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"Taxonomia de procariontes isolados de cnidários"

Tese apresentada ao Instituto de Biologia para obtenção de Título de Doutor em Genética e Biologia Molecular na área de Genética de Microrganismos

Orientador: Prof. Dr. Fabiano Lopes Thompson

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"A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável."

Galileu Galilei

“The true taxonomist is a man with a mission, he often leads a cloistered life, protected from vexations and frustations of the everyday world, and he may well wear blinkers as opaque as any worn by a horse....

Living a life of seclusion, safe in his small laboratory, and surrounded by his books, his microscope (and perhaps his computer tape), he affects an unconcern for the mundane application of his work...”

S.T. Cowan, 1965

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RESUMO

Microrganismos têm um papel fundamental na saúde de cnidários, estabelecendo relações ecológicas diversas, de parasitismo até mutualismo. O conhecimento acerca da diversidade de procariontes associados com cnidários e corais no Brasil ainda é escasso. Este é o primeiro estudo enfocando a caracterização taxonômica de bactérias heterotróficas incluindo fixadoras de nitrogênio (N_2) e especialmente vibrios isolados do muco do coral endêmico *Mussismilia hispida* e de zoantídeos simpátricos *Palythoa caribaeorum, Palythoa variabilis* e *Zoanthus solanderi* da região de São Sebastião-SP, Brasil. Ao todo, foram obtidos 488 isolados bacterianos destes cnidários, empregando-se os meios de cultivo Ágar Marinho (AM), Ágar Fixação de Nitrogênio (NFb) e Ágar Tiosulfato-Citrato-Bile-Sacarose (TCBS). Os cnidários foram coletados em três praias (Grande, Portinho e Preta) na região de São Sebastião em 2005 e 2006. A maioria dos isolados obtidos pertenciam à classe *Gammaproteobacteria*. Mais da metade foram identificados como vibrios com base em sequências parciais do gene 16S rRNA. Vibrios do grupo core corresponderam a 80 % das bactérias potencialmente fixadoras de N_2 e revelaram alta similaridade de sequência no gene *pyrH* com *V. harveyi* e *V. alginolyticus*. Dezenove isolados representativos, pertencentes às espécies *V. harveyi, V. alginolyticus, V. campbellii* e *V. parahaemolyticus* foram capazes de crescer 6 vezes sucessivas em meio NFb e alguns deles mostraram forte atividade da nitrogenase pelo teste de redução de acetileno (ARA). Cerca de 150 vibrios isolados em TCBS foram caracterizados por meio de sequências de 16S rRNA, *recA* e *pyrH*. Os taxa mais abundantes foram *V. harveyi, V. rotiferianus, V. campbellii, V. alginolyticus, V. mediterranei* (=*V. shillonii*), *V. chagasii*, *V. tubiashii* e uma espécie nova de *Vibrio*. Com exceção de *V. chagasii* que foi encontrado apenas no muco de *M. hispida,* as demais espécies foram observadas nos diferentes hospedeiros, não havendo fortes evidências da presença de espécies hospedeiro-específicas. A variabilidade intra-específica dos vibrios foi avaliada por meio de padrões de banda de elementos palindrômicos extragênicos repetitivos (rep-PCR). Esta análise revelou alta diversidade genômica entre os isolados. Cada isolado apresentava um padrão de rep-PCR distinto, indicando uma grande diversidade de populações co-existindo no mesmo habitat. As identificações geradas pelo rep-PCR concordaram com as identificações obtidas pelo MLSA (Multilocus Sequence Analysis). Diversos isolados de diferentes grupos microbianos apresentaram menos de 97 % de similaridade em sequências do gene 16S rRNA com espécies conhecidas, indicando que elas seriam possíveis espécies novas. Ao todo, sete espécies novas foram descritas por meio da taxonomia polifásica: *Marinobacterium coralli* sp. nov., *Marinomonas brasilensis* sp. nov., *Photobacterium swingsii* sp. nov., *P. jeanii* sp. nov., *Vibrio communis* sp. nov., *V. variabilis* sp. nov. e *V. marinum* sp. nov. Os dados fenotípicos, quimiotaxonômicos, genotípicos e filogenéticos obtidos a partir da descrição das sete espécies novas demonstraram que há uma ampla variabilidade fenotípica intra-específica. Este estudo determinou a identidade dos principais grupos de bactérias cultivadas do muco de corais, sobretudo, os vibrios. Uma das espécies de vibrios mais abundantes no muco dos corais é *V. communis* sp. nov. A fixação de N_2 representa um fenótipo comum entre as espécies de vibrios estudadas, sugerindo uma função positiva no holobionte.

ABSTRACT

Microorganisms have a key role in the health of cnidarians establishing ecologic relationships, of parasitism to mutualism. The knowledge on the diversity of prokaryotes associated with corals in Brazil is very limited. This is the first study on the taxonomic characterization of heterotrophic bacteria, including putative N_2-fixing and specially vibrios isolated from mucus of endemic coral *Mussismilia hispida* and the sympatric zoanthids *Palythoa caribaeorum, Palythoa variabilis* and *Zoanthus solanderi*. Cnidarian specimens were isolated in the beaches Grande, Portinho and Preta from São Sebastião region, SP, Brazil, in 2005 and 2006. A total of 488 isolates were obtained using different culture media, i.e. Marine Agar (MA), Nitrogen Fixation (NFb) and Thiosulphate-Citrate-Bile-Sucrose Agar (TCBS). The majority of the isolates fell within the class *Gammaproteobacteria* and more than half of them were vibrios based on 16S rRNA gene sequences. Based on *pyrH* gene sequences 80 % of the putative N_2-fixing bacteria clustered with the *Vibrio* core species group (i.e. *V. harveyi* and *V. alginolyticus*). Nineteen representative isolates of *V. harveyi, V. alginolyticus, V. campbellii* and *V. parahaemolyticus*) were capable of growing six successive times in nitrogen-free medium and some of them showed strong nitrogenase activity by means of the acetylene reduction assay (ARA). About 150 vibrios isolated on TCBS were characterized by means of 16S rRNA, *recA* and *pyrH* gene sequences. The most abundant taxa were *V. harveyi, V. rotiferianus, V. campbellii, V. alginolyticus*, *V. mediterranei* (=*V. shillonii*), *V. chagasii*, *V. tubiashii* and a new *Vibrio* species. With the exception of *V. chagasii* which was found only in the mucus of *M. hispida*, the other species appeared in different hosts with no strong evidence for the presence of host-specific species. A high genomic diversity was observed using rep-PCR. Each vibrio isolate represented a different co-occuring population, suggesting huge intra-especific diversity. There was a complete agreement between the grouping based on rep-PCR and identification based on MLSA (Multilocus Sequence Analysis). Several isolates had less than 97 % similarity towards know species based on 16S rRNA gene sequence, indicating that they are possibly new species. In total, seven new species were described based on polyphasic taxonomy: *Marinobacterium coralli* sp. nov., *Marinomonas brasilensis* sp. nov. *Photobacterium swingsii* sp. nov., *P. jeanii* sp. nov., *Vibrio communis* sp. nov., *V. variabilis* sp. nov. and *V. marinum* sp. nov. The new species were characterized by polyphasic taxonomy. Based on phenotypic, chimiotaxonomic, genotypic and phylogenetic data obtained from description of new species, it was demonstrated a huge intra-specific phenotypic variability. This study determined the taxonomic identity of heterotrophic bacteria, especially *Vibrio*, isolates from cnidarians. *V. communis* sp. nov. is one of the most frequently found species in the corals. Nitrogen fixation is a common phenotypic trait among vibrios, suggesting that these bacteria may have a positive function in the holobiont.

INTRODUÇÃO

O filo Cnidaria é formado por aproximadamente 10.000 espécies representadas principalmente pelas hidras, anêmonas-do-mar, águas-vivas ou medusas e corais. *Mussismilia hispida* é um importante coral formador de recifes, endêmico e amplamente distribuído na costa do Brasil. O coral e uma infinidade de outros organismos como os zoantídeos *Palythoa caribaeorum, Palythoa variabilis e Zoanthus solanderi,* compartilham o mesmo habitat nos recifes de corais e em outros ambientes coralíneos, tais como os costões rochosos. Corais que habitam costões rochosos participam da transferência de matéria e energia na cadeia trófica, favorecem o aumento da diversidade genética por meio da geração de habitats e microhabitats, e são fontes de compostos bioativos. Notoriamente, os costões rochosos, quando comparados com seus arredores, apresentam uma vasta diversidade de espécies que se abrigam e vivem nestes sistemas. Além disto, corais são os principais construtores de recifes. Diversos fatores locais e globais vêm colaborando para a degradação dos ambientes recifais, sejam costões rochosos ou recifes de corais, sobretudo, o aquecimento global, impactos antropogênicos (como por ex. esgoto doméstico) e as doenças infecciosas (branqueamento, praga branca, banda amarela, entre outras).

Os corais são mais bem descritos como holobiontes. O holobionte compreende o hospedeiro coral, as microalgas zooxantelas, e microrganismos (microbiota). Zooxantelas fazem associações simbióticas com diversos cnidários, como zoantídeos e corais, fornecendo a eles grande parte da nutrição e energia. Além das zooxantelas, outros tipos de microrganismos (bactérias, fungos e vírus) também estabelecem associações com corais, formando o holobionte. Os microrganismos desempenham um importante papel na sobrevivência e funcionalidade dos holobiontes, fornecendo nutrientes, tais como nitrogênio, fósforo, e outros compostos que não podem ser fornecidos somente pelas zooxantelas, além de terem um papel central na manutenção da saúde de seus hospedeiros, atuando como um sistema de defesa capaz de produzir antimicrobianos contra microrganismos invasores.

Microrganismos do holobionte (bactérias, protistas, fungos e vírus) têm um papel fundamental na saúde dos hospedeiros, corais, estabelecendo relações ecológicas diversas, de parasitismo até mutualismo. Mudanças nas populações que compõem a microbiota desses organismos podem resultar em distúrbios na relação microbiota-hospedeiro especialmente quando os corais estão sob estresse, causados principalmente pelo aquecimento das águas e a poluição. Estes fatores favorecem a proliferação ou a colonização de microrganismos patogênicos e conseqüentemente o desenvolvimento de processos infecciosos e doenças. Por exemplo, o branqueamento, uma doença infecciosa, onde populações de vibrios parecem ser dominantes. A perda da coloração do coral devido à perda do simbionte zooxantela caracteriza o branqueamento. Esta doença tem dizimado corais em todo o mundo nos últimos anos. *Mussismilia* aparece hoje na lista das espécies ameaçadas de extinção. Vibrios representam uma fração considerável da

microbiota de diferentes espécies de corais (10^7 UFC/mL no muco de coral), tanto em espécimes saudáveis quanto doentes. O papel que estes microrganismos desempenham no holobionte parece ser controverso e tem sido objeto de investigação. Alguns estudos demonstraram que determinadas espécies de vibrios, tais como *V. coralliilyticus* e *V. shilloni*, são patogênicas para corais. A primeira induz fotoinibição em *Acropora palmata*, enquanto que a segunda causa o branqueamento em *Oculina patagonica*.

O conhecimento sobre a diversidade de bactérias associadas aos corais e a importância dessas associações ainda é muito escasso. Estudos pioneiros visando a taxonomia microbiana da microbiota de corais brasileiros resultarão em um melhor entendimento dos processos ecológicos envolvendo holobiontes. A informação gerada pela análise comparativa da biodiversidade microbiana em regiões e micro-habitats diferentes poderia também ser usada para fins de manejo de ambientes recifais. Os estudos de caracterização taxonômica servem como o ponto de partida para análises de genética bacteriana, como por exemplo, estudos de genes de virulência, epizootias, e transferência gênica lateral. A coleção de microrganismos constituída neste trabalho poderá servir também de base para estudos futuros, como por exemplo, a determinação de genes e elementos extragênicos específicos de cada linhagem que caracterizam as variações genômicas intra-específicas que foram observadas.

A classificação e a identificação de bactérias se baseiam em uma estratégia polifásica, que compreende o estudo de um grupo de bactérias por meio de análises fenotípicas, quimiotaxonômicas, genotípicas, filogenéticas e ecológicas. Um dos principais objetivos da taxonomia polifásica é integrar diferentes tipos de dados e fornecer de forma reprodutiva esquemas de classificação úteis, permanentes, preditivos, objetivos e altamente informativos e que possam ser usados por vários cientistas e outros profissionais em aplicações práticas. A aplicação de técnicas de sequenciamento baseando-se principalmente na comparação das sequências de nucleotídeos possibilitou o entendimento das relações filogenéticas, formando ainda um crescente banco de dados para referências futuras. Sequências de genes que codificam proteínas essenciais do metabolismo celular bacteriano (*housekeeping*) permitem a inferência taxonômica precisa, com a identificação e classificação de isolados. O presente trabalho demonstrou que para determinados grupos bacterianos, como por exemplo, *V. harveyi*, a sequência de um único gene (*pyrH*) seria suficiente para a identificação na maioria dos casos. Quando casos difíceis são encontrados, um segundo gene (como por ex. *topA*, *ftsZ* e *mreB*) pode ser incluído na análise para aumentar o poder de resolução taxonômica. De qualquer modo, a hibridização DNA-DNA ainda é o padrão-ouro na taxonomia, sendo normalmente necessária para a descrição de espécies novas. O sucesso no uso de sequências de vários loci reside na praticidade em avaliar vastas coleções bacterianas.

OBJETIVOS

1. Caracterizar taxonomicamente as bactérias heterotróficas, incluindo fixadoras de nitrogênio e vibrios isolados do muco dos cnidários, aparentemente saudáveis, *Mussismilia hispida*, *Palythoa caribaeorum, Palythoa variabilis* e *Zoanthus solanderi*, da região de São Sebastião-SP, por meio da taxonomia polifásica, possibilitando a identificação dos principais grupos microbianos;

2. Determinar a variabilidade genômica espaço-temporal de vibrios a partir de isolados obtidos de três pontos geográficos do canal de São Sebastião-SP (Praias Preta, Portinho e Grande) em dois anos consecutivos (2005 e 2006) e;

3. Classificar as espécies novas de bactérias por meio da taxonomia polifásica.

II.1. PRINCIPAIS CARACTERÍSTICAS DOS CNIDÁRIOS

O filo Cnidaria é representado principalmente pelas hidras, águas-vivas ou medusas, anêmonas-do-mar e corais. Etimologicamente, Cnidaria (ni-DA-re-a) é a forma latinizada da palavra de origem grega (**κνιδοσ)** que significa urtiga ou urticante**.** O nome deste filo está principalmente relacionado à presença de uma célula chamada de cnidoblasto (cnidócito) que contém na estrutura intracelular, as cnidas, uma das principais características que compõem os organismos deste grupo. Esta estrutura localizada dentro do nematocisto (cápsula) quando estimulada pelo cnidocílio, por meios físicos ou químicos, libera seus filamentos que transportam toxinas podendo ser usados para defesa, secreção de substâncias urticantes e paralisantes, adesão e penetração às superfícies de objetos e coleta de alimentos (Marques & Collins, 2004).

Três diferentes tipos de cnidas são encontrados nesse filo: espirocistos, pticocistos e nematocistos (Hyman, 1940). Nematocistos são usados principalmente para adesão e liberação de toxinas e estão presentes em todos os grupos do filo Cnidaria, enquanto espirocistos e pticocistos possuem funções de adesão e agregação de partículas de sedimento respectivamente e são restritos a poucos indivíduos no filo. A presença e eficiência das cnidas na captura de alimentos faz com que eles sejam quase que exclusivamente carnívoros (Migotto *et al*., 2004) sua principal fonte de alimento é obtida através do zooplâncton (Gili & Hughes, 1995).

Cnidários podem ser encontrados em diferentes habitates, incluindo superfícies e costões rochosos, regiões entre marés e zonas abissais nos oceanos, rios e lagos (Hyman, 1940; Marques, 1997). Os organismos que compõem este filo possuem simetria radial e podem ser indivíduos solitários ou coloniais (Castro, 1994). Sua estrutura corporal é representada por duas formas morfológicas distintas o pólipo (séssil) e a medusa (livre-natante). Algumas espécies podem desenvolver as duas formas no seu ciclo de vida enquanto outras possuem uma das formas reduzida ou ausente. O pólipo é uma estrutura cilíndrica tubular com uma extremidade fixa, uma cavidade interna e uma única abertura superior, que serve de boca e ânus, circundada por tentáculos que são usados para

alimentação e proteção, sendo que neles se encontram também os cnidócitos (Casto 1994). A medusa possui um corpo mole em forma de sino com aspecto gelatinoso e transparente devido à abundante camada de mesogléia, além disso, possuem tentáculos com células urticantes (cnidócitos) e a estrutura bucal inserida na região central da porção inferior do sino ou zona côncava. O esqueleto desses organismos pode estar presente na forma de um exoesqueleto, secretado na superfície externa do corpo ou endoesqueleto, formado por peças separadas (escleritos) ou em massa contínua (Hyman, 1940). A reprodução pode ser assexuada ou sexuada, sendo freqüente a alternância de fases assexuadas e sexuadas no ciclo de vida destes animais. Em sua maioria os organismos deste filo são dióicos, porém há muitas espécies hermafroditas (Campbell, 1974).

Cnidários, como os antozoários formadores ou não de recifes de corais, possuem associações com diversos organismos, como as que ocorrem com as algas zooxantelas (Odum, 1988), peixes (Schlichter, 1976), moluscos (Morton & Scott), poliquetas (Randal & Eldredge, 1976), poríferos (West, 1976), crustáceos (Guinot *et al*., 1995) entre outros. Esses organismos podem viver sobre e dentro das colônias ou se associar a pólipos isolados.

II.2. SISTEMÁTICA DO FILO CNIDARIA

O filo Cnidaria é formado por aproximadamente 10.000 espécies (Migotto *et al*., 2004) e sua taxonomia ainda é bastante controversa. Atualmente o filo está dividido em cinco classes de organismos: Anthozoa (anêmonas-do-mar e corais verdadeiros), Scyphozoa (verdadeiras água-vivas), Cubozoa (medusas em forma de cubo), Hydrozoa (hidras, algumas medusas e corais-de-fogo), Staurozoa composta por estauromedusas, que habitam as profundezas do oceano fixando-se pelos tentáculos, e conulados fósseis (Marques & Collins, 2004) (**Tabela 1**).

Phylum Cnidaria
Class Anthozoa
Subphylum Medusozoa
Class Staurozoa nov.
Order Stauromedusae
Order Conulatae (extinct)
Class Cubozoa
Class Scyphozoa
Order Coronatae
Subclass Discomedusae
Order Semaeostomeae
Order Rhizostomeae
Class Hydrozoa
Order Limnomedusae
Subclass Trachylina
Order Actinulida
Order Trachymedusae
Order Narcomedusae
Order Laingiomedusae
Subclass Hydroidolina
Order Leptothecata
Order Siphonophorae
Order Anthoathecata

Tabela 1. Classificação filogenética do filo Cnidaria sugerida por Marques & Collins. (2004). Dados baseados em análise cladística e sequências do gene 18S.

A maior classe dentro do filo dos cnidários é representada pelos antozoários com mais de 6.000 espécies. Dentro da classe Anthozoa a ordem Scleractinia é a maior, com cerca de 650 espécies representadas pelos corais verdadeiros ou pétreos, com esqueleto calcário secretado por células da base do pólipo, sendo estes uns dos principais formadores de recifes (Dorit *et al*., 1991; Zorzetto & Falcão, 2004).

II.3. IMPORTÂNCIA DOS CNIDÁRIOS

Mussismilia hispida, Palythoa caribaeorum, Palythoa variabilis e Zoanthus solanderi pertencem ao filo Cnidaria, e fazem parte da classe Anthozoa. A espécie *Mussismilia hispida* foi descrita por Verril (1868) e está alocada dentro da ordem Scleractinia, ela é um dos mais importantes corais formadores de recife do oceano Atlântico Sul. Dentre as aproximadamente 20 espécies de corais (Scleractinia) formadoras de recifes no Brasil *M. hispida* possui a mais ampla distribuição geográfica, estendendo-se desde o estado do Rio Grande do Norte até Santa Catarina. A capacidade de *Mussismilia* sobreviver em diferentes regiões indica sua ampla habilidade de adaptação a diferentes gradientes ambientais, como temperatura, turbidez da água e poluição. O gênero *Mussismilia* constitui um grupo endêmico de corais escleractíneos

massivos e representa cerca de 70 % da massa formadora de recife em algumas áreas (Leão & Kikuchi, 2001; Nunes *et al*., 2008). Entretanto, um estudo recente sugere que esse grupo está ameaçado, sendo sua extinção prevista em menos de um século (Francini-Filho *et al.*, 2008). Dá-se então a importância de conhecer e entender melhor os processos que mantêm a sobrevivência desses indivíduos.

Palythoa caribaeorum, Palythoa variabilis e Zoanthus solanderi pertencem à ordem Zoantharia, também chamada de Zoanthidea ou Zoanthiniaria (Sinniger *et al*., 2005; Sinniger & Häussermann 2009). Estas espécies zooxanteladas de águas rasas são simpátricas na costa brasileira e compartilham o mesmo habitat com *M. hispida.* A ordem Zoanthidea é um grupo importante no ecossistema recifal, sendo os organismos dominantes no infralitoral (Ryland, 1997). O gênero *Palythoa* é amplamente distribuído ao redor do mundo, sendo encontrado principalmente em rochas da costa ou em recifes de corais. *P. caribaeorum* pode cobrir até 50 % da área total das rochas litorâneas em algumas regiões da costa brasileira (Oigman-Pszczol *et al*., 2004) e são organismos conhecidos pela habilidade em produzir uma potente toxina chamada palitoxina (Wattenberg, 2007; Nordt *et al*., 2009), além de produzir abundante quantidade de muco. Características que possibilitam a ocupação desses indivíduos em áreas com período de dessecação e estresse de intensa luz solar.

A investigação microbiológica dessas diferentes espécies simpátricas no recife de coral nos permitirá inferir se cada um destes cnidários tem microbiotas específicas sugerindo co-evolução ou se eles compartilham uma composição microbiana comum.

II.4. CORAIS E OS RECIFES DE CORAIS

Corais são organismos abundantes no meio marinho, sendo encontrados desde zonas costeiras, até mesmo águas frias e profundas (Cortés, 2003; Penn *et al*., 2006). Os corais scleractíneos zooxantelados são um dos principais organismos construtores de recifes (Castro, 1994), juntamente com os hidrocorais e as algas calcárias (Kikuchi, 2000). Entretanto, em muitos locais os corais não produzem quantidades de carbonato de cálcio suficientes para formar estruturas recifais, devido à influência dos fatores ambientais como temperatura da água, salinidade, sedimentação, luminosidade,

hidrodinamismo e nutrientes (Birkeland, 1997; Hubbard, 1997). No entanto, essas condições podem não ser limitantes para a existência dos corais, que lá ocorrem como comunidades coralíneas, como as que encontramos em costões rochosos em Arraial do Cabo (Rio de Janeiro) e nas ilhas oceânicas brasileiras (Castro & Pires, 2001). No Brasil a fauna de corais é caracterizada pela baixa diversidade de espécies, sendo a maioria endêmica, concentradas em áreas altamente ameaças (Leão & Kikuchi, 2005; Dutra *et al*., 2006) e relíquias remanescentes do terciário. Esta fauna é composta, predominantemente, por formas massivas, praticamente dominadas pela ordem Scleractinia, com formato de cogumelos, conhecidos como chapeirões (Leão *et al*., 2003).

Recifes são considerados uma estrutura rochosa rígida do ponto de vista geomorfológico (Leão, 1994). Sua formação ocorre através da deposição de sedimentos (grãos de areia, carapaças ou esqueletos calcários de organismos), propiciando o assentamento de organismos fundadores que retêm e compactam o sedimento, permitindo a ocupação e crescimento dos construtores de recife que secretam carbonato de cálcio e edificam a estrutura recifal (Glynn, 1997). Entretanto, para que a formação do recife ocorra é necessária ação mútua de diversos seres que se relacionam entre si numa complexa teia de inter-relações e de eventos em sucessão. Os recifes cobrem aproximadamente 0,1 a 0,5 % do assoalho oceânico com áreas que variam de 255.000 Km^2 (Spalding & Grendel, 1997) a 1.500.000 Km^2 (Copper, 1994). Na região equatorial/tropical do globo a distribuição dos recifes ocorre na forma de um anel que se estende apenas para o lado ocidental dos oceanos Atlântico e Pacífico, geralmente, em águas rasas, quentes e claras (Thurman, 1997). O Brasil apresenta os únicos recifes de coral do Atlântico Sul. Dos oito mil quilômetros de costa litorânea do Brasil, dois mil e quatrocentos apresentam recifes, distribuídos de forma descontínua. Essa distância corresponde à extensão da Grande Barreira de Corais, na Austrália, que possui a maior formação recifal do mundo, entretanto lá os recifes ocupam uma área contínua, de 350.000 Km^2, sendo possível observá-la do espaço (http://acd.ufrj.br/~prjcoralvivo).

II.5. IMPORTÂNCIA DOS CORAIS E RECIFES NO MEIO MARINHO

Corais possuem uma relação simbiótica extremamente importante com as microalgas zooxantelas que fornecem, através do processo de fotossíntese, grande parte

dos compostos orgânicos nutritivos e oxigênio que os corais necessitam para sobreviverem. Por sua vez os corais oferecem as zooxantelas os produtos gerados pelo seu metabolismo (compostos nitrogenados, CO_2 e P) (Sorokin, 1990). No entanto, alguns nutrientes importantes que não podem ser oferecidos pelas microalgas parecem ser adquiridos pela associação com outros microrganismos (Sebens *et al.,* 1998). Esses eventos fazem com que o coral participe do processo de transferência de matéria e energia, possibilitando o desenvolvimento de uma grande, diversa e complexa cadeia trófica, em água muitas vezes pobre em nutrientes (oligotróficas) (Odum, 1988). Estas relações culminam na presença de uma alta diversidade genética de espécies.

Por serem compostos por uma estrutura irregular com reentrâncias, buracos e elevações o recife oferece moradia, abrigo, e local adequado para alimentação e reprodução de diversos seres, estabelecendo habitat para comunidades diversas e abundantes de organismos que formam ecossistemas com alta produtividade e biodiversidade extremamente importantes. Corais e recifes podem servir como excelentes observatórios microbianos e indicadores da saúde do meio marinho, pois qualquer alteração de sua homeostase afeta diretamente o ecossistema a eles envolvidos. Os recifes são considerados o segundo ecossistema em diversidade de espécies do mundo (Bellwood *et al.,* 2004; Zorzetto & Falcão, 2004) sendo nomeados as florestas tropicais úmidas dos oceanos (Sebens, 1994). Esta alta diversidade encontrada nos recifes de corais faz desses ecossistemas uma fonte de alimento para o homem desde tempos remotos, principalmente para populações costeiras. Além de grande potencial pesqueiro, diversos organismos recifais (gastrópodes, polvos, lulas, lagostas, bivalves, entre outros) são também utilizados como alimento, movendo a economia de diversos países no mundo via pesca. A diversidade recifal também representa potencial fonte de substâncias químicas, e outros compostos bioativos (por ex. antimicrobianos e antivirais) de interesse para o homem (Cortés, 2003).

Do ponto de vista físico, estruturas recifais próximas à costa agem como barreiras contra ação das ondas, evitando alagamentos e erosão costeira, além de protegerem ecossistemas costeiros, como os manguezais. Além disso, recifes de corais são conhecidos pela sua extraordinária beleza natural, caracterizada pela grande riqueza de

espécies presentes em águas quentes e claras (Rohwer *et al.,* 2001, 2002; Rosenberg & Loya, 2004), o que atraí grande quantidade de mergulhadores. Em algumas regiões do mundo, o turismo em áreas recifais tornou-se a principal atividade econômica, gerando renda e emprego.

Milhões de pessoas se beneficiam de recursos e serviços que os recifes de corais provêm. Mudanças na biodiversidade de recifes, como diminuição da fauna de corais, podem causar grandes impactos ecológicos e consequentemente diminuição ou perda do desenvolvimento social e econômico de muitas zonas marítimas que dependem da pesca e do turismo (Bellwood *et al.,* 2004). O custo da destruição de 1 quilômetro de recifes de corais foi estimado em mais de 1 milhão de dólares devido à perda de divisas pelo turismo, pesca e outros fatores (Hoegh-Guldberg, 2004). O valor gerado pelas atividades econômicas provenientes de recursos e serviços desse ecossistema é estimado em cerca de 375 bilhões de dólares por ano (Constanza *et al*., 1997). Somente no Caribe o turismo nos recifes gera cerca de 90 bilhões de dólares anualmente (Hoegh-Guldberg, 1999).

II.6. IMPACTOS RESPONSÁVEIS PELA DEGRADAÇÃO DE CORAIS E RECIFES

Em todo mundo vários trabalhos revelam a redução na abundância e diversidade de corais (Harvell *et al.,*1999; 2002; Weil, 2004; Rosenberg & Loya, 2004, Chadwick-Furman, 2006; Rosenberg *et al*., 2007; Knowlton & Jackson, 2008; Leão *et al*., 2008; Francini-Filho *et al*., 2008; Bourne *et al*, 2009; Mydlarz *et al*., 2010) e indicam que um terço de todas as espécies de corais estão ameaçadas de extinção (Carpenter *et al*., 2008). Estima-se que mais de 30 % dos recifes já estejam severamente danificados e que dentro de trinta a quarenta anos aproximadamente 60 % das áreas recifais do mundo estarão totalmente degradadas (Hoegh-Guldberg, 2004; Knowlton & Jackson 2008).

Pequenas mudanças ambientais podem causar grandes danos aos sistemas coralíneos, já que grande parte deles localiza-se próximo aos trópicos onde as condições ambientais são relativamente estáveis (McClanahan *et al*., 2002). O efeito do estresse, provocado pelas variações ambientais, afeta frequentemente a capacidade de recuperação desse ecossistema, altera a distribuição das espécies em vários pontos do globo (Walther

et al., 2002) e causa sérias mudanças em sua estrutura (Bellwood *et al.*, 2004). As principais causas responsáveis pela degradação dos corais estão relacionadas a mudanças climáticas (Harvell *et al.*, 2002, Hoegh-Guldberg *et al*., 2007, Doney *et al*., 2009), impactos antropogênicos (poluição, pesca demasiada, etc) (Szmant, 2002; Hughes *et al*., 2003; Feely *et al*, 2004; Cooley & Doney, 2009) e doenças infecciosas causadas por microrganismos (Rosenberg *et al*., 2007; Bourne *et al*., 2009).

II.6.1. MUDANÇAS CLIMÁTICAS

Mudanças climáticas como aumento da temperatura global, efeito estufa entre outros fenômenos ocorridos na Terra fazem parte do crescente impacto humano no planeta, que agridem os corais. Os processos ecológicos nos recifes coralíneos são altamente sensíveis a variações de temperatura (Sheppard, 2003; Hughes *et al.,* 2003; Graham *et al.,* 2006) e condições físico-químicas da água do mar (Drollet *et al.,* 1994). A elevação da temperatura pode provocar aumento da atividade fotossintetizante dos simbiontes, estimulando o aumento das taxas metabólicas do hospedeiro e resultando em formação de compostos tóxicos (peróxidos), que podem danificar as células do hospedeiro e interferir nas suas vias bioquímicas (http://www.co2science.org/education/reports/corals/coralreefs.pdf). Os efeitos do aumento de radiação ultravioleta (UV) ainda são amplamente desconhecidos, entretanto, eles parecem danificar os propágulos planctônicos de coral na água de superfície do oceano, diminuindo taxas de fluxo de genes entre populações de corais. Este fenômeno resulta em aumento da extinção de espécies locais, impactando fortemente pequenas populações de corais que compõem recifes amplamente separados (Chadwick-Furman, 2006; Shick *et al*., 2006).

O oceano é o mais amplo reservatório natural de carbono, absorvendo cerca de 30 % do carbono emitido na atmosfera por ano. Porém, esta função tem sido prejudicada pelo efeito de acidificação oceânica. Quando CO_2 dissolve na superfície da água do mar ele reage com H_2O para formar ácido carbônico (H_2CO_3), que é dissociado em íons bicarbonato (HCO_3^-) e íons hidrogênio (H^+). O aumento de H+ diminui o pH, aumentando a acidez da água. O excesso de íons H^+ reage com íons carbonato (CO_3^{-2}) para formar mais íons bicarbonato (HCO_3^-). Portanto o aumento de CO_2 nos oceanos

causa aumento das concentrações de íons (HCO_3^-) e (H^+) e conseqüentemente diminui a disponibilidade de íons CO_3^{-2}, necessários para a formação do carbonato de cálcio ($CaCO_3$), aragonito e calcita, que compõem o esqueleto e a carapaça de diversos organismos marinhos, como corais e crustáceos além de servirem como edificação para muitas plantas e animais marinhos (Feely *et al*., 2004; Doney *et al*., 2009). As concentrações de íons carbonato nos oceanos estão realmente mais baixas do que durante os últimos 800.000 mil anos. Como os oceanos tornam-se menos saturados com minerais de carbonato, ao longo do tempo é esperado que os organismos marinhos construam fracas estruturas de esqueletos e carapaças, com taxa de crescimento lenta, tornando-os presas fáceis na competição com outros organismos marinhos (Kleypas *et al*., 2006; Fine & Tchernov, 2007). Além disso, com a diminuição da disponibilidade de minerais de carbonato na água do mar o meio pode tornar-se corrosivo, induzindo as carapaças e esqueletos de organismos calcificadores à completa dissolução (Guinotte *et al*., 2006; Turley *et al*., 2007).

Uma das mais notáveis mudanças climáticas que afetam os organismos marinhos perece ser o El Niño. Este fenômeno causa elevação da temperatura da água do mar. Eventos de branqueamento de corais vêm sendo amplamente observados ao redor do mundo em períodos de El Niño concomitantemente a mortalidade massiva de corais (Harvell *et al.,* 1999). Antes de 1980, todos os casos de branqueamento relatados se referiam à regiões geograficamente limitadas, causados por estresses claramente locais, como em recifes atingidos por furacões. Desde o início da década de 80 eventos de branqueamento em larga escala ao redor do mundo têm se tornado mais intensos e frequentes. No Brasil os primeiros episódios de branqueamento de corais relacionados principalmente com o aumento da temperatura provocado pelo El Niño foram observados na região sudeste durante o verão de 1994 e no início de 1996 (em São Sebastião, SP), afetando principalmente o coral *Mussismilia hispida* e o zoantídeo *Palythoa caribaeorum* (Migotto, 1997). Este autor observou que o branqueamento desses cnidários se deu alguns dias após a ocorrência de anormalidades térmicas da água superficial do mar, da ordem de 2,4 °C acima da média normal, durante o verão. Outros pesquisadores relataram casos subsequentes em vários recifes ao longo da costa nordestina, em graus mais intensos durante os anos quando houve variações térmicas da água do mar iguais ou

superiores a 1 ^{o}C (Castro & Pires, 1999; Zorzetto & Falcão, 2004; Leão *et al.,* 2003, 2008).

II.6.2. IMPACTOS ANTROPOGÊNICOS

Variações na transparência da água, aumento do acúmulo de sedimento sobre os recifes, redução na salinidade, contaminação por efluentes domésticos e industriais, derramamento de óleo, são problemas que podem causar distúrbios no ecossistema e deprimir o crescimento dos corais (Dodge & Vaysnis, 1977; Brown & Howard, 1985; Rogers, 1990; Glynn, 1997; Brown, 1997; Hodgson & Dixon, 2000). Os impactos humanos, tais como poluição pela agricultura, aquacultura, metais pesados, esgotos domésticos e derramamento de óleo, assim como o desenvolvimento urbano, turismo inadequado, pesca demasiada e métodos destrutivos de pesca, como o uso de dinamites, estão entre os principais fatores responsáveis pela destruição dos recifes de corais, resultando em uma acelerada degradação dos ecossistemas costeiros, cuja estrutura trófica fica seriamente comprometida (Jackson *et al.*, 1989, 2001; Newton *et al*., 2007).

Microrganismos inseridos nos oceanos por derramamento de esgotos têm ocasionado surtos de doenças ao meio marinho (Harvell *et al*., 1999; Dinsdale *et al*., 2008; Sutherland *et al*., 2004, 2010). Estes microrganismos patogênicos podem ser transferidos ao homem, através de consumo de peixes e crustáceos contaminados ou durante recreação. Patterson *et al*. (2002) identificaram através de análises de sequências do gene 16S rRNA, uma cepa de *Serratia marcescens*, como o agente causador da doença Mancha Branca no coral *Acropora palmata,* no Caribe, sugerindo que esta bactéria está associada com poluição de origem fecal lançada nos oceanos. Recentemente, estes pesquisadores conseguiram recuperar a mesma linhagem patogênica em molusco marinho (*Coralliophila abbreviata*), água recifal e duas espécies de corais *Siderastrea siderea* e *Solenastrea bournoni,* suportando a prévia conclusão de que humanos são, provavelmente, fonte de doenças no meio marinho (Sutherland *et al*., 2010). Microrganismos detectados na microbiota de corais doentes sugerem que efluentes de esgotos, poluição terrestre e doenças derivadas de outros organismos marinhos contribuem significativamente para o desenvolvimento de doenças nos corais (Frias-Lopez *et al.*, 2002; Voss & Richardson; 2006; Sutherland *et al.*, 2004, 2010).

II.6.3. DOENÇAS INFECCIOSAS EM CORAIS

Durante as duas últimas décadas foi observado um grande aumento na frequência e distribuição de doenças em corais (Hoegh-Guldberg, 1999; Ward & Lafferty, 2004, Rosenberg *et al*., 2007; Cervino *et al*., 2008; Kim *et al*., 2009). Cerca de 30 doenças infecciosas já foram descritas (Weil, 2004) e elas são consideradas uma das principais causas de degradação e extinção de corais em diversas partes do mundo (Rosenberg & Loya, 2004; Rosenberg *et al*., 2007; Bourne *et al*., 2009, Johnson *et al*., 2010).

As bactérias patogênicas envolvidas em doenças de corais pertencem a grupos microbianos variados (Rosenberg *et al*., 2007), mas notoriamente inclui o gênero *Vibrio* (Thompson *et al*., 2006). Doenças como a banda amarela descrita em diversas espécies de corais (*Montastrea* spp., *Fungia* spp., *Herpolitha* spp. e *Diploastrea heliopora*) têm sido associadas com a presença de várias espécies de *Vibrio*, incluindo *V. rotiferianus*, *V. harveyi*, *V. alginolyticus* e *V. proteolyticus*. Esta doença causa manchas amarelas no tecido, formando bandas de crescimento lento (0.5-1 cm por mês). As bactérias atacam diretamente as zooxantelas intracelulares causando degeneração e deformidade de suas organelas que culmina em decréscimo de clorofila e pigmentos acessórios, interferindo na reprodução dos corais (Cervino *et al*., 2004, 2008; Weil *et al*., 2009).

Uma das doenças mais sérias e prevalentes da atualidade é conhecida como branqueamento de corais (Stone, 2007; Causey, 2008). O branqueamento aparece quando há uma ruptura da associação simbiótica entre o coral e a alga zooxantela (do gênero *Symbiodinium*), devido à perda da alga endosimbiôntica ou de seus pigmentos que dá cor aos tecidos coralíneos (Thurman, 1997). Quando a zooxantela é expulsa ou torna-se despigmentada, o tecido fica transparente exibindo o esqueleto de carbonato de cálcio branco, induzindo um jejum compulsório ao hospedeiro, uma vez que a alga simbionte supre a maior parte das necessidades nutricionais do coral. Aquecimento global, temperaturas extremas (choque térmico, quente ou frio), alta irradiação solar, escuro prolongado, metais pesados (cobre e zinco) e altas taxas de sedimentação têm sido relatados como responsáveis pelo surgimento do fenômeno de branqueamento em corais (Hoegh-Guldberg, 1999; Brown *et al*., 2000; McClanaham *et al*., 2007). Entretanto,

bactérias especialmente do gênero *Vibrio* parecem ter um papel relevante nas mortes causadas em corais durante esse fenômeno (Rosenberg *et al*., 2007).

Diversos trabalhos vêm relatando que o aumento da temperatura da água do mar parece ser o principal estímulo para desencadear a expressão de genes de virulência em algumas espécies de *Vibrio* iniciando processos patogênicos que não se manifestavam em condições normais (Bem-Haim *et al*., 2003; Rosenberg *et al* 2007; Sussman *et al*., 2009; Thurber *et al*., 2009). As espécies *Vibrio shilonii* e *Vibrio coralliilyticus* têm se mostrado um importante patógeno de corais no desenvolvimento do branqueamento. Elevações da temperatura no meio marinho (>25 °C), particularmente durante o verão e em períodos de El Niño, induzem a atração química de *V. shilonii* ao muco do coral, provocando adesão a um receptor β-galactosidase para então penetrarem na camada epitelial do coral, onde eles se multiplicam, atingindo uma densidade de 10^9 bactérias por cm^3 de tecido. Nesta fase a bactéria expressa toxina P (PYPVYAPPPWP), que inibe a fotossíntese da zooxantela intracelular levando a perda do simbionte ou de seu pigmento. Com a diminuição da temperatura da água os vibrios não produzem essas adesinas, toxinas ou superóxido dismutase (SOS) necessárias a sua invasão e acabam sendo destruídos por radicais de oxigênio gerados pelo coral (Rosenberg *et al*., 2007).

Variações na composição de comunidades bacterianas associadas aos corais observadas antes, durante e após o fenômeno de branqueamento mostraram um aumento de 200 % no número de indivíduos da população de vibrios presente no muco do coral *Montastraea annularis,* durante surto da doença (Ritchie *et al*., 1994). Estas variações parecem estar relacionadas com mudanças nos parâmetros físico-químicos da água, como pH e tensão de oxigênio, aumento da temperatura e disponibilidade de fontes de carbono favoráveis ao crescimento de vibrios (Ritchie & Smith*.,* 1995ab, 2004, Ritchie, 2006). Aumento da temperatura é apontado como um dos mais importantes fatores que regulam quantitativamente a população de vibrios assim como sua patogenicidade (Cervino *et al*., 2004; Sawabe *et al*., 2007a; Fukui *et al*., 2009). Estudos sobre diversidade de vibrios associados com o branqueamento em Davies Reef e Magnetic Island (barreira de corais, AU) e em Kaneohe Bay (Hawaii) indicam que espécies, como ex. *Enterovibrio coralii, Photobacterium rosenbergii, V. fortis, V. campbellii, V. harveyi, V. mediterranei* e *V.*

rotiferianus podem estar envolvidas no processo de branqueamento (Thompson *et al.,* 2005a). Aquaculturas poderiam servir como reservatórios e/ou um foco de distribuição de linhagens patogênicas de vibrios, pois durante certos períodos do ano vibrios patogênicos suportariam condições ambientais adversas dentro dos cultivos e quando as condições ambientais fossem favoráveis (isto é, disponibilidade de nutrientes e temperatura elevada), eles seriam capazes de causar doença em outros seres (Bem-Haim *et al.,* 2003).

O branqueamento atinge a saúde do coral porque ele provoca uma redução nas taxas de calcificação do seu exoesqueleto, diminui crescimento e afeta a reprodução de corais (Goreau & Macfarlane, 1990; Michalek-Wagner & Willis, 2001), reduzindo também as camadas de tecido e muco do hospedeiro (Rosenberg *et al*., 2009). Estes danos representam um impacto significativo nos recifes que pode comprometer seriamente a manutenção e o desenvolvimento da estrutura recifal (VanOpen & Lough, 2009), já que os seus principais construtores sofrem déficit energético e podem tornar-se vulneráveis a ação de predadores e doenças que podem levá-los à morte (Glynn, 1993; Hoegh-Guldberg, 1999; CoRIS, 2007), favorecendo a transição de um ecossistema dominado por coral a um ecossistema composto apenas por macroalgas (Bahartan *et al*., 2010).

Conhecer a composição das comunidades microbianas associadas com corais, como elas mudam ao longo do tempo e entender como elas colaboram na saúde dos seus hospedeiros são a chave para a preservação de corais e recifes. Mudanças na composição das comunidades bacterianas podem afetar a saúde do coral assim como sua susceptibilidade a doenças (Bourne & Munn, 2005). Pantos *et al.* (2003), demonstraram que a comunidade microbiana de todas as colônias do coral é afetada mesmo quando realmente uma pequena parte da colônia mostra sinais de doenças. Estes resultados monstram que mudanças na microbiota normal, observadas antes de sinais visíveis de estresse, podem ser usadas como um bioindicador de ambos, mudanças ambientais e doenças. Desta forma o mais completo entendimento da variabilidade na comunidade bacteriana de corais saudáveis é claramente essencial, antes mesmo de avaliarmos os fatores ambientais que controlam estas variações (Rohwer & Kelley, 2004; Guppy & Bythell, 2006).

II.7. CONCEITO DO HOLOBIONTE

Apesar da interação mutualística entre as algas do gênero *Symbiodinium* (zooxantelas) e os corais serem de extrema importância para sobrevivência destes organismos, pesquisas baseadas em métodos independentes de cultivo têm demonstrado que uma abundante, diversa e específica população de outros microrganismos simbióticos incluindo bactérias, arquéias, vírus, fungos e protozoários associados aos corais (Kellog, 2004; Bourne & Munn, 2005, Wegley *et al*., 2007, Rosenberg *et al*., 2007; Bourne *et al*., 2009) também possuem funções de grande importância para manutenção da sua homeostase e parecem ter co-evoluído com o hospedeiro (Ritchie & Smith, 2004; Rohwer & Kelly 2004).

A colônia do coral e todas as suas associações simbióticas com outros microrganismos foi proposta por Rowher *et al*. (2002) como um holobionte. Essas inter-relações parecem ter um papel fundamental para sobrevivência dos organismos envolvidos, pois há uma constante troca de nutrientes necessários a manutenção do equilíbrio destes seres vivos. Além disso, essa microbiota parece co-evoluir juntamente com o hospedeiro (Rosenberg, *et al*., 2007; Weiss & Alleme, 2009) (**Figura 1**).

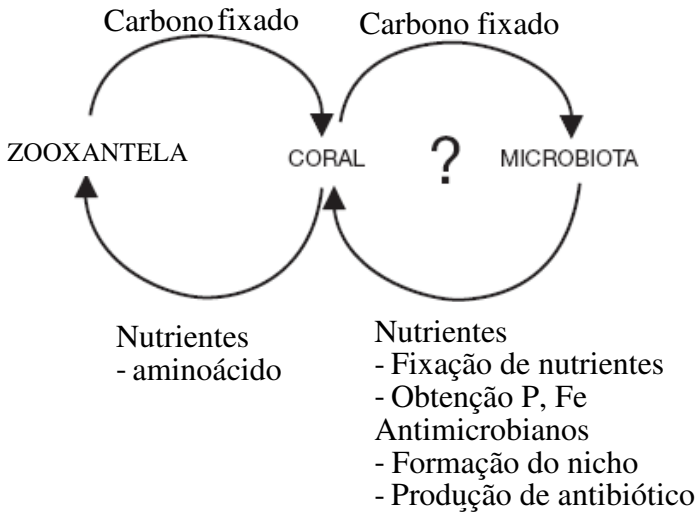

Figura 1. Modelo mostrando as inter-relações do holobionte (coral + zooxantela + microrganismo). Modificado de Rohwer *et al*. (2002).

O papel desses microrganismos na saúde (proteção e nutrição) e doença de corais tem sido amplamente investigado no campo da microbiologia de corais. Diversas formas de vida microbiana representadas por organismos de todos os três domínios Bacteria, Archaea e Eucarya, assim como muitos vírus, estão interagindo nas estruturas que compõem o coral. Microrganismos habitam três principais estruturas que constituem o coral: o muco, o tecido do coral (incluindo a cavidade gastrodérmica) e o esqueleto de carbonato de cálcio ($CaCO_3$) (**Figura 2)**.

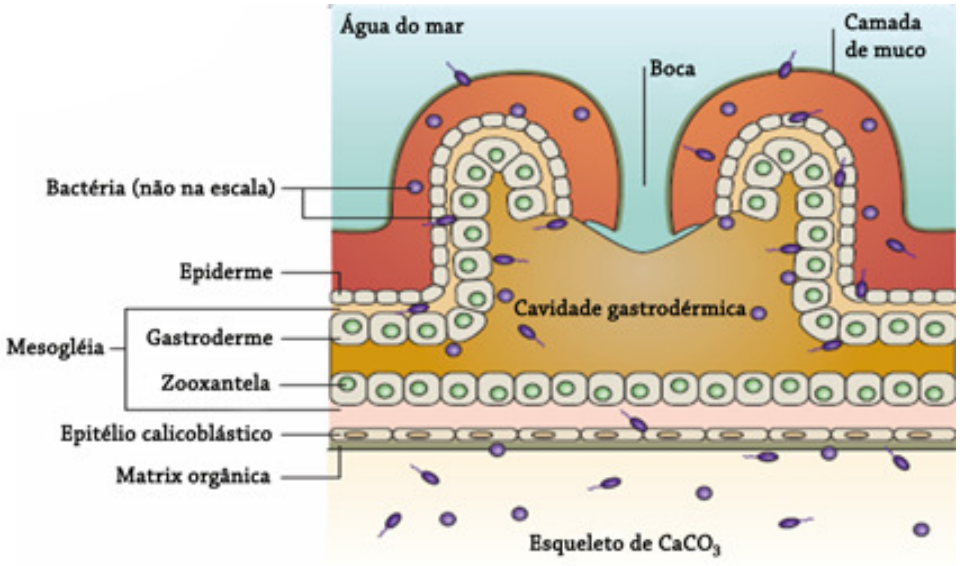

Figura 2 – A estrutura do tecido do coral representado pela espécie *Oculina patagonica*. Duas camadas de células, a epiderme e a gastroderme, cobertas por uma camada de muco na superfície, estão conectadas a um largo esqueleto poroso de carbonato de cálcio. Zooxantelas estão na gastroderme (em verde) enquanto as bactérias estão representadas pelas estruturas roxas (Modificado de Rosenberg *et al.*, 2007).

Distúrbios ou mudanças em algum desses parceiros podem comprometer a saúde do holobionte como um todo e desencadear o surgimento de doenças. Esse parece ser, portanto, o resultado do reflexo de um complexo conjunto de interações entre uma variedade de parceiros associados que afetam a adaptação do holobionte coletivamente. Desta forma, para o entendimento dos processos de doenças dentro do coral, faz-se necessário obter um profundo conhecimento da biologia básica de cada membro do coral holobionte. Para tanto a informação sobre a composição da microbiota de corais

saudáveis torna-se fundamental. Estudos da microbiota associada aos corais têm despertado o interesse de pesquisadores (Rohwer *et al*., 2002, Bourne & Munn, 2005; Littman *et al*., 2009) o qual ajudará o entendimento do papel mutualista dessas múltiplas espécies além de auxiliar a identificar quais espécies desempenham papéis chaves na manutenção da saúde do coral (Bourne *et al*., 2009). O conhecimento das interações na microbiota do holobionte é também importante porque as relações comensais entre diversas espécies determinam a distribuição de populações microbianas nas estruturas do coral capazes de estabelecer o nicho dessas comunidades que podem atuar na defesa do seu hospedeiro (Reshef *et al.,* 2006, Ritchie, 2006; Mydlarz *et al*., 2008).

II.8. DIVERSIDADE DE MICRORGANISMOS NO HOLOBIONTE

II.8.1. *SYMBIODINIUM.*

O gênero *Symbiodinium* (zooxantela), descrito na década de 1950, é um importante simbionte do coral. Este gênero é excepcionalmente diverso contendo múltiplos taxa (Baker *et al*., 2003) que podem estar associados com diferentes organismos marinhos como corais, anêmonas, medusas, ascídias, esponjas, moluscos entre outros. Uma única espécie de coral pode abrigar múltiplas espécies de *Symbiodinium* (Rowan & Knowlton, 1995; Baker, 2001), sendo que diferentes taxa podem habitar diferentes profundidades (LaJeunesse *et al*., 2003). *Symbiodinium* fornece uma grande parte de energia requisitada pelo seu hospedeiro através da transferência de carbono fixado fotossinteticamente **(**Fallowski *et al*., 1984), além de grande quantidade de oxigênio que permite respiração eficiente do coral e dos procariontes associados. Adicionalmente altas concentrações de O_2 resultam em formação de radicais de O_2 que oferecem aos corais proteção contra infecções bacterianas e podem atuar como força seletiva para o simbionte (Banin *et al*., 2003). Até o presente não está claro como fatores ambientais, como temperatura, afetam a distribuição desse simbionte, mas é conhecido que em processos de branqueamento ocorre ruptura da associação entre o coral e o hospedeiro, por perda da alga ou de seu pigmento que expõe o esqueleto do hospedeiro. Esse fenômeno também parece ser mais facilmente observado em períodos mais quentes do ano (Goreau *et al*., 1997) ou quando ocorre aquecimento da água (Hoegh-Guldberg, 1999), porém, nos dois casos o branqueamento resultou da infecção de bactérias

dependentes de temperatura (Kushmaro *et al*., 1996; Ben-Haim *et al*., 2003). Na água existem, no entanto, diferentes espécies de algas zooxantelas que podem vir a reocupar o tecido do coral. A capacidade de recuperação do hospedeiro dependerá da duração e da intensidade do distúrbio. Surge, então, a hipótese de que o branqueamento possa ser um mecanismo adaptativo que permita o coral trocar linhagens de *Symbiodinium* sensíveis por outros mais resistentes (Buddemeier & Fautin, 1993).

II.8.2. MICRORGANISMOS EUCARIONTES

Fungos endolíticos são frequentemente encontrados associados com corais vivos ou no esqueleto calcário de seus fósseis (Bentis *et al*., 2000). Doze gêneros, incluindo *Aspergillus,* têm sido relatados na literatura associados com corais (Kendrick *et al*., 1982). Micróbios protistas identificados como *stramenopiles* também são relatados associados ao muco de corais, mostrando 92 % de similaridade nas sequências do gene 18S rRNA em direção a *Hypochytrium catenoides* com possível papel ecológico no holobionte (Kramarsky-Winter *et al*., 2006).

II.8.3. VÍRUS

Vírus é a mais abundante entidade biológica no planeta e eles são responsáveis por estruturar as comunidades planctônicas marinhas, eucarióticas e procarióticas, uma vez que vírus são capazes de influenciar os ciclos biogeoquímicos globais. Pesquisas com vírus e seus efeitos no coral tornaram-se um emergente campo de estudos, mas que ainda estão em sua infância, especialmente pela dificuldade de cultivá-los, já que técnicas para cultura desses vírus ainda não foram estabelecidas. Entretanto, metodologias como microscopia eletrônica e impregnação por SYBR gold revelam a presença de numerosos vírus e partículas virais (VLPs) no muco e tecido de corais, associados aos endosimbiontes (*Symbiodinium*) e na água marinha próxima de seu habitat (Davy *et al*., 2006; Van Oppen *et al*., 2009). Até o presente não há relatos de vírus como patógeno de corais. Entretanto, a combinação das metodologias de metagenômica e PCR em tempo real usadas por Thurber *et al*. (2009) mostraram que a presença de sequências do tipo herpes-vírus aumentava rapidamente no coral *Porites compressa* quando ele foi submetido a situação de estresse (temperatura, pH e aumento de nutrientes). Além disso,

relatos de anomalias como tumores em corais são comuns e os vírus parecem estar envolvidos (Kaczmarsky & Richardson, 2007) já que eles são responsáveis pelas desordens de proliferação celular ou neoplasma em muitos outros organismos (Cuzich, 2000).

Vírus podem também ter um papel benéfico na saúde e manutenção do holobionte. A população proliferante de bacteriófagos líticos pode estabilizar a comunidade de bactérias associadas ao coral, pois, um aumento na abundância de uma população dominante de bactérias eleva seu contato com fagos conduzindo a um aumento significativo da infecção e conseqüentemente na lise bacteriana, controlando então sua abundância (Bourne *et al*., 2009). Desta forma, bacteriófagos fornecem uma opção para controle da dispersão de doenças infecciosas de corais, uma vez que fagos líticos marcados contra patógenos de corais como *Vibrio coralliilyticus* e *Thalossomonas loyana* preveniram a transmissão e subseqüentemente, a infecção da doença em testes experimentais controlados (Efrony *et al*., 2007, 2009). Um estudo demostrou que o uso de fagos controla doenças infecciosas em corais porque eles infectam e lisam patógenos e podem prevenir infecções subseqüentes, porque permanecem associados com o coral (Efrony *et al*., 2007). Outro exemplo bem documentado no meio marinho é a sazonalidade de cólera, pois fagos levam a eliminação da linhagem de *Vibrio cholerae* que está causando a epidemia, refletindo em queda no número de casos em humanos (Faruque *et al*., 2005).

II.8.4. PROCARIONTES

Técnicas independentes de cultivo têm revelado a presença de uma ampla e diversa população de arquéias em corais (Kellog, 2004; Wegley *et al*., 2004, Bourne *et al*., 2009) e sugerem que elas estejam em quantidade maiores que 10^7 células por cm^2 em sua superfície. Evidências sugerem, entretanto, que elas não parecem ter uma relação de especificidade com o hospedeiro, uma vez que a maioria das sequências encontradas associadas aos corais estavam presentes também na coluna de água ao redor dos hospedeiros (Kellog, 2004). O papel biológico desses organismos ainda é pouco conhecido no coral holobionte.

Bactérias que habitam o coral podem ser encontradas associadas à camada de muco, ao tecido (incluindo a cavidade gastrodérmica) e ao esqueleto de carbonato de cálcio, entretanto, cada uma das camadas abriga uma população bacteriana distinta no mesmo fragmento de coral (Bourne & Munn, 2005; Koren & Rosenberg, 2006). Além disso, é provável que dentro de cada habitat existam micro nichos colonizados por diferentes espécies bacterianas (Ritchie & Smith, 2004). Apesar de o coral possuir seus próprios mecanismos de defesa, como movimento ciliar, aumento da produção de muco, fagocitose (Bigger & Hildemann, 1982; Mullen *et al*., 2004) e produção de antimicrobianos, por exemplo, quando induzido ao estresse mecânico causado pela mordida de um predador (Kim, 1994; Kelman *et al.*, 2006; Geffen *et al*., 2009). Essa específica composição microbiana também atua na defesa do holobionte, pois, por competição elas podem impedir que patógenos oportunistas se instalem e se multipliquem (Rypien *et al*., 2009), ou ainda pela liberação de antimicrobianos capazes de eliminar bactérias invasoras (Reshef *et al*., 2006, Ritchie, 2006).

Esqueleto de corais são estruturas porosas habitadas por uma variedade de bactérias que parecem ser responsáveis por fornecer 50 % do nitrogênio total necessário ao coral (Ferrer & Szmant, 1988). Cianobactérias encontradas nessa camada fornecem também compostos orgânicos ao tecido do coral, auxiliando crucialmente na sobrevivência do hospedeiro quando ocorre perda da zooxantela (Fine & Loya, 2002). A camada de mucopolissacarídeo ou muco, que se estende poucos milímetros acima da superfície dos tecidos do coral, é constituída principalmente por fucose, arabinose, manose, galactose e resíduos de glucose, secretados por células especializadas presentes na epiderme do coral (Paul *et al.,* 1986). Essa camada sustenta alto crescimento bacteriano gerado, provavelmente, a partir da degradação dos constituintes do próprio muco, sendo que, os produtos desta degradação ou as próprias bactérias podem servir de alimento para corais e outros organismos (Ducklow & Mitchell, 1979). O muco é usado também para capturar presas ou para proteção e nutrição do coral (Sorokin, 1978; Ducklow, 1990; Bak *et al.,* 1998; Brown & Bythell, 2005; Jatkar *et al*., 2010).

Pesquisas utilizando métodos dependentes de cultivo, focadas na investigação da microbiologia da camada de muco dos corais, demonstraram que esta camada suporta

uma diversa, abundante e benéfica comunidade bacteriana (Ducklow & Mitchel, 1979; Ritchie & Smith, 1995; Rohwer *et al*., 2001) incluindo as fixadoras de nitrogênio (Williams *et al*., 1987, Shashar *et al*., 1994; Lesser *et al*., 2004, Olson *et al*., 2009) e as decompositoras de quitina, que é o segundo biopolímero mais abundante do planeta depois da celulose e correponde a uma importante fonte de carbono e nitrogênio no meio marinho (Ducklow & Mitchel, 1979; McCarthy *et al*., 1997). A abundância de bactérias na camada de muco tem sido estimada em 10^8 unidades formadoras de colônia (ufc) por ml (Koren & Rosenberg, 2006) e sua população representa um tamanho de 100 a 1000 vezes maior do que as observadas na água do mar (Rosenberg *et al*., 2007). Esses valores, entretanto, representam aproximadamente apenas 0,2 % do total de bactérias determinada microscopicamente através de marcação por SYBR gold (Koren & Rosenberg, 2006). As quantidades de bactérias cultiváveis que colonizam o tecido são similares às encontradas na camada de muco (Koren & Rosenberg, 2006). Porém, as espécies abundantes no tecido diferem das espécies abundantes do muco (Bourne e Munn, 2005; Koren & Rosenberg, 2006). Rohwer *et al*. (2002) sugeriu através de análises independentes de cultivo que composições de populações bacterianas similares associavam-se com as mesmas espécies de coral mesmo que separadas geograficamente e que diferentes populações microbianas colonizavam espécies diferentes de corais. Recentemente, análises da diversidade bacteriana, utilizando método independente de cultivo, feita por Littman *et al*. (2009) mostraram que corais de espécies diferentes, próximas geograficamente, possuiam comunidades bacterianas semelhantes, enquanto que espécimes da mesma espécie de coral, geograficamente distantes, apresentaram comunidades microbianas diferentes.

Embora a composição da população de bactérias não cultiváveis possa mostrar-se distintas das populações cultiváveis (Rosemberg *et al*., 2007), diversos estudos têm revelado a presença de vibrios associados tanto a espécimes saudáveis de corais, quanto a doentes (Lampert *et al*., 2006; Koren & Rosenberg, 2006; Weil *et al.,* 2006; Kooperman *et al.,* 2007; Luna *et al*., 2007; Dinsdale *et al.,* 2008; Sussman *et al.,* 2009, Alves Jr *et al*., 2009; Reis *et al.,* 2009). Koren & Rosenberg (2006) analisaram a população microbiana no muco e no tecido saudável de *Oculina patagonica*, que possui habilidade de crescer sob condições muito variáveis de temperatura (16 a 41°C) e salinidade (28 a 50 %). Estes

autores verificaram que vibrios identificados como *V. splendidus* e *V. halioticoli* apareceram em ambos os métodos dependentes e independentes de cultivo. Lampert *et al.* (2006) também mostraram através de análises independentes de cultivo que vibrios parecem pertencer a microbiota natural de *Fungia scutaria.* Entretanto, outros autores têm sugerido, com base em estudos de contagem de colônias e presença de alta proporção de genes que correlacionam aos vibrios (N-acetylglucosaminases), que o aumento de vibrios no muco de corais e na água ao seu redor pode ser um indicador de más condições ambientais (Dinsdale *et al.,* 2008).

Vibrios estão presentes em uma fração considerável da microbiota do muco de diferentes espécies de corais e em *Pocillopora damicornis* são encontrados 2 x 10^7 vibrios/cm^3 em espécimes doentes e 6 x 10^6 vibrios/cm^3 em espécimes saudáveis do coral (Luna *et al.*, 2007). Eles são gamaproteobactérias importantes e ubíquas no meio marinho, compreendendo cerca de 10 % das bactérias marinhas facilmente cultiváveis (Eilers *et al*., 2000), além de desempenharem papel importante na saúde humana e animal. O gênero *Vibrio* possui espécies muito versáteis metabolicamente, sendo encontradas tanto em vida livre, quanto colonizando diversos animais marinhos (Fukui *et al*., 2009). Alguns vibrios estabelecem relações mutualísticas com invertebrados marinhos (Ruby, 1996), enquanto outros são patogênicos para vários animais como camarão (Liu *et al*., 2004), diversas espécies de peixes, moluscos (Lightner & Redman, 1998; Gomez-Gil *et al*., 2004; Thompson *et al*., 2004; Sawabe *et al*., 2007a) e corais (Rosenberg *et al.*, 2007).

Vibrios simbiontes de corais também poderiam ter um papel positivo no coral, por meio da disponibilização de nutrientes, moléculas bioativas e energia para o hospedeiro, permitindo a ocupação de diferentes nichos no holobionte. A formação de biofilmes por vibrios na superfície externa de cnidários e de outros organismos marinhos pode constituir uma estratégia para sua sobrevivência durante períodos escassos de alimento ou de outros estresses ambientais (Wolfe *et al*., 2004; Tait *et al*., 2010). Nos biofilmes estas bactérias podem ser capazes de absorver nutrientes, resistir a antibióticos e estabelecer associações favoráveis com outras bactérias ou hospedeiros. Por outro lado o biofilme pode servir de proteção e de alimento para o próprio coral.

II.9. FUNDAMENTOS BÁSICOS DA FIXAÇÃO BIOLÓGICA DO NITROGÊNIO (FBN) E O PAPEL DA NITROGENASE

Diversos pesquisadores têm trabalhado com a hipótese de que corais estabelecem uma relação de mutualismo com bactérias fixadoras de nitrogênio (BFN) (Williams *et al*., 1987; Shashar *et al*., 1994; Rohwer *et al*., 2002; Wegley *et al*., 2007) e essa relação parece auxiliar a sobrevivência desses organismos em águas oligotróficas onde frequentemente o nitrogênio é limitado (Capone, 2001). Desta forma a associação entre o coral e as BFN parece oferecer ao hospedeiro uma grande vantagem na obtenção da quantidade necessária de nitrogênio utilizada pelo coral (Shashar *et al*., 1994).

A capacidade de fixar nitrogênio é encontrada na maioria dos grupos filogenéticos entre as bactérias, incluindo sulfo-bactérias, firmibactéria, actinomicetos, cianobactérias, proteobactérias e arqueobactérias (Dixon & Kahn, 2004), assim como em grupos associados aos corais (Lesser *et al*., 2004, Hewson *et al*., 2007; Olson *et al*., 2009). Vibrios desempenham papéis importantes no ciclo de nutrientes em ambientes marinhos (Thompson *et al*., 2004). Algumas espécies como *V. diazotrophicus*, *V. natriegens* e *V. cincinnatiensis* (patógeno humano oportunista) são classificadas como fixadoras de nitrogênio atmosférico (N_2) (Thompson *et al*., 2006), mais recentemente *V. parahaemolyticus* (patógeno humano) mostrou ser capaz de fixar N_2 em águas de estuário (Criminger *et al*., 2007).

O ciclo do nitrogênio marinho é dirigido principalmente por dois processos complementares, a fixação do nitrogênio (FN) e a desnitrificação (Capone & Knapp, 2007). O nitrogênio é o constituinte essencial de aminoácidos, proteínas, bases nitrogenadas, ácidos nucléicos, hormônios e clorofila, entre outras moléculas que têm função limitante no crescimento de microrganismos, plantas e animais. No entanto, para ser utilizado pela maioria dos seres vivos o nitrogênio deve ser reduzido à amônia (NH_3) pelo processo de FN, por meios físico-químicos ou biológicos. O processo biológico pode funcionar por auto-regulação, uma fez que quando há nitrogênio suficiente disponível, o processo de FN ($= N_2 \rightarrow NH_3$) pode ser desativado (Döbereiner, 1992).

BFN são seres capazes de fixar N_2 em condições de vida livre, em seu habitat natural ou em interações com outros organismos, incluindo desde associações não-obrigatórias até associações de simbiose estrita (Döbereiner, 1995). O Sistema de regulação do nitrogênio (Ntr) consiste em uma cascata regulatória bastante complexa. Em *Escherichia coli* este sistema foi inicialmente descrito com seis proteínas: NtrB, NtrC, UTase/UR (GlnD), P_{II} (GlnB), ATase (GlnE) e glutamina sintetase (GS ou GlnA) (Merrick & Edwards, 1995). A descoberta de uma segunda proteína da família P_{II} (GlnK) em *E. coli* e em outros microrganismos aumentou a complexidade do sistema. Desta forma algumas proteínas se tornam chaves no processo de ativação da fixação biológica de nitrogênio (FBN) uma delas é a NtrC que quando fosforilada ativa a transcrição de genes envolvidos na assimilação de fontes alternativas de nitrogênio, ativando a expressão de um conjunto de genes denominado *nif* (*nitrogen fixing*), que codificam proteínas diretamente envolvidas no processo de fixação, enquanto outros genes tais como: *ntr*A, *ntr*B, *fix*, *fdx*, *rnf* e *nod* codificam proteínas que, indiretamente, desempenham função essencial na FBN. Um total de 20 genes *nif,* organizados em 7-9 *operons*, já foram identificados em *Klebsiella pneumoniae* (Streicher *et al*., 1972; Arnold *et al*., 1988) e 14 deles têm sido frequentemente encontrados em BFN. Esses genes apresentam bastante variabilidade entre os grupos microbianos fixadores de nitrogênio e isso se deve a influência dos diferentes microambientes no qual eles estão envolvidos (Zehr *et al*., 2003).

O fator chave da biofixação é a enzima nitrogenase, que catalisa a quebra das moléculas de N_2. O gene *nif*H codifica para a unidade estrutural da dinitrogenase redutase e os genes *nif*D e *nif*K para as subunidades estruturais da dinitrogenase. O complexo nitrogenase é, desta forma, composto por duas proteínas: a dinitrogenase-MoFe (um tetrâmero complexado a molibdênio e ferro) ou NifDK e a dinitrogenase redutase-Fe (um dímero complexado a ferro) ou NifH, ambas capazes de transportar elétrons. A proteína NifH é um dímero γ_2 (produto do gene *nifH*), e sua função é transportar elétrons até proteína NifDK (Burris, 1991). Os genes *nif*, principalmente o gene *nif*H, têm sido utilizados como marcadores no estudo de organismos fixadores de nitrogênio. O estudo da sequência desses genes também pode ser empregado para caracterizar a diversidade genética de bactérias diazotróficas.

Para que a reação de fixação ocorra, é necessário que elétrons sejam transportados por moléculas aptas a realizá-lo. A redução do substrato pela nitrogenase envolve três tipos básicos de transferência de elétrons: 1) redução da Fe-proteína por carreadores de elétrons; 2) transferência de um elétron a partir da Fe-proteína para a MoFe-proteína através de um processo dependente de Mg-ATP e 3) transferência de elétron para o substrato ligado ao sítio ativo da MoFe-proteína. Portanto, em condições ótimas, a estequiometria da reação catalítica, responsável pela redução do N_2 para a formação de duas moléculas de amônia, é usualmente descrita como mostrado por Simpson & Burris (1984).

$$\mathbf{N_2 + 8}\boldsymbol{e}\mathbf{ + 8H^+ + 16Mg\text{-}ATP \xrightarrow{N_{itrogenase}} 2NH_3 + H_2 + 16Mg\text{-}ADP + 16Pi}$$

Uma característica peculiar da nitrogenase é a capacidade de reduzir diferentes substratos: 1) nitrogênio para amônia ($N_2 \rightarrow NH_3$); 2) acetileno para etileno ($C_2H_2 \rightarrow C_2H_4$); 3) óxido nitroso para nitrogênio e água ($N_2O \rightarrow N_2 + H_2O$) entre outros (Sprent & Sprent, 1990). Com base nesses conceitos Hardy *et al*. (1968) propuseram um método eficiente para o estudo da atividade enzimática da nitrogenase. Uma vez que os gases acetileno e etileno podem ser quantificados em cromatografia gasosa através do teste de redução de acetileno (ARA) é possível utilizar acetileno como substrato na incubação de células vivas e quantificar o produto (etileno) gerado pela ação enzimática. A medida dessa redução pode ser utilizada como estimativa da fixação do nitrogênio em diferentes habitates (Odum, 1983).

TAXONOMIA E FILOGENIA DE PROCARIONTES

Sistemática é a ciência dedicada ao estudo da diversidade dos organismos e suas relações, que unem conceitos de taxonomia e filogenia. A taxonomia é a ciência que estuda a classificação, nomenclatura e identificação dos seres vivos, que é à base das ciências biológicas e um dos seus ramos mais antigo. A classificação consiste no ordenamento de organismos em grupos taxonômicos (taxa) com base na similaridade. A nomenclatura refere-se à definição do nome do organismo enquanto a identificação é o processo que determina se o organismo pertence a um dos grupos já classificados e nomeados anteriormente na literatura. Um dos principais objetivos da taxonomia é fornecer esquemas de classificações úteis, permanentes, preditivos, objetivos e informativos, de maneira que possam ser usados por cientistas e por outros profissionais, como por exemplo, em propostas práticas de interesse médico (Goodfellow, 2000). Filogenia é uma ferramenta usada para classificação e elucidação de relações evolutivas entre organismos.

II.10. ASPECTOS HISTÓRICOS DA TAXONOMIA POLIFÁSICA

Antigamente a classificação e identificação de linhagens bacterianas eram tradicionalmente feitas utilizando métodos clássicos de caracterização fenotípica (morfologia, características fisiológicas e bioquímicas), com base nos conceitos herdados da botânica. Entretanto, métodos fenotípicos são limitados a um restrito número de características, que quando avaliadas podem gerar resultados duvidosos devido a variações na expressão dos genes (Busch & Nitschko, 1999). Além disso, análises de dados fenotípicos não apresentam uma correlação coerente com características filogenéticas e pouca informação é obtida sobre as relações evolutivas entre os organismos estudados (Hugenholtz & Pace, 1996). Um exemplo clássico do problema de uma classificação baseada no fenótipo é a criação do gênero *Shigella,* originado a partir de isolados clínicos de *Escherichia coli* e com base em caracteres fenotípicos (Lan & Reeves, 2002). Isolados de *Shigella* são identificados genomicamente como membros da espécie *E. coli*.

Atualmente a taxonomia de bactérias é praticada com base nos conceitos de taxonomia polifásica, introduzidos por Colwell e colaboradores na década de 70, que usa um conjunto diversificado de dados, incluindo propriedades fenotípicas, genotípicas, químicas e ecológicas para classificar, nomear e identificar isolados bacterianos (Colwell *et al.*, 1970; Vandamme *et al.,* 1996).

Desde 1987 a metodologia de Hibridização DNA-DNA (HDD) é considerada o pilar para delineação de espécie bacteriana na taxonomia polifásica (Wayne *et al*., 1987, Stackebrandt *et al* 2002). De fato HDD confronta o DNA total entre linhagens bacterianas e provou ser pela primeira vez a mais forte ferramenta para identificar e categorizar procariotos de forma não ambígua. Em todos os casos de hibridização o DNA alvo é fixado e o DNA marcado é adicionado para hibridização. Depois de retirar o excesso de DNA marcado que não se ligou ao DNA fixado, as cinéticas de hibridizações heterólogas são medidas e interpretadas (Ludwig, 2007). Entretanto para que os resultados de HDD sejam confiáveis faz-se necessário a experiência de laboratórios especializados na preparação e purificação de alta qualidade de DNA, inclusão de linhagens de referência em cada experimento e na sua técnica propriamente dita uma vez que nenhum dos passos é aplicável sem eficiente treinamento (Stackebrandt & Ebers, 2006). Condições que tornam as HDD limitadas a um número restrito de laboratórios internacionais (Thompson *et al*., 2005b). Valores obtidos por diferentes metodologias de HDD dificultam a comparação dos resultados obtidos em diferentes estudos, além disso, inviabilizam a construção de um banco de dados centralizado.

Com o desenvolvimento das técnicas de sequenciamento dos ácidos nucléicos e análise de proteínas Woese (1987) introduziu informações de sequências de gene à sistemática microbiana e permitiu o esclarecimento das relações filogenéticas dos organismos procarióticos, baseando-se principalmente na comparação das sequências de 1500 nucleotídios do gene 16S ribossomal RNA (rRNA) que codificam a subunidade 30S ribossomal e está presente em todos os procariontes.

DNA ribossomal é um dos melhores alvos para estudos filogenéticos porque ele esta distribuído universalmente, possui constante funcionalidade e tem estrutura em mosaico com domínios variáveis e domínios altamente conservados através da evolução

(Woese, 1987). Entretanto a precisão das inferências filogenéticas das sequências de rRNA, depende do número de bases comparadas. A utilização de sequências, 1000 a 1500 nucleotídeos, evita relacionar os organismos equivocadamente por convergência de seus fenótipos e para que sejam efetivas devem ser consideradas no mínimo 500 bases para cada organismo (Fry *et al.,* 1991). Embora em diversos grupos microbianos a informação obtida pelas sequências do 16S rRNA revela uma limitada resolução taxonômica tanto em clones bacterianos, já que a presença de diversos operons dos genes rRNA encontrados em bactérias pode causar cerca de 1 % de variabilidade entre sequências de clones bacterianos (Acinas *et al*., 2004), quanto em espécies irmãs, ou seja espécies diferentes mas muito próximas filogeneticamente, devido a alta similaridade entre as sequências do 16S (Thompson *et al*., 2007).

De qualquer forma, a classificação filogenética baseada na análise de sequências de 16S rRNA apresenta boa correlação com características fenotípicas e quimiotaxonômicas em bactérias incluindo dados de composição de parede, padrões de ácidos graxos (FAME) e proteínas (Goodfellow & O'Donnel, 1994; Stackebrandt & Goebel, 1994). Desta forma, baseando-se na afiliação de sequência do 16S rRNA com espécies descritas é possível inferir algumas características fisiológicas, fenotípicas e químicas para novos grupos de microrganismos (Goodfellow & O'Donnel, 1994).

O conteúdo de informação obtida através de sequências gênicas do 16S rRNA possibilita a determinação das relações taxonômicas e filogenéticas tanto supra-específicas (classificação de isolados em gênero e famílias), quanto infra-específicas (distinção de espécies), assim como afiliação filogenética de organismos representantes de novas espécies (Goodfellow & O'Donnell, 1994). Além disso, dados de sequências podem ser facilmente armazenados em bancos de dados que servirão de base para comparações futuras (Pace *et al*., 1986).

II.11. SISTEMÁTICA DE PROCARIONTES

Como o estabelecimento de sequências do gene 16S rRNA possibilitou a determinação da posição filogenética de organismos procarióticos por uma análise mais rápida, a utilização desta metodologia tornou-se cada vez mais ampla. Contudo estudos

comparativos de sequências do gene conservado e de seu produto revelaram claras limitações do seu uso na determinação das relações filogenéticas em alguns grupos microbianos e estes só puderam ser solucionados com dados obtidos através HDD. Deste modo HDD se torna a metodologia chave capaz de delinear espécie em procariotos (Wayne *et al*., 1987; Stackebrandt & Goebel, 1994). Embora dados obtidos por HDD sejam altamente discriminatório seu uso é muito laborioso e o emprego desta metodologia torna-se restrito a poucos laboratórios no mundo.

Stackebrandt & Goebel (1994) conseguiram estabelecer, desta forma, uma correlação entre as metodologias de HDD e sequências do gene 16S rRNA de maneira a reduzir a carga de trabalho. Estes autores sugeriram que a execução dos experimentos de reassociação do DNA ou HDD é necessária somente entre linhagens que apresentam > 97 % de similaridade em suas sequências do gene 16S rRNA. Este princípio tem sido aceito para todos os procariontes. Ele define que organismos que compartilham < 97 % de similaridade na sequência do gene 16S rRNA resulta geralmente em HDD com < 70 % de reassociação, representando diferentes espécies. Do mesmo modo organismos que compartilham > 97 % de similaridade na sequência do gene 16S rRNA pode representar diferentes espécies (quadro vermelho, < 70 % similaridade HDD) ou pertencer a mesma espécie (quadro azul, > 70 % similaridade DDH) **Figura 3.**

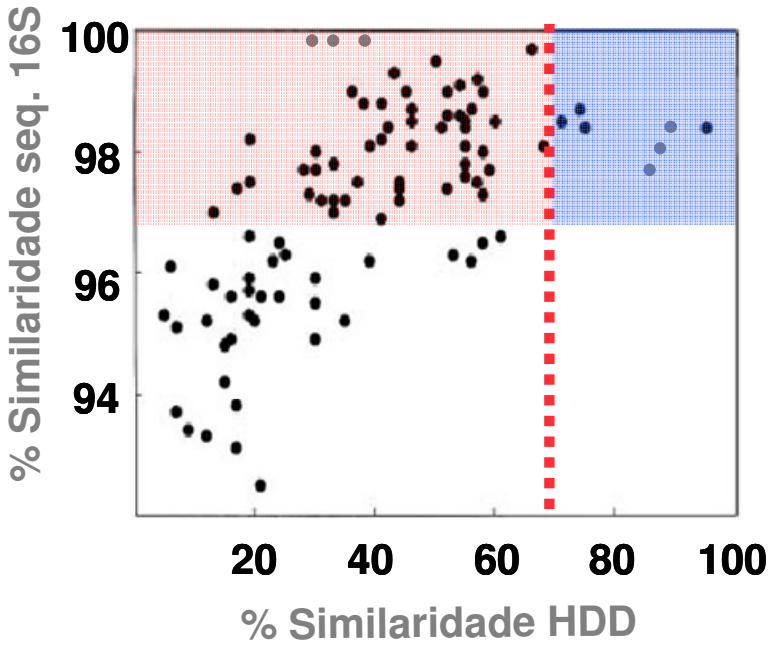

Figura 3. Relação entre similaridade de sequências do gene 16S rRNA e valores de hibridização DNA-DNA entre diferentes pares de organismos (modificado de Stackebrandt & Goebel, 1994; Rosselo-Mora & Amann, 2001).

II.12. DEFINIÇÃO E CONCEITOS DE ESPÉCIE BACTERIANA

O conceito biológico de espécie estabelecido por Mayer (1942) relata que: "Espécies são grupos de populações naturais que estão ou têm o potencial de estar se intercruzando, e que estão reprodutivamente isolados de outros grupos". Entretanto, esses princípios tornam-se impossíveis de serem aplicados dentro da microbiologia uma vez que procariotos se reproduzem de forma assexuada. Para tanto se faz necessário uma distinta definição de espécie procariótica. Uma espécie tem sido descrita como o melhor grupo de indivíduos, isolados/ linhagens, coerentes genomicamente que compartilham um alto grau de similaridade em (muitas) características independentes, comparativamente testadas sob condições altamente padronizadas (Stackebrandt *et al.,* 2002).

A definição de espécie não envolve processos biológicos responsáveis por especiação (Gevers *et al*., 2005). Com base na definição de espécie amplamente empregada hoje, linhagens da mesma espécie apresentam fenótipos (por exemplo, a expressão de diferentes tipos de enzimas, capacidade de usar diferentes tipos de compostos como fonte de energia, crescimento em diferentes temperaturas e concentrações de sal), genótipos (por exemplo, rep-PCR e AFLP) e características quimiotaxonômicas (por exemplo, FAME e poliaminas) muito semelhantes e formando grupos distintos de vizinhos próximos (Gillis *et al*., 2005). Uma espécie é definida como um grupo de linhagens (incluindo a linhagem-tipo) que apresentam > 70 % de similaridade genômica obtida por HDD, < 5 °C de variação na temperatura de "melting" (Tm) que desnaturam as fitas de DNA, < 5 % de diferença no conteúdo G+C do DNA genômico total e > 97 % de identidade em sequências do gene 16S rRNA (Wayne *et al*, 1987; Stackebrandt & Goebel, 1994; Ursing *et al*., 1995; Vandamme *et al*, 1996). Essa definição de espécie bacteriana é pragmática e operacional e visa o estabelecimento de um sistema taxonômico rápido, confiável, reprodutível e útil. Este sistema reflete de certa forma a evolução microbiana. A definição é útil para uma variedade de aplicações (como

por exemplo, medicina e agricultura), sendo um consenso em microbiologia (Gillis *et al*., 2005; Gevers *et al*., 2005).

A questão sobre a existência de espécie em procariontes e de um único conceito que explicaria a formação de espécies é amplamente debatido (Cohan, 2001, 2002; Gevers *et al*., 2005, Konstantinidis & Tiedje 2005ab; Konstantinidis *et al*., 2006a; Thompson *et al*., 2009). Três conceitos de espécie são mais amplamente aceitos (Cohan &, Koeppel, 2008; Lawrence & Retchless, 2009). O principal objetivo destes conceitos seria explicar o processo de especiação a partir de processos biológicos. Um conceito de espécie bastante aceito é o ecótipo estável. Este conceito defende que as espécies são formadas a partir de mutações adaptativas, que permitem um determinado ecótipo ocupar um determinado nicho ecológico. Com o tempo, este ecótipo passa por sucessivas mutações e seleções até estar totalmente diferenciado geneticamente do ecótipo ancestral (Cohan, 2001; Madigan *et al*., 2004). Outro conceito seria da espécie biológica, onde um grupo de linhagens permanece dentro de uma mesma espécie por meio da recombinação homóloga. Devido às diferenças gênicas, a recombinação homóloga não ocorreria entre linhagens de espécies distintas. Em suma, em contraste com a definição pragmática de espécie com base na abordagem polifásica que é amplamente aceita, não há um consenso sobre um conceito de espécie bacteriana. Possivelmente um único conceito talvez não seja suficiente para explicar a complexidade da evolução do genoma bacteriano. Como mutação, recombinação homóloga e recombinação não-homóloga podem ocorrer em um único genoma em níveis diferentes, o cenário é complexo.

II.13. TAXONOMIA GENÔMICA

Com o advento das técnicas de biologia molecular um número cada vez mais amplo de metodologias, que se baseiam em informações contidas no DNA dos organismos, como proteínas e principalmente ácidos nucléicos vem sendo introduzidas em estudo de biodiversidade microbiana (Sawabe *et al*, 2007b; Thompson *et al*., 2004, 2005; Reis *et al*., 2009). O emprego dessas metodologias aumenta a confiabilidade da resolução taxonômica capaz de delinear família, gênero, espécie e linhagem em grupos microbianos, uma vez que as informações obtidas são valiosas para uma nova percepção da composição da estrutura populacional de algumas espécies microbianas, além de

possibilitarem também um meio de identificar estes organismos. Metodologias que se baseiam em informações genômicas, com diferentes resoluções taxonômicas, são comumente empregadas no estudo taxonômico e integram juntamente com os dados fenotípicos, quimiotaxonômicos e ecológicos a abordagem da taxonomia polifásica em procariontes (**Figura 4**).

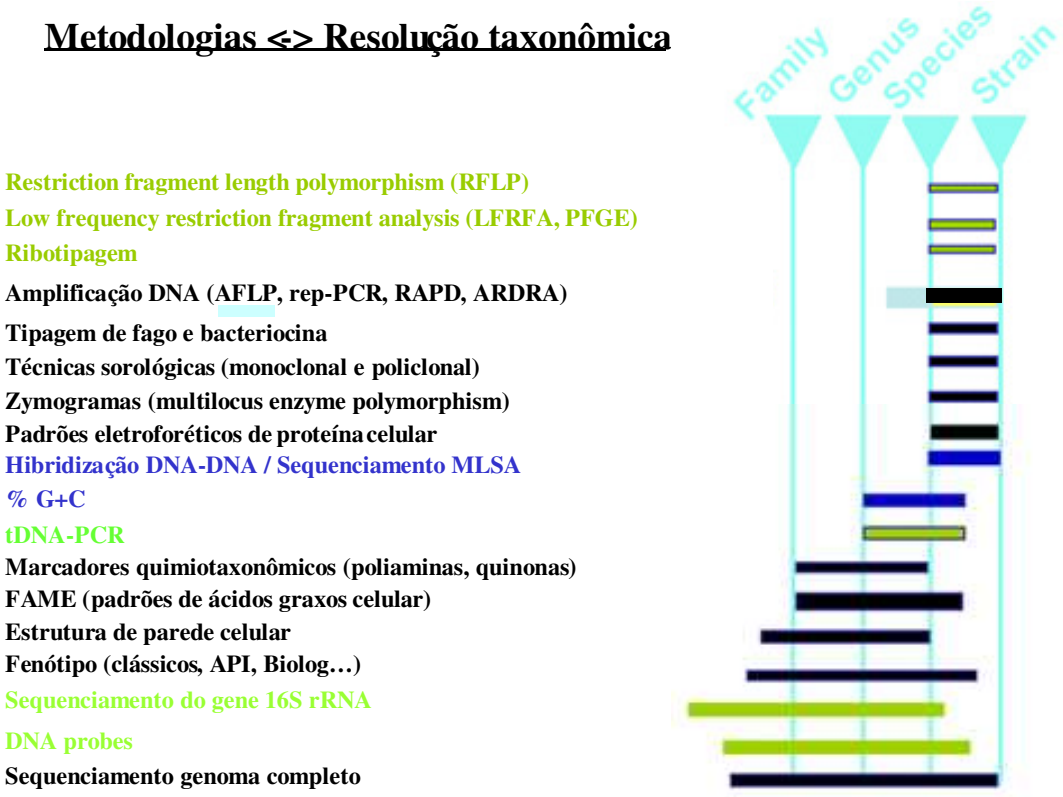

Figura 4. Resolução taxonômica das técnicas mais usadas na classificação de microrganismos. (Modificado a partir de Vandamme *et al.*, 1996).

Diversos estudos vêm apontando sucessos na classificação e identificação de microrganismo com base em dados obtidos de múltiplas sequências de genes (Sawabe *et al*, 2007b; Thompon *et al*., 2005, 2007, 2008). Rápida e robusta a classificação por MLSA (Multilocus Sequence Analysis) consiste de um conjunto de genes universais que estão presentes em cópia única no genoma, com variações suficientes dentro do grupo que esta sob investigação, mas não devem sofrer recombinação gênica e devem representar um sinal filogenético do grupo. Os valores de similaridade de sequências obtidos são usados para análise das relações filogenéticas dos taxa relacionados.

Com o avanço nos estudos obtidos com dados de MLSA focados em reavaliação de espécie em procariotos, tem sido sugerido que análise de pelo menos 5 genes que codificam proteínas essenciais para o metabolismo celular (*housekeeping*), obtidos de diferentes loci cromossômicos e distribuídos amplamente entre os taxa, podem oferecer informações suficientes e capazes de distinguir espécies bacterianas intimamente relacionadas (Zeigler, 2003). Além disso, uma vez definida que uma espécie é estabelecida através desta metodologia, informações de sequências de um único membro do conjunto de genes estudados podem ser suficientes para designar uma linhagem adicional como membro desta espécie (Stackebrandt *et al.,* 2002; Zeigler, 2003). Zeigler (2003) declarou que 5 genes são na verdade mais do que suficientes para igualar ou mesmo superar o poder de HDD na alocação de isolados microbianos em espécies bacterianas. O conjunto de genes candidatos capaz de predizer uma espécie com exatidão foi determinado com base em 4 critérios prioritários: (i) os genes devem ser amplamente distribuídos nos genomas bacterianos, (ii) os genes devem ser únicos dentro de um dado genoma, (iii) as sequências dos genes individuais devem ser longas o suficiente para conterem informações suficientes, mas devem ser curta o suficiente para permitirem o sequenciamento conveniente (900 - 2250 nt), (iv) as sequências devem predizer as relações do genoma completo com alta precisão e exatidão (Zeigler, 2003).

O uso de sequências obtidas de genes *housekeeping* tem ampliado a visão sobre a diversidade e evolução de bactérias (Cohan, 2002) e uma de suas principais vantagens é obtida pela indexação de diferenças entre as sequências dos genes estudados, pois, sua evolução ocorre mais rapidamente do que o gene 16S rRNA, além disso, eles atuam como um tampão contra os efeitos de distorção causados por recombinação gênica, que podem ocorrer com o uso de um único locus genético (Gevers *et al*., 2005).

Em 2006, análises de sequências filogenéticas de múltiplos genes (6-8) representaram a mais favorável metodologia para estudo de diversidade em espécies (Konstantinidis *et al*., 2006b). Este estudo avaliou quatro grupos microbianos importantes e concluiu que três é o número mínimo de genes para aplicações em práticas de MLSA, pois, se houver uma possível transferência gênica lateral (TGL) presente em um dos

genes de uma ou de poucas linhagens estudadas, o sinal filogenético que permanece dos outros dois genes é capaz de encobrir o evento de TGL.

O emprego de MLSA especialmente na taxonomia de *Vibrio* vem sendo amplamente difundido (Sawabe *et al*, 2007b; Thompon *et al*., 2005, 2007, 2008, 2009) devido às características de baixo custo de ensaios, facilidade de construção de bases de dados de acesso público, incorporação de novos dados e recursos de análise computacional. Atualmente estão disponíveis, em acesso livre na internet, esquemas de MLSA para a maioria dos patógenos humanos do gênero *Vibrio* (http://www.taxvibrio.lncc.br/).

Com o advento das técnicas de sequenciamento do genoma de forma ultra-rápida, torna-se possível obter sequências do genoma microbiano quase que por completo em menos de um dia (Andersson *et al.*, 2008; Eid *et al*., 2009). A análise de sequências de genomas completos têm despertado interesse nos taxonomistas pela possibilidade de explorar relações evolutivas entre espécies bacterianas (Wolf *et al*., 2002) e estabelecer sistemática com base no genoma total (Coenye *et al*., 2005). Essas informações conduzirão a taxonomia microbiana para uma nova era, já que, é possível se obter informações contidas em todos os genes do genoma, como assinatura ampla dos genomas (Coenye *et al*., 2003; van Passel *et al*., 2006; Phillippy *et al*., 2007), identidade de aminoácidos (Goris *et al*., 2007) e toda composição genética que possa ser útil, como a construção de uma robusta árvore taxonômica (supertrees) baseadas em todos os genes que compõe a essência do genoma (Brown *et al*., 2001; Konstantinidis *et al*., 2005b; Thompson *et al*., 2009).

Com a nova geração de sequenciadores de DNA será possível obter mais que uma dúzia de genomas procarióticos em menos de uma hora, o que possibilitará provavelmente o sequenciamento do genoma completo de forma mais barata e rápida do que múltiplos genes por MLSA (Thompson *et al*., 2009). No futuro MLSA poderá ser usada apenas como metodologia discriminatória (Mahenthiralingam *et al*., 2006).

III.1. ESTRATÉGIA DE ESTUDO

O presente estudo foi desenvolvido seguindo 4 principais estratégias que estão listadas abaixo:

1. Seleção das espécies-alvo e locais de coleta.

2. Coleta do material biológico e isolamento microbiano em meios de cultivo: Ágar Marinho (MA), Ágar Fixação de Nitrogênio (NFb) e Ágar Tiosulfato-Citrato-Bile-Sacarose (TCBS).

3. Triagem dos isolados purificados, caracterização taxonômica, análise do padrão de distribuição espaço temporal e especificidade bactéria-hospedeiro.

4. Caracterização e descrição de novas espécies bacterianas por meio da taxonomia polifásica.

III.2. METODOLOGIAS UTILIZADAS

Sequenciamento de fragmentos do DNA genômico microbiano

DNA genômico dos isolados ambientais foram extraídos e usados para amplificação dos genes de interesse de acordo com Chimetto *et al.* (2008, 2009). Mutilocus Sequence Analysis (MLSA) foram realizadas de acordo com Thompson *et al.* (2005b). Os produtos amplificados foram purificados pelo KIT GFX e enzima EXOSAP (GE Healthcare), de acordo com instruções do fabricante, sendo posteriormente sequenciados (Chimetto *et al.*, 2008, 2009) em um MegaBace.

As sequências foram analisadas com o auxílio da ferramenta *Genebuilder* do programa *Kodon* package 2.03 e do ChromasPro. Análises filogenéticas foram feitas com o auxílio do software Mega, Kodon e Bionumerics onde matrizes de similaridade e árvores filogenéticas foram criadas, baseadas nos métodos de Máxima Parcimônia e Neighbor Joining (Saitou & Nei, 1987). Sequências obtidas foram submetidas ao BLAST (Basic Local Alignment Search Tools) (Altschul *et al.,* 1997), para comparação com sequências

conhecidas depositadas (GenBank-http://www.ncbi.nlm.nih.gov) a fim de determinar a posição filogenética dos diferentes grupos microbianos estudados.

Elementos palindrômicos extragênicos repetitivos (rep-PCR)

Isolados representativos pertencentes ao grupo dos vibrios foram caracterizados por rep-PCR como descrito previamente (Gomez-Gil *et al*., 2004, 2007). DNA genômico foi extraído com Kit para extração de DNA Promega Wizard (Promega) de acordo com instruções do fabricante. O DNA obtido foi ajustado através de spectrophotometro para 50 ng ml^{-1}. DNA fingerprinting das linhagens analisadas por rep-PCR foi realizado através do primer $(GTG)_5$. O produto de rep-PCR foi amplificado com enzima AmpliTaq® DNA polymerase (Applied-Biosystems, USA) e corridos em eletroforese em gel de agarose 2.25 % 20 x 20 cm por 18 hr a 55 V e 4-8 °C. O gel obtido foi corado com brometo de etídeo e visualizado em sistema de documentação de gel (UVP). Arquivos no formato TIFF obtidos foram analisados com GelCompar II software (Ver. 4.5, Applied-Maths), calculando a matriz de similaridade com coeficiente Jaccard e dendrograma construído com Ward (posição tolerante de 0.59 %). Linhagens de referência de todas as espécies válidas de vibrios foram incluídas.

Polimorfismo do comprimento de fragmentos amplificados (AFLP)

Esta etapa foi realizada como relatado por Beaz Hidalgo *et al*. (2008). Resumidamente, 1 μg de DNA foi digerido com *Taq*I (5'TCGA3) e *Hind*III (5'AAGCTT3) (Amersham Pharmacia Biotech, Suécia) e, posteriormente, ligado com adaptadores dupla fita complementares às extremidades dos fragmentos de restrição, com T4 ligase (Amersham Pharmacia Biotech), para gerar modelo de DNA para amplificação por PCR. Uma PCR seletiva foi então realizada com os primers H01-6FAM (5'GACTGCGTACCAGCTTA3`, marcado no final 5 'com o corante fluorescente 6-FAM) e T13 (5'GTTTCTTATGAGTCCTGACCGAG3 "), utilizando as condições descritas por Thompson *et al*. (2001a), em um GeneAmp termociclador PCR System 9700 (Applied Biosystems, E.U.A.). Separação dos produtos de PCR seletiva foi realizada através de um capilar ABI Prism 3130XL sequenciador de DNA (Applied Biosystems). A normalização dos padrões eletroforéticos resultante foi feita através do Gene Mapper 4.0 software (Applera Co., Norwalk, CT). Para posterior análise,

fragmentos de 20 a 600 pares de base foram transferidos para a BioNumerics ™ 4,61 software (Applied Maths, Belgium). Para a análise numérica foi usado a zona compreendida entre 40 - 580 pb. Valores de similaridade foram calculados utilizando o coeficiente de Dice (o valor da tolerância 0,15%), e um dendrograma foi construído usando UPGMA.

Redução de acetileno (ARA)

Os vibrios isolados em meio seletivo, Nitrogen-free medium (NFb), foram inoculadas em frascos de 10 ml contento 4 ml de meio NFb (Baldani *et al.,* 1997) com 0,01 % de sal e com 3 % Nacl, semi-sólido, ou seja de 1,75 a 1,8 g ágar/L. O Ph do meio foi ajustado entre 6,5 ou 6,8. Para cada concentração de NaCl foram feitos tubos em duplicata. Em seguida elas foram incubadas a 28ºC por 48h, após este período os tubos foram vedados e retirou-se de cada frasco 1 ml de ar. Em seguida, foram colocados em cada frasco 0,6 ml de acetileno, deixando novamente na estufa 28 ºC por 48h. Atividade de nitrogenada foi avaliada através da produção de etileno, testada qualitativamente através do método de redução de acetileno por cromatografia gasosa (ARA). Foram injetados 0,1 ml da fase aérea das culturas em frascos em cromatógrafo a gás Shimadzu GC-14A, com coluna PorapaK-N 80/100 – INOX a 70 ºC (Turner & Gibson, 1980). A temperatura do injetor e detector foi ajustada para 180 ºC a 230 ºC respectivamente. Cada isolado foi testado três vezes independentemente.

Ésteres metílicos de ácidos graxos (FAME)

A análise de ésteres metílicos de ácidos graxos das células bacterianas foram realizadas através de cromatografia gasosa como descrito por Huys *et al.* (1994).

Análise do conteúdo G+C do DNA

O conteúdo G+C (mol%) que compõe a molécula de DNA dos microrganismos foram analisados através de HPLC de acordo com Mesbah *et al*. (1989).

Hibridização DNA-DNA (HDD)

A hibridização DNA-DNA foi executada sob condições rigorosas usando a técnica de microplaca com DNA marcado por fotobiotina como descrito por Ezaki *et al*.

(1989), com modificações sugeridas por Willems *et al*. (2001). Linhagens de referência, consideradas vizinhas filogenéticas mais próximas das linhagens investigadas, foram incluídas em todos os testes. Reações recíprocas foram realizadas para cada par de DNA e todas as variações estiveram dentro do limite sugerido para este método (Goris *et al.,* 1998).

ANÁLISE DA BIODIVERSIDADE MICROBIANA ASSOCIADA A CNIDÁRIOS DE SÃO SEBASTIÃO, SP, BRASIL.

IV.1. DIVERSIDADE MICROBIANA ASSOCIADA AOS CNIDÁRIOS.

Foram obtidos 488 isolados bacterianos, **Tabela 1**, do muco dos cnidários (*Mussismilia hispida, Palythoa caribaeorum, Palythoa variabilis* e *Zoanthus solanderi*) na região de São Sebastião, São Paulo, em três diferentes pontos (praias Grande, Portinho e Preta), em dois verões consecutivos (03/02/2005 e 01/02/2006).

Grupos taxonômicos	**1ª. COLETA**	**2ª. COLETA**
	(03/02/2005)	(01/02/2006)
Bactérias heterotróficas - (MA)	50	79
Putativas fixadoras de N_2 - (NFb)	95	-
Putativos vibrios - (TCBS)	123	141
Total de isolados	**268**	**220**

Tabela1. Número de isolados obtidos por grupo microbiano nas duas coletas.

Todos os isolados obtidos neste projeto foram inicialmente analisados através do sequenciamento do gene 16S rRNA. Os dados obtidos revelaram que o grupo microbiano mais frequentemente encontrado pertence à família *Vibrionacea*. Abundância de vibrios é relatada como 10^6 – 10^7 UFC/mL muco (2 a 3 ordens de grandeza maior do que os encontrados na água do mar). A maioria (92 %) das bactérias heterotróficas isoladas do muco do coral *M. hispida* e do zoantídeo *P. caribaeorum* agrupou-se na classe *Gammaproteobacteria*, seguidos de 4,5 % *Epsilonproteobacteria,* 2,5 % *Alphaproteobacteria* e 1 % *Actinobacteria*. Dentro de *Gammaproteobacteria,* 54 % dos isolados obtidos agruparam-se no gênero *Vibrio* e 23 % a *Pseudoalteromonas.* Os demais grupos encontrados foram *Photobacterium, Shewanella*, *Marinomonas, Erythrobacter, Arcobacter, Thalassolituus, Ferrimonas, Marinobacterium* e "Actinobacteria". Grupos nomeados como A1-A6, possuem isolados que apresentam menos de 97 % de similaridade com espécies conhecidas baseados em sequências do gene 16S rRNA, indicando possivelmente novas espécies de bactéria (**Figura 1 e Tabela 2**).

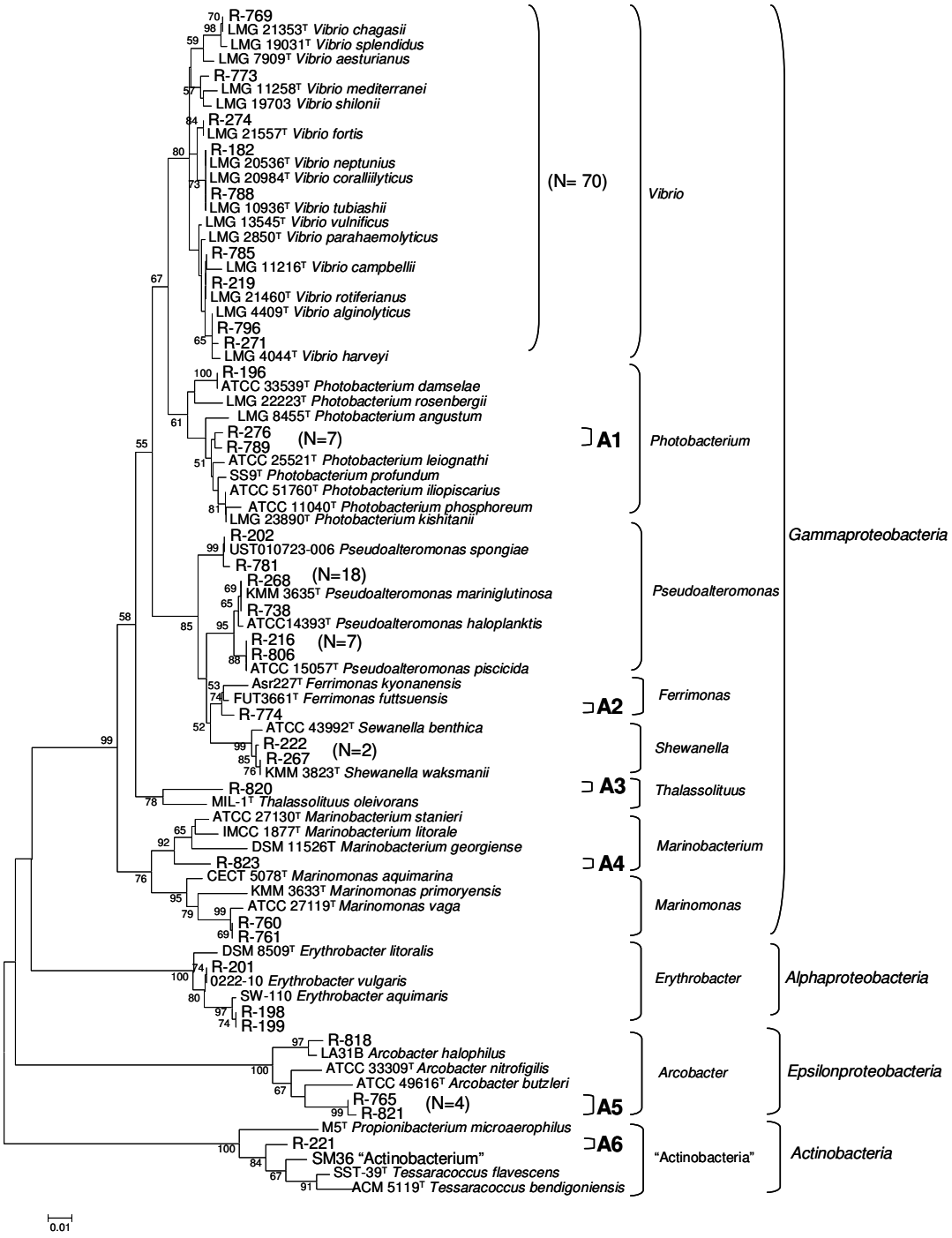

Figura 1. Árvore filogenética baseada em sequências parciais do rRNA 16S (500bp), usando método de Neighbour joining e modelo de correção Kimura-2P. Boostrap são mostrados após 1000 repetições. Barra de escala corresponde a 1% de divergência. Valores de Bootstrap inferiores a 50 % não são mostrados. A1, A2, A3, A4, A5, e A6 correspondem às possíveis espécies novas.

Tabela 2. Lista detalhada de bactérias heterotróficas isoladas em MA.

Hospedeiro	Ano isolamento	Local coleta	Grupo microbiano	Nº do isolado	Morfotipo
P. caribaeorum	2005	Praia Preta	*Pseudoalteromonas* sp.	R-180	3.6a
			Vibrio sp.	R-179	3.6e
				R-181	3.6d
				R-182	3.6c
				R-183	3.6c
				R-184	3.5a
				R-185	3.5b
				R-186	3.5c
				R-187	3.5d
		Praia Portinho	Perdeu viabilidade	R-200	2.11b
			Vibrio sp.	R-188	2.7b
				R-203	2.7d
				R-204	2.7c
				R-205	2.7a
				R-206	2.3c
				R-207	2.3b
				R-208	2.3a
				R-272	2.8
		Praia Grande	*Vibrio* sp.	R-189	1.5c
				R-190	1.5b
				R-191	1.5a
				R-192	1.3b
				R-193	1.3a
				R-195	1.5d
				R-273	1.9b
				R-274	1.8a
				R-275	1.8b
			Photobacterium sp.	R-276	1.7a
	2006	Praia Preta	Perdeu viabilidade	R-736	N2
				R-737	N1
			Vibrio sp.	R-788	M1
			Photobacterium sp.	R-789	M2
		Praia Portinho	*Photobacterium* sp.	R-744	Q4
				R-758	P2
				R-787	R1
				R-797	S1
			Pseudoalteromonas sp.	R-745	Q3
			Vibrio sp.	R-741	P1
				R-747	Q1
				R-749	P1.1
				R-750	P5
				R-752	O2
				R-762	O1
				R-766	P3
				R-796	S2
				R-817	Q1

M. hispida	2005	Praia Preta	*Erytrobacter* sp.	R-198	3.4d
				R-199	3.4d
				R-201	3.4c
			Pseudoalteromonas sp.	R-202	3.4b
		Praia Grande	*Photobacterium* sp.	R-196	1.1b
			Pseudoalteromonas sp.	R-216	1.6a
			Vibrio sp.	R-194	1.1c
				R-197	1.1a
				R-217	1.10b
				R-218	1.11
				R-219	1.12b
				R-220	1.12a
			"Actinobacteria" sp.	R-221	1.14a
			Perdeu viabilidade	R-223	1.15a
				R-224	1.1ba
			Shewanella sp.	R-222	1.15b
				R-225	1.6b
				R-267	1.10a
			Pseudoalteromonas sp.	R-268	1.16d
			Vibrio sp.	R-269	1.16c
				R-270	1.16b
				R-271	1.13
	2006	Praia Preta	*Vibrio* sp.	R-782	I1
				R-739	J1
				R-756	L2
				R-757	L1
				R-798	J4b
				R-805	I2
				R-807	L4
				R-812	K2
				R-824	17A2
			Photobacterium sp.	R-755	L3
			Arcobacter sp.	R-765	I3
			Ferrimonas sp.	R-774	K4
			Pseudoalteromonas sp.	R-738	J4a
				R-784	J6
				R-790	J2b
				R-809	J5b
				R-810	J5a
				R-811	J3
				R-813	K1
				R-815	J2a
			Marinobacterium sp.	R-823	K3
		Praia Portinho	*Vibrio* sp.	R-743	H3
				R-763	D4
			Pseudoalteromonas sp.	R-740	F2
				R-754	F4b
				R-777	D2

M. hispida	2006	Praia Portinho	*Pseudoalteromonas* sp.	R-778	D1
				R-779	*1
				R-781	F4a
				R-803	F1
				R-804	H1
				R-806	F3b
				R-808	F3a
			Vibrio sp.	R-780	*2
				R-785	G2
				R-825	6 E1
			Thalassolituus sp.	R-820	H4
			Arcobacter sp.	R-818	*3
				R-819	H2
				R-821	G3
				R-822	D3
		Praia Grande	*Pseudoalteromonas* sp.	R-759	A3
				R-770	B6
				R-791	C4
				R-793	C3
			Marinomonas sp.	R-760	A2
				R-761	A2.1
			Vibrio sp.	R-742	A3.1
				R-768	A1.1
				R-769	B7
				R-771	B1
				R-772	C6
				R-773	C5
				R-786	A4
				R-794	C2
				R-799	B5
				R-800	B4
				R-801	B3
				R-802	B2
			Perdeu viabilidade	R-792	C1
				R-795	A1
Praia Preta (coordenadas 23°49´10´´ sul e 45°24´37´´oeste). Canal de São Sebastião, Brazil					
Praia Portinho (coordenadas 23°50´25´´ sul e 45°24´22´´oeste). Canal de São Sebastião, Brazil					
Praia Grande (coordenadas 23°50´59´´ sul e 45°24´59´´oeste). Canal de São Sebastião, Brazil					

Legenda - Tabela 2. A tabela nos mostra que as bactérias heterotróficas mais frequentemente encontradas associadas a *M. hispida* e *P. caribaeorum* nos dois anos de coleta foram *Vibrios* sp., *Pseudoalteromonas* sp. e *Photobacterium* sp., enquanto todos os demais grupos microbianos foram encontrados associados apenas com *M. hispida*. Entretanto, a diversidade encontrada em *M. hispida* em 2005 mostra-se diferente da encontrada em 2006. Além disso, não houve um claro padrão de distribuição microbiana entre os diferentes pontos de coleta e os diferentes hospedeiros associados.

IV.2. Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral *Mussismilia hispida.*

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Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral *Mussismilia hispida*

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Abstract

Taxonomic characterization was performed on the putative N_2-fixing microbiota associated with the coral species *Mussismilia hispida*, and with its sympatric species *Palythoa caribaeorum*, *P. variabilis*, and *Zoanthus solanderi*, off the coast of São Sebastião (São Paulo State, Brazil). The 95 isolates belonged to the *Gammaproteobacteria* according to the 16S rDNA gene sequences. In order to identify the isolates unambiguously, *pyrH* gene sequencing was carried out. The majority of the isolates ($n = 76$) fell within the *Vibrio* core group, with the highest gene sequence similarity being towards *Vibrio harveyi* and *Vibrio alginolyticus*. Nineteen representative isolates belonging to *V. harveyi* ($n = 7$), *V. alginolyticus* ($n = 8$), *V. campbellii* ($n = 3$), and *V. parahaemolyticus* ($n = 1$) were capable of growing six successive times in nitrogen-free medium and some of them showed strong nitrogenase activity by means of the acetylene reduction assay (ARA). It was concluded that nitrogen fixation is a common phenotypic trait among *Vibrio* species of the core group. The fact that different *Vibrio* species can fix N_2 might explain why they are so abundant in the mucus of different coral species.

Keywords: N_2-fixing bacteria; Vibrios; *V. alginolyticus*; *V. harveyi*; Coral; *Mussismilia hispida*; *Palythoa caribaeorum*; *P. variabilis*; *Zoanthus solanderi*

Introduction

Coral reefs are among the most productive and diverse ecosystems within coastal tropical environments, mainly in oligotrophic regions [9]. All the coral reefs of the South Atlantic Ocean are spread throughout the northeastern coast and continental shelf of Brazil. The diversity of coral fauna is low, and mainly consists of relics from the Tertiary period. Brazilian coral reefs show initial growth as a mushroom-like structure, with a considerable amount of incrusting coralline algae [15,14]. Their fauna is composed mainly of cnidarians from the class *Anthozoa*, order *Scleractinia*, family *Mussidae*, and genus *Mussismilia* [6].

Mussismilia hispida is one of the seven scleractinian species and it has the widest geographic distribution. It inhabits from Santa Catarina to Rio Grande do Norte

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(ca. 5000 km), which indicates its adaptation to wide environmental gradients, such as temperature, water turbidity and pollution. *M. hispida* is endemic to Brazil, and one of the major reef-builders along the north-eastern Brazilian coast. The Brazilian corals of the genus *Mussismilia* are in danger of extinction [11], possibly due to a variety of stressors, including infection. However, so far, no data is available on the taxonomic composition of the microbiota of *M. hispida*.

Corals and coral reefs have experienced a tremendous decline in recent decades. Global warming, pollution, and infectious diseases, particularly those caused by vibrios, are among the main causes of the increasing stress that they are suffering worldwide [10,13,32]. Culture-independent studies based on 16S rDNA clone libraries and metagenomics have shown that vibrios are abundant in the mucus of different coral species, with significant increases in vibrio populations immediately before massive bleaching events, leading to a dominance of vibrio sequences in the sequence libraries [4,5]. Indeed, *Vibrio alginolyticus*, *V. coralliilyticus*, *V. harveyi*, and *V. shilonii* (= *V. mediterranei*) have been shown to be coral pathogens [12,19,26] and are found in association with different coral species [4,18].

On the other hand, some vibrios may also establish mutualistic partnerships with corals by providing nutrients and secondary metabolites (e.g. bacteriocins) to their hosts [18]. Corals may harbor a variety of N_2-fixers which may provide a substantial amount of the total nitrogen needed by the host metabolism [16,23,31]. Nitrogen fixation, the production of NH_3 by the reduction of N_2, is carried out by nitrogenases and is a tightly regulated process under the control of the NtrC activator protein. This protein is a response regulator and its phosphorylated form will induce transcription of nitrogenases when NH_3 is not available. Coral reefs occur in oligotrophic areas possibly because of the N_2 fixation activity occurring in the corals themselves [7].

In the present study, taxonomic characterization was performed on the dominant culturable N_2-fixing microbiota associated with *M. hispida* and its sympatric species *Palythoa caribaeorum*, *P. variabilis*, and *Zoanthus solanderi*. The genera *Palythoa* and *Zoanthus* belong to the phylum *Cnidaria*, class *Anthozoa*, order *Zoanthidea* and family *Zoanthidae*, comprising shallow water zooxanthelate species [6]. The genera *Palythoa* and *Zoanthus* appear to be widespread in different continents. By examining these sympatric cnidarian species, the host specificity of the microbiota of each taxon was evaluated. In order to confirm that *V. alginolyticus*, *V. campbellii*, *V. harveyi*, and *V. parahaemolyticus* isolates were able to fix N_2, successive passages in nitrogen-free medium were undertaken and, subsequently, representative isolates were subjected to the acetylene reduction test.

Materials and methods

Thirty-two cnidarian specimens belonging to four species were collected on 3 February 2005 at three sites: Grande (23°50′25″S; 045°24′59″W), Portinho (23°50′25″S; 045°24′22″W) and Preta (23°49′10″S; 045°24′37″W) beaches located near the Centro de Biologia Marinha-USP (CEBIMAR-USP; São Sebastião Channel, São Paulo, Brazil) by SCUBA diving between depths of 3 and 7 m. The beaches Grande, Portinho and Preta are about 2 km apart from each other, the latter being on the continental side of the São Sebastião Channel and the first two are opposite facing CEBIMAR-USP. The cnidarian specimens were associated with rocky shores at these sites. Intact colonies of *M. hispida* and fragments of zoanthids were placed in sterile plastic bags and kept at ca. 10 °C for 6 h prior to microbiological examination. Samples were taken to the University of Campinas for isolation, purification, and characterization of the microorganisms.

Isolation and preservation of strains

The isolation of putative N_2 fixers from the cnidarian mucus was performed using the nitrogen-free (NFb) selective medium supplemented with 3% NaCl [2]. The mucus was drained from the coral samples using a sterile syringe. Tenfold dilutions of coral mucus were obtained in sterile saline solution (3% NaCl). A total of 100 ml aliquots of the dilutions were plated onto NFb and 2–8 representative colony morphotypes were picked for further purification from the highest dilution (10^4) after 4 days of incubation at 28 °C. Isolates obtained in this study are listed in Table 1. Pure cultures were maintained in vials with 20% glycerol at −80 °C.

Taxonomic characterization

The preliminary characterization of all pure cultures was obtained by 16S rDNA gene sequences, as described previously but with minor modifications [25]. The reactions were composed of 37.5 μl sterile MilliQ water, 5.0 μl PCR buffer (10 ×), 1.5 μl Mg_2Cl (1.5 mM), 0.4 μl dNTP's (0.2 mM each), 1 μl forward primer p27f (5′AGA GTT TGA TCM TGG CTC AG3′, 20 μM), 1 μl reverse primer (5′CGG TGT GTA CAA GGC CCG GGA ACG3′, 20 μM), 0.4 μl AmpliTaq DNA Polymerase (2 U/μl), and 1 μl template DNA (0.02 μg/μl). The thermal program consisted of (1) 2 min at 95 °C, (2) 30 cycles of 1 min at 94 °C + 1 min at 55 °C and 3 min at 72 °C, and (3) 3 min at 72 °C. PCR was performed using an Eppendorf thermocycler. The PCR products were purified using a solution of PEG8000 (20%)/2 M NaCl. Purified PCR products were eluted in 50 μl sterile MilliQ water. Subsequently, 5.0 μl of purified PCR product were mixed with 4.0 μl ET Terminator™ Mix

Table 1. Strain list

Species name	Strain no.	Colony morphotype	Source
V. alginolyticus	R-228	3.3.a	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-232	3.2.b	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	**R-234****	**1.16.d**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	**R-235****	**3.3.b**	***Zoanthus solanderi*, Praia Preta**
V. alginolyticus	**R-262****	**2.5.c1**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-263	1.13.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-265****	**1.3.b1**	***Palythoa caribaeorum*, Praia Grande**
V. alginolyticus	R-283	3.2.a	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-284	3.4.b	*Mussismilia hispida*, Praia Preta
V. alginolyticus	R-287	1.11.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-288	1.1.c2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-289	1.15.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-290****	**2.4.b**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-291	1.1.b1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-292	1.1.a1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-293	1.1.d2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-294	3.3.c	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-295	2.10.b	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	R-296	2.3.	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-297	1.1.a2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-298	2.2.a	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-299	1.2.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-300****	**1.4.c**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	R-301	2.9.a2	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	**R-302****	**2.2.b**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-303	2.5.c2	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-304	1.6.d	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-306	1.6.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-308	3.3.d	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-309	1.12.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-310***	**1.15.b**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	R-312	1.10.b2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-313	2.8.a	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	R-314	2.4.a	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-315	2.4.c	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-316	2.6.c	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-317	2.6.b	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-318	1.1.c1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-319	1.3.b2	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-320	1.3.a	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-321	1.14.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-322	1.4.c	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-323	1.1.b2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-324	1.1.d1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-325	1.8.a	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-326	1.6.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-329	3.3.e	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-331	2.8.b	*Palythoa variabilis*, Praia Portinho
V. fortis	R-248	3.4.c	*Mussismilia hispida*, Praia Preta
V. fortis	R-254	1.11.b	*Mussismilia hispida*, Praia Grande
V. harveyi	**R-227****	**1.12.b**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-230****	**1.5.c**	***Palythoa caribaeorum*, Praia Grande**
V. harveyi	**R-233***	**1.16.d**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-239****	**1.6.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-242	1.7.a	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-243	2.7.a	*Palythoa caribaeorum*, Praia Portinho

Table 1. (*continued*)

Species name	Strain no.	Colony morphotype	Source
V. harveyi	**R-246****	**1.2.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-253****	**1.11.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-255	3.5.a	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-257	2.7.c	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-259	3.5.b	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-260	3.5.c	*Palythoa caribaeorum*, Praia Preta
V. harveyi	**R-264***	**1.4.a**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-280	2.9.a1	*Palythoa variabilis*, Praia Portinho
V. harveyi	R-285	2.7.b	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-286	2.6.a	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-305	1.9.c	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-307	1.8.b	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-311	3.6.a	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-327	1.9.b1	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-328	1.9.b2	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-330	1.8.c	*Palythoa caribaeorum*, Praia Grande
V. parahaemolyticus	**R-241****	**1.9.a**	***Palythoa caribaeorum*, Praia Grande**
V. tubiashii	R-229	1.5.b	*Palythoa caribaeorum*, Praia Grande
V. tubiashii	R-252	3.6.c	*Palythoa caribaeorum*, Praia Preta
Vibrio sp.	R-240	1.7.b	*Palythoa caribaeorum*, Praia Grande
Alteromonas sp.	R-250	1.16.c	*Mussismilia hispida*, Praia Grande
Alteromonas sp.	R-251	3.6.b	*Palythoa caribaeorum*, Praia Preta
Marinomonas sp.	R-236	1.14.d	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-237	1.14.d	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-238	3.1.	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-247	3.4.d	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-249	1.16.a	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-256	3.4.a	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-261	1.15.c	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-278	1.13.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-231	1.15.d	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-244	2.5.a	*Mussismilia hispida*, Praia Portinho
Pseudoalteromonas sp.	R-245	1.4.b	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-258	2.10.a	*Palythoa variabilis*, Praia Portinho
Pseudoalteromonas sp.	R-266	1.2.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-277	1.10.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-279	1.10.b1	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-281	2.9.b	*Palythoa variabilis*, Praia Portinho
Pseudoalteromonas sp.	R-282	2.5.b	*Mussismilia hispida*, Praia Portinho

Representative strains tested for nitrogenase activity are indicated in bold.
The code used for the colony morphotypes is composed of three digits. The location (1, Praia Grande; 2, Praia Portinho; and 3, Praia Preta), specimen number (16 specimens from Praia Grande, 10 specimens from Praia Portinho, and six specimens from Praia Preta), and the colony type (a, b, c, onwards).
*Indicates growth after six successive passages in the minimal medium NFb.
**Denotes isolates with both phenotypes positive in the ARA test and growth after six successive passages in the minimal medium NFb.

(GE Health Care), 0.2 μl sequencing primer 782*r* (5′ACC AGG GTA TCT AAT CCT GT3′, 20 μM), and 0.8 μl MilliQ water. The thermal program consisted of 30 cycles of 20 s at 95 °C + 15 s at 50 °C + 1 min at 60 °C. The purification of the sequencing products was obtained by mixing 1 μl ammonium acetate (7.5 M) and 27.5 μl absolute ethanol, followed by incubation in the dark for 30 min and subsequent centrifugation at 3700 rpm for 75 min at 4 °C. Separation of the DNA fragments was obtained in a Megabace 1000 system (GE Health Care). Voltage and time of injection were 3 kV and 80 s. Running was performed at 9 kV for 100 min at 44 °C.

Vibrios were identified using the identification marker *pyrH*, as described previously with minor modifications [21,28,29]. Briefly, PCR was composed of 38.2 μl sterile

MilliQ water, 1.5 µl Mg_2Cl (1.5 mM), 5.0 µl PCR buffer (10 ×), 0.4 µl dNTP's (0.2 mM each), 1.2 µl forward primer (pyrH80F-5′GAT CGT ATG GCT CAA GAA G3′, 20 µM), 1.2 µl reverse primer (pyrH530R-5′TAG GCA TTT TGT GGT CAC G3′, 20 µM), 0.4 µl AmpliTaq DNA Polymerase (2 U/µl), and 2.0 µl template DNA (0.05 µg/µl). The thermal program consisted of (1) 5 min at 95 °C, (2) 3 cycles of 1 min at 95 °C + 2 min 15 s at 55 °C and 1 min 15 s at 72 °C, (3) 30 cycles of 30 s at 95 °C + 1 min 15 s at 55 °C and 1 min 15 s at 72 °C, and (4) a final 7 min at 72 °C. *pyrH* PCR products were purified with the enzyme Exosap, according to the instructions of the manufacturer (GE Health Care). Sequencing was performed as described above using 0.6 µl of each PCR primer (pyrH80F and pyrH530R, 20 µM) and an annealing temperature of 50 °C.

Raw sequence data were transferred to the Gene Builder module within the Kodon package version 2.03 (Applied Maths, Belgium), where consensus sequences were determined. Similarity matrices and phylogenetic trees were constructed in the Mega version 4.0 software [24], using partial 16S rDNA (ca. 500 bp) and *pyrH* (ca. 450 bp) sequences. Trees were drawn using the neighbor-joining method [20] based on Kimura-2P distances with bootstrap analysis using 1000 repetitions. The gene sequence data obtained in this study is also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The gene sequences were deposited in the GenBank under the accession nos. EU251514-EU251689.

Nitrogen fixation: acetylene reduction test (ARA) and growth in minimal medium

Nineteen representative *Vibrio* isolates were grown six times successively in semisolid NFb medium at 28 °C for 48 h in anaerobic conditions. *Klebsiella* sp. ICB375 and *Escherichia coli* ICB250 were included as positive and negative controls, respectively. N_2 fixation was also evaluated using the acetylene reduction test with the same set of isolates. Cultures were incubated in semisolid NFb medium with 0.6 ml of acetylene in sealed tubes and kept at 28 °C for 48 h. The production of ethylene was quantified by injecting 0.1 ml of the air phase produced in the sealed tubes into a gas chromatographer (Shimadzu GC-14A, equipped with a column PorapaK-N 80/100 – INOX) at 70 °C [30]. The injector and detector were set at 180 and 230 °C, respectively. Each isolate was tested independently by acetylene reduction assay three times.

Results and discussion

The 95 isolates belonged to the *Gammaproteobacteria* according to the partial (ca. 500 bp) 16S rDNA gene sequences (Fig. 1). The majority of the isolates ($n = 76$) fell within the *Vibrio* core group, with highest 16S rDNA gene sequence similarity towards *Vibrio harveyi*, and *V. alginolyticus*. In order to identify these isolates unambiguously, *pyrH* sequences were obtained and compared with a large database [21]. It was shown in a previous study that the *pyrH* gene is a reliable identification marker for vibrios [27–29]. Clearly, most of the *Vibrio* isolates belonged to the species *V. alginolyticus* and *V. harveyi*, according to *pyrH* gene sequences (Fig. 2), although some isolates grouped with the reference strain *V. harveyi* LMG 20370. This latter

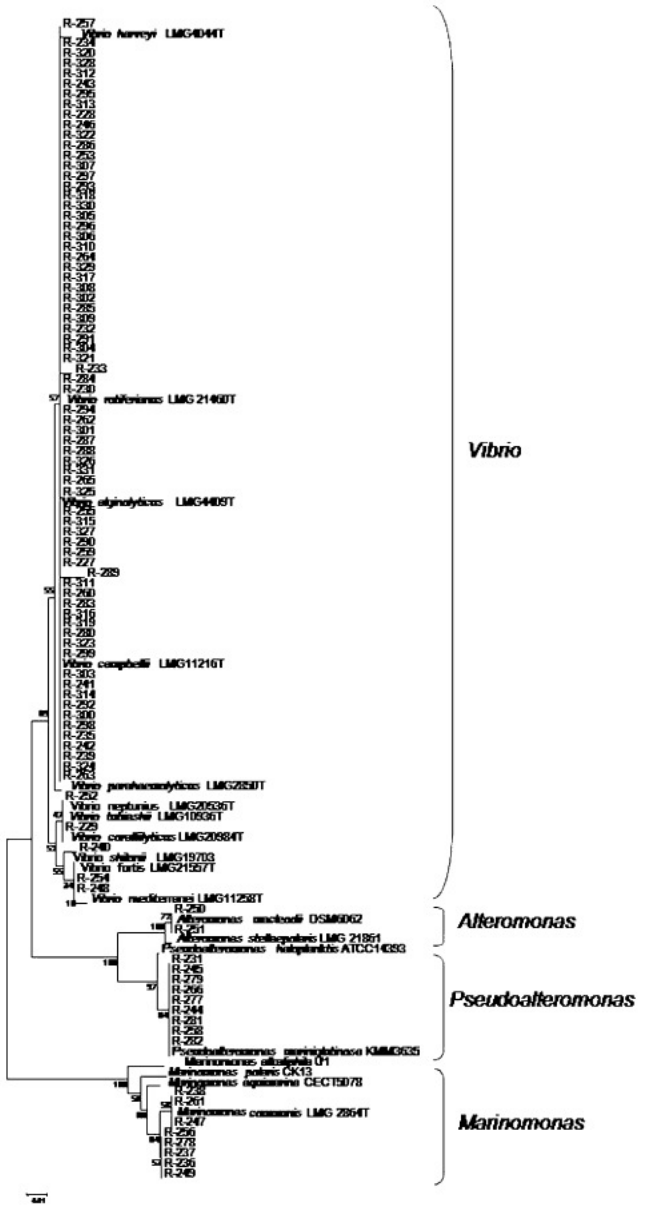

Fig. 1. Phylogenetic tree based on partial 16S rDNA sequences (ca. 500 bp) using the neighbor-joining method and Kimura-2P distances. Bootstrap analysis after 1000 repetitions is shown. Scale bar corresponds to 1% sequence divergence.

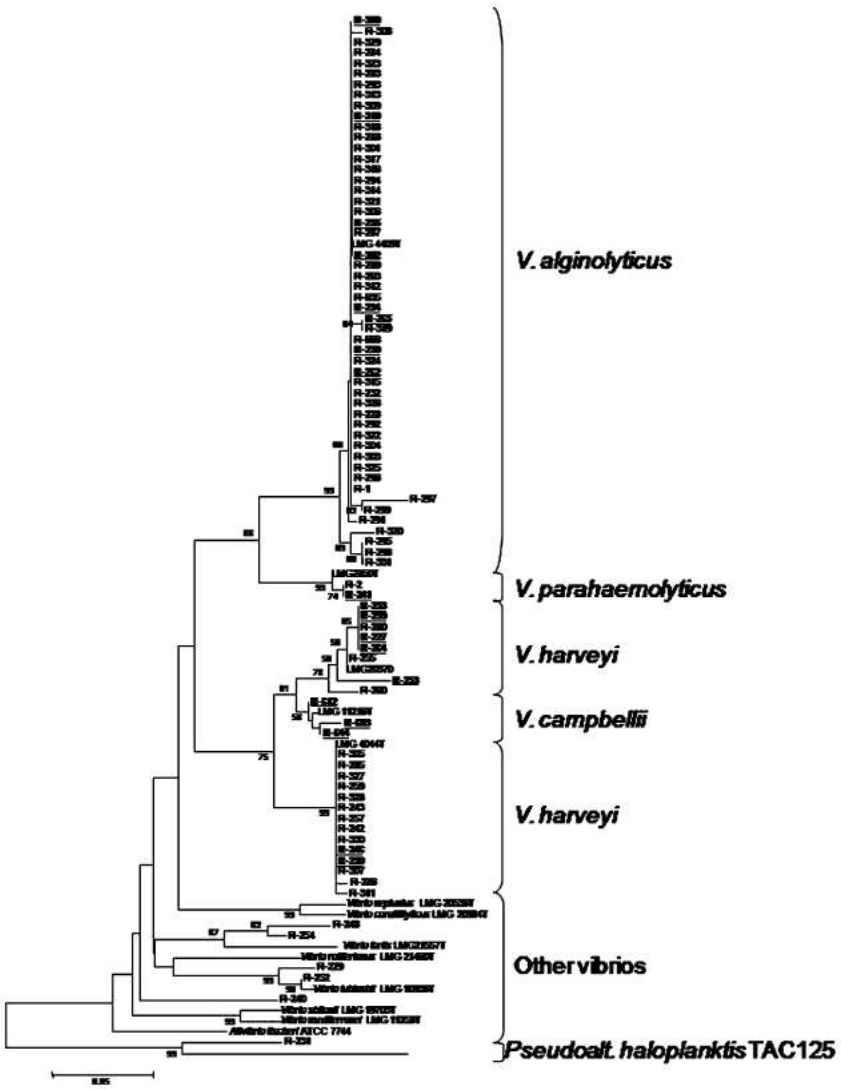

Fig. 2. Phylogenetic tree based on *pyrH* sequences (ca. 450 bp) of vibrios using the neighbor-joining method and Kimura-2P distances. Bootstrap analysis after 1000 repetitions is shown. Scale bar corresponds to 5% sequence divergence. *V. campbellii* R-603, R-612, R-644, and *V. parahaemolyticus* R-2 were isolated from *M. hispida* by using TCBS and were included as controls in this study [28]. Representative strains tested for growth six successive times in NFb and ARA are underlined.

strain formed a separate group by AFLP; the so called AFLP group 31 described by Thompson et al. [25]. The group comprising LMG 20370 possibly represents a new species, but further taxonomic work is needed in order to confirm this hypothesis. Several isolates within the species *V. alginolyticus* and *V. harveyi* were indistinguishable by *pyrH* sequences, suggesting the low taxonomic resolution of this gene for discriminating strains. One single *M. hispida* colony harbored up to eight different colony morphotypes. Overall, the different morphotypes belonged to the same species. Isolates R-288, R-291, R-292, R-293, R-297, R-318, R-324, and R-323 belonged to *V. alginolyticus*, indicating colony variation in this species (Table 1).

All 19 representative isolates (eight *V. alginolyticus*, seven *V. harveyi*, three *V. campbellii* and one *V. parahaemolyticus*) were able to grow six successive times in the nitrogen-free medium, indicating that they indeed fixed N_2 (Table 1; Fig. 2). *V. alginolyticus* isolates showed prolific growth in the nitrogen-free medium. The acetylene reduction test indicated nitrogenase activity in *V. harveyi* R-227, R-230, R-239, R-246, and R-253, the latter of which was an unexpected phenotype for this species. The fact that both *V. alginolyticus* and *V. harveyi* fix N_2 might explain their dominance amongst other species in the mucus of the cnidarians examined in this study. In the holobiont, these vibrios may obtain N_2 via microbial-mediated denitrification within the coral mucus [31].

A single *M. hispida* colony harbored isolates belonging to different species, as was the case for *V. alginolyticus* R-289 and R-310, *Marinomonas* sp. R-261, and *Pseudoalteromonas* sp. R-231. Isolates

allocated to the genus *Alteromonas* were also retrieved. *Alteromonas* sp. R-250 and R-251 appeared in *M. hispida* and *P. caribaeorum*, respectively, and in different locations. *Marinomonas* and *Alteromonas* isolates showed <97% 16S rDNA similarity towards known species. These microorganisms are abundant in the marine environment and are commonly retrieved in culture-dependent diversity studies worldwide, but the isolates obtained in this study differ from currently described species. The isolates associated with the Brazilian cnidarians probably belong to new species yet to be described taxonomically. Work is under way to describe the new taxa and to understand better their role in coral health. With the exception of the new *Marinomonas* isolates that showed some sort of host specificity for *M. hispida*, all other bacterial species appeared in multiple cnidarian hosts, indicating that these sympatric cnidarians share their N_2-fixing microbiota.

One of the most notorious groups of coral-associated bacteria is the *Vibrio* core group, which are pathogens [1,3,8,12,17]. We show here that several *Vibrio* isolates are able to fix N_2 and in doing so they might be considered coral mutualists. It is well known that vibrios of the core group may respond swiftly to changes in environmental conditions. For instance, the doubling time in vibrios of the core group may be below 15 min, particularly at temperatures higher than 25 °C and in carbon-rich environments such us the coral mucus [22]. The results presented in this study suggest that *V. alginolyticus*, *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* isolates might have a positive effect on coral health by fixing N_2, but further work in aquaria is needed to confirm if the same strains can both fix N_2 and cause infection in corals. It might be that under stressful conditions such as high sea water temperature and high nutrient loads (i.e. high concentrations of dissolved ammonia, phosphate, and organic matter) these vibrios will act as opportunistic pathogens, outcompeting other species present in the coral mucus [10]. Our taxonomic study suggests that N_2 fixation is a common phenotypic characteristic among different *Vibrio* species, including putative coral pathogenic species. Work is currently under way to unravel the genomic properties of representative N_2-fixing isolates obtained in this study.

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IV.3. Genomic diversity of vibrios associated with the Brazilian coral *Mussismilia hispida* and its sympatric zoanthids (*Palythoa caribaeorum, Palythoa variabilis* and *Zoanthus solanderi*).

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ORIGINAL ARTICLE

Genomic diversity of vibrios associated with the Brazilian coral *Mussismilia hispida* and its sympatric zoanthids (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*)

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Keywords
bacteria, Brazil, coral mucus, *Mussismilia hispida*, *Palythoa caribaeorum*, *Palythoa variabilis*, *Vibrio*, *Zoanthus solanderi*.

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Abstract

Aims: A taxonomic survey of the vibrios associated with the Brazilian endemic coral *Mussismilia hispida* and the sympatric zoanthids (i.e. *Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*).

Methods and Results: Mucus of 54 cnidarian specimens collected in three different places at São Sebastião in two consecutive years (i.e. 2005 and 2006) was used for taxonomic characterization of the cnidarian microbiota. Ninety-eight of the 151 vibrio isolates fell within the vibrio core group according to partial 16S rDNA sequences. We performed the sequencing of *recA* and *pyrH* genes of all vibrio isolates. The most abundant taxa belonged to the vibrio core group (*Vibrio harveyi*, *Vibrio rotiferianus*, *Vibrio campbellii* and *Vibrio alginolyticus*), *Vibrio mediterranei* (=*Vibrio shillonii*) and *Vibrio chagasii*. With the exception of *V. chagasii* which was found only in the mucus of *M. hispida*, the other species appeared in different hosts with no evidence for the presence of host-specific clones or species. Using rep-PCR analysis, we observed a high genomic heterogeneity within the vibrios. Each vibrio isolate generated a different rep-PCR fingerprint pattern. There was a complete agreement between the grouping based on rep-PCR and concatenated sequences of *pyrH*, *recA* and 16S rDNA, but the pyrH gene has the highest discriminatory power for vibrio species identification.

Conclusion: The vibrio core group is dominant in the mucus of these cnidarians. There is a tremendous diversity of vibrio lineages within the coral mucus. *pyrH* gene sequences permit a clear-cut identification of vibrios.

Significance and Impact of the Study: The taxonomic resolution provided by *pyrH* (but not *recA*) appears to be enough for identifying species of vibrios and for disclosing putative new taxa. The vibrio core group appears to be dominant in the mucus of the Brazilian cnidarians. The overrepresentation of these vibrios may reflect as yet unknown ecological functions in the coral holobiont.

Introduction

There has been a growing number of studies on the diversity of coral microbiota in the last 10 years (Rohwer *et al.* 2001; Rosenberg *et al.* 2007; Dinsdale *et al.* 2008). Corals have a key role in the construction of reefs and form the benthic fauna in a variety of rocky shores worldwide. The reef Biome has a key role for the health of the Oceans, but it is also extremely important for the economy of several countries via fishing, ecotourism and

nursery place. It became evident that corals harbour an enormous diversity of bacteria, both well-known cultivable taxa (e.g. vibrios) and novel groups awaiting formal taxonomic characterization. Rohwer *et al.* (2001, 2002) studied the diversity of the coral microbiota by culture-independent methods using 16S rRNA libraries and observed that each of the sympatric coral species *Montastraea franksi*, *Diploria strigosa* and *Porites astreoides* had a different microbiota. In addition, they found that the microbiota of the same coral species was similar even in specimens collected in different years and >3000 km apart from each other. Based on these data, these authors argued that each coral species has its own microbiota, possibly acquired via host-microbe co-evolution. Apparently, the composition of the coral mucus has a considerable effect on the composition of the microbiota. On the other hand, it was shown more recently that some bacterial species (e.g. in the *Rickettsiales*) are widespread in multiple species of corals (Casas *et al.* 2004). So far, the host-specific hypothesis put forward by Rohwer *et al.* (2001, 2002) has not been evaluated for the microbiota of the Brazilian corals.

Vibrios appear as a considerable fraction of the microbiota of different coral species (with counts of up to 10^7 cells ml^{-1} of coral mucus), in both healthy (Koren and Rosenberg 2006) and diseased specimens (Weil *et al.* 2006; Bourne *et al.* 2007; Kooperman *et al.* 2007). Vibrios appear to belong to the indigenous microbiota of *Fungia scutaria* according to culture-independent analysis (Lampert *et al.* 2006). Based on high vibrio colony counts and a high proportion of vibrio-related genes (e.g. *N*-acetylglucosaminases) in coral reefs, some authors suggested that an enrichment of vibrios in coral mucus and in the surrounding waters may be an indication of unhealthy environmental conditions (Dinsdale *et al.* 2008). A number of studies have revealed the presence of the vibrios associated in corals (Koren and Rosenberg 2006; Bourne *et al.* 2007; Kooperman *et al.* 2007; Dinsdale *et al.* 2008). Indeed, vibrios may represent a large proportion of the coral microbiota (up to 38%) in *Pocillopora damicornis* (Bourne and Munn 2005), and up to 68% in *Oculina patagonica* (Koren and Rosenberg 2006). However, the role of these micro-organisms in the coral holobiont seems to be controversial. Because some vibrios establish mutualistic relationships with marine invertebrates (e.g. *Aliivibrio fischeri* and the squid *Euprymna scolopes*) (Ruby 1996), we cannot rule out the possibility that some *Vibrio* species associated with corals will have a beneficial effect on the health of their hosts. Beneficial effects might include nitrogen fixation (Shashar *et al.* 1994; Lesser *et al.* 2004; Chimetto *et al.* 2008), food resource (Kooperman *et al.* 2007), chitin decomposition (Ducklow and Mitchell 1979), and production of antimicrobials (Ritchie 2006).

This is the first study aiming at the taxonomic characterization of the heterotrophic microbiota, particularly vibrios, associated with the Brazilian endemic coral *Mussismilia hispida* and the sympatric zoanthids (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*). These shallow water zooxanthellated species are sympatric on the Brazilian coast, sