic pathways lacking in the necessary genetic material to encode for essential nutrients, frequently rely on close symbiotic relationships with other bacteria for survival and may therefore be recalcitrant to cultivation in purity. Then, the absence of some complex conditions of cultivation in clinical microbiology laboratories has contributed to numerous isolation failures of fastidious species of bacteria that were considered "unculturable". To this end, providing environmental and/or nutritional conditions like those in the natural habitat of these bacteria could be an interesting option for the improvement of culture and the success of isolation. As an example, we have shown with genomic studies that *Gemmata* spp. lack a complete set of genes involved in iron acquisition and on the basis that in the environment and human microbiota, *Gemmata* organisms live in community with the Proteobacteria prototype *Escherichia coli*, suggesting an iron-based cooperation between them. We tested therefore whether co-culture of *Gemmata* spp. with the well-known Proteobacteria *Escherichia coli* filtrate to improve the growth of *Gemmata* spp.

Thus, our results showed that the number of both *G. obscuriglobus and Gemmata massiliana* colonies where significantly higher on basic medium supplemented with *E. coli* filtrate than on the standard medium ($p<0.0001$) (personal data submitted for publication). Hence, cooperating groups exist between planctomycetes and proteobacteria as *E. coli* for siderophore, which causes the interests of these individuals to be associated with those of the

group and other such as Verrucomicrobia, Actinobacteria. In addition, (Ramos-Padrón et al., 2011) using a molecular approach to assess the prokaryotic community composition as a function of tailings depth in Pond, including a surface water sample, authors found that the surface water harboured entirely different taxa as *Methyloversatilis* ,*Azospirillum* and *Gemmata* from samples analysed from deeper strata where strictly anaerobic taxa were identified. These association may be relying on oxygen requirement or bacterial nutrition cooperation. In summary, in the clinical laboratory, most yet uncultured planctomycetes lack these environmental conditions like those like "*Chlamydia* or virus are need host to express it life". The need to recovery yet uncultured planctomycetes should take these process account to complemented current culture media with other bacterial filtrates.

VIII.3. d. Planctomycetes associated with natural sponges

In the marine environments, Planctomycetes are often associated with sponge surfaces where they form multispecies assemblages which are called biofilms. While these assemblages can form on basically every surface, bacteria associated with aquatic eukaryotic phototrophs, such as macroalgae or seagrass and marine sponges (Pimentel-Elardo et al., 2003), were found to play important roles in supporting the growth of their hosts while they at the same time live off the nutrients provided by the host organism. A great number of studies have revealed that Planctomycetes are commonly associated with marine sponges (Izumi et al., 2013; Mohamed et al., 2010; Ouyang et al., 2010; Pimentel-Elardo et al., 2003; Sipkema et al., 2011; Webster and Taylor, 2012; Zhu et al., 2008). Sponges are hosts to diverse microorganisms that can constitute up to 60% of the total sponge biomass (Hentschel et al., 2006; Taylor et al., 2007; Vacelet and Donadey, 1977; Wilkinson, 1978). Most marine sponges establish a persistent

association with a wide array of phylogenetically and physiologically diverse microbes. Sponge–microbe associations involve a diverse range of heterotrophic bacteria, including Planctomycestes, verrucomicrobia cyanobacteria, facultative anaerobes, unicellular algae and archaea (Hoffmann et al., 2005; Pimentel-Elardo et al., 2003; Scheuermayer, 2006; Schmitt et al., 2012). Symbiotic relationships between sponges and microorganisms contribute to the sponges' health and nutrition. These relationships can involve more than one partner and can vary from mutualism to commensalism to parasitism. In contrast, sponges may offer nourishment and protection to their symbionts (Bultel-Poncé et al., 1999), and the symbionts may benefit the nutrition of their host by translocation of metabolites through for example nitrogen fixation, nitrification and photosynthesis (Ribes et al., 2012; Schläppy et al., 2010; Wilkinson and Fay, 1979; Wilkinson and Garrone, 1980). On the surface of marine sponges, a large diversity of planctomycetes has been observed among bacteria that are constantly resident in the microbial biofilms of the marine environment. These interactions between the two organisms are either promoted by bacterial attachment via planctomycetes holdfasts and / or via chemotaxis or a symbiotic relationship. Indeed, culture-dependent and independent methods have revealed the existence of many cultured and uncultivated species in the epibacterial communities of the marine sponge. Several factors have been implicated in the colonization of sponge surfaces by planctomycetes, including the adhesion factors present on the surface of certain species of planctomycetes (Stalks, holfasts, frimbriae) that favor their attachment to marine sponges. On the other hand, these associations could be explained by the fact that the sponges which secrete various unknown molecules, or sulphated polysaccharides (which are the substrate of the abundant sulfatases produced by the planctomycetes)(Wegner et al., 2013) via the algae hosted by the sponge, or molecules such as siderophores secreted by Proteobacteria associated with biofilms. There appears to be some specificity between certain

planctomycete species that frequently associate with sponges (Pimentel-Elardo et al., 2003). The nature of this association could also be related to the chemical nature of the sulphated polysaccharides produced by each alga associated sponge residing in the biofilms. In addition, since planctomycetes are resistant to many antibiotics (Cayrou et al., 2010), this property would allow them to resist the bactericidal action of several antimicrobial compounds produced by sponges (Doshi et al., 2011) against other bacteria associated in these biofilms community. This selects *Planctomycetes* to the detriment of other bacterial species. Despite increasing knowledge of the successful association between *Planctomycetes* and sponges, considerable effort is required to fully understand this relationship. Accordingly, we have conducted a study on this interaction and observed that the heat-aqueous extract and small tissues of the marine sponges *Spongia officinalis* were able to improve the growth of *Gemmata massiliana* and *Gemmata obscuriglobus* through mechanical and growth factors mechanisms (data submitted for publication in scientific report). In conclusion, an identification and purification of these molecules should be carried out in the perspective to enrich planctomycetes culture media.

VIII.3.e. Planctomycetes associated with human microbiota

At our knowledge, until now, there are no published data that reports the isolation of Planctomycetes in pure culture from polymicrobial samples such as human microbiota. However, *Planctomycetes* organisms are part of the human microbiota and the genus *Gemmata* spp. is the most commonly (50 %) associated with human as their sequences have been found in 6/12 individuals (Cayrou et al., 2013; De Hertogh et al., 2006; Maldonado-Contreras et al., 2011), and recently, their sequences have been detected in the blood

collected from two immunocompromised aplastic patient (Drancourt et al., 2014), although we failed to isolate any planctomycetes in pure culture. Cayrou et *al*. 2010 observed that when patients received peptidoglycan inhibitor antibiotics they had the high prevalence of Planctomycetes, which data are in agreement with the demonstrated resistance of peptidoglycan-less Planctomycetes organisms to such antibiotics (Cayrou et al., 2010; Liesack et al., 1986). Similar opportunistic behaviors have been also reported with the neibouring clade-*Verrucomicrobia* from PVC superphylum (Dubourg et al., 2013, 2017). Originally known as typical environmental bacteria and neglected in clinical microbiology laboratories, recent year, Planctomycetes have gain many interests (Aghnatios and Drancourt, 2016). However, these data reported in humans so far could only represented the tip of the iceberg. Indeed, their DNA remain problematic as the 16S rRNA gene PCR primers routinely used to detect bacteria in microbiology laboratories failed to detect some Planctomycetes organisms (Vergin et al., 1998). In addition, Christen et *al*.2010 on the basis of the detection on rpo*B* gene argue that Planctomycetes escape standard clinical diagnostics and certain methods were suggested to overcome this limitation (Christen et al., 2018). Nowadays, the diversity and variation investigations (antibiotherapy) of human microbiota presents several challenges to microbiologists. Originally dominated by culture-dependent methods for exploring this ecosystem, the advent of molecular tools has revolutionized our ability to investigate these relationships. Microbial culturomic, a concept based on a use of several culture conditions with identification by MALDI-TOF followed by the genome sequencing of the new species cultured had allowed a complementarity with metagenomics but Planctomycetes have been neglected by most human microbiota studies using culturomic approaches. This is due in large part to the fact that they grow very slowly compared to ordinary bacteria (such as *E. coli*) and their low nutrient requirement in the

culture media where they usually grow compared to enriched media commonly used in clinical microbiology. In the future, studies using complementary methods from a broad range of both culture-based and molecular tools will increase our knowledge of the repertoire of this complex ecosystem and host-Planctomycetes mutualism. So far, it remains enigmatic whether Planctomycetes play an active role in human pathogenesis and today, there is no conclusive statistically significant evidence, that members of the phylum Planctomycetes are responsible for any kind of known disease.

IX. Discussion and perspectives of methods and sources for the enrichment and isolation of Planctomycetes in pure culture

With the golden era of the PCR, sequencing and bioinformatic revolution, the abundance, diversity, and ecology of microorganisms gained another dimension. But the true knowledge of bacterial taxonomy, physiology and pathogenicity has long time based only on cultured organisms and presents several challenges for clinical microbiologists. Attempts to the recovery of *Gemmata* organisms from blood samples by manipulating growth media have not met with success (Drancourt et al., 2014), and so far, it remains enigmatic whether Planctomycetes play an active role in human pathogenesis because, there is no conclusive significant evidence, that members of the phylum Planctomycetes are responsible for any kind of known disease unless we bring proof by culture in several patients. Moreover, knowledge on certain aspect of the biology of microorganisms cannot be reached unless the organisms are available in culture but the problem of uncultivability remains a major challenge. Here, we reasoned that uncultivable recalcitrant microorganisms might grow in pure culture if provided with the chemical components of their natural environment. Hence, after going through the

literature data, we found common points about the isolation, culture methods of the planctomycetes strains that have been isolated by the authors, and certain perspective to help both clinical and environmental biologist to improve Planctomycetes cultivability. Thus, James T. Staley is the one who laid the first foundation of Planctomycetes culture using a low nutrient-media. (Staley, 1973). Since then, after great technological accomplishments, several culture methods and media formulations for the isolation of planctomycetes were subsequently performed. Theses breakthrough were especially due to Dr. Heinz Schlesner's work (Schlesner, 1994). The difficulties related to the culture of planctomycetes are due to their lack of Fstz (partly), their particular mode of reproduction by budding and their generation time relatively very long (13h for *G. obscuriglobus* - 2 weeks for anammox, see above) compared to 20 minutes for *E. coli*) (Fuerst, 2004, 2017; Lee et al., 2009). In addition, the need of cell to attach to a solid support (since most budding forms lived in the attached state) to ensure their budding (Henrici and Johnson, 1935) in the natural environment would make the task difficult on ordinary solid agar. Hence, for the success of isolation, several enrichment techniques should be tried, and some enrichment experiments should take several months. The technology of prior enrichment on cover glass and the so-called "Petri-dish method"(Hirsch et al., 1977) have allowed Schlesner's to recover many isolates (257 strains) from various habitats in pure culture after several months, using diluted media (Schlesner, 1994). The petri dish technique, taking a great advantage of attachment of *Planctomycetes* to glass surfaces, was especially important for increasing markedly the numbers of *Planctomycetes* for further isolation (Schlesner, 1994). Indeed, allowing samples with or without addition of low concentrations of substrata to stand for a long time or generally employing nutrient-poor media were most successful. Dilute media were preferred and taking a great advantage since Planctomycetes are overgrown by faster growing bacteria (Hirsch and

Müller, 1985). Also, in a polymicrobial sample such as stool, competition between planctomycetes and fast-growing microorganisms occurs and hampers their isolation by agar invasion and depletes nutrients. However, the fact that Planctomycetes are peptidoglycan-less involves the use of antibiotics (see above) targeting the peptidoglycan to solve this problem and allow Planctomycetes to form colonies on the isolation plates (Cayrou et al., 2010; Godinho et al., 2019). Besides the overgrowth of rapid growing bacteria, another problem, commonly faced when isolating bacteria from clinical samples, is the rapid and invasive growth of fungi (*Aspergillus*, personal data) despite the addition of amphotericin B (Christen et al., 2018). Some authors commonly use cycloheximide to inhibit fungal growth (Schlesner, 1994; Wang et al., 2002; Winkelmann and Harder, 2009) but some others prefer fungicides like pevaryl (econazole nitrate; 1%) and benlate (benomyl, or methyl l-(butylcarbamoyl)-2-benzimidazole carbamate;4 mg·mL−1) for its effectiveness (Lage and Bondoso, 2011, 2012). According to strains isolated so far, the most common technique of isolating planctomycetes in general, and *Gemmata* in particular still consists of taking the natural habitat (depending on whether the habitat is fresh water, marine, brackish, earth extract, tap water ...) where Planctomycetes have detected by molecular tools, to then transport the sample (liquid-solid) of this natural habitat to the laboratory which will serve as a basis for the preparation of enrichment and isolation media. Once in the laboratory, the sample of the natural habitat will be filtered (0.22 µm) and this filtrate will serve as a basis for the preparation of enrichment and isolation media (Aghnatios and Drancourt, 2015; Aghnatios et al., 2015; Fuerst et al., 1997; Schlesner, 1994). Then, the resulted sterile filtrate usually supplemented by the following components (gram per liter of sterile filtrate): peptone (0.05% or 0.025%), yeast extract (0.1 g or 0.025%), glucose as carbon source, N-acetylglucosamine (1g / l, C and N_2 sources) and Staley vitamins solution (see media section above for more details). According

to vitamin addition, only vitamin B12 (cyanocobalamin) and Vitamin B6 (biotin), used together have proved their effectiveness to potentiate the growth of *Gemmata obscuriglobus* in recent study and remain sufficient to restore colony growth to comparable rates as other commonly used media (Mishek et al., 2018; Schlesner, 1994; Staley, 1968). For the addition of micro- and macronutrients belonging from the Hutner's basal salts, (component of Staley'medium) some authors state that it produced favorable results (Cohen-Bazire et al., 1957; Schlesner, 1994), however, for the fresh water bacteria such *Gemmata massiliana* and *Gemmata obscuriglobus* (salinity must be <0.6%), its addition does not proved its effectiveness compared to basic Caulobacter medium (personal data submitted in Frontiers in Microbiology and our longtime observation). In order to improve the Planctomycetes isolation from clinical and environmental laboratory, we recommended, on the basis of the literature data, we suggest that i) sphagum and lichen gained a great attention to be help researchers to isolate *Gemmata*-like planctomycetes and other yet- uncultured planctomycetes from clinical samples as most *Gemmata*-like have been isolated in these natural habitats (*Zavarzinella Formosa*, *Telmocola sphagniphila* and others) from Sphagum-peat bog and lichens (Ivanova et al., 2016a; Kulichevskaya et al., 2008, 2009, 2012b, 2017a, 2017b). ii) Furthermore, many Planctomycetes colonies have been recovered from the surface of small portions of macroalgae and algae water-extracts have proved that the growth of Planctomycetes can be supported by macroalgae compounds (Lage and Bondoso, 2011). This hypothesis has been supported by the nutritional role of macroalgae for Planctomycetes (Lage and Bondoso, 2011, 2012). Indeed, water-soluble extracts (used as macroalgal macerated) of Ulva sp. and *F. spiralis* have proved their effectiveness to improve the growth of some Planctomycetes as certain planctomycetes possess many sulfatases to metabolize the sulfated heteropolysaccharides produced by algae. This explain their strong association with algal

species and has allowed Lage and Bondoso to obtain a large collection of culturable Planctomycetes, essentially from the surface of macroalgae (Lage and Bondoso, 2014). ii) In addition, marine sponge heat- aqueous extracts sterilized by autoclaving should be constitute a potential novel source of growth factor and basic micro-and macronutrient belonging from natural environment to revolutionize Planctomycetes culture. Prior to plated the inoculum into agar plate, the sample should be enriched at 3 or 4 days and more (2-4 month, see (Schlesner, 1994) until a turbidity occurred (Personal experiment performed using natural marine sponge and data submitted in Frontiers in Microbiology). This has been supported by previous studies (Pimentel-Elardo et al., 2003; Webster and Taylor, 2012; Winkelmann and Harder, 2009). Better, the liquid culture should be associated with a solid phase for stalked or budding bacteria attachment during enrichment period. The highest number of cells were usually found on the walls of the vessels and the majority of mature cells are attached to surfaces (sponge, sphagnum and macroalgae). As an example, after obtaining the media, a portion of the sample should be concentrated (*e.g*, 2 grams of wet peat suspended in 10 ml of sterile water and treated in a laboratory stomacher at 240 rpm for 5 min has allowed to isolate *Telmatocola sphagniphila* (Kulichevskaya et al., 2012b) to have a small volume of bacteria to be inoculated. Then, the resulting suspension can be used to inoculate serum bottles containing 90 ml of sterile dilute medium described above (after pH adjustment as close as possible to the natural habitat). The enrichment media should be incubated in the dark or light at room temperature (mesophilic) for at least 2-4 weeks or even 2-7 months (Schlesner, 1994). Method of coverslips or petri-dish described above will provide support of attachment, necessary for the bacteria of interest that are planctomycetes for their attachment. 20-µl aliquots of the resulting enrichment cultures could be plated onto solid medium containing appropriate antibiotic and fungicide described above. The microbial cells that will develop in contact with

the glass can be observed microscopically to detect the presence of cells with a morphology similar to that of planctomycetes in the enrichment broth (budding cells) before seeding on a solid version of the same liquid medium (agar addition). Some plates could be incubated in gastight jars containing 5% CO2 (v/v) in air. Humidification of petri dishes should be controlled to prevent premature drying of agar plates. This humidification could be ensured by introducing the petri dishes in a jar with Kleenex paper soaked in water. Colonies that developed on plates must be screened microscopically for the presence of cells with planctomycete-like morphology. The selected cell material should be re-streaked onto the same medium and this procedure must be repeated until the target microorganism was obtained in a pure culture. Finally, as an alternative, another isolation technique which are relevant and commonly used is the Micromanipulation using the methods and apparatus described by (Skerman, 1968) to separate a *Planctomycetes* isolate from a co-cultured non *Planctomycete* isolate after the failure of conventional sub culturing method.

Conclusions

Planctomycetes are fascinating bacteria with unusual traits which recall those of the eukaryote cell and emerge as a good model for evolutionary studies. (Fuerst, 1995). Indeed, they have managed to intrigue and inspire the scientists from the very start. The morphological and cell biological characteristics, and in some cases, even misguided us researchers. The confusing etymology of Planctomycetes, meaning“floating fungus” (Gimesi, 1924). Far from being a well-known group of bacteria, although their first observation goes back to 1924, it is only since the 1970–1980s that more regular published data began to appear (Bauld and Staley, 1976; Staley, 1973). In this review, culture aspects have been highlighted, providing a summary of the work carried out over recent years with the objective of isolating Planctomycetes from various clinical and environmental samples in pure culture. New isolation approaches by combining a new combination of new media formulation (including sponge extracts, macroalgae macerate, lichens and mosses extracts, *E. coli* filtrate and iron, vitamin B12 and vitamin B 6), culture approaches (Petri-dish techniques, sponge extract-tissues as solid support), antibiotics and fungicides, the use of low nutrient media should be essential for successful isolations. We hope that our review will help researchers achieve this goal.

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PARTIE II :

Diagnostic bactériologique des septicémies et des infections respiratoires à Gemmata spp. à partir d'échantillons cliniques (Sang et Lavages Brocho-Alvéolaires).

INTRODUCTION

Les bactéries du genre *Gemmata* sont des *Planctomycetes* appartenant au superphylum PVC (*Planctomycetes-Verrucomicrobia-Chlamydiae*)(Ward et al., 2006b), regroupant des bactéries environnementales très réfractaires à la culture. Elles sont très fastidieuses avec une croissance très lente, mais surtout un grand nombre d'espèces restent incultivées (Wiegand et al., 2018). Au sein du phylum des *Planctomycetes*, le genre *Gemmata* est le seul ayant suscité un grand intérêt pour la microbiologie clinique (Aghnatios and Drancourt, 2016). En effet, les bactéries de ce genre ont été suspectées d'être responsables d'infections opportunistes chez deux patients fébriles atteints de leucémie associant un tableau clinique de neutropénie, pneumonie et diarrhée (Drancourt et al., 2014). Ce genre englobe non seulement un vaste ensemble de bactéries incultivées dont les séquences nucléotidiques ont été détectées dans divers habitats et chez l'homme, mais également deux espèces cultivées : *Gemmata obscuriglobus* et *Gemmata massiliana* (Aghnatios et al., 2015; Cayrou et al., 2013; Franzmann and Skerman, 1984; Fuerst et al., 1997). Cette dernière a été récemment isolée à partir de l'eau chlorée du réseau hospitalier à proximité des deux patients précédemment cités ; ce qui réconforte l'hypothèse d'une ingestion de ces bactéries via l'eau du réseau hospitalier suivie de leur translocation intestinale occasionnée par la perturbation du microbiote intestinal (antibiothérapie et radiothérapie). Connues pour leurs multirésistances vis-à-vis des antibiotiques utilisés en clinique aux posologies usuelles (Cayrou et al., 2010) et leurs associations récemment démontrées chez l'homme (Cayrou et al., 2013b; Drancourt et al., 2014), le pouvoir des *Gemmata* spp. de se comporter comme des potentiels pathogènes

opportunistes (Aghnatios and Drancourt, 2016) devrait donc justifier des investigations plus poussées.

Ainsi, nous nous proposons dans cette étude, de rechercher les bactéries du genre *Gemmata* dans le sang et les liquides bronchoalvéolaires (LBA) des patients par une approche de biologie moléculaire et par culture bactérienne.

MATÉRIELS ET MÉTHODES.

Cadre et Période d'étude

L'étude a été réalisée à l'Institut Hospitalo-Universitaire de Marseille (Novembre 2016 à Aout 2017) et au Centre Hospitalier Universitaire de Montpellier Arnaud de Villeneuve (Septembre 2017 à Aout 2018).

Echantillons cliniques et Comité d'Ethique : Après obtention de l'avis du comité d'éthique, les échantillons de sang contenus dans des flacons d'hémoculture de patients suspects d'aplasie et/ou de translocation digestive ont été collectés en routine (Marseille uniquement). Egalement, les recherches ont porté sur des LBA pour une éventuelle infection respiratoire opportuniste à *Gemmata* spp. Pour les LBA, il n'y avait pas de critères d'inclusion et tous les LBA de routine étaient collectés et analysés (Marseille et Montpellier). Une petite partie du lot de LBA (92) de Montpellier était congelé (– 80°) avant les analyses.

PCR basée sur le gene *rpo*B

Un système PCR en temps réel, a été utilisé pour la détection spécifique du gène *rpo*B de *Gemmata* spp. en présence de contrôles (positifs et négatifs). Contrôles positifs (*G. massiliana* et *G. obscuriglobus*) réalisés à distance des échantillons pour éviter toute contamination.

Les amorces et sondes

Trois contrôles négatifs (contenant le mélange de PCR sans ADN) et deux contrôles positifs (contenant le mélange de PCR et l'ADN de *G. massiliana* et le mélange de PCR et l'ADN de *G. obscuriglobus*) ont été utilisés pour la validation et l'interprétation des résultats.

Les PCR en temps réel ont été effectuées dans un volume final de 20 µL ; contenant 5 µL d'ADN, 10 µL de mélange de PCR (Eurogentec, Angers, France), 3,5 µL d'eau stérile, 0,5 µL de l'amorce sens 5'-GCAAGCTCAACTCGCTCAAC-3' (40 nmol/L), 0,5 µL de l'amorce antisens 5'-CTTCGAGATGACGCCCTTGT-3' (40 nmol/L) (Eurogentec), et 0,5 µL de sonde TaqMan MGB marquée par le fluorophore FAM 6 5'-ATGGTGAAGGTCTACGTCGC-3' (6 nmol/L) (Applied Biosystems, Courtaboeuf, France). Le cycle d'amplification consistait en une étape de pré-incubation de 2 min à 50°C, suivie d'une étape de dénaturation de 15 minutes à 95°C et de 46 cycles de 30 s à 95°C et 1 min à 58°C. L'échantillon était ensuite refroidi à 45°C pendant 30 s. (Christen et al., 2018)

Extraction d'ADN

Toutes les extractions d'ADN ont été réalisées, en prélevant 200 µL de l'échantillon de sang ou de LBA à tester, sur EZ1 advanced XL en utilisant le kit EZ1 DNA Investigator (Qiagen, Courtaboeuf, France). Le volume d'élution choisi était de 50 µL.

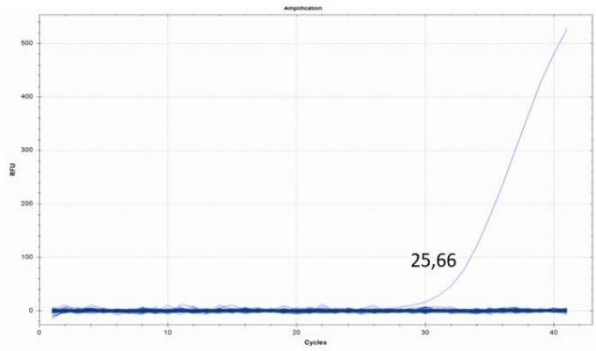
Culture et conditions de culture : les milieux Caulobacter DSMZ 595 et de Staley DSMZ 629 préparés comme décrit sur le site Web (http : //www.dsmz.de) étaient ensemencés avant

la réalisation de la PCR. Ces milieux étaient sélectifs et contenaient 32 mg/L d'amphotéricine B, 40 mg/L de vancomycine et 100 mg/L d'imipenème. Les prélèvements ont été cultivés sur ces milieux solides incubés en aérobiose à 30°C pendant 30 jours. L'observation se faisait chaque 3-5 jours au début (surveillance des contaminations), puis chaque 7 jours avec humidification de la jarre avec du papier Kleenex imbibé d'eau pour éviter l'assèchement prématuré des géloses. Les géloses contaminées (*Aspergillus*) étaient réensemencées systématiquement jusqu'à ce que les 30, jours de culture soient atteints. Des témoins négatifs des LBA (sérum physiologique) étaient ensemencés et incubés en parallèle des LBA.

RÉSULTATS ET DISCUSSION

Il a été récemment rapportée, la détection par PCR et séquençage d'ADN de Planctomycetes, des séquences proches du genre *Gemmata* dans le sang total prélevé chez deux patients (2/100 testés) atteints de leucémie et d'une neutropénie aplasique, en présence de contrôles négatifs (100 donneurs de sang) (Drancourt et al., 2014). Cette unique observation a suscité l'hypothèse que les bactéries du genre *Gemmata* pourraient être des pathogènes opportunistes responsables de bactériémie par translocation digestive, puisque qu'un tel mécanisme est décrit pour d'autres bactéries du tractus digestif chez de tels patients (Tancrède and Andremont, 1985). Afin de conforter cette hypothèse, il était impératif pour nous de conduire cette étude sur des échantillons cliniques afin d'isoler ces bactéries en culture pure (Gold standard). Nous avons donc collecté 426 échantillons cliniques dont 34 échantillons de sang contenus dans des flacons d'hémoculture (anaérobie et aérobie pour chaque patient) de patients supposés atteints aplasiques et/ou d'avoir une translocation digestive (Marseille) et

392 liquides de lavage broncho-alvéolaire (touts patients confondus à Marseille et à Montpellier) afin de rechercher les *Gemmata* par qPCR (basé sur le gèene *rpo*B) et par culture. Chez un patient, une qPCR positive (**Figure 1**) a été obtenue à partir du sang d'hémoculture. Une PCR standard a alors été réalisée mais sans possibilité d'obtenir de l'ADN. Les températures d'hybridation ont ensuite été ajustées et repéter l'opération sans succès. Un séquençage en aveugle a alors été tenté avec les produits de la qPCR et de la PCR standard mais sans possibilité d'obtenir des séquences. Également, aucun isolat de Planctomycetes n'a été obtenu en culture pure à partir de tous les échantillons, autant pour les LBA que les prélèvements sanguins. Les contrôles positifs de *Gemmata obscuriglobus* et de *Gemmata massiliana* étaient positifs respectivement au bout de 2 semaines et 3 semaines de cultures alors que les contrôles négatifs (Serum physiologique, LBA) étaient négatifs au bout des 30 jours d'incubation dans les mêmes conditions.



Quantification Data

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
A07	FAM		Unkn		29,39	29,39	0,000
A08	FAM		Unkn		36,81	36,81	0,000
B01	FAM		Unkn	2	25,66	25,66	0,000

Figure 1 : Courbe qPCR positive d'un patient suspect d'aplasie suivie de translocation.

Actuellement, aucune bactériémie à *Gemmata* spp. n'a été découverte en pratique clinique. Ainsi, si elles existent comme nous le supposons chez des patients en aplasie fébrile, cela signifie qu'elles ne sont pas détectées par la méthode standard appliquée actuellement dans nos laboratoires de routine. Les systèmes automatisés ne s'y prêtent pas pour de telles bactéries. La température de l'automate (37°C) n'est pas optimale, mais elle ne devrait pas être létale. Une des causes la plus plausible serait le bouillon contenu dans le flacon d'hémoculture (Christen et al., 2018). En effet, ces bouillons d'hémoculture sont des milieux enrichis et inhiberaient la croissance de ces germes fastidieux. Les Planctomycetes sont reconnues pour n'avoir qu'une croissance optimale qu'en milieux dilué avec de faibles nutriments, conformément aux observations de Staley, qui a réussi à isoler puis cultiver un Planctomycetes pour la première fois de l'environnement (Staley, 1973). Aussi, Christen *et al*., 2018 ont montré par des modèles expérimentaux que les bactériémies à *Gemmata* spp. présentent une meilleure sensibilité par culture directe sur milieu solide Caulobacter du sang n'ayant pas séjourné dans les bouillons d'hémoculture.

La PCR également semble avoir de moins bonnes performances après un bref séjour dans les flacons d'hémocultures. Les limites de la PCR sur les *Gemmata* et les Planctomycetes en général ont déjà été soulevées par des auteurs (Christen et al., 2018; Pollet et al., 2011), ce qui réduirait aussi le taux de détection. Outre l'action bactéricide du sang par les phagocytes, qui détruisent les *Gemmata* (Kaboré et al., 2018), le séjour des ces bactéries dans le bouillon d'hémoculture est un mauvais pronostic pour la réussite d'une bonne PCR et l'isolement en culture. Une alternative de choix serait donc de préférer un ensemencement direct de sang juste après le prélèvement, au mieux à partir d'un tube hépariné que sur un tube EDTA (Christen et al., 2018).

Par ailleurs, la taille de notre échantillon de sang très réduite par la faible inclusion de patients suspects d'aplasie ne permet pas à présent de remettre en cause la possible d'existence de bactériémies à *Gemmata* spp. En outre, il s'agissait de sang humain non circulant contenu dans des flacons d'hémoculture à dans des conditions ne permettant pas sa conservation. Celui-ci était donc altéré, et la mort bactérienne dans ces conditions ne pourrait préjuger de leur viabilité lors d'une bactériémie chez un patient.

En conclusion, dans ce travail, excepté un échantillon positif de qPCR dans le sang d'un patient avec un résultat positif sans une possibilité d'obtenir de l'ADN à la PCR standard (manque spécificité/sensibilité) et ni des séquences au séquençage en aveugle, nous n'avons pas pu isoler un Planctomycetes en raison de la faible performance de nos outils diagnostiques actuels. L'échec de l'isolement, même avec la durée d'incubation prolongée, implique nécessairement le développement d'autres méthodes de détection et de culture des bactériémies à *Gemmata* spp. Dès lors, nos futurs axes de recherche devront intégrer : i) une amélioration des conditions pré-analytiques (prélèvements sanguins sur tube hépariné et ensemencés le plus rapidement possible dans un délai < 2h, ii) l'augmentation des possibilités d'inclusion de patients neutropéniques (le caractère très bactéricide des phagocytes pour les *Gemmata* spp. écartant tout axe de recherche à explorer chez des sujets immunocompétents) et enfin iii) la recherche d'outils innovants de culture pour l'amélioration des outils diagnostiques de culture pour une croissance accélérée de ces germes très fastidieux.

PARTIE III :

Modèles d'étude des Gemmata comme pathogènes opportunistes à travers la phagocytose.

Les *Gemmata* sont des bactéries essentiellement isolées et détectées dans divers habitats aquatiques et telluriques. Les amibes y résident également. Il a été démontré que les amibes pouvaient se comporter comme un « cheval de Troie » en hébergeant en leur sein d'autres microorganismes comme les Légionnelles, les Mycobactéries et surtout les *Chlamydia.* Ces dernières, membres du superphylum des PVC sont connues comme étant le genre ayant le plus grand nombre endosymbiotes. Ainsi, la survie de ces organismes au sein des amibes permet d'utiliser ces dernières à des fins d'isolement (mycobactéries et légionelles). Cette méthode a déjà montré son efficacité pour isoler de nouvelles bactéries. Nous avons donc testé cette stratégie en co-cultivant les deux souches de *Gemmata* cultivables (*Gemmata massiliana* et *Gemmata obscuriglobus*) avec les cellules monocytaires THP-1 et 3 espèces d'amibes *Acanthamoeba polyphaga. Acanthamoeba castellanii et Acanthamoeba griffini* de façon indépendante. Cette étude présentait trois intérêts majeurs. Premièrement, une coculture avec trois types d'*Acanthamoeba* pour simuler leurs rapports avec les phagocytes environnementaux qui pourraient les héberger et infecter l'homme (cas des légionnelles). Deuxièmement, les *Gemmata* étant des bactéries très fastidieuses à cultiver, nous avons voulu voir si l'amibe permettrait d'offrir un environnement favorable pour la croissance rapide des *Gemmata* et servir comme un outil de diagnostic. Troisièmement, avec les cellules THP, elles ont été co-cultivées avec les *Gemmata* dans le but d'explorer, *in vitro,* la phagocytose pour simuler les conditions d'immunodépressions chez les patients leucémiques (personnes immunodéprimés). Les co-cultures étaient surveillées par la microscopie et des UFCs à chaque temps pendant 72 heures.

Ainsi, nos observations ont révélé qu'après une période d'incubation de 2 heures de temps, les bactéries étaient rapidement internalisées par les phagocytes et au bout de 72 heures de co-culture aucune bactérie n'était plus visible au sein du cytosol des amibes et des

THP-1. Ces observations microscopiques ont été supportées par l'absence de bactérie viable après la mise en culture des lysats du produit de co-culture sur gélose solide (0 CFU) à 72 heures post-coculture.

En conclusion, les données rapportées dans cette étude montrent que la co-culture des deux espèces de *Gemmata* étudiées avec les trois espèces amibiennes entraîne l'internalisation rapide, la lyse et la mort des bactéries *Gemmata*. Les amibes du genre *Acanthamoeba* testées dans notre travail, sont donc peu susceptibles d'héberger des *Gemmata* spp. dans l'environnement. En conséquence, ces amibes ne pourraient donc pas être utilisées pour l'isolement des bactéries *Gemmata* au laboratoire selon les conditions de co-culture utilisées dans cette étude. Enfin, les interactions entre les cellules THP-1 et *Gemmata* spp. pourraient donner un aperçu de l'action des monocytes – macrophages contre *Gemmata* spp. au cours de la colonisation et de l'infection. Seuls les patients présentant une activité macrophagique compromise (immunodépression), tels que les patients aplasiques, pourraient être infectés par *Gemmata* spp.

Article 2

Coculture models illustrates *Gemmata spp*. to phagocytosis.

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Co-culture models illustrate the digestion of *Gemmata* spp. by phagocytes

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Gemmata **spp. bacteria thrive in the same aquatic environments as free-living amoebae. DNA-based detection of *Gemmata* spp. sequences in the microbiota of the human digestive tract and blood further questioned the susceptibility of *Gemmata* spp. to phagocytes. Here, *Gemmata obscuriglobus* and *Gemmata massiliana* were co-cultured with the amoebae *Acanthamoeba polyphaga*, *Acanthamoeba castellanii*, *Acanthamoeba griffini* and THP-1 macrophage-like phagocytes. All experiments were performed in five independant replicates. The ratio amoeba/bacteria was 1:20 and the ratio THP-1/bacteria was 1:10. After a 2-hour co-culture, extracellular bacteria were killed by kanamycin or amikacin and eliminated. The intracellular location of *Gemmata* bacteria was specified by confocal microscopy. Microscopic enumerations and culture-based enumerations of colony-forming units were performed at T = 0, 1, 2, 3, 4, 8, 16, 24, 48 and 72 hours post-infection. Then, *Gemmata* bacteria were engulfed into the phagocytes' cytoplasmic vacuoles, more than (98 ± 2)% of *Gemmata* bacteria, compared to controls, were destroyed by phagocytic cells after a 48-h co-culture according to microscopy and culture results, and no positive culture was observed at T = 72-hours. Under our co-culture conditions, *Gemmata* bacteria were therefore susceptible to the environmental and host phagocytes here investigated. These data suggest that these *Acanthamoeba* species and THP-1 cells cannot be used to isolate *G. massiliana* and *G. obscuriglobus* under the co-culture conditions applied in this study. Although the THP-1 response can point towards potential responses that might occur *in vivo*, these responses should first bevalidated by *in vivo* studies to draw definite conclusions.**

Free-living amoebae (FLA) are unicellular eukaryotes commonly found in natural aquatic and soil environments[1–3]. FLA and bacteria interact in biofilms in aquatic habitats where amoebae can act as "Trojan horses" for bacterial pathogens[4–8]. Indeed, some environmental bacteria designated as Amoeba-Resisting Bacteria (ARB) have seen their mechanisms evolve to resist amoebae and use amoebae as replicative niches[4–6]. In 1980, Robowtham demonstrated that the bacterial pathogen *Legionella pneumophila* was a prototype ARB[4] and several ARB have been further discovered, including, among others, *Coxiella burnetii*[5], several mycobacteria[6–12] and *Parachlamydia acanthamoebae*, a member of the so-called *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* (PVC) superphylum[13,14].

Planctomycetes are members of terrestrial and aquatic microbial communities[15–20]. These organisms have been regularly isolated from various habitats including soil, freshwater lakes, seawater, brackish water lagoon, wastewater habitats and river biofilms[15–23]. In humans also, the DNA of *Planctomycetes* has been detected in the gut microbiota[24] where *Akkermancia mucophila* has been isolated[25,26]. Only two representative organisms of the genus *Gemmata* have been cultured, including *Gemmata obscuriglobus*, first isolated in 1984 from a freshwater dam in Australia[17], and *Gemmata massiliana*, that we recently isolated from a hospital water system in close proximity to patients[18]. Therefore, patients may be exposed to these microorganisms when drinking freshwater or water used for health care such as hydrotherapy baths. Accordingly, *Gemmata* DNA sequences have been detected in the human digestive microbiota[24] as well as in the blood collected from two aplastic leukemic febrile patients with neutropenia[27]. *Gemmata* spp. bacteria are multimultidrug-resistant *Planctomycetes*[28] and may be opportunistic pathogens in selected patient populations[27].

In the environment, *Gemmata* may be in close contact with FLA, which normally feed on bacteria by phagocytosis. Amoeba co-culture has been used to isolate some fastidious microorganisms such as members

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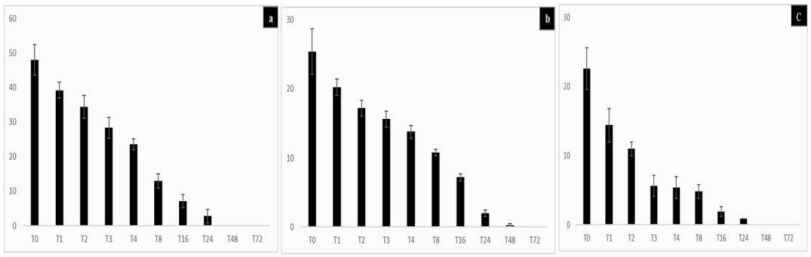

Figure 1. (**a**–**c**) Percentage of intracellular *G. obscuriglobus* bacteria observed by fluorescence microscopy after acridine orange staining in trophozoites of *A. polyphaga* (**a**), *A. castellanii* (**b**) and *A. griffini* (**c**). X axis figures time (hours) of coculture, Y axis figures the percentage of intracellular bacteria per 100-amoebae counted, standard errors are figured by error bars.

of *Legionella*[4] and waterborne mycobacteria including *Mycobacterium massiliense*[12]. In addition, amoebae may be used as model organisms to investigate the relationship between bacteria and phagocytes, including macrophages[29]. Some studies reported the relationship between planctomycetes and aquatic microbial communities such as sponges[23,30] and macroalgae[31–33], but the interactions between amoebae and *Gemmata* bacteria have not been specifically explored.

Here, we investigated the relationship between phagocytic amoebae and THP-1 cells with the two cultured *Gemmata* species. After preliminary experiments ensuring the viability of both *Gemmata* species in the medium used to culture amoebae and in the medium used to culture macrophages, both *Gemmata* species were exposed to amoebae and macrophages and the intracellular uptake and survival of bacteria were observed by optic and confocal microscopy, and colony-forming unit measurement.

Results

Preliminary experiments. Prior to co-culture experiments, we assessed the survival of both *Gemmata* species in the media used to culture amoebae and THP-1 cells. As for the survival of 2.10^7 *Gemmata*/mL in PAS (Page's Amoebal Saline) and RPMI 1640 (Roswell Park Memorial Institute), the number of medium colony-forming-units (CFU)/mL was $(1.84 \pm 0.28) \times 10^7$ and $(1.92 \pm 0.13) \times 10^7$ for *G. obscuriglobus* and *G. massiliana*, respectively, whereas it was $(2.01 \pm 0.31) \times 10^7$ for *G. obscuriglobus* and $(2.03 \pm 0.17) \times 10^7$ CFU/mL for *G. massiliana*. Likewise, the number of *G. obscuriglobus* CFU/mL obtained with the suspension contained in PAS was $(1.78 \pm 0.98) \times 10^7$ and $(1.87 \pm 0.24) \times 10^7$ for *G. massiliana* ($P > 0.05$) at day 3. Co-culture experiments were performed including an optimized (after preliminary experiments) two-hour inoculation of phagocytes with *Gemmata* bacteria to allow for a greater duration of bacterial uptake. Extra-phagocyte bacteria were killed by antibiotic treatment with $82 \pm 5\%$ and $92 \pm 2\%$ of *G. obscuriglobus* death in PAS and RPMI medium, respectively; and $86 \pm 7\%$ and $89 \pm 4\%$ of *G. massiliana* death in PAS and RPMI medium, respectively. Furthermore, inoculated phagocytes were washed three times and the third washing was microscopically observed and cultured on solid agar. Microscopic observation showed $24 \pm 8/\mu L$ phagocytes and $31 \pm 13/\mu L$ *Gemmata* bacteria. Culture remained sterile except for seven *G. obscuriglobus* colonies observed in one of the replicates of the *G. obscuriglobus-A. castellanii* co-culture. Light microscopy of co-culture at $T = 0$ ("time of beginning"after the third wash) showed intra-phagocyte *Gemmata* spp. and a few extracellular bacteria. Heat-shock procedure lysed 96–100% amoebae and 98–100% THP1-cells (trypan blue staining). The effects of thermal shock on *Gemmata* spp. viability included a loss rate of (2.48 ± 1.02) % $(1.95 \pm 0.09) \times 10^7$ CFU/mL remaining alive) for *G. obscuriglobus* and (7.15 ± 2.24) % $(1.88 \pm 0.18) \times 10^7$ CFU/mL remaining alive) for *G. massiliana*, using an initial suspension of 2.10^7 CFU/mL ($P > 0.05$). The sterility of the third washing and of *G. obscuriglobus* and *G. masiliana*-inoculated amoebae as well as that of the THP-1 cells not submitted to thermal shock confirm that further culture observations from the thermal shock lysates indeed derive from intracellular bacteria.

***Gemmata* spp.-amoebae co-culture.** The reproducibility of the results here reported was ensured by five successive and independant experiments in which all the non-infected, negative-control amoebae remained *Gemmata*-free. In these co-culture experiments, the number of non-inoculated, negative-control amoebae and *Gemmata* spp.-containing amoebae did not change significantly over time ($p < 0.05$). Accordingly, no amoebal lysis and no cysts were observed by microscopy after day 3 of the experiment in negative controls and *Gemmata* spp. -containing amoebae. After a 2-hour co-culture and antibiotic treatment followed by series of washes at T0, the percentage of *G. obscuriglobus*-containing amoeba trophozoites was of $(48.2 \pm 4.5)\%$ for *A. polyphaga* (Fig. 1a), $(25.4 \pm 3.4)\%$ for *A. castellanii* (Fig. 1b) and $(22.6 \pm 3.2)\%$ for *A. griffini* (Fig. 1c) ($P > 0.05$) and the percentage of *G. massiliana*-containing amoeba trophozoites was of $(41 \pm 4.4)\%$ for *A. polyphaga* (Fig. 2a), $(30.4 \pm 2.4)\%$ for *A. castellanii* (Fig. 2b) and $(17.6 \pm 2.1)\%$ for *A. griffini* ($P > 0.05$) (Fig. 2c). At $T = 0$, *Gemmata* spp.-containing amoebae then contained between 1 and 7 *G. obscuriglobus* bacteria and between 1 and 5 *G. massiliana* bacteria per amoebal trophozoite. Some bacteria were located into vacuoles, as confirmed by confocal microscopy. Three-dimensional (3D) reconstruction after z-stack acquisition using confocal laser microscopy (Zeiss LSM 800) showed the presence of internalized green fluorescent bacteria within vacuoles inside infected

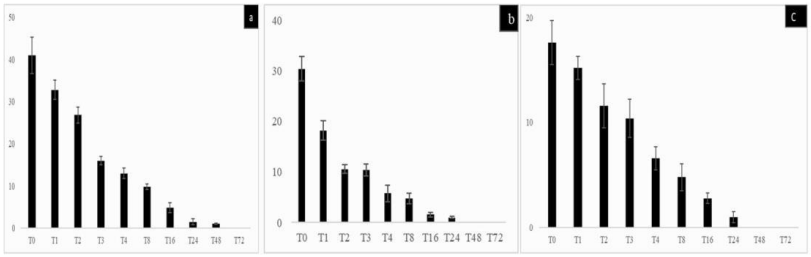

Figure 2. (**a**–**c**) Percentage of intracellular *G. massiliana* bacteria observed by fluorescence microscopy after acridine orange staining in trophozoites of *A. polyphaga* (**a**), *A. castellanii* (**b**) and *A. griffini* (**c**). X axis figures time (hours) of co-culture, Y axis figures the percentage of intracellular bacteria per 100-amoebae counted, standard errors are figured by error bars.

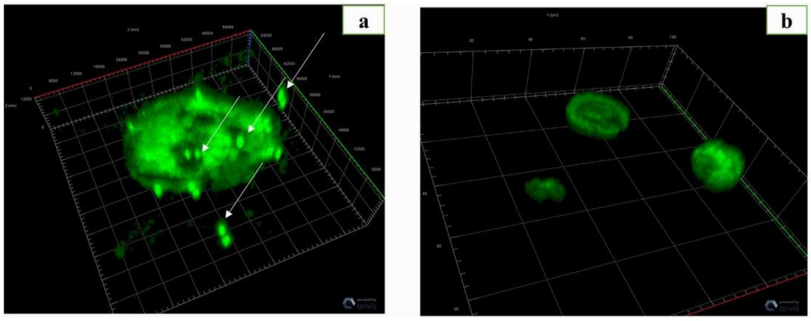

Figure 3. (**a,b**) Confocal microscopy after acridine orange staining showing *G. massiliana* (white arrows) within and outside of *A. griffini* trophozoite at T0 h (**a**) and negative control (uninfected *A. griffini* (**b**) at T0 h. Magnification 63X/1.4 oil objective, 3D reconstruction after z-stack in Zeiss LSM 800 confocal microscopy.

amoebae. However, no bacteria were observed within uninoculated amoebae. In *A. griffini*, the amoeba contained 3.67 ± 2.51 *G. massiliana* and 4.26 ± 2.77 *G. obscuriglobus*, including 2 ± 1 *Gemmata* spp. located in vacuoles (Fig. 3a), whereas non-inoculated control amoebae did not exhibit bacteria or vacuoles (Fig. 3b). In successive time-point observations, the number of intra-amoebal bacteria decreased, resulting in $98 \pm 2\%$ of *Gemmata* spp. bacteria being digested by amoebae after a 48-h co-culture. In parallel, the number of *Gemmata* spp. CFUs performed with the lysate after thermal shock decreased significantly ($P < 0.0001$) from T = 0 -hour to T = 48 -hours. Indeed, in *G. massiliana*-A. *polyphaga* co-culture, the number of CFUs decreased from $(5.21 \pm 0.29) \times 10^4$ at T = 0 -hour to $(0.32 \pm 0.12) \times 10^4$ at T = 16 -hours, $(0.11 \pm 0.02) \times 10^4$ at T = 24 ($P < 0.0001$) and limit of detection at T = 48 -hours for. For *G. obscuriglobus-A. polyphaga* co-culture, CFUs decreased from $(7.32 \pm 0.50) \times 10^4$ at T = 0 -hour to $(1.70 \pm 0.40) \times 10^4$ at T = 16 -hours, $(0.13 \pm 0.07) \times 10^4$ at T = 24 ($P < 0.0001$) and limit of detection at T = 48 hours. In *A.castellanii-G.massiliana* co-culture, the number of CFUs decreased from $(6.30 \pm 0.30) \times 10^4$ at T = 0 -hour to $(1.10 \pm 0.26) \times 10^4$ at T = 16 hours, $(0.38 \pm 0.03) \times 10^4$ at T = 24 ($P < 0.0001$) and limit of detection at T = 48 hours. In *G. obscuriglobus-A.castellanii* co-culture, CFUs decreased from $(8.30 \pm 0.61) \times 10^4$ at T = 0 -hour to $(1.32. \pm 0.43) \times 10^4$ at T = 16 hours, $(0.73 \pm 0.17) \times 10^4$ at T = 24 and $(0.16 \pm 0.06) \times 10^4$ at T = 48 hours ($P < 0.0001$). In *A. griffini* co-culture, CFUs decreased from $(4.30 \pm 0.27) \times 10^4$ for *G. massiliana* at T = 0 -hour to $(0.90 \pm 0.34) \times 10^4$ at T = 16 -hours, $(0.20 \pm 0.02) \times 10^4$ at T = 24 ($P < 0.001$) and limit of detection at T = 48 hours and for *G. obscuriglobus* from $(4.10 \pm 0.15) \times 10^4$ at T = 0 -hour to $(2.42 \pm 0.39) \times 10^4$ at T = 16 -hours, $(0.15 \pm 0.05) \times 10^4$ at T = 24 and $(0.09 \pm 0.05) \times 10^4$ at T = 48 hours ($P < 0.0001$). No positive culture was observed at T = 72 -hours for the two *Gemmata* species co-cultured with any amoebal lysate (Figs. 4a, 4b). Compared to controls not submitted to thermal shock, the amoebae did not lead phagocyted *Gemmata* bacteria out of their cytosol, since no growth was observed. More than $(98 \pm 2\%)$ of the *Gemmata* spp. organisms were internalized and destroyed by amoeba after a 48-h co-culture according to microscopic results, and no positive culture was observed at T = 72 -hours. Overall, no replication occured under the experimental conditions here reported. Compared to non-inoculated amoebae controls, almost all amoebae possessed many vacuoles without bacteria at T = 72 -hours. *Gemmata* spp. bacteria became indetectable by microscopic examination and by sub-culturing on agar plates at day 3. No discrepancy was observed between microscopy and culture results.

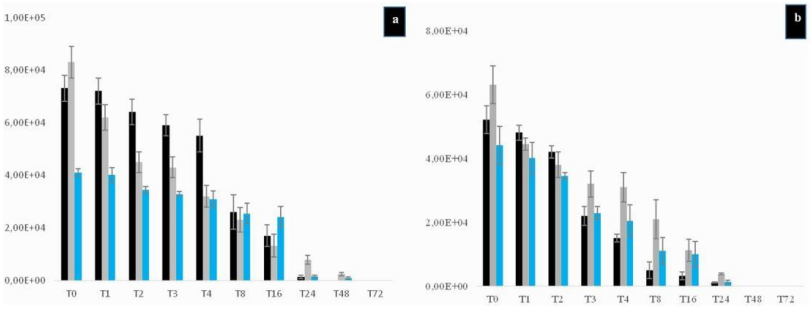

Figure 4. (**a,b**) Cultures of lysate performed after the thermal shock of *G. obscuriglobus* (**a**) and *G. massiliana* (**b**) with *A. polyphaga* trophozoites (blackbars), *A. castellanii* (gray bars) and *A. griffini* (blue bars). X axis figures time (hours) of co-culture, Y axis figures the number of CFUs, standard errors are figured by error bars.

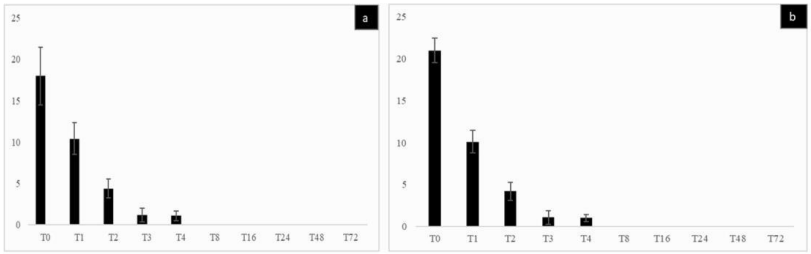

Figure 5. (**a,b**) Intracellular survival of *G. obscuriglobus* (**a**) and *G. massiliana* (**b**) within THP-1 cells. X axis figures time (hours) of co-culture and Y axis figures the percentage of infected THP-1 cells, standard errors are represented by error bars.

***Gemmata* spp.-THP-1 cells co-culture.** The reproducibility of the results here reported was ensured by five successive and independant experiments in which all the non-infected, negative-control THP-1 cells remained *Gemmata*-free. In a first step, we observed that *Gemmata* spp. bacteria survived for 72 hours in RPMI. Then co-culture experiments and microscopic examination of THP-1 cells after trypan blue staining indicated no significant difference in the viability of THP-1 cells over time, whether they be *Gemmata* spp. -containing THP-1 or not. THP-1 cells viability was of (95.6 ± 2.7) % (non- inoculated) and (96 ± 2.1) % (inoculated THP-1 cells) at day 1, *versus* (93.2 ± 2.4) % (non- inoculated) and (91 ± 3.2) % (inoculated THP-1cells) at day 3 post-inoculation ($P > 0.05$). After a 2 -hour co-culture and antibiotic treatment followed by series of washes at T0, (18 ± 4) % of THP-1 cells were found to be *G. obscuriglobus*.-containing THP-1 and (21 ± 2) % were found to be *G. massiliana*-containing THP-1 ($p > 0.05$). Then, at T = 1, T2, T3, T4 and T8 until 72 -hours post-culture, the number of *G. obscuriglobus*-containing THP-1 cells decreased from (10.4 ± 2)%, (4.4 ± 2)%, (1.2 ± 1)% and (1.1 ± 1) % to limit of detection at T = 8 until day 3 of co-culture, respectively (Fig. 5a). For *G. massiliana*, At T = 1, T2, T3, T4 and T8 until 72 hours post-culture, the number of *G. massiliana* -containing THP-1 cells decreased from (10.1 ± 1.4) %, (4.2 ± 1.4) %, (1.1 ± 1) %, and (1.0 ± 0.4) % to limit of detection at T = 8 until day 3 of co-culture, respectively, as showed by Fig. 5b. The number of *Gemmata* organisms per THP-1 cell varied from 1 to 2 whereas negative controls remained *Gemmata*–free. The number of non-inoculated (negative-control) and *Gemmata* spp.-containing THP-1 cells did not change significantly during the experiment. Likewise, the number of *G.massiliana* CFUs performed with culture lysate decreased from (3.2. ± 2.4) × 10^4 at T = 0 -hour, to (2.2 ± 1.8) × 10^4 at T = 1 to (1.2 ± 0.4) × 10^4 at T = 2 -hours ($P < 0.001$), tolimit of detection from T = 3 to T = 72 hours. For *G. obscuriglobus*, the number of CFUs decreased from (2.3. ± 1.8) × 10^4 at T = 0 -hour to (1.3 ± 0.2) × 10^4 at T = 1 and limit of detection from T = 2 to T = 72 hours (Fig. 6). THP-1 cells did not allow for the multiplication of *Gemmata* organisms that became indetectable by means of microscopic examination of phagocytic cells or by subculturing on agar plates at day 3 of co-culture.

Discussion

Amoebae and THP-1 cells are phagocytic cells which were previously used to probe microorganism-phagocyte interactions[34–39]. In the present study, we investigated such interactions between only two culturable representatives of the bacterial genus *Gemmata*, i.e. *G. obscuriglobus* and *G. massiliana*. In these series of experiments, culture of the washing product and controls not submitted to thermal shock action of amoebae and THP-1

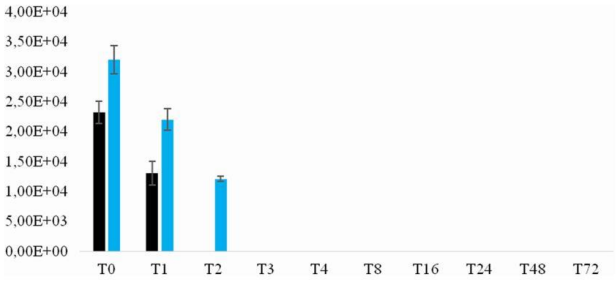

Figure 6. Cultures of lysate of THP-1 cells infected with *G. obscuriglobus* (black bars) or *G. massiliana* (blue bars). X axis figures time (hours) of co-culture, Y axis figures the number of CFUs, standard errors are figured by error bars.

cells inoculated with *G. obscuriglobus* or *G. masiliana* remained sterile, indicating that no extracellular bacteria remained alive or escaped from the cytosol of phagocytic cells, and that all further observations in culture of lysate obtained after thermal shock derived from intracellular bacteria. Also, the data presented in this study were interpreted as authentic because negative controls remained negative in each experimental step. At last, the observations were reproduced five times.

In every co-culture experiment, amoebae and THP-1 cells were found to be bactericidal against the two investigated *Gemmata* spp. The fact that results obtained with amoebae mirror results obtained with THP-1 cells is not surprising, as demonstrated previously with the similar actions of these phagocytic cells in numerous bacteria such as *Legionnela*, many mycobacteria and *Chlamydia*, which can resist both amoebae and macrophages[4,6,13].

Results obtained with the amoebae here investigated suggest that these unicellular eukaryotes are unlikely hosting *Gemmata* species in the aquatic environments where they could thrive together. *Gemmata* are mainly found in soil and aquatic environments along with FLA but have also been found in the human gut microbiota, in the blood of immune-compromised aplastic leukemic patients[17,18,24,26] and hospital water networks in close proximity to patients[18]. These recent observations in our laboratory led us to conduct also *Gemmata* spp-TPH-1 cells co-culture to obtain insights into the link between phagocytose and monocyte-macrophage cells in human infection. Using a low (1:10) multiplicity of infection (M.O.I) with THP-1 and hight M.O.I (1:20) with amoebae, we obtained reproducible results in five independant experiments. We observed that *Gemmata* spp. were rapidly internalized and digested by amoebae and THP-1.

This high susceptibility of *Gemmata* spp. to bactericidal phagocytosis contrasted with the fact that *Gemmata* spp. are multidrug-resistant bacteria[28] and have a high ability to adapt to harsh environments. Moreover, these bacteria may thrive in the human body[24,27]. However, under the co-culture conditions of our experiments, *Gemmata* bacteria did not resist to amoebae and THP-1 bactericidal action despite their panoply of attack and defense mechanisms[40]. The rapid and significant phagocytosis of *Gemmata* bacteria could be explained by the presence of holdfasts of glycoproteic nature on their outer membrane[16,18] which could facilitate adhesion to phagocytic cell receptors and increase cell-to-cell contact and rapid internalization. Consequently, *Gemmata* spp. life cycle requires an attached state. Their proliferation starts when they attach through their holdfast to a bracket. Then, flagellated budding new cells are formed which move freely in the water until developing their holdfast and returning to the attached form[41]. FLA and THP-1 cells do not offer these survival and proliferation conditions compared to macroalgae, marine sponges and crustaceans, which are closely related to planctomycetes in aquatic environments and biofilms[31].

In conclusion, the data here reported show that coculturing the two *Gemmata* species under investigation with *Acanthamoeba* amoebae results in *Gemmata* death. *Acanthamoeba* ameobae are unlikely hosting *Gemmata* spp. in the environment. Moreover, these amoebae could not be used for the tentative isolation of *Gemmata* bacteria in the laboratory under the co-culture conditions tested in this study. Moreover, the interactions between THP-1 cells and *Gemmata* spp. could provide insight into the action of the monocytes–macrophages against *Gemmata* spp. cells during the colonization and infection. Though unlikely, patients with compromised macrophage activities, such as aplastic patients, could be infected by opportunistic *Gemmata* spp. Although the THP-1 response can hint to potential responses that might occur *in vivo*, these reponses should first be validated by *in vivo* studies before drawing more definite conclusions.

Materials and Methods

Preliminary experiments. The methodological procedures here presented have been designed and standardized after a series of preliminary studies. Preliminary experiments were used to define the phagocyte/bacteria ratio; the duration of co-culture of 6 h, 4 h, 3 h and 2 h before T0; antibiotic treatment conditions (concentration and duration of treatment) to kill rapidly extracellular bacteria, and the effectiveness of the wash in removing extra-cellular bacteria (later controlled by microscopy and culture). Also, different types of staining (Giemsa, Gimenez, Hemacolor, acridin orange) were tested to choose the most appropriate staining, and the thermal shock procedure was evaluated and standardized before the experiments. Then, the protocol was successively applied with each amoeba species and to THP-1 cells.

Bacterial strains and culture conditions. *G. obscuriglobus* DSM 5831^T and *G. massiliana* DSM 26013^T (CSUR P189^T) were obtained from the Collection de Souches de l'Unité des Rickettsies, Marseille, France, and the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Bacteria of both species were sub-cultured on Caulobacter medium DSMZ 595 supplemented by 5% *Escherichia coli* filtrate or Staley's maintenance medium DSMZ 629 prepared as described on the website (http: //www.dsmz.de). Bacteria were grown on these solid media incubated aerobically at 30 °C for 7 to 14 days. Identification of colonies was ensured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis as previously described[42]. Prior to co-culture, colonies were harvested in a 15-mL tube containing 5 mL of sterile phosphate-buffered saline (PBS), the tube was rigorously vortexed and the suspension was passed three times through a 29-Gauge needle in order to separate aggregates. The inoculum was adjusted at 2×10^7 cells colony-forming units (CFU)/mL after calibration by Kovas slide 10 (Hycor Biomedical, Indianapolis, IN, USA) for co-culture. In parallel, this inoculum was maintained in RPMI 1640 and PAS for three days to check *Gemmata* spp viability after one to three days and compared with Staley's liquid medium as reference. Culture-based microbial enumeration on Staley's solid agar has been performed in order to assess bacterial survival in these co-culture liquid media.

Amoebae and culture conditions. *Acanthamoeba polyphaga* (strain Linc AP1), *Acanthamoeba castellanii* (strain ATCC 30234) and *Acanthamoeba griffin* (strain ATCC 50702) were cultured independently in axenically Peptone Yeast-extract Glucose (PYG) medium placed in 75-cm^2 culture flasks. The flasks were then incubated for 48 hours at 30 °C for *A. polyphaga* and 28 °C for *A. griffini* and *A. castellanii*. Trophozoites were suspended by tapping the flasks, centrifuged in 50-mL tubes and the pellet was suspended in Page's amoebal saline (PAS). Then, the amoebal cells were adjusted at 10^5 cells/mL using Kovas slide 10 and we checked their viability using trypan blue staining before co-cuture. Amoeba viability and growth were assessed at the end of co-culture (day 3) using trypan blue staining.

Human monocytic THP-1 cell lines and culture conditions. THP-1 cells were retreived from the blood of a patient with acute monocytic leukemia. Since their establishment in 1980[43], THP-1 cells have become one of most widely used cell lines to investigate the function and regulation of monocytes and macrophages in the cardiovascular system. After exposure to phorbol-12-myristate-13-acetate (PMA, also known as TPA, 12-O-tetradecanoylphorbol-13-acetate), nearly all the THP-1 cells started to transform into macrophages[44]. In this study, unstimulated THP-1 cells were preferred over macrophages (present in the tissues) in order to mimic the behavior of peripheral blood monocytes cells. Indeed, *Gemmata* DNA has been previously detected in the blood collected from two aplastic leukemic febrile patients but not in tissues. THP-1 cells were kindly provided by Dr. E. GHIGO, IHU Méditerranée Infection, Marseille, France. Cells were grown in RPMI 1640 (Gibco™, Eggenstein, Germany) tissue culture medium supplemented with 2% glutamine and 10% heat-inactivated fetal bovine serum in 75-cm^2 tissue culture flasks, incubated at 37 °C in 5% CO_2. The culture medium was refreshed every 3 days. Prior to co-culture, cells were harvested and washed thoroughly twice with PBS and adjusted at 2×10^6 cells/mL using Kovas slide 10. We checked their viability using trypan blue staining before co-cuture. The viability and growth were assessed at the end of co-culture (day 3).

Amoeba-*Gemmata* species co-culture conditions. All experiments were performed in five independant replicates during 72 hours in 12-well tissue culture plates (Becton Dickinson, Le Pont-de-Claix, France) and repeated five times for ensuring reproducibility. Each species of amoeba was co-cultured with each *Gemmata* species. Each plate contained six wells with amoebae co-cultured with each *Gemmata* species and six wells with amoebae as negative control wells (bacteria-free). More precisely, 1.8 mL of the amoebae-containing suspension was pipetted in each well of a 12 well-plate, 200 µL of *Gemmata* suspension at 2×10^7 CFU/mL in PBS (ratio amoebae/bacteria was 1:20) was added in challenged wells and 200 µL of PBS in control wells. Plates were incubated at 30 °C for two hours. After a 2 -hour co-culture, in order to eliminate extracellular bacteria, the supernatant was removed, the amoeba monolayer was washed twice and two milliliters of modified PAS containing 150 g/L kanamycin was added in each well, including negative control wells. After a 30-min incubation period with the antibiotic, the amoeba monolayer was rinsed twice with PBS to eliminate the extracellular bacteria, this operation was repeated once with a 30-min incubation period and then rinsed twice to obtain a third washing. Antibiotic treatment protocol in 2×30 min was chosen to combine inactivation and physical removal of extracellular bacteria to have low extracellular bacteria in the last rinse product. Antibiotic inactivation tests of *Gemmata* spp were performed in PAS in the presence or absence of phagocytic cells for the interpretation of culture results. Finally, after a series of antibiotic rinsing, the amoeba monolayer was covered with two mL of PAS in each well and the plates were incubated at 30 °C for *A. polyphaga* and 28 °C for *A. griffini* and *A.castellanii* for the rest of the experiment. Negative controls (uninfected) of each amoeba were cultured separately in PAS medium as described above.

THP-1 cells-*Gemmata* spp.co-culture conditions. Co-culture was performed in RPMI 1640 medium without fœtal bovine serum and glutamine in order to deplete the stored nutrients and slow down the growth of THP-1 cells. The experiments were performed in five independent replicates during 72 hours in 12-well tissue culture plates, each containing independently *Gemmata* species, as previously described for amoebae. Each plate contained six wells with THP-1 cells co-cultured with each *Gemmata* species and six wells with THP-1 cells as negative control wells (bacteria-free). More specifically, 1.8 mL of 2.10^6 cells/mL THP-1 cells (calibrated as described above) suspension was pipetted in each well of a 12-well plate, 200 µL of *Gemmata* suspension at 2×10^7 CFU/mL (ratio THP-1 cells/bacteria was 1:10) was added in challenged wells and 200 µL of PBS in control wells. Plates were incubated at 37 °C with 5% CO_2 for two hours. After a 2 -hour co-culture, in order to eliminate

extracellular bacteria, the supernatant was removed, THP-1 cells were washed with PBS and two mL of RPMI containing 150 g/L of amikacin was added in all wells, including negative control wells for 30 min. After a 30-min incubation period at 37 °C under 5% CO_2 with the antibiotic, the cells were rinsed to eliminate the extracellular bacteria, then antibiotic treatment was repeated once with a 30-min incubation period and then rinsed twice to obtain the last rinse called washing product. 100 µL of this washing product was cultured in Staley's medium to ensure the absence of viable extracellular bacteria. Microscopic controls (using Kova slide) at the fresh state of the washing product were performed, as described above, before being plated on solid agar. Finally, THP-1cells were covered with 2-mL of RPMI in each well and the preparations were incubated at 37 °C in the presence of 5% CO_2 for 72 hours. Uninfected THP-1 cells were cultured in the same conditions as negative controls.

Microscopy and culture conditions of lysate obtained after thermal shock. After a 2 -hour co-culture duration, antibiotic treatment and a series of rinsing, microscopic enumerations and culture-based enumerations of colony-forming units were performed at T = 0 (time beginning after the last rinse of antibiotic), 1, 2, 3, 4, (close kinetic counting to see more intracellular bacteria phagocyted at the beginning) and T 8, 16, 24, 48, 72 -hour post-inoculation (procedure standardized after preliminary sudies).

Microscopy. The phagocyte cells and the supernatant were removed from each well, 200-µL volume was used to prepare a Cytospin (smears) centrifuged at 44 g during 5 mins for microscopy analysis. Smears were pre-fixed with 90° ethanol. Amoeba-*Gemmata* spp coculture smears were examined by fluorescence microscopy after acridine orange staining as described on the website http://www.memobio.fr/html/bact/ba_te_acr.html. In order to precise the intracellular location of bacteria, smears were observed under a Zeiss LSM 800 confocal microscope using a 488 nm excitation laser (Carl Zeiss S.A.S., Marly-le-Roi, France). The 63X/1.4NA oil immersion objective was used for image acquisition. 3D reconstruction was performed using Zen software (Zeiss) from a z-stack acquisition of 10 images with a z-spacing of 1.2 µm. Images were post-processed using ImageJ software by adjusting contrast and brightness. For THP-1-*Gemmata* co-culture, slides were stained by the Gimenez method and obseved by light microscopy. Microscopic results were expressed by counting the number of intracellular bacteria per 100-amoeba or THP-1 counted. Means and standard errors of five independent experiments have been calculated using Excel 2013 software. The phagocytic cells and the supernatant were removed from each well, a 200-µL volume was used to prepare a Cytospin (smears) centrifuged at 44 g during 5 mins for microscopy analysis. Smears were prefixed with 90° Ethanol. Amoeba-*Gemmata* spp co-culture smears were examined by fluorescence microscopy after acridine orange staining as described on the website http://www.memobio.fr/html/bact/ba_te_acr.html. In order to observe intrabacteria localization in vacuoles or in the cytoplasm, smears were observed under a Zeiss LSM 800 confocal microscope using a 488 nm excitation laser (Carl Zeiss S.A.S., Marly-le-Roi, France). The 63X/1.4NA oil immersion objective was used for image acquisition. 3D reconstruction was performed using Zen software (Zeiss) from a z-stack acquisition of 10 images with a z-spacing of 1.2 µm. Images were post-processed using ImageJ software by adjusting contrast and brightness. For THP-1-*Gemmata* co-culture, slides were stained by the Gimenez method and obseved by light microscopy. Microscopic results were expressed by counting the number of intracellular bacteria and extracellular bacteria (remaining bacteria not removed by last rinsing) per 100-amoebae or THP-1 counted. Means and standard errors of five independant replicates have been calculated using Excel 2013 software.

Culture conditions of lysate obtained after thermal shock. To determine the viability of intra-phagocyte bacteria, 200 µL of the phagocytic monolayer and the supernatant were removed from the bottom of wells and transferred in 1.5-mL polypropylene tubes. The preparation was submitted to thermal shock. It was frozen in liquid nitrogen at −196 °C, submitted to direct defrosting in a water bath at 40 °C for two minutes and then vortexed for 30 seconds. This operation was repeated once to ensure more lysis of host cells (Phagocytic lysis was checked using Trypan blue staining). A 100-µL volume of the lysate and 100 µL of a one-fold dilution in PBS were cultured for two weeks in Staley's medium and incubated at 30 °C to quantify CFU. A 100-µL volume of culture product not subjected to the thermal shock action was cultured in parallel for control. Colonies were counted after 2 weeks of growth at 30 °C. Futhermore, in order to assess the impact of thermal shock on the viability of *Gemmata* spp. bacteria, the *Gemmata* inoculum at 2.10^7 bacteria/mL was subjected to the thermal shock procedure and then plated on staley's agar medium to check its viability. Data are expressed as CFU per milliliter. Means and standard errors have been calculated using Excel 2013 software.

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Author Contributions

O.D.K. performed the experiments and drafted the manuscript. A.L. performed confocal microscopy observations. S.G. conceived the study and drafted the manuscript. M.D. conceived the study and wrote the main manuscript. All authors reviewed the manuscript.

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PARTIE IV :

Amélioration et développement de nouveaux milieux de culture des Gemmata.

Chapitre 1

Amélioration de la culture des *Gemmata* par les extraits d'éponges marines.

Dans l'environnement marin, une grande partie de *Planctomycetes* (autres que *Gemmata*) résident en permanence dans les éponges marines et y cohabitent par des relations symbiotiques hautement intégrées. Ces associations pouvant être mécaniques et/ou nutritionnelles via une chimiotaxie (des nutriments et/ou facteurs de croissance) qui attirent les bactéries. Ces dernières viennent donc s'attacher à l'éponge marine par l'intermédiaire de glycoprotéines présentes à leur surface pour former des jonctions temporelles ou permanentes afin d'assurer leur bourgeonnement. L'objectif de ce travail était donc de mimer ces conditions environnementales et de tendre vers l'habitat naturel le plus proche possible de ces bactéries sessiles et bourgeonnantes, afin de concevoir de nouveaux milieux pour améliorer leur croissance et isolement. Ainsi, nous avons comparé un nouveau milieu de culture A, incorporant des tissus d'éponge marine de *Spongia officinalis* au milieu de culture standard ; et un milieu de culture B incorporant le filtrat aqueux de *S. officinalis* dans le milieu A. Les trois milieux (standard, A et B) ont été inoculés avec *Gemmata obscuriglobus* (G.o) et *Gemmata massiliana* (G.m) en présence de contrôles négatifs. La croissance bactérienne a été quantifiée quotidiennement par le dénombrement des Unités Formant Colonies (UFCs). Le nombre d'UFCs dans le milieu standard (1.363 ± 115 pour *G.o*, 1.288 ± 83 pour *G.m*) était significativement plus faible que ceux comptés à partir du milieu B (2,552 ± 128 pour *G.o*, 1.870 ± 112 pour *G.m*) et du milieu A (2.851 ± 137 pour *G.o*, 2.035 ± 163 pour *G.m*) après 2 jours d'incubation. Au 3ème jour, les UFCs comptées à partir des milieux A et B ont augmenté à plus d'un log comparativement au milieu standard ($p < 0.10^{-3}$). Ces nouveaux milieux enrichis A et B pourront être implémentés au laboratoire de microbiologie clinique pour tenter l'isolement et la culture des bactéries du genre *Gemmata* à partir de prélèvemenrs cliniques, en particulier le sang des patients aplasiques fébriles.

Article 3

Improved culture of fastidious *Gemmata* spp. bacteria using marine sponges

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Improved culture of fastidious *Gemmata* spp. bacteria using marine sponge skeletons.

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Abstract

Gemmata are Planctomycetes bacteria recalcitrant to traditional cultivation in the clinical microbiology laboratory and they have been seldom documented in patients. Based on previously known relationships of Planctomycetes with marine sponges, we designed a new culture medium A incorporating marine sponge skeleton of *Spongia* sp. to the standard culture medium; and culture medium B incorporating *Spongia* sp. skeleton heat aqueous filtrate into medium A; and inoculating the three culture media (standard, A and B) with *Gemmata obscuriglobus* DSM 5831^T and *Gemmata massiliana* DSM 26013^T in the presence of negative controls. Cultures were observed by naked eyes for 7 days and bacterial growth was quantified by microscopic observations and culture-based enumerations. Macroscopic observations at day-3 revealed a pink bacterial pellet in medium B tubes while standard medium tubes remained limpid until day-8. Growing *Gemmata* spp. bacteria in medium A yielded air bubbles released by bacterial respiration, whereas control tubes remained bubble-free. The number of colonies in standard medium (1.363 ± 115 for *G. obscuriglobus,* 1.288 ± 83 for *G. massiliana*) was significantly lower than those counted from medium B (2.552 ± 128 for *G. obscuriglobus,* 1.870 ± 112 for *G. massiliana*) and from medium A (2.851 ± 137 for *G. obscuriglobus,* 2.035 ± 163 for *G. massiliana*) ($p<0.10^{-4}$) at day-2 incubation. At day-3 incubation, the number of colonies counted from supplemented media A and B increased up to one log than those counted from the control medium ($p<0.10^{-4}$). Along the following day-4-7 incubation, the number of colonies counted from media A and B remained significantly higher compared to standard medium ($p<0.10^{-4}$). These data indicate that incorporation of spongin-based marine sponge skeleton and heat aqueous

filtrate of sponge skeleton significantly improved growth of *Gemmata* spp. bacteria. These observations pave the way towards improved isolation and culture of *Gemmata* spp. from environmental and clinical specimens.

Keywords: Planctomycetes*, Gemmata* organisms, sponge skeleton, sponge filtrate, sponge small fractions, unsupplemented medium, culture.

INTRODUCTION

Bacteria of the genus *Gemmata*, phylum Planctomycetes[1] form a group of organisms of interest in environmental sciences and medicine[2]**.** In the environment, these organisms have been detected in soil, freshwater and marine habitats[3–7]. However, environmental *Gemmata* spp. form a vast world of mainly uncultured organisms as only *Gemmata obscuriglobus* has been isolated from freshwater[8] and *Gemmata massiliana* from an hospital water network in close proximity with patients[9]. In the clinical microbiology laboratory also, *Gemmata* spp. organisms are recalcitrant to traditional cultivation, although they have been seldom documented in patients[10,11]**.** Using a PCR-sequencing approach, we recently found some *Gemmata*-like sequences in the blood collected from two patients with febrile aplasic neutropenia and leukemia, although we failed to isolate any Planctomycetes organism from these patients' blood specimens in pure culture, despite several attempts[11]. Indeed, *Gemmata* organisms are fastidious bacteria requiring highly specific culture medium[12–14]. Accordingly, conventional automated microbial detection of blood culture system is not appropriate to detect such bacteria (undetected) and less sensitive than culturing mock-infected blood on Caulobacter agar[15]. Nevertheless, their resistance to most of the routinely used antibiotics[16] and the recently demonstrated association with humans[10,11] support the potential of *Gemmata* organisms to behave as opportunistic pathogens warranting furthur investigations[2].

It has been reported that the lack of complex factors/conditions in the laboratory contributed to the inability to isolate some fastidious bacterial species[17,18]**.** Accordingly, providing environmental and nutritional conditions similar to those existing in the natural habitat where yet uncultured bacteria are detected, may be an

option for tentative isolation and culture[17,18]. In marine environments, a large fraction of Plantomycetes reside permanently in sponges pointing to highly integrated symbiotic relationships between the host sponge and Planctomycetes[19]. The reasons for such a symbiosis may be mechanical or nutritional relationships and chemotaxis as nutrients released by marine sponge surfaces may attract bacteria[19] and provoke their attachment via glycoproteins and uncharacterized holdfast structures. The holdfast of marine bacteria can form temporal or permanent junctions to stabilize the biofilm[20,21] and may help *Gemmata* organisms to ensure a rapid growth and their reproduction by budding[8,22,23].

Based on these knowledges, we hypothesized that creating in the laboratory an attached-living style using dead marine sponge tissues (spongin) may act as a growth-promoting condition to improve the culturabily of *Gemmata* organisms. We sought to test the growth-enhancement effect of complementing *Gemmata* species standard culture medium with marine sponge filtrate and sponge small fractions as a solid phase to mimic planctomycetes natural environment in order to develop a new biphasic culture system for *Gemmata* spp. bacteria.

RESULTS

Organoleptic characteristics of the liquid media and macroscopic-microscopic structures of dead sponge skeleton: By observing the organoleptic traits of the three different media after autoclaving, we observed a more yellow color of the media supplemented with marine sponge extracts while the unsupplemented Caulobacter standard liquid medium remained very light straw yellow. It is probable that the yellow color resulted from the diffusion of chemical compounds from sponge after

autoclaving since the sponge, heated in deionized water alone (without Caulobacter components) showed a slight yellow color. However, the autoclaving process did not significantly alter the macroscopic and microscopic structures of the sponge skeleton compared to control unautoclaved sponge skeletons.

Macroscopic observations. In stationary broth cultures, the macroscopic appearance of the supplemented broth changed considerably during the course of culture. Indeed, growth occured in the form of a pink pellet that developed at the bottom of the tube. The pink pellet appeared at day-3 (*G. obscuriglobus*) and day-4 (*G. massiliana*) in the medium B, while it appeared at day 7-8 for *G. obscuriglobus* and at day 10-11 for *G. massiliana* in control medium. In medium A growth occured with proportional increase of the air bubbles in the medium but the pink pellet did not appear in parallel in medium B. Indeed, for medium A, at day-1 post-inoculation, we observed air bubbles that increased significantly in number at days 3-4 and throughout the experiment up to a peak at day 6-7 for inoculated media while the tubes containing non-inoculated medium A exhibited no air bubbles. Also, after being vortexed, the enriched media showed a stable foam of at least 2.5 cm above the liquid (inoculated and uninoculated) for 5 to 7 min while a small layer of the foam of 2 mm disappeared after 30 seconds in the unsupplemented medium (inoculated and non-inoculated tubes). At day-14 post-inoculation, this foam did not appear after the vortex process for inoculated supplemented media while it always appeared from non-inoculated medium. These observations suggested the possible presence of amphiphilic molecules from the sponge skeleton medium that have been dissolved in the standard medium. Also, the fact that the foam that appeared at the beginning of the experiment did not appear at day-14 post-inoculation suggested that some

molecules, which formed this foam, were completely consumed by the bacteria or bacterial growth has induced an inhibition of this foam, due to the releasing of acid toxic products pH (3.5- 4) in the medium. By vortexing the tubes, the supplemented media appeared to be in well-clouded at the 3 rd day of the experiment compared to the non-supplemented media that remained limpid until the small pink layer began to appear at the day 7-8 post-inoculation (*G. obscuriglobus)* and day 10 post-inoculation (*G. massiliana)*.

Microscopic observations. Under our microscopic visualization, all non-inoculated, negative control tubes remained sterile over the entire experiments, and culture on soild media provided confirmation. The composition of the medium was an important factor affecting both the rosettes formation (*G. massiliana)* and the latent period before the apparition of budding cells (*G. massiliana* and *G. obscuriglobus*). In inoculated tubes, both strains were ovoid with a pleomorphism characteristic of bacteria from supplemented broth and usually occurred in pairs (mother cell with small bud located at one pole) *versus* singly from unsupplemented medium at day-1 post-inoculation. Indeed, at one day post-inocalation, in the medium supplemented with small fractions of sponge as solid support, 40 ± 7 % of *G. massiliana* cells appeared to possess one daughter cell (small bud) located near the mother cell and 46 ± 13 % of *G. obscuriglobus* bacteria possessed daughter cell located near the mother cell. Also, budding bacteria counted from the medium containing sponge filtrate alone represented at one day post-inoculation, 38 ± 12% for *G. massiliana* and 43 ±11 % for *G. obscuriglobus* while bacteria visualized from the control media showed a lower proportion of 10 ± 4 % for *G. massiliana versus* 15 ± 6 % for *G. obscuriglobus* bacteria with one bud located at a pole of mother cell. On solid agar,

these small buds after their growth can be seen near one of the two poles of the mother cell (which appears bigger). Without the small fractions of sponge as solid support in the medium supplemented with marine sponge filtrate and the standard Caulobacter medium, the pellet resuspended contained small and large rosettes (8-20 cells per rosette for sponge filtrate medium and 4-12 cells per rosette for control medium compared to medium containing sponge fractions ($P < 0.001$) where the cells appeared separated, attached to sponge skeletons, singly, pairs, pleiomorphic or forming small rosettes (4 to 6 cells). These observations suggest that in the absence of sponge as a solid support, bacteria tend to form large rosettes and thus constituting their own "self-support" to ensure their budding. In contrast, with the presence of sponge as a solid support, bacteria tend to cling, preferably, to the sponge fragments to ensure their budding.

Colonies-based microbial enumeration on Caulobacter solid medium. The negative control plates remained sterile over the entire experiments. In the media containing sponge extracts, growth yield was not proportional to the sponge extract concentration from 0.5 g to 1g. At sponge extract concentrations of 0.5% (M/V), the viscosity remained roughly similar compared to the standard medium and did not compromise the bacterial enumeration as standard deviation values were optimum in the medium containing sponge fractions (preliminary studies, data not shown). *Gemmata obscuriglobus* colonies sub-cultured in each broth of the three tested liquid media, were seeded at each time (as described in the method section) on Caulobacter solid agar and then counted after a 14-day incubation period at 30°C. Thus, colonies enumerations revealed that the cultures of *G. obscuriglobus* from both supplemented broth, *i.e* sponge filtrate broth, and sponge small fractions immersed in

sponge filtrate broth (biphasic system) were significantly higher than cultures obtained with Caulobacter standard broth ($p<0.0001$). Indeed, from standard Caulobacter broth, the number of *G. obscuriglobus* colonies counted was (1.162 ± 51) at day-1, (1.363 ± 115) at day-2, (2.390 ± 427) at day-3, and (6.800 ± 810), (270.000 ± 2.100) at day-4 and day-7 post-inoculation respectively. Supplementation of the standard Caulobacter broth with sponge filtrate increased significantly the number of *Gemmata obscuriglobus* colonies ($p<0.0001$) and the colonies counted after seeding onto solid agar plates were (1.792 ± 86 colonies) at day-1, (2.552 ± 128) at day-2, (17.000± 1.120) at day-3, (130.000 ± 1.270), (2.775.000 ± 35.000) at day-4 and 7 post-inoculation respectively. Likewise, the addition of sponge small fractions in the standard broth supplemented with sponge filtrate (biphasic system) showed that the number of *Gemmata obscuriglobus* colonies was (1.146 ± 66) at day-1, (2.851 ± 137) at day-2, (19.750 ± 1.300) at day-3, and (279.600 ± 1.277), (3.220.000 ± 46.000) at day-4 and 7 post-inoculation, respectively (**Figure 1a)**. In parallel to colonies enumerations from agar plates, microscopic monitoring showed the rosette of cells and attached bacteria to sponge despite the 10 secondes vortex process. We had then increased the vortex process until the low standard deviations values (described in method section) has been obtained (4 ×10 s). Thus, the following enumerations after the serial vortex resulted to the very high increasing of colonies from the medium supplemented with small fractions of marine sponge (biphasic system), as shown in (**Figure 1b)**, compared to the two other media (monophasic systems).

Likewise, the same effects of the addition of sponge extracts were observed with *Gemmata massiliana.* Indeed, bacteria sub-cultured in each broth of the three tested

liquid media were plated on Caulobacter solid agar and then counted after a 21-day incubation period. Thus, the number of *G. massiliana* colonies enumarated from Caulobacter standard medium was (1.193 ± 96 colonies) at day-1, (1.288 ± 83) at day-2, (1.480 ± 122) at day-3, and (4.100 ± 314), (28.000 ± 2.550) at day-4 and day-7 post-inoculation respectively, whereas the number of colonies counted from medium supplemented with sponge filtrate was (1.527± 89 colonies) at day-1, (1.870 ± 128) at day-2, (11.000 ± 2.100) at day-3, and (62.000 ± 2.260), (220.000 ± 4.200) at day-4 and 7 post-inoculation respectively. The addition of sponge small fractions for bacterial attachment in the standard broth supplemented with sponge filtrate (biphasic system) increased the growth rate and colonies enumerations were (1.166± 72 colonies) at day-1, (2.035 ± 128) at day-2, (12.600± 2.300) at day-3, and (93.000 ± 3.900), (280.000 ± 5.600) at day-4 and day-7 post-inoculation respectively (**Figure 2a)**. As described for *G. obscuriglobus*, the enumeration of *G. massiliana* colonies after the serial vortex resulted in a very high increasing of colonies from the biphasic medium supplemented with small fractions of marine sponges than the two other monophasic media, as show the (**Figure 2b)**. From day-7 to day-14 post-inoculation, the depletion of nutrients and potential growth factors thus marks the stationary phase of bacterial growth while the bacterial growth progressed in the unsupplemented medium.

Growth on solid media.

On the surface of both media, both colonies were circular, convex and had entire margins with sometime a litle bud (daugher cell) near to mother cell. *G. massiliana* organisms formed colorless colonies (pink), while *G. obscuriglobus* colonies were pink-red. Relatively, the growth of both organisms from medium supplemented with

marine sponge filtrate was more rapid, with small colonies becoming visible at day 5 (*G. obscuriglobus*) and days 7 (*G. massiliana*) *versus* day 8 (*G. obscuriglobus*) to day 11 (*G. massiliana*) in unsupplemend solid medium at 30°C (size<0.5 mm). The maximal colony diameter varies somewhat with the strain, but usually reaches 1 to 3 mm after 14-day (*G. obscuriglobus)*, 21-day (*G. massiliana*) incubation from medium supplemented with sponge extract *versus* 1 to 2 mm diameters from unsupplemented solid medium.

Discussion

Although *Gemmata* spp. sequences have been detected in stool specimens[10] and in the blood of two patients with febrile neutropenia[11], no isolate issued from tentative culture of these clinical specimens. Failure to isolate any *Gemmata* organism from these specimens may rely on the fact that PCR-based detected organisms were dead and indeed, no characterization of their viability has been attempted. Alternatively, failure to isolate and culture may be due to inappropriate culture conditions of the clinical specimens. Blood culture remains the gold standard for diagnosing bloodstream infections but conventional cultivation of *Gemmata* microorganisms from blood culture is requiring specific conditions for blood collection and culture[15].

Mimicking culture strategies by environmental microbiologists, we aimed to incorporate marine sponge skeleton fragments and spongin-based sponge skeleton extract in the culture medium routinely used to grow *G. obscuriglobus* and *G. massiliana* organisms, because of some other Planctomycetes have been shown to have tight relationships with marine sponges[19,26,27]. Indeed, our observations

revealed a significant growth-promoting effect after complementing Caulobacter standard medium with marine sponge skeleton filtrate. These results imply that some yet unknown thermosoluble growth factors are strongly and possibly bound to the sponge skeletons and have been dissolved by heat (autoclaving) in supplemented media. The existence of an intimate relationship between planctomycetes and marine sponges[19] can be ascribed (partly) to these observations via a mounting molecular evidence, stimulating the rapid growth of slow-growing bacteria like planctomycetes.

Spongia sp.(Demospongiae: Porifera) [26] consist of sponges whose skeleton is mostly made up of a composite of natural biomaterials containing organic constituents like protein spongin (a network of organic collagenous analogous to collagen type XIII), polysaccharides and/or inorganic compounds, which may have been incorporated into the spongin structure from the environment [27–31]. Spongin consists mostly of carbon, nitrogen, oxygen and hydrogen and the the presence of sulfuris connected with the disulfide bonds of cysteine has also been reported in spongin structure [32].Sponginous collagens analysis of the sponge *Spongia officinalis obliqua* revealed that not only proteinogenic amino acids but also halogenated (Brominated) tyrosines were occured in these sponges [33]. More reports have pointed that sponge collagen is a good biomaterial for medical[34], pharmaceutical[35], nutraceutical[36] and cosmeceutical[36–38] applications (bone tissue regeneration, moisturizer in cosmetic formulations). Indeed, collagen has the properties related to its gel formation, surface behavior, which includes emulsion, foam formation, stabilization (stable foam occurred in our tested media, see above for details), adhesion, protective colloid function and film-forming capacity. In addition, collagen is a good surface-active agent and has an ability to penetrate a lipid-free interface[39,40].

All these propreties make it a good component which are likely contributed to improve the *Gemmata* nutrition, which are bacteria well-known to uptake of such macromolecules by their endocytosis-like process[41,42]

Additionnaly, some of sponge skeletons solubilized molecules by heat in our enriched media are likely Glycosaminoglycans (a source of N-acetyl-glucosamine, N-acetyl-galactosamine, uronic acid), a well-known polysaccharide from sponginous skeletons of *Spongia officinalis* and *S. lamella* with a total content of 0.367 ± 0.028 and 0.460 ± 0.081 (µg hexuronate/mg dry weight), respectively[33,37]. The N-acetyl-glucosamine from these Glycosaminoglycans represents a good source of both carbone and nitrogen for planctomycetes nutrition [12,13] and this can explain (partly) the molecular evidence stimulating the rapid growth of slow-growing *Gemmata* spp. Hence, sponge skeleton filtrates are a good source of several well-known molecules but, it seems that spongin chemistry is very complex by the presence of many yet unknown molecules which have never been reported and which have certainly contributed to enrich again our supplemented media.

On the other hand, we observed that adding marine sponge small fractions (medium A) to culture of *Gemmata* spp. bacteria resulted in highly significant increases in bacterial growth (P<0.0001), compared to controls. We interpreted these observations as indicative of a mechanical effect of promoting growth. In the nature, the planctomycetes (genus *Rhodopirellula*) exhibit an attached life style to Mediterranean sponge *Aplysina aerophoba*[14,19].The presence of a holdfast of glycoproteic nature favors attachment and, thus, the colonization of surfaces[43]. The marine sponge *Spongia* sp. skeleton resemble in its organization and composition the connective tissue of vertebrates in that it is composed of collagen fibers and fibrils

embedded in an amorphous matrix containing carbohydrates[28], which support the adhesion and have already proven to promote growth and differentiation of human mesenchymal stem cells into osteoblast cells[44–46]. It is the mechanical and materials properties of the skeleton of these sponges which would favor *Gemmata* holdfasts attachment and likely could activate a signal transduction through a second messenger to accelerate the budding, which would result of an increasing cell division.

In summary, the very rapid growth kinetics observed from supplemented media may be due to some well-known (N-acetyl-d-glucosamine) and/or unknown soluble chemicals released by hot water from the sponge skeleton tissues for *Gemmata* metabolism. The sponge may also have promoted mecanically the rapid growth by providing a microenvironment for bacterial attachment and an increasing of bacterial division. Despite the 2-3 days gained after the enriched solid media by the sponge skeleton filtrate, the growth of these fastidious bacteria remains slow on this enriched solid medium. Further works should be undertaken to lyophilize these sponge skeleton filtrates or by grinding fresh sponge tissues to enrich fastidious germs culture media. A culture in a sponge skeleton liquid medium is necessary as enriched medium before plating on sponge filtrate solid agar for isolation.

In conclusion, our study provids here, a very promising technique which could be easily implemented in microbiology laboratories. Although scientists can enrich slow-growing microorganisms using several methods as diffusion chambers[47]or soil substrate membranes[48], most enriched bacteria (uncultured *Gemmata*-likes) could not grow on agar plates for isolation and further cultivation because, in fact, during

the enrichment step, fast-growing microorganisms may overcome slow growing bacteria, leading to reduced diversity of bacterial species. This technique may help confirm the involvement of *Gemmata*-like microorganisms in infectious processes in aplastic patients or in pulmonary disease in the intensive care unit, which are not documented microbiologically yet.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *G. obscuriglobus* DSM 5831^T and *G. massiliana* DSM 26013^T (CSUR P189^T) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the Collection de Souches de l'Unité des Rickettsies (Marseille, France), respectively. Bacteria were sub-cultured on solid Caulobacter medium DSMZ 595 and in liquid Caulobacter medium DSMZ 595, prepared as described on the website (http://www.dsmz.de). These two culture media were used as standard control media in next experiments. Bacteria were grown on these media incubated aerobically at 30°C for 7 to 14 days. Identification of colonies was then ensured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis as previously described[33]

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Marine sponges. Natural dead sponges skeleton *Spongia* sp. (**Fig.3a**) were purchased from three local suppliers in Marseille, France (local suppliers are a shop selling natural arts objects such as unbleached marine sponge and other products) at different times for successive experiments. Researchers should be careful, the color of the sponge must be dark yellow to maroon color and unscented, compared to

skeletons of natural sponges totally bleached and sold in soap shops and pharmacies (very white and purified, personal comparison). Sponges were soaked in 9.6 % bleach (sodium hypochlorite, Laboratoire Oxena, Romans-sur-Isère, France) solution for 3 minutes and then washed in deionized water as described elswhere[50]. Sponges were then rinsed three times by immersion in deionized sterile water for one hour and then dried at room temperature. Afterwards, sponges were cut into 0.5 g pieces (**Fig.3b**).

Experimental procedures. All experiments incorporated five replicates, and were repeated five times for ensuring reproducibility, and were done independently with each one of the two cultured strains.

Experiments on liquid media (see Fig.3). Three liquid media were prepared: (i) five glass tubes (Bio-Rad Laboratories, Hercules, California) containing 15 mL Caulobacter liquid medium as control medium (ii) five glass tubes containing 15 mL Caulobacter liquid medium plus accurately weighed 0.5-g fractions (in order to increase the surface of bacterial attachment) of marine sponges, immersed in the Caulobacter liquid medium, a liquid–solid biphasic medium refered as medium A (iii) five glass tubes containing 15 mL of medium A except that the 0.5-g pieces of sponge tissues were removed after autoclaving and filtered to yield a liquid monophasic medium refered as medium B (**Fig.3c,d,e**). All media were sterilized by autoclaving at 121°C for 15 min. and the pH was adjusted to 6.8 by the dropwise addition of KOH (10 M). Also, the viscosity of the media was grossly assessed by depositing a drop on a solid agar, inclined at an angle of about 30 degree and allowing the liquid to flow. In each experiment, batches comprised of five inoculated tubes and three negative controls, non-inoculated tubes. Prior to inoculation,

Gemmata spp. bacteria were suspended in sterile water, calibrated at 10^3 bacteria/ mL using a Kovas slide 10 (Hycor biomedical, Germany) and 10 µL of this suspension were inoculated into each one of 5 tubes containing control Caulobacter medium, 5 tubes containing medium A and 5 tubes containing medium B (**Fig.3f**). In parallel, 10 µL of sterile water were added in the 3 negative control tubes. The preparations were then incubated at 30º C in an aerobic atmosphere for seven days. Bacteria growth was examined daily by microscopic observations and culture-based enumerations of the Colony-Forming Units (CFUs), from day 1 to day 7. At each time-point, each tube was vortexed for 10 seconds (**Fig.3g**) and broth was removed to perform microscopic observations (**Fig.3h**) and serial dilutions of 1, 1/10, 1/100, 1/1.000, 1/10.000 followed by subculture onto Caulobacter solidified medium on Petri dishes (Greiner, Frickenhausen, Germany) (**Fig.3i**). In order to unhook and to disperse bacteria, vortexing was repeated for 4 times until a constant enumeration was obtained (low standard deviation values). Only the results of the first vortexing (1x10 s) and 4th vortexing (3 x10 s) were presented. Bacteria were then counted after two-week incubation at 30º C under aerobic atmosphere. Optical microscopic observations were carried-out on the sponge tissues before and after autoclaving. Also, microscopic observations were performed in the presence or without trypan blue staining at fresh state using Kova slide, in order to monitor bacterial attachment to marine sponges' small fractions, bacterial abundance, rosettes formation and their morphology from each medium.

Experiments on solid media. Experiments were also conducted to evaluate the time of growth and the colonies features on solid media (distilled water containing 0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4$1S 7H20 and 1.5 % agar). Indeed,

for control medium and medium A described above, 100-mm Petri dishes containing 15g/L of solid agar were prepared to contain each component reported above (**Fig.3j**). For medium A, 500 µL of marine sponge filtrate were added on the plate and dried for 30 minutes in a laminar flow cabinet and 500 µL of Caulobacter liquid medium were added in the control Petri dishes. Non-inoculated, negative control Petri dishes were manipulated in parallel. Agar plates were incubated at 30°C for 2-3 weeks under aerobic conditions and monitored every day. *G. massiliana* and *G. obscuriglobus* were cultured independently in the same manner.

Statistical analysis. Means and standard deviation values were calculated at each time (five replicates, n=5). All the experiments were reproduced independently in the same manner with *G. obscuriglobus* in parallel to *G. massiliana*. Results are expressed as CFU per mL. Statistical analysis one line (http://marne.u707.jussieu.fr/biostatgv/) was used to conduct an analysis of variance and least significant difference mean separation tests (P <0.05).

Figure legends:

Figure 1. Growth-enhancement effect of complementing *Gemmata obscuriglobus* standard culture medium (red bar) with marine sponge filtrate (green bar) and sponge small fractions as a solid phase (yellow bar). Bacteria were counted on Caulobacter solid agar plate after 1 x 10 s **(a)** and 4 x 10 s **(b)** of vortex process. The number of *G. obscuriglobus* colonies (Y axis) was monitored along a 7 days period (X axis). Data presented are for a 7 days period culture. Each data point represents the mean ± SD of five experiments. Standard errors are represented by error bars.

Figure 2. Growth-enhancement effect of complementing *Gemmata massiliana* standard culture medium (red bar) with marine sponge filtrate (green bar) and sponge small fractions as a solid phase (yellow bar). Bacteria were counted on Caulobacter solid agar plate after 1 x 10 s **(a)** and 4 x 10 s **(b)** of vortex process. The number of *G. obscuriglobus* colonies (Y axis) was monitored along a 7 days period (X axis). Data presented are for a 7 days period culture. Each data point represents the mean ± SD of five experiments. Standard errors are represented by error bars.

Figure 3. The main steps of experimental procedure: a. Dead *Spongia* Sp. skeleton were washed and dried. **b**. Sponges were cut into small pieces of uniform size and 0.5 g were introduced in glass tubes before adding 15 mL Caulobacter liquid medium (CLM). **c.** The three-liquid media before autoclaving: ten glass tubes containing 0.5 g small fractions of sponges immersed in 15 mL CLM (medium B), and five glass tubes containing 15 mL. **d.** Marine sponges immersed in the CLM after autoclaving at 121°C / 15 min **e.** The three media after autoclaving: at left to right:

control medium, medium B (prepared as described for medium A except that the 0.5 g small pieces of sponge tissue have been removed after autoclaving and filtered and medium A (a liquid–solid biphasic system). The final volume of culture at 15 mL was then completed after an adjustment with each corresponding liquid medium after autoclaving in order to compensate the liquid evaporated. **f**. The characteristics of the three liquid media prepared to be inoculated. (**S**) for sponge medium, (**F**) for filtrate and (**C**) for CLM. **g.** Procedure of vortex. Each tube was vortexed for 1x10 s and broth was removed to perform serial dilutions followed by subculture onto agar solidified medium on 100-mm Petri dishes. Vortex procedure was repeated (4 x10 s) until a constant enumeration was obtained. Bacterial growth was monitored by microscopy (using kova slides) (**h**) and CFUs enumeration **(i)** in parallel. **j.** Culture on solid media. For control medium and sponge filtrate medium, 15 g/L of agar were added to prepare corresponding solid media: sponge filtrate (**F**) solid medium, and (C) Caulobacter solid medium.

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Competing Interests

The authors have declared that no competing interests exist.

Authors' contributions

ODK performed experiments and interpreted the experimental data. SG and MD designed the experiments. ODK, SG and MD drafted the manuscript. All authors read and approved the final manuscript.

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Figure 1 (a,b)

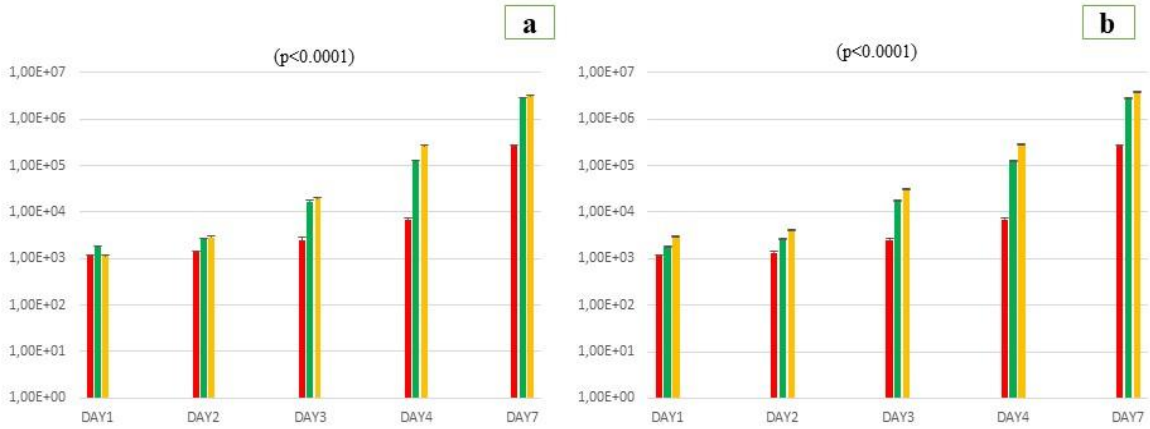
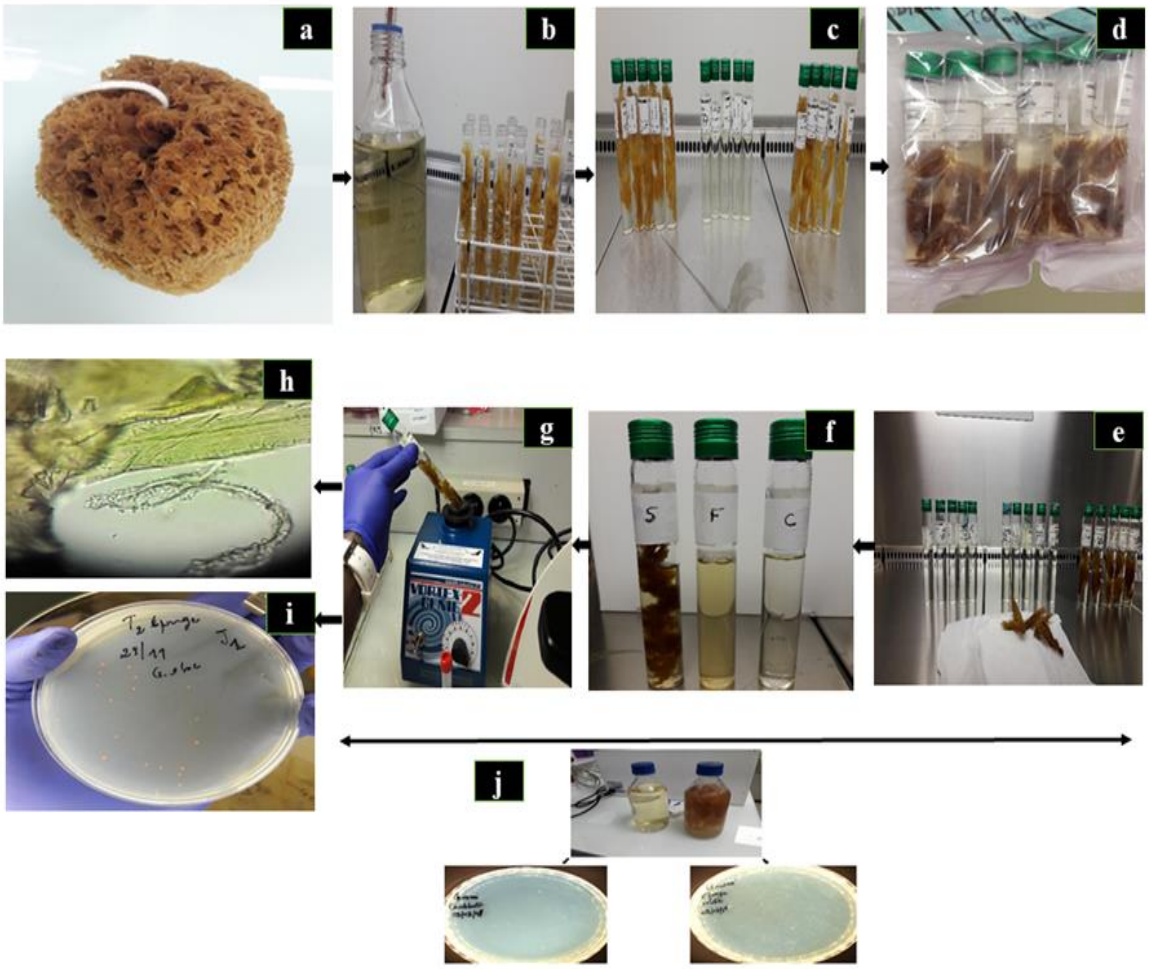

a
(p<0.0001)
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
DAY1
DAY2
DAY3
DAY4
DAY7
b
(p<0.0001)
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
DAY1
DAY2
DAY3
DAY4
DAY7

Figure 2 (a, b)


a
(p<0.0001)
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
DAY1
DAY2
DAY3
DAY4
DAY7
b
(p<0.0001)
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
DAY1
DAY2
DAY3
DAY4
DAY7

Figure 3



Chapitre 2

Amélioration de la culture des *Gemmata* spp. par co-culture avec le filtrat d'*Escherichia coli*- métabolisme du fer.

Cette étude a été guidée par le fait que beaucoup de planctomycetes (genre *Planctomyces*) possèdent de nombreuses inclusions d'hydroxyde ferrique qui se cristallisent à l'un des pôles de la cellule ou dans l'anammoxosome (van Niftrik et al., 2008; Razumov, 1949; Schmidt et al., 1981, 1982; Skuja, 1964; Storesund and Øvreås, 2013; Wawrik, 1952, 1956; Zavarzin, 1961). Aussi, de nombreux planctomycetes autres que *Gemmata* ont un tropisme pour les niches écologiques contenant des sédiments de fer (Benson et al., 2011; Storesund and Øvreås, 2013). Ainsi, dans une perspective d'améliorer les milieux de culture, nous avons poursuivi les travaux initiés par nos prédécesseurs qui avaient exploré le génome des deux espèces de *Gemmata*, à la recherche d'une voie métabolique incomplète ou déficiente qui pourrait entraver leur croissance sur milieu axénique. La voie d'acquisition du fer a été identifiée comme incomplète chez les deux bactéries du genre *Gemmata* en particulier, et les Planctomycetes en général : ni *G. obscuriglobus* ni *G. massiliana* ne codent pour les sidérophores ni l'enzyme ferriréductase. Par la suite, nous avons appliqué cette connaissance pour complémenter les milieux de culture (Caulobacter et Stanley) des *Gemmata* avec du filtrat d'*Escherichia coli,* forcé de produire des sidérophores et l'enzyme ferriréductase extracellulaire en bouillon liquide. En outre, l'étude de la relation cause à effet a été conduite en présence d'antagoniste du Fer (Deferioxamine) et du fer ferreux et férrique. La croissance des deux souches a ensuite été évaluée en milieu liquide suivie d'un dénombrement de CFUs sur milieu solide. Les expériences ont également été conduites sur des versions solides des milieux liquides. Nos observations ont révélé une croissance significativement améliorée par comparaison aux bactéries *Gemmata* cultivées sur un milieu standard non supplémenté par le filtrat-fer d'*Escherichia coli*. Les résultats de ce travail ont fait l'objet d'un article qui a été soumis au journal Frontiers in Microbiology pour publication.

Article 4

Escherichia coli culture filtrate co-culture enhances growth of *Gemmata* spp.

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Soumis pour publication dans Frontiers in Microbiology et en Révision

REVISED VERSION

Escherichia coli culture filtrate enhances the growth of *Gemmata* spp.

Running title: Iron-demanding *Gemmata* spp.

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Number of tables: 0

Number of figures: 4

ABSTRACT

Background: Genomics revealed that *Gemmata* spp., are lacking iron acquisition genes. Their lifestyle in communities with Proteobacteria suggests iron-based cooperation. We tested co-culturing *Gemmata* spp. with *Escherichia coli* culture filtrate to improve growth and isolation of *Gemmata* spp.

Materials and Methods: *Gemmata obscuriglobus* and *Gemmata massiliana* were cultured in Caulobacter and Staley's medium supplemented or not with *E. coli* filtrate, containing growth factors such as siderophores and extracellular ferri-reductases or unknow molecules. Non-inoculated controls were manipulated in parallel. We completed iron metabolism studies with $FeSO_4$, $FeCl_3$ and deferoxamine in the presence of *E. coli* filtrate and controls. Bacterial growth was monitored for seven days.

Results and discussion: Non-inoculated, negative controls remained sterile all the experiments. The number of *G. obscuriglobus* colonies was significantly higher on Caulobacter medium supplemented with *E. coli* filtrate than on the standard medium ($p<0.0001$). Likewise, the number of *G. obscuriglobus* colonies was significantly higher on Staley's medium supplemented with *E. coli* filtrate than on the standard medium ($p<0.0001$). For *G. massiliana*, the number of colonies was significantly higher on the medium supplemented with *E. coli* filtrate compared to the standard medium ($p<0.0001$). Further studies revealed a significant growth-enhancement effect after a complementing Caulobacter medium with *E. coli* filtrate and 10^{-4}M $FeSO_4$ compared to Caulobacter medium supplemented with *E. coli* alone ($p<0.0122$). Finally, the intracellular iron concentration measured in *G. obscuriglobus* and *G. massiliana* cultured in an iron-depleted broth, supplemented with *E. coli*

filtrate was respectively of 0.63 ± 0.16 µmol/L and 0.78 ± 0.12 µmol/L whereas it was of 1.72 ± 0.13 µmol/L in *G. obscuriglobus*, 1.56 µmol/L ± 0.11µmol/L for *G. massiliana* grown in broth supplemented with *E. coli* filtrate and $FeSO_4$. In the other conditions of culture, the iron concentration was 0.66± 0.17 µmol/L for *G. obscuriglobus* and 0.52 ± 0.14 µmol/L for *G. massiliana*. Deferoxamine slow down and prevented *Gemmata* spp growth. The data here reported indicated that in an iron-deprived environment, the iron uptake regulator stops iron usage, favours iron storage and slows down cellular division. Media supplemented with *E. coli* filtrate and $FeSO_4$ in these circumtances act as growth factors for *Gemmata* spp. suggesting new culturomics approaches.

Keywords: Planctomycetes*, Gemmata obscuriglobus, Gemmata massiliana, E. coli* filtrate, siderophore, iron and culture*.*

Background

Gemmata obscuriglobus (**Franzmann and Skerman 1984**) and *Gemmata massiliana* (**Aghnatios and Drancourt 2015**) are currently the sole cultured representatives of the Planctomycetes genus *Gemmata.* These orgarnisms have been detected in soil and aquatic habitat (**Hu, Cao and Zhang 2014; Pizzetti *et al.* 2011; Kirkpatrick *et al.* 2006; Pollet, Humbert and Tadonleke 2014; Shu and Jiao 2008; Pollet, Tadonleke and Humbert 2011; Buckley *et al.* 2006**), including hospital water networks in close proximity with patients (**Aghnatios and Drancourt 2015**). Using a PCR-sequencing approach, we demonstrated the presence of *Gemmata*-like sequences in the blood collected from two aplastic leukemic febrile patients with neutropenia, although we failed to isolate any Planctomycetes from the patients' blood specimens (**Drancourt *et al.* 2014**). Due to their multi-antibiotic-resistance (**Cayrou, C., Raoult, D., and Drancourt, M. 2010)** and their recent demonstrated association with humans, the potential of *Gemmata* organisms to behave as opportunistic pathogens should be more widely recognized (**Aghnatios and Drancourt 2016**).

Both *G. obscuriglobus* and *G. massiliana* are fastidious organisms, with *G. obscuriglobus* exhibiting a 13-hour doubling time and requiring a highly specific culture medium (**Lage and Bondoso 2012; Schlesner 1994; Winkelmann and Harder 2009**). Accordingly, most knowledge regarding the environmental and host-associated microbiota niches of *Gemmata* organisms is derived from DNA-based studies including PCR-sequencing-based analyses and metagenomics studies (**Bacci *et al.* 2015; Vega-Avila *et al.* 2015; Zhang *et al.* 2015**).

In the environment and human microbiota, *Gemmata* organisms live in communities with Proteobacteria (**Revetta *et al.* 2010**). Genomic studies revealed that *Gemmata* bacteria lack siderophores for their metabolism, partially explaining their fastidiousness when grown on culture media (**Jeske *et al.* 2013; Doern 2000**). Here we hypothesized that co-culture of *Gemmata* bacteria with *Escherichia coli* as a Proteobacteria organism could improve the growth of *Gemmata* bacteria using culture media.

Materials and methods

Bacterial strains

G. obscuriglobus DSM 5831^T and *G. massiliana* DSM 26013^T (CSUR P189^T) were obtained from the Collection de Souches de l'Unité des Rickettsies (Marseille, France) and the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Bacteria of both species were sub-cultured on Caulobacter medium DSMZ 595 or Staley's maintenance medium DSMZ 629 prepared as described on the website (**http: //www.dsmz.de**). Bacteria were grown on these solid media incubated aerobically at 30°C for 7 to 14 days. Identification of colonies was ensured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis as previously described (**Cayrou, C., Raoult, D., & Drancourt, M.** 2010).

***Escherichia coli* filtrate preparation:** *E. coli* strain CIP 7624 (Collection de l'Institut Pasteur, Paris, France) was initially cultured on blood agar (bioMérieux, Marcy l'Etoile, France) for 24 hours at 37°C and its identification was ensured by using MALDI-TOF-MS as previously described **(Seng).** Kovas slide 10 (Hycor biomedical, Germany) and microscopic examination were used to calibrate the bacterial cell

counts at 10^{12} Colony Forming Units (CFU) /mL. One mL of this suspension was then sub-cultured in 75-cm2 culture flasks contained 49 mL of autoclaved GLD medium (1 g glucose, 1.4 g peptone, 0.3 g NaCl, 20 mL Hutner's salt (DSMZ 590), 10 mL Staley vitamins (DSMZ 600, added after filter-sterilized) and 970 mL distilled water) incubated aerobically with shaking at 250 rpm for two days at 30°C in order to elicit the release of *E. coli* siderophores in a low-iron environment (**Miethke M, and Marahiel MA. 2007**). Sonication was used in order to increase release of *E-coli* siderophores, as previously described (**Kwon, Y. C., & Jewett, M. C. 2015**). Finally, sonication broth was 0.2 µm-filtered (Sigma-Aldrich, Saint-Quentin Fallavier, France) to obtain the *E. coli* filtrate named solution A. Solution B was prepared in the same manner as solution A except that a 10^{-4} M ferrous sulfate heptahydrate (Sigma-Aldrich) was added to the GLD medium followed by 3-day incubation and filtration. Solution B was aimed at inducing the production of extracellular iron reductase by *E. coli* in an iron-rich environment. As a negative control an autoclaved non-inoculated GLD medium was treated in the same conditions in parralel to inoculated culture flasks. Finally, flask 10-µL of solution A, solution B and control GLD medium were then seeded in blood, Staley's and Caulobacter solid agar to ensure sterility.

Culture of *Gemmata* spp. on Caulobacter and Staley's liquid media with *E. coli* filtrate.

G. obscuriglobus and *G. massiliana* were cultured independently in five replicates, in Caulobacter liquid medium in a final volume of 15 mL. In detail, five (5) tubes containing 9 mL of Caulobacter liquid medium were supplemented with 5 mL of *E-coli* filtrate (2.5 mL solution A + 2.5 mL solution B) and five (5) tubes containing 9 mL of Caulobacter liquid medium were supplemented with 5 mL of GLD medium (negative

controls). One mL of an inoculum of 3.10^2 CFUs/mL, suspended in sterile distilled water (Bio-Rad Laboratories, Hercules, California) was inoculated in each one of the ten (10) tubes (5 test tubes and 5 control tubes). Moreover, 2 test tubes and 2 control tubes were manipulated in the same conditions in parallel as negative control tubes with only 1-mL sterile distilled water (no inoculated tubes). The preparations were then incubated at 30ºC in an aerobic atmosphere for seven days. At day 1; 2; 3; 4; and day 7 post-inoculation, each tube was shaken, and 1-mL of the broth was removed to perform serial dilutions of 1, 1/10, 1/100, 1/1.000, 1/10.000 in sterile distilled water for culture-based microbial enumerations. CFUs were enumerated on 100-mm Petri dishes containing Caulobacter solid agar. Colonies were counted using scanning software (ImageJ, Interscience, Saint Nom, France). Means and standard errors were calculated at each time (five replicates, n=5). All the experiments were reproduced independently in the same manner with Staley's liquid medium with *G. obscuriglobus* in parallel to *G. massiliana*.

Culture of *Gemmata* spp. in iron-repleted and iron-depleted conditions in the presence of *E-coli* filtrate and deferoxamine

The experiments were performed only in Caulobacter liquid medium (*Gemmata* spp. standard medium iron-free, compared with Staley's medium which contains $FeSO_4$) in five independent replicates. The iron metabolism was studied in assay tubes containing a Caulobacter liquid medium in iron-repleted and iron-depleted conditions in the presence of *E-coli* filtrate and controls. Ferrous iron heptahydrate, $FeSO_4$•7 H_2O (Sigma Aldrich), ferric chloride, $FeCl_3$ (Sigma Aldrich) and deferoxamine (Novartis, Rueil-Malmaison, France) were used to probe iron assimilation. Each one of these components was added at a final concentration at 10^{-4} M in a final volume at

15-mL. In detail, the first tube contained 10^{-4}M $FeSO_4$, the second contained10^{-4} M $FeCl_3$, the third contained10^{-4}M deferoxamine, the fourth contained 10^{-4}M $FeSO_4$ +10^{-4}M deferoxamine, the fifth tube contained 10^{-4}M $FeCl_3$+10^{-4}M deferoxamine dissolved each one in Caulobacter liquid medium and the last tube contained only Caulobacter liquid medium. In parallel, six other tubes contained 9 mL of Caulobacter liquid medium supplemented with 5 mL of *E. coli* filtrate (2.5 solution A+2.5 solution B) and each one of these components as descibed above, was added at a final concentration at 10^{-4}M in a 15-mL final volume. Then, 1 mL of an inoculum of 3.10^2 CFUs/mL suspended in Caulobacter liquid medium was added in each one of the twelve tubes and incubated aerobically at 30°C for 7 days. One non-inoculated, negative control tube for each one of the twelve tubes was manipulated in parallel. At day 1; 2; 3; 4; and day 7 post-inoculation, each tube was shaken, and 1 mL was removed to perform serial dilution of 1, 1/10, 1/100, 1/1000 and 1/10.000 in distilled sterile water for culture-based CFU enumerations on Caulobacter solid agar. Furthermore, for each tube, 100-mm Petri dishes containing solid agar were prepared to contain each component reported above with the same final concentration in parallel in order to monitor the experiment on solid media (colonies features, color and time of growth in the presence or not *E. coli* filtrate. For the Petri-dishes; prepared to contain *E. coli* filtrate, 500µL solution A and 500µL solution B were added on the plate and dried at room temperature for 30 minutes in a laminar flow cabinet. Non-inoculated, negative control tubes and Petri dishes were manipulated in parallel. Bacteria were then counted using scanning software. *G. massiliana* and *G. obscuriglobus* were cultured independently in the same manner. Finally, intracellular iron was quantified after one-day and seven-day incubation. 10

µL of each liquid culture were inoculated on a Caulobacter solid medium and a Caulobacter solid medium complemented with each component as described above to monitor the bacterial features, survival and contamination. After seven day-incubation, the liquid medium was centrifuged at 1.1g for five minutes and the pellet was washed thrice with 10^{-4}M deferoxamine. A colorimetric ferrozine method was used to measure the concentration of iron as previously described (**Riemer J, Hoepken HH, et *al*. 2004**). Briefly, 200 µL of 50 mM NaOH, 200 µL of 10 mM HCl and 200 µL of iron releasing solution were added to the specimens and incubated further for two hours at 60ºC. All solutions were then 0.2µm filtered and iron concentration was measured in a 350 µL-aliquot using an Iron2 cobas kit (Cobas, Meylan, France).

Results and discussions

Non-inoculated, negative controls remained sterile all the time of experiments. The number of *G. obscuriglobus* colonies was significantly higher on Caulobacter medium supplemented with *E. coli* filtrate (126 ± 13 colonies on day 1; 787 ± 38 colonies on day 7) than on the standard medium (62 ± 10 colonies on day 1; 261 ± 27 colonies on day 7), ($p<0.0001$). Likewise, the number of *G. obscuriglobus* colonies was significantly higher on Staley's medium supplemented with *E. coli* filtrate (75 ± 11 colonies on day1; 247 ± 20 colonies on day7) than on the standard medium (32 ± 6 colonies on day1; 82 ± 18 colonies on day 7) ($p<0.0001$) (**Fig.1**). For *G. massiliana*, the number of colonies was significantly higher on the medium supplemented with *E. coli* filtrate (Caulobacter medium 170 ± 29 colonies at day 1; 694 ± 35 colonies at day 7; Staley medium 74 ± 12 colonies at day 1 and 246 ± 21 colonies at day 7) compared to the standard medium (Caulobacter medium 89 ± 11 colonies at day 1

and 329 ± 37 colonies at day 7; ($p<0.0001$), Staley medium 54 ± 8 colonies at day 1 and 148 ± 17 colonies at day 7), ($p<0.0001$)(**Fig.2**). Altogether, the number of *Gemmata* spp. colonies was significantly higher after enrichment of the reference culture medium with *E. coli* filtrate ($p<0.0001$). Surprisingly, *Gemmata* spp. grew better in Caulobacter medium supplemented with *E. coli* filtrate than in Staley's medium supplemented with *E. coli* filtrate (**Fig.1;2)** although Staley's medium contains more components such as Staley's vitamins (see medium DSZM 600) and Hutner's salts (see medium DSZM 590), which contains 99mg/L $FeSO_4$. The pH of the two liquid media was maintained at pH= 7 at 25°C to respect the *Gemmata* spp. optimal pH for growth. At day3-7; the pH of Caulobacter liquid medium measured at 4.6 ± 0.2 (day-3-7) and 4.2 ±0.4 (day-3-7) for the Staley's liquid medium. At this acid pH = 4, it is known that the chemical oxidation of ferrous iron contained in aqueous environment is no longer significant (**Maitte, B., Grgic, D., & Jorand, F. P.** 2016) and therefore likely to release more ferrous iron than its oxidized form. More iron did not enhance the *Gemmata* growth. It is possible that other factors may inhibit the growth of *Gemmata* spp. and decrease the iron activity in Staley's medium. We then completed our experiments by complementing Caulobacter liquid medium with 10^{-4}M ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), 10^{-4}M ferric chloride ($FeCl_3$), and deferoxamine in the presence or not of *E. coli* fitrate to gain insight on the variety of iron which promotes more *Gemmata* spp. growth. (**Fig.3;4**). Our observations revealed a high growth-enhancement effect after complementing Caulobacter medium with *E. coli* filtrate and 10^{-4}M $FeSO_4$ (189 ± 22 colonies on day1; 1,091 ± 53 colonies on day7 for *G. obscuriglobus,* and 248 ± 19 colonies on day1; 1,029 ± 32 colonies on day 7 for *G. massiliana*) compared to Caulobacter medium supplemented

with *E. coli* filtrate alone (134 ± 17 colonies on day1, 783 ± 31 colonies on day7 for *G. obscuriglobus*; ($p<0.0016$); 166 ± 18 colonies on day1, 713 ± 27 colonies on day 7 for *G. massiliana* ($p<0.0122$). The same trend was obtained by complementing Caulobacter medium with *E. coli* filtrate in the presence of $FeCl_3$ and showed that $FeSO_4$, promote *Gemmata* growth more than $FeCl_3$ contained in the liquid medium (**Fig.3, 4**). These results might depend on the interaction between the variety of iron and its binding with siderophores contained in *E. coli* filtrate or the pH and iron potential-oxydo-reduction. Indeed, these two varieties of iron have different behaviors depending on the pH, oxygen concentration and bacteria metabolism. The chemical oxidation of aqueous ferrous iron increases with increasing pH and oxygen concentration. At pH = 4, the chemical oxidation of ferrous iron is no longer significant and ferrous iron is then used by acidophilic ferrooxidizing bacteria as a source of energy. In aqueous environments at neutral pH (6 to 8, this reaction starts $Fe^{2+}+2OH^{-}\rightarrow Fe(OH)_2\downarrow$, and is complete at pH 7.8.) and saturated with oxygen (8 mg / l) the chemical oxidation of iron is rapid and the bacterial populations do not represent a significant part of the oxidation. However, in the presence of a low oxygen concentration (2 mg / l), the chemical oxidation rate decreases and ferrooxidant neutrophilic bacteria are able to use ferrous iron as a source of energy, and we are in the presence of a competition (**Maitte, B., Grgic, D., & Jorand, F. P.** 2016).Also, siderophores (contained in *E. coli* filtrate) form high-spin, kinetically labile chelates with ferric ion which are characterized by exceptional thermodynamic stability (**Schwarzenbach G. and Schwarzenbach K., 1963 ; Raymond K. N. and Carrano C. J. 1979**) and they have strong affinity for only the higher oxidation state of iron and set this natural complexing agent apart from molecules such as heme,

which serve effectively as electron shuttles. At the same time, the relatively weak complexing of Fe (II) affords an efficient means of release, via reduction, inside the cell. This large discrepancy in the binding constants for Fe (II) and Fe(III) drives down the oxidation-reduction potential, and there has been some discussion that the actual value may be beyond the range of natural reducing agents. This aspect of the problem requires clarification and elucidation at the enzyme-ferrireductase level. Probably the significant feature is the oxidation-reduction potential of the enzyme-ferric siderophore complex rather than the potential of the free ferric chelate.With few exceptions, the “hard” acid ion, Fe(III), is linked to hard base atoms, such as oxygen, which accounts for the preference for ferric ion (**Neilands, J. B. 1995**). Then, due to it reduced form maintained in caulobacter medium at pH= 4.4 ± 0.3 (day-3-7) than 3.1 ±0.6 (day-3-7) for the medium supplemented with $FeCl_3$, ferrous iron was hence sufficiently available for *Gemmata* spp at this reductive state (**Fig.3;4**).Intracellular iron concentration measured in *G. obscuriglobus* and *G. massiliana* cultured in an iron-depleted broth, supplemented with *E. coli* filtrate alone were respectively 0.63 ± 0.16 µmol/L and 0.78 ± 0.12 µmol/L and whereas its were 1.72 ± 0.13 µmol/L in *G. obscuriglobus*, 1.56 µmol/L ± 0.11µmol/L for *G. massiliana* in grown in a iron-rich broth supplemented with *E. coli* filtrate and $FeSO_4$. In the other conditions of culture, the iron concentration was 0.66± 0.17 µmol/L for *G. obscuriglobus* and 0.52 ± 0.14 µmol/L for *G. massiliana*. These data may be indicated that in an iron deprived environment, the iron uptake regulator stops iron usage, favours iron storage and slows down cellular division. Media supplemented with *E. coli* filtrate and $FeSO_4$ in these circumtances act as growth factors for *Gemmata* spp. Futhermore, deferoxamine added on these media containing iron slows down both *G. massiliana*

and *G. obscuriglobus* growth. *E. coli* filtrate added then on the media containing deferoxamine promote *Gemmata* spp growth, observed with the two-iron variety and more in the media deferoxamine-free (**Fig.3.4**). These last observations have been confirmed on solid medium complemented with *E. coli* filtrate, $FeSO_4$, $FeCl_3$ and deferoxamine. Otherwise, the features of colonies observed on solid caulobacter agar compared with colonies observed in caulobacter solid agar complemented with *E-coli* filtrate (500µL solution A and 500µL of solution B), $FeSO_4$, $FeCl_3$ and deferoxamine showed different colors of colonies (colonies grown on Caulobacter from iron-enriched broth were bigger and redder than colonies grown in the other culture conditions, which appeared small and pale pink. This phenomenon was more marked for *G.obscuriglobus* than for *G.massiliana*) and times of growth (5-7days for *G. obscuriglobus* while it was 6-7 days for *G. massiliana* in the presence of *E. coli* filtrate and FeSO4) compared to 8-9 days with the media supplemented by $FeSO_4$ or $FeCl_3$ without *E-coli* filtrate, observed with *G. massiliana*. Also; It grew moderately on Caulobacter solid agar when previously incubated in a broth containing deferoxamine (**Fig.3, 4**). The fact that the presence of deferoxamine in the medium supplemented by iron slows down *Gemmata* spp growth suggest that iron improve the *Gemmata* spp. growth. The results obtained with deferoxamine as indicated in figure3 and 4 suggest that *E-coli* contains siderophores which have a high affinity for iron than deferoxalmine. Indeed, *Gemmata* spp. growth have been improved in the media previously contained deferoxamine and iron by *E.coli* fitrate supplementated, suggesting that *E. coli* siderophore is able to shift the balance between deferoxamine and iron and make iron reduced more availaible for the cells. Finally, there had a lightweight growth enhancement effect in the medium supplemented with *E. coli*

filtrate and $FeCl_3$, which might suggest a presence of extracellular ferrireductase in *E. coli* filtrate to reduce ferric iron to ferrous iron (**Fig3,4**).

Genomic trait relevant to this observation

In order to probe any genomic trait relevant to this observation, we annotated the genome of *Planctomycete* KSU-1 [Genbank: ASM29679v1], *Pirellula staleyi* DSM 6068 [Genbank: NC_013720.1], *Phycisphaera mikurensis* NBRC 102666 [Genbank: NC_017080.1], *Planctomyces maris* DSM 8797 [Genbank: NZ_ABCE00000000.1], *G. obscuriglobus* UQM 2246 [Genbank: NZ_ABGO00000000.1], *G. massiliana* [Genbank: CBXA000000000.1], *Planctomyces limnophilus* DSM 3776 [Genbank: NC_014149.1] and *Blastopirellula marina* DSM 3645 [Genbank: NZ_AANZ00000000.1] using the Rapid Annotation Subsystem Technology server **(Meyer *et al.* 2008**). Using the seed viewer, we then searched in the iron acquisition and metabolism subsystem for genes coding for siderophores, iron acquisition and metabolism and iron transport. Additionally, in the stress response and oxidative stress subsystem, we searched for genes that played a double role as protectors from oxidative stress and iron related functions; ferric iron uptake regulation protein, heme oxygenase and ferroxidase. We searched specifically for bacterioferritin and ferrous iron transport protein in the NCBI *G. obscuriglobus* proteomic database as *G. massiliana* protein database is not annotated. All detected genes were double checked using NCBI BLAST **(Geer *et al.* 2010**). *Planctomycete* KSU-1, *P. maris*, *G. massiliana*, *P. limnophilus* and *B. marina* genomes do not encode siderophores, iron acquisition, transport and metabolism genes; *P. staleyi* genome contained *ton*B gene, a component of the ferric iron siderophore transport system; and *P. mikurensis* encodes *pit*A, *pit*C and *pit*D genes responsible for ferric iron ABC transport. Both

Gemmata species encode a heme oxygenase that could release ferrous iron from hosts' heme and ferroxidase that oxidizes ferrous iron to ferric iron. In the *G. obscuriglobus* protein database we spotted a ferric iron uptake regulation protein bacterioferritin, ferrous iron transport protein A and ferrous iron transport protein B. Altogether, these genomic data suggest that *Gemmata* spp. have the ability to extract heme-bound ferrous iron by heme oxygenase, to transport and to oxidize ferrous iron into ferric iron and to stock ferric iron in bacterioferritin; while *Gemmata* spp. lack the ability to transport, to capture and to reduce ferric iron to a ferrous form. Therefore, we observed that both *Gemmata* species lack a complete set of genes involved in iron acquisition.

Iron is a trace metal necessary for bacterial survival and growth since several metalloproteins use iron as a cofactor (**Schalk, Hannauer and Braud 2011**). Iron found in soil and sedimentary and more rarely in ocean water (**Andrew, Robinson and Rodriguez-Quinones 2003**) is extracted from the environment and transported into the bacterial cell by siderophores that are repressed in an iron-rich environment. Also, environmental ferric iron has to be reduced into ferrous iron by extracellular bacterial reductase to be assimilated by the bacteria **(Guan, Kanoh and Kamino 2001; Miethke and Marahiel 2007; D'Onofrio *et al.* 2010; Vartivarian and Cowart 1999)**. Ferric uptake regulator protein controls iron acquisition through a ferrous iron mediated repression of iron-regulated promoters, since an excess of intracellular iron engenders reactive oxygen species via the Fenton reaction **(Escolar, Pérez-Martín and De Lorenzo 1999)**. Therefore, several bacteria lacking siderophores depend on other bacteria to provide them with iron **(D'Onofrio *et al.* 2010; Posey and Gherardini 2000; Reeves *et al.* 1983**) thus explaining in part their fastidiousness

when grown on a synthetic medium (**D'Onofrio *et al.* 2010**). Accordingly, a great diversity of Planctomycetes lineages including *Gemmata*-Isosphaera, Planctomyces, Phycisphaerae, Pirellula-Rhodopirellula-Blastopirellula and the "OM190" lineage have been detected in iron-hydroxide deposits associated with Anammox bacteria of the Brocadiales lineage, which synthetize bacterioferritin that captures and stores ferric iron. The high diversity of Planctomycetes in these Anammox-rich environments contrasts with the restricted diversity of Planctomycetes in other environments, suggesting an iron-based cooperation between Anammox and the other Planctomycetes members **(van Niftrik and Jetten 2012; Zhao *et al.* 2014; Storesund and Ovreas 2013**).

In this study, *G. obscuriglobus* and *G. massiliana* genome analysis revealed that both species have an incomplete iron pathway. Indeed, only a ferric uptake regulator protein, a heme oxygenase and a ferroxidase were detected in their genomes and a bacterioferritin and ferrous iron transport proteins in the annotated genome of *G. obscuriglobus*. Accordingly, we observed that *G. obscuriglobus* did not grow on a medium containing deferoxamine. The addition of *E. coli* supernatant significantly increased the growth of both *Gemmata* species. These data suggest that in the environment, as well as in human microbiota, *Gemmata* organisms might rely on neighbouring bacteria to get required ferrous iron. At the opposite, axenic media limit the capacities of *Gemmata* bacteria for iron acquisition. It should be taken into consideration that ferrous iron oxidizes into ferric iron when the pH is superior to 5, making the available ferrous iron in an axenic medium very scarce.

In conclusion, our results indicate that genome analysis can be used to progressively improve the Planctomycetes empirical culture media as illustrated for *Gemmata*

species in this report. This has been the case in the genome-based assessment of the bacterial nutriment requirement for the culture of fastidious, facultative intracellular bacteria *Rickettsia*, *Tropheryma* and *Coxiella* (**Renesto and Raoult 2003; Renesto *et al.* 2003; Renesto *et al.* 2005; Fournier, Drancourt and Raoult 2007; Omsland *et al.* 2009**). This culture medium design strategy helped improve the culture of fastidious bacteria, allowing to switch from empirical medium design to more specialized ones. Likewise, investigating nutrients requirements for *Gemmata* organisms may help designing new culture media for their recovery from both environmental and host microbiota (**Drancourt *et al.* 2014**).

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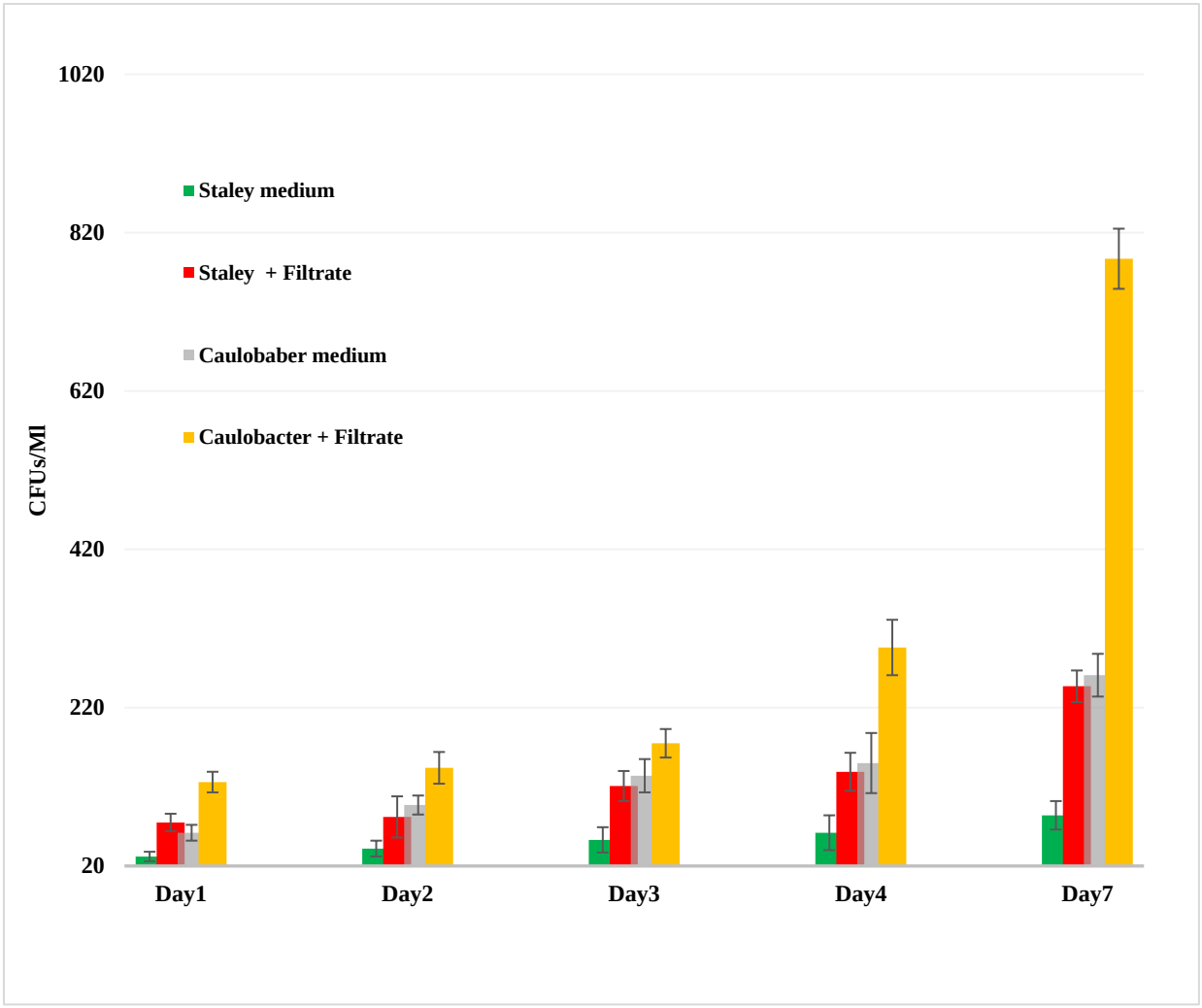
Figure legends:

Figure 1. *G. obscuriglobus* growth in Caulobacter standard medium (gray coloured bar) and Staley standard medium (green coloured bar) compared to Caulobacter (yellow coloured bar) and Staley medium (red coloured bar) supplemented with *E. coli* filtrate. Number of *G. obscuriglobus* colonies per millilitre (Y axis) was monitored on solid agar medium along a 7 days period (X axis).

Figure 2. *G. massiliana* growth in Caulobacter standard medium (gray coloured bar) and Staley standard medium (green coloured bar) compared to Caulobacter (yellow coloured bar) and Staley medium (red coloured bar) supplemented with *E. coli* filtrate. Number of *G. massiliana* colonies per millilitre (Y axis) was monitored on solid agar medium along a 7 days period (X axis).

Figure 3. *G. obscuriglobus* growth in Caulobacter standard medium in the presence of *E. coli* filtrate, $FeSO_4$, $FeCl_3$, Deferoxamine and controls. Number of *G. obscuriglobus* colonies per millilitre (Y axis) was monitored on solid agar medium along a 7 days period (X axis).

Figure 4. *G. massiliana* growth in Caulobacter standard medium in the presence of *E. coli* filtrate, $FeSO_4$, $FeCl_3$, Deferoxamine and controls. Number of *G. massiliana* colonies per millilitre (Y axis) was monitored on solid agar medium along a 7 days period (X axis).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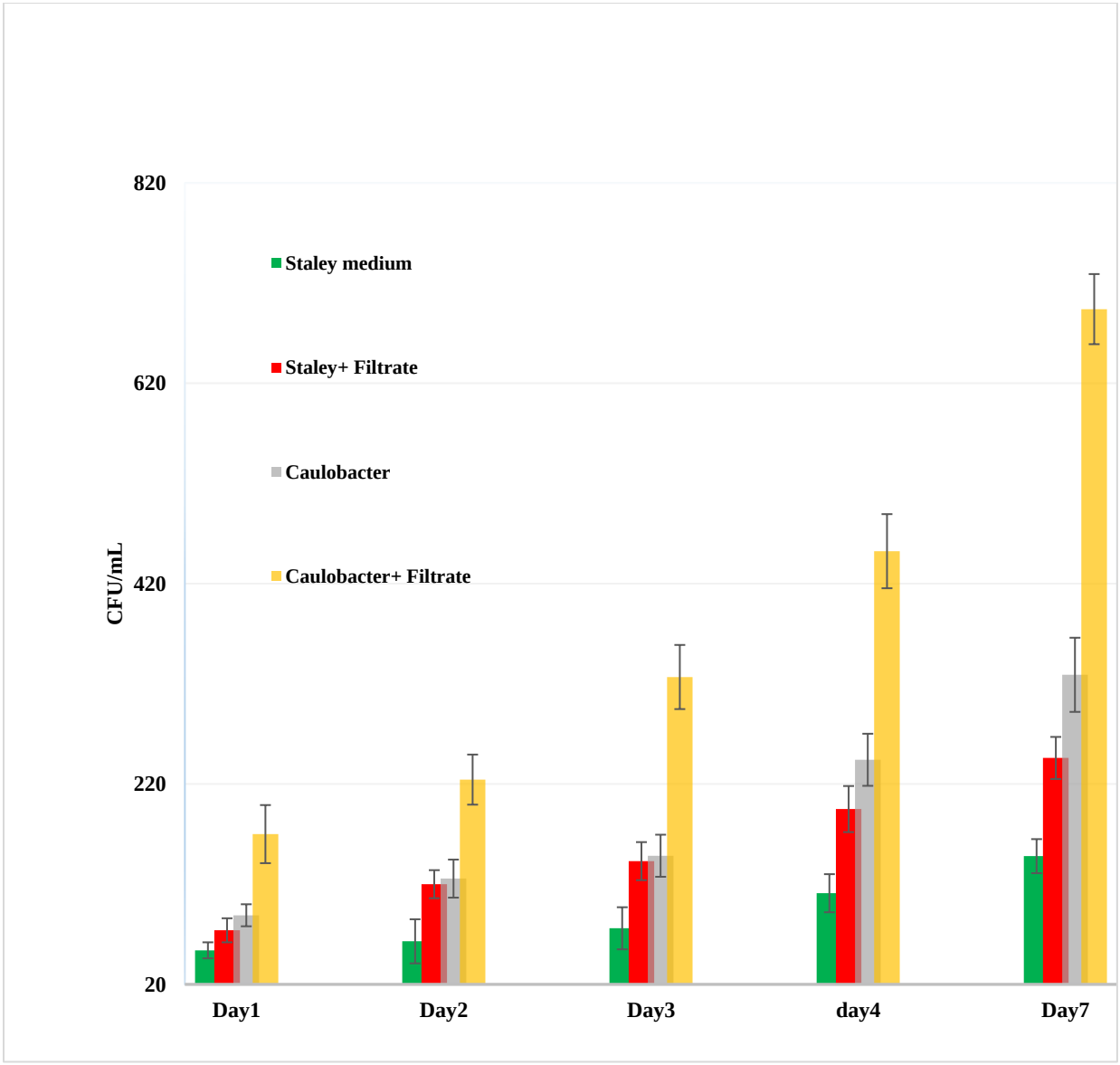
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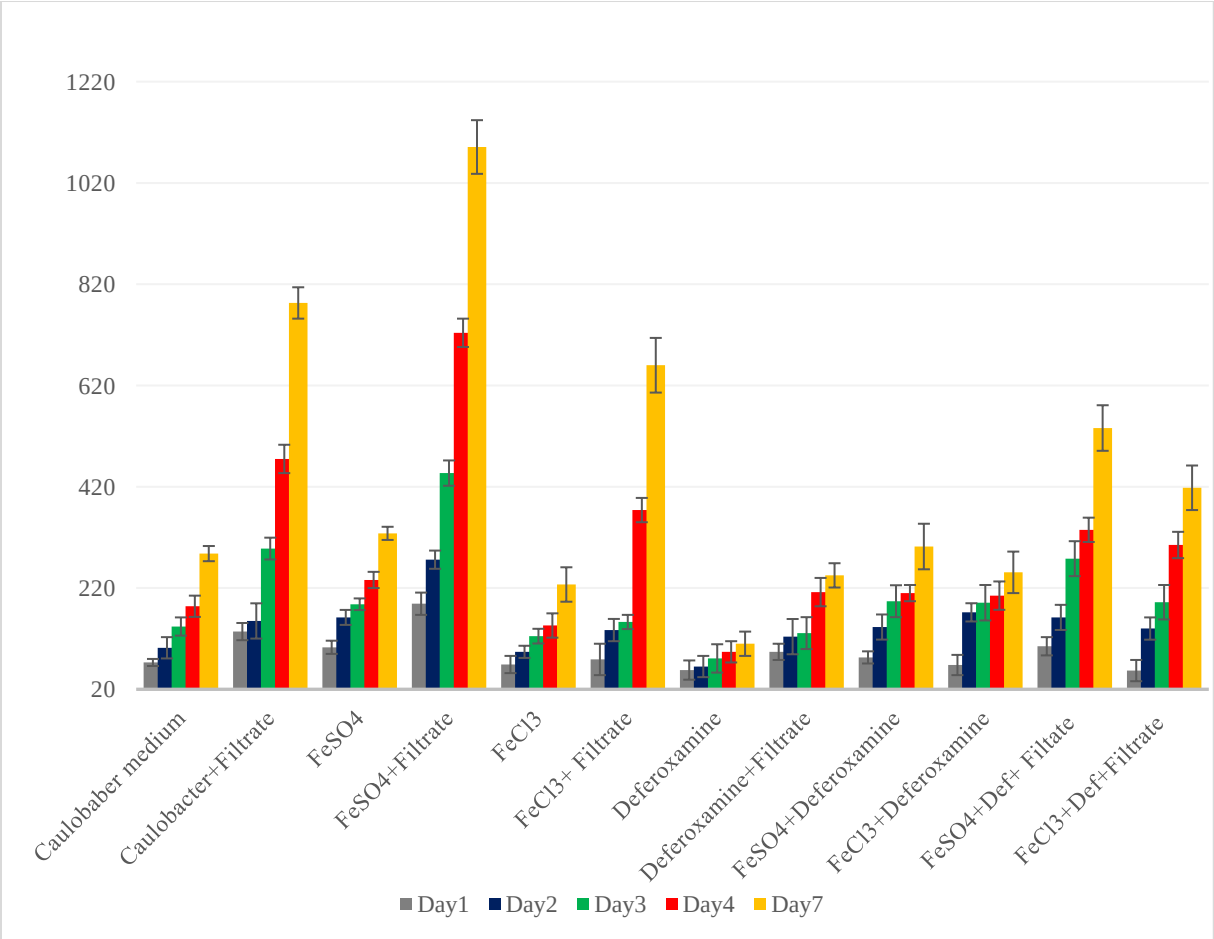
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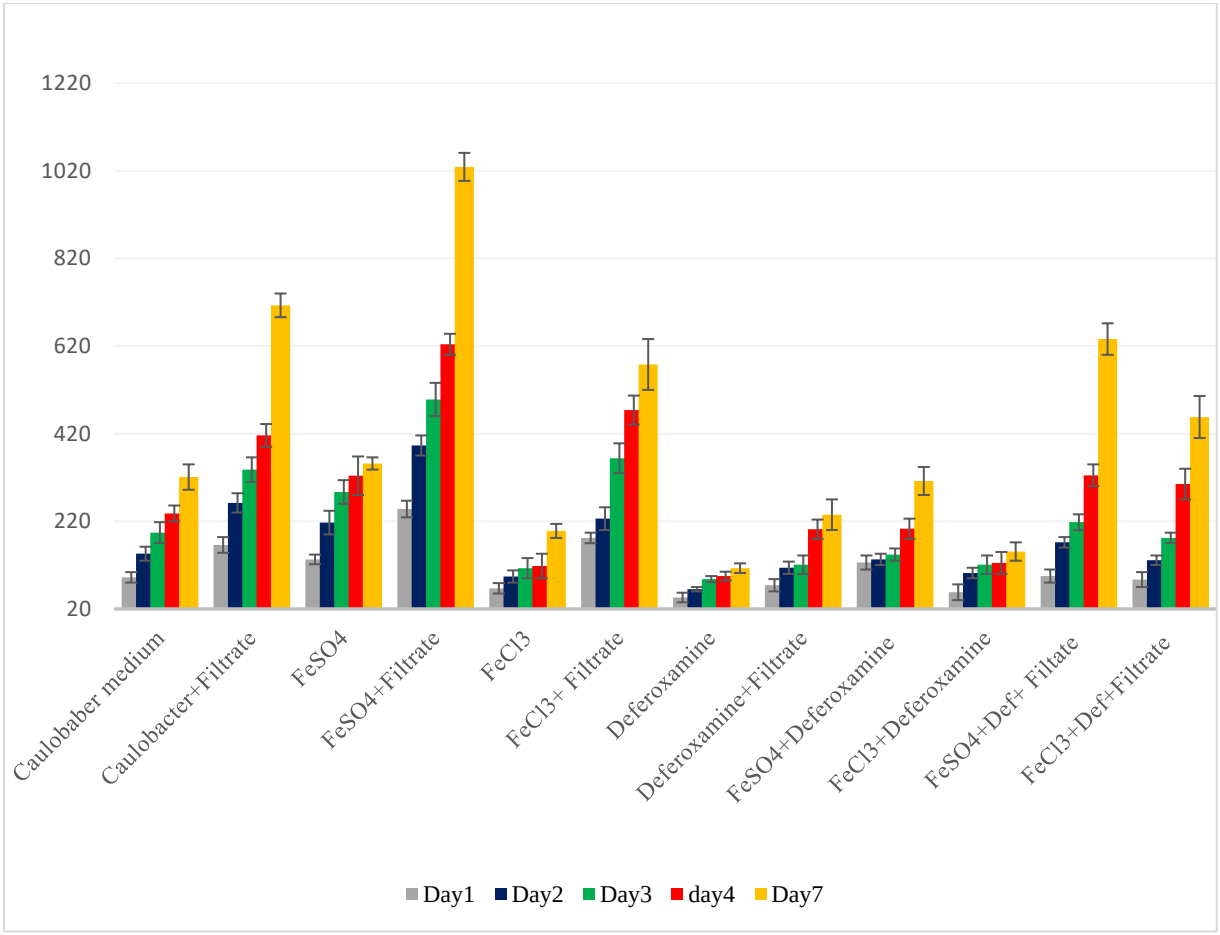
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FeCl3+Def+Filtrate
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PARTIE V :

Gemmata et translocation : Influence des Rayons X sur le microbiote intestinal.

L'isolement de *G. massiliana* (Aghnatios and Drancourt, 2015, 2015) dans le réseau d'eau hospitalier à proximité des patients (Drancourt et al., 2014) nous a conforté dans notre hypothèse, d'une possibilité d'entrée digestive des *Gemmata* par ingestion d'eau contaminée, suivie de leur translocation dans le sang des 2 patients leucémiques (immunodéprimés). Un tel mécanisme a déjà été rapporté pour d'autres bactéries du tractus digestif (Tancrède and Andremont, 1985). Cette translocation aurait été induite à la suite d'une perturbation de la flore résidente, occasionnée par la radiothérapie chez ces patients leucémiques. En outre, les bactéries *Gemmata* sont connues pour leur résistance à de hautes doses de rayonnements. En effet, ces bactéries possèdent un nucléoïde entouré d'une double membrane, équipé d'un mécanisme de réparation de l'ADN, et leur antibiorésistance (Aghnatios and Drancourt, 2016; Cayrou et al., 2010; Fuerst and Webb, 1991; Lieber et al., 2009) sont autant de facteurs qui auraient favorisé la prolifération des *Gemmata* opportunistes dans un habitat compétitif comme le microbiote intestinal au détriment de la flore résidente, d'où leur translocation par suite de la rupture de l'effet barrière.

En vue de vérifier l'hypothèse de l'influence des rayons X sur la flore intestinale, nous avons irradié, en une dose cumulative de Gray 40, des selles de 8 patients. Une approche de Culturomic en pré et post-irradiation nous a permis d'observer une forte perturbation de la flore intestinale. Cette étude est en cours d'écriture sous forme d'un article scientifique.

Article 5

Gemmata and translocation: impact of X-rays on the human gut microbiota.

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En cours d'écriture

Gemmata and translocation: impact of X-rays on the human gut microbiota.

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Abstract

Introduction

Planctomycetes bacteria are well-known to be a part of human gut microbiota (Cayrou et al., 2013). Their DNAs have been also detected in the blood samples collected from two patients with aplastic neutropenia, leukemia, pneumonia and diarrhea and there are currently suspected to have an opportunistic behavior under certain conditions. Indeed, the isolation of *Gemmata. massiliana* (Aghnatios and Drancourt, 2015; Aghnatios et al., 2015) from the hospital water network in close proximity to these two patients (Drancourt et al., 2014) has supported the possibility of *Gemmata* bacteria ingestion by the patients via contaminated hospital water, followed by their translocation into the blood of these two immunocompromised patients. Such mechanism has already been reported for other bacteria belonging from human gut microbiota. (Tancrède and Andremont, 1985). Furthermore, we have hypothesized that this translocation would have been induced after a disturbance of the resident flora caused by the long-term empirical antibiotic/anticancer drugs therapies and radiotherapy applied in these immunocompromised patients. This hypothesis has been greatly strengthened by the fact *Gemmata* bacteria exhibit properties of radioresistance (Lieber et al., 2009) as *Planctomycetes* exhibit a distinctive nucleoid structure in which chromatin is encapsulated within a discrete membrane-bound compartment (Fuerst, 2004; Fuerst and Webb, 1991) and some authors argue that *Gemmata obscuriglobus* is equipped with a DNA repair mechanism to resist a high dose of radiation (Lieber et al., 2009). We have hypothesized hence, that the radiotherapy and radioresistance properties of such bacteria would have favored the proliferation of the opportunistic *Gemmata* , living in

a competitive habitat like the gut microbiota at the expense of the resident flora, followed of their translocation due to the breaking of the gut microbiota barrier effect. Despite increasing evidence of the relationship between gut microbiota and radiotherapy, no analyses have been performed to investigate the influence of radiation on human gut microbiota by using direct action of X-rays on stool samples. Therefore, the aim of this study was to test the hypothesis of the influence of X-rays on the gut microbiota through a unique dose of X-ray on stool samples using culturomic approaches. Culture have been performed before and after irradiation to assess the direct impact of X ray on the gut microbiota.

Materials and Methods

Ethics committee approval and consent. This sudy was approved by the Research Ethical Committee in health of Science at Bobo-Dioulasso, Burkina Faso under (N/Ref.002-2018-CEIRS). In addition, the consent was obtained from all participants for stool specimens' collection and subsequent analysis.

Stool samples. The stool samples were obtained from the patients belonging to Bobo Dioulasso, Burkina Faso. All investigations have been performed in Marseille, France.

High-Throughput Bacterial Culture of stool samples before irradiation

Stool samples were analysed in anaerobic encloser using two culture conditions for anaerobic bacteria. One condition is from 18 preselected culture condition previoulsy described (Lagier et al., 2015) with some modifications. Each gram of

stool is diluated in 1 mL of Dulbecco's Phosphate-Bufered Saline (DPBS 1X) and the suspension was preincubated in anaerobic blood culture bottle with rumen fluid and sheep blood in anaerobic condition at 37°C. At each 03 hours, 06 hours, 09 hours, 7 days and 14 days; 1mL of the culture was diluted with 900 µL DPB 1X in serial dilution from 1/10 to $10/10^{10}$, inoculated on 5% sheep blood Agar (Biomérieux, France) and incubated at 37°C for 3 days in anaerobic condition. The second condition was performed using YCFA (DSMZ) medium modified for solid and liquid medium. All reagents used for medium preparation was filtered at 0.22 µm only agar was autoclaved for solid medium. The anaerobic blood culture bottles were empted and replaced with YCFA liquid medium using a vacuum system to remove any oxygen. A volume of 3mL of blood and rumen was added and the same procedure was used for the first condition. Also, all sample after 10-fold serial dilutions were plated directly on 5% sheep blood Agar and incubated in anaeobic condition 37°C for 3 days. A subculture of colonies isolated were performed to obtain pure colonies and MALDI TOF was used for colonies identifications.

In parallel, aerobic bacteria were cultured in the same maner but under aerobic conditions. For the research of *Planctomycetes* bacteria, the stool samples were plated on Caulobacter medium, prepared as described by (Christen et al., 2018) containing 40 mg/L vancomycin, 100 mg/L imipenem and 32 mg/L amphotericin B and incubated aerobically at 30 °C for four weeks.

Identification by MALDI TOF

The colonies are identified by MALDI TOF MS with microflex from Bruker Daltonics (Bremen, Germany) according to the manufacturer's instructions. Each

colony was deposited in duplicate on a 96 MSP microplate and covered with 1.5 µL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid). The spectrum of each colony was compared with IHU data base spectra. Colony was identified at species level with a score >1.9. Non identified colony was verified at least 3 times, if the colony is remained not identified, then the16s RNA was used for identification as previously described (Lagier et al., 2016).

Irradiation procedure and High-Throughput Bacterial Culture of stool samples after irradiation

In 15-mL falcon tubes, 1g of each patient's stool was diluted in 1.5 mL of sterile Dulbecco's Phosphate-Bufered Saline (DPBS 1X) to prepare the bacterial suspensions to be irradiated. After triturating and homogenizing the stools, this supension was then introduced into a 1.5- mL Eppendorf tube and filled completely to drive out the vacuum. Four (4) sterility controls containing only DPBS were prepared under the same conditions to be irradiated. All Eppendorf tubes containing the stool suspensions and controls were then transported in a cooler containing ice at the service of Radiotherapy and Oncology of APHM, Marseille, France to be irradiated. The 12 tubes were conveniently mounted on a single polystyrene plate containing wells uniformly prior to irradiation. An X-ray dose was then delivered to the 12 tubes (8 stools + 4 controls) until the 40 Gy dose was reached. After irradiation, the bacteria were later cultured under the same conditions as the stool before irradiation, including controls. Irradiated stool culture and colonies identifications were done as described above for the same non-irradiated stool samples (Lagier et al., 2016).

Results

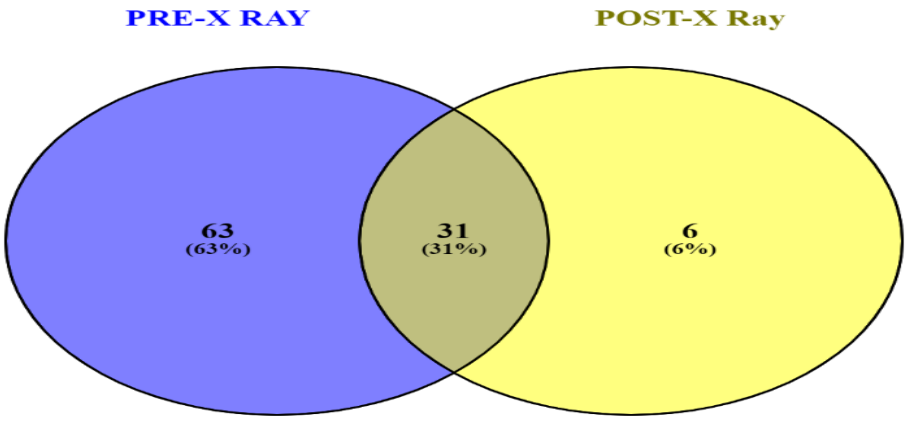

Figure 1: The Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/) shows the effect of irradiation on bacteria of the gut microbiota. In purple, we have 63 bacteria cultured before irradiation, in yellow we have 6 bacteria that were grown after irradiation and 31 bacteria that were not influenced by the action of X-rays.

Bacterial species cultured before and after irradiation.

6 elements included exclusively in "POST-X Ray":

Adlercreutzia equolifaciens

Bacillus subtilis

Butyricimonas virosa

Clostridium difficile

Gordonibacter urolithinfaciens

Lachnoclostridium edouardi

31 common elements in "PRE-X RAY" and "POST-X Ray":

Bacillus cereus

Bacteroides fragilis

Bifidobacterium longum

Bifidobacterium pseudocatenulatum

Butyricimonas phoceensis

Clostridium bifermentans

Clostridium perfringens

Clostridium sordellii

Clostridium sporogenes

Clostridium tertium

Collinsella aerofaciens

Drancourtella massiliensis

Eggerthella lenta

Enterococcus asini

Enterococcus faecalis

Enterococcus faecium

Enterococcus hirae

Enterococcus mundtii

Escherichia coli

Flavonifractor plautii

Lactobacillus gasseri

Lactobacillus ruminis

Lactococcus garvieae

Marseillibacter massiliensis

Pediococcus acidilactici

Pediococcus pentaceus

Streptococcus equinus

Streptococcus gallolyticus

Streptococcus lutetiensis

Weissela cibaria

Weissella confusa

63 elements included exclusively in "PRE-X RAY":

181022-IF4-Thao25 P8842

Aeromonas bestiarum

Aeromonas salmonicida

Agathobaculum massienlesis

Alistipes jeddahensis

Alistipes putredini

Alistipes senegalensis

Allisonella histaminiformans

Bacteroides bouchesdurhonensis

Bacteroides ovatus

Bacteroides thetaioteomicron

Bacteroides uniformis

Bacteroides vulgatus

Barnesiella intestinihominis

Bifidobacterium dentium

Cellulomonas timonensis

Clostridium butyricum

Clostridium intestinalis

Clostridium paraperfringens

Collinsella bouchesdurhonensis

Cutibacterium acnes

Cutibacterium eggermontii

Dorea longicatena

Enterococcus avium

Enterococcus casseliflavus

Enterococcus raffinosus

Finegoldia magna

Gordonibacter pamelaeae

Holdemanella biformis

Ihuella massiliensis

Intestinomonas butyriciproducens

Lactobacillus agilis

Lactobacillus brevis

Lactobacillus crispatus

Lactobacillus fermentum

Lactobacillus frumenti

Lactobacillus garvieae

Lactobacillus mucosae

Lactobacillus oris

Lactobacillus plantarum

Lactobacillus salivarius

Mobilobacillus massiliensis

Murdochiella asaccharolytica

Negativicoccus succinicivorans

Ninae P9525

Olsenella cagae

Olsenella uli

Parabacteroides distasonis

Parabacteroides merdae

Peptoniphilus gorbachii

Peptoniphilus harei

Peptoniphilus lacrimalis

Peptoniphilus vaginalis

Staphylococcus capitis

Staphylococcus epidermidis

Staphylococcus pasteuri

Staphylococcus warneri

Streptococcus infantarius

Sutterella massiliensi

Sutterella wadsworthensis

Turicibacter sanguinis

Varibaculum cambriense

Yersinia intermedia

Table 1. Comparison of bacterial communities before and after irradiation. Relative abundances of phylum-level taxa are compared.

	PRE X RAY	POST X RAY
FIRMICUTE	63	27
ACTINOBACTERIA	11	6
BACTERIODETES	12	3
PROTEOBACTERIA	6	1
UNCLASSIFIED	2	0
TOTAL	94	37

Discussion

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Conclusions Générales et Perspectives.

Bien que la première description des Planctomycetes remonte à 1924 (Gimesi, 1924), ces bactéries constituent un des groupes bactériens méconnus, plus particulièrement en Microbiologie Clinique. Leurs caractéristiques morphologiques et biologiques très particulières ont intrigué les microbiologistes dès leur découverte. Les Planctomycetes et les autres membres du superphylum PVC (Chlamydia) ont déconcerté et, dans certains cas, même induit les chercheurs en erreur et ont suscité de nombreuses controverses ayant remis en question des dogmes formulés à leur propos. Ceci est illustré par l'identification initiale erronée de *Chlamydia trachomatis* en tant que virus par Von Prowazek et Halberstadter en 1907 (Von Prowazek and Halberstadter, 1907), et par l'étymologie déroutante des « Planctomycetes », initialement considérées comme des champignons planctoniques (Gimesi, 1924). Ce n'est que dans les années 1970-1980 que des données de plus en plus régulières ont commencé à voir le jour avec l'isolement des toutes premières espèces en culture pure, grâce à l'utilisation de milieux dilués par James Staley (Bauld and staley, 1976; Staley, 1973).

Bien qu'un net changement de paradigme ait eu lieu dans le domaine de la recherche sur les Planctomycetes ces dernières années, la position taxonomique des Planctomycetes parmi les bactéries reste encore discutée du fait de caractéristiques en commun avec les eucaryotes. En effet, les bactéries du phylum Planctomycetes sont très distinctes et non-conformistes en ce sens que leurs mécanismes de division cellulaire « inhabituels » (abscence du système de division cellulaire universel basé sur FtsZ) les rendent uniques parmi les bactéries et certains auteurs suggèrent que les Planctomycetes constituent une branche profondément enracinée au sein des bactéries du superphylum PVC. Les Planctomycetes sont considérées aujourd'hui comme des bactéries-clés pour l'étude de l'évolution (Fuerst, 1995) et ce phylum si passionnant mérite donc plus d'attention en ce qui concerne leur métabolisme primaire et secondaire car il a été rapporté que ces bactéries constituent des « producteurs

talentueux » de molécules bioactives comme les antibiotiques et des cytotoxiques (anticancéreux). Cependant, comme il n'existe encore que peu de représentants en cultures axéniques (0,6% de la diversité au niveau des OTU), les informations sur les Planctomycetes restent toujours peu étendues. Plusieurs espèces n'ont été décrites que sur la base d'une simple observation microscopique ou d'une détection moléculaire de courtes séquences d'ADN en raison de leur résistance à l'isolement et à la culture. Ainsi les bactéries Planctomycetes restent assez peu explorées et il serait hautement souhaitable d'améliorer leurs techniques de culture et d'isolement afin de disposer de plus d'espèces en culture car compte tenu de la profondeur phylogénétique de ce phylum on pourrait s'attendre à une nouvelle biologie passionnante si plus d'espèces étaient cultivées.

Longtemps considérées comme des bactéries typiquement environnementales, ce n'est que dans un passé très récent (2014) que les Planctomycetes ont attiré l'attention des microbiologistes du domaine médical avec un rapport anecdotique de bactériémies opportunistes chez deux patients en aplasie fébrile, associant une leucémie aigüe myéloïde, fièvre, éruption cutanée, diarrhée, pneumonie micronodulaire et colite neutropénique par suite d'une translocation intestinale vers le sang chez ces patients (Drancourt et al., 2014). Les Planctomycetes sont présentes dans le microbiote digestif humain de sujets sains et malades avec une prévalence de 0,4% à 1,8% (avec une majorité (50%) proche du genre *Gemmata*) et une hétérogénéité variable en fonction des pays (Cayrou et al., 2013). Ces bactéries sont également présentes dans l'eau des réseaux hospitaliers avec une fréquence de 2,2% pour les points d'eau filtrée contre 11,3% des points d'eau non-filtrée (P <0,05), constituant ainsi un risque pour les patients en situations d'immunodépression (Aghnatios and Drancourt, 2015). Cependant, ces bactéries échappent aux techniques de diagnostic microbiologique de routine et il s'agit de bactéries d'isolement et de culture très fastidieuses (Christen et al., 2018). Elles

constituent donc un groupe de microorganismes négligés chez l'homme et à notre connaissance aucun laboratoire de microbiologie clinique ne s'y intéresse. Jusqu'à nos jours, aucune publication scientifique n'a encore rapporté un isolement en culture axénique d'un planctomycetes à partir d'un prélèvement clinique.

Au cours de notre travail de thèse, nous nous sommes particulièrement intéressés au genre *Gemmata* du fait de leur association à l'homme et au milieu hospitalier. En effet, l'étude des *Gemmata* spp., colonisant l'eau chlorée du réseau hospitalier, multirésistants aux antibiotiques utilisés dans les traitements empiriques, et membres du microbiote digestif de l'homme ; présentent un grand intérêt en ce sens que leur détection dans le sang de patients fébriles conforte l'hypothèse de leur rôle comme pathogènes opportunistes. Nos travaux de Thèse s'inscrivaient dans une perspective générale de diagnostic de laboratoire de microbiologie clinique et consistaient en l'isolement des bactéries *Gemmata* des prélèvements humains (sang, LBA). Le but initialement fixé, n'a cependant pas été concrétisé bien que nous ayons détecté un échantillon positif par qPCR dans le sang d'un patient, non confirmé par la PCR standard ni par séquençage qui a échoué. L'échec de l'isolement malgré plusieurs tentatives, même avec la durée d'incubation prolongée, implique nécessairement le développement d'autres méthodes de culture des bactériémies à *Gemmata* spp. Ainsi, il ne nous restait qu'une seule option visant à améliorer les conditions de culture des *Gemmata* afin d'optimiser l'isolement à partir d'échantillons cliniques. C'est dans ce cadre que nous avons rédigé notre revue sur les possibilités d'améliorer leur culture et isolement, en se basant sur la richesse des planctomycetes dans certains habitats naturels de prédilection afin de mimer leurs conditions environnementales au laboratoire pour développer de nous nouveaux outils. Ainsi, nous avons noté que ces bactéries présentaient une très forte association avec les macro-algues, les éponges marines, les sphaignes des tourbières, les alpha-protéobactéries, et certains genres

étaient fortement associés aux dépôts d'hydroxyde ferrique (genre *Planctomyces)* dans certains habitats. Notre revue de la littérature, nous a également permis de mettre en évidence les aspects de la culture de ces bactéries fastidieuses, les travaux effectués au cours des dernières années qui ont permis d'isoler de nouvelles espèces de Planctomycetes dans divers échantillons environnementaux en culture pure et de proposer de nouvelles approches de culture. Ainsi, nos travaux nous ont permis :

- D'évaluer la possibilité d'isoler les *Gemmata* par coculture avec les amibes. Nos travaux ont cependant montré que cette approche était inappropriée. En effet, les deux espèces de *Gemmata* testées ont été phagocytées et lysées après 72 heures d'incubation. Les données présentées dans ces trois modèles de coculture *Gemmata*-phagocytes environnementaux (*Acanthamoeba*) suggèrent que ces trois types d'amibes n'offrent pas un environnement convenable à la croissance des *Gemmata* et ne pourront donc pas être utilisées à des fins d'isolement.

- Savoir également que ces bactéries sont sensibles aux cellules phagocytaires THP-1, suggérant par analogie qu'elles ne pourront pas échapper à la phagocytose par les cellules du système monocytes-macrophages chez des sujets immunocompétents ; ce qui reconforte l'hypothèse selon laquelle les *Gemmata* seraient de pathogènes opportunistes chez des patients présentant un déficit congénital ou acquis de l'immunité de première ligne, tels que les patients neutropéniques. Cependant, cette hypothèse mérite d'être explorée sur des modèles animaux pour une confirmation *in vivo*.

- En outre, deux nouvelles approches de culture basées sur l'étude des niches écologiques des Planctomycetes ont été mises au point : Un milieu biphasique tout à fait original constitué de tissus d'éponges marines-filtrat d'éponge (système biphasique). En effet, un protocole de culture des Planctomycetes à partir des éponges marines a permis d'améliorer significativement la croissance des *Gemmata* par rapport à la culture « traditionnelle » en se basant sur leur besoin d'attachement à un support pour accélérer leur bourgeonnement. L'utilisation de filtrat d'éponge marine (monophasique) a également montré son efficacité pour réduire le temps de dédoublement sur milieux liquide et solide par rapport au milieu de culture standard, suggérant que les éponges marines peuvent constituer des stocks (lyophilisat) de facteurs de croissance pouvant servir à supplémenter les milieux standards des Planctomycetes et de faciliter leur culture et isolement à partir d'échantillons cliniques
- Un milieu enrichi supplémenté par le filtrat d'*Escherichia coli*-fer mettant en exergue l'intérêt des sidérophores et l'enzyme ferriréductase extracellulaire produite par E. coli dans le métabolisme sidérique de ces bactéries. En effet, l'analyse du génome de G. *obscuriglobus* et de *G. massiliana* a révélé que les 2 espèces ne codaient pas certains composants essentiels dans la voie du métabolisme et d'acquisition du fer (sidérophores et enzymes ferriréductase) d'où leur fortement association symbiotique ou parasitique à d'autres bactéries dans différents habitats pour bénéficier de ces composants afin d'assimiler le fer. Cette observation nous a permis de désigner un nouveau protocole de culture pour l'isolement des *Gemmata*, qui a montré que la culture de *G. obscuriglobus* et *G. massiliana* en présence du filtrat d'*Escherichia coli*-fer, améliorait significativement la croissance des *Gemmata* par rapport aux bactéries *Gemmata* cultivées en l'absence du filtrat d'*Escherichia coli*. L'ajout de la

deferoxamine inhibait cet effet en chélatant le fer. Ainsi cette stratégie de culture montre l'intérêt des sidérophores et l'enzyme ferriréductase extracellulaire produite par *E. coli* et le fer. Ces composants pourront être lyophilisés en vue de préparer des additifs de ces molécules à utiliser en complément des milieux de culture standard. Cette innovation du milieu de culture facilitera l'isolement des souches de Planctomycetes à partir d'échantillons cliniques, de préférence dans des échantillons où aucun autre organisme n'a été identifié. Également cette méthode pourrait aussi être extrapolée à d'autres bactéries déficientes en voie métabolique du fer.

– Aussi, nos travaux nous ont permis d'observer que la radiothérapie induit un déséquilibre de la flore microbienne instestinale (dysbiose), qui faciliterait la croissance lente des Planctomycetes probablement à cause de la diminution des bactéries compétitrices dans un habitat très compétitif tel que le microbiote digestif. La grande tolérance aux stress et aux hautes doses de radiations des *Gemmata* leur permettrait de résister à ces conditions adverses et de passer facilement la barrière intestinale dans toutes les situations de dysbiose. Cependant, il serait également utile de travailler sur des modèles animaux afin de confirmer notre hypothèse (translocation) in vivo après infection des animaux de laboratoire. par les *Gemmata* suivie d'une irradiation.

Les nouvelles approches de culture testées dans cette étude, fondent les futurs travaux de microbiologie clinique dans la perspective d'isolement afin d'affirmer de manière concluante que les *Gemmata* sont des pathogènes opportunistes car :

- Il est prévu que ces travaux soient poursuivis au sein de l'IHU, en mixant ces deux milieux pour rechercher les *Gemmata* dans des échantillons de sang hépariné collectés chez les patients aplasiques.

- En outre, il est prévu de tester la sensibilité des *Gemmata* vis-à-vis des anticancéreux pour avoir un aperçu sur ce paramètre qui aurait pu inhiber la croissance des *Gemmata* et empêcher leur isolement en culture chez les patients leucémiques soumis à la chimiothérapie.

- Enfin, sur la base des données de la littérature, nous comptons tester la croissance des *Gemmata* avec les extraits et des macérats d'algues, de lichens et de mousses (surtout la sphaigne présente dans les tourbières, qui s'est avérée être la source la plus prolifique ayant permis l'isolement du plus grand nombre de planctomycetes et plus interessant qu'il s'agissait de *Gemmata*-like, membre de la famille des *Gemmatacea*)(Kulichevskaya et al., 2009, 2017), par la même approche de culture que celle utilisée pour les éponges (filtrat - support solide). En effet, ces associations sont très fortes en raison des molécules sulfatées produites par les hôtes, qui sont des sources de nutriments et de facteurs de croissance pour les Planctomycetes qui ont un contenu génétique en sulfatases. Il conviendrait donc aussi d'explorer cette possibilité d'améliorer la culture des *Gemmata* par ces sources de nutriments afin de mimer le plus proche possible leur environnement naturel au laboratoire de microbiologie clinique.

En définitive, nos futurs axes de recherche devront intégrer :

- i) une amélioration des conditions pré-analytiques (prélèvements sanguins sur tube hépariné et ensemencés le plus rapidement possible dans un délai < 2 heures pour éviter que le contact trop prolongé avec le peu de phagocytes des patients aplasiques dans le tube

- ii) l'augmentation des possibilités d'inclusion de patients neutropéniques (le caractère très bactéricide des phagocytes pour les *Gemmata* spp. écartant tout axe de recherche à explorer chez des sujets immunocompétents) et enfin

- iii) l'implémentation des outils innovants de culture cités plus haut pour une croissance accélérée de ces germes très fastidieux afin de mieux aborder l'étude de pathogénicité en facilitant l'isolement à partir d'échantillons cliniques. Toutes ces données seront déposées dans la littérature afin d'attirer l'attention des autres laboratoires sur la recherche de ces bactéries négligées en clinique car à notre connaissance, aucun laboratoire de microbiologie médicale ne s'intéresse aux Plantomycetes en routine.

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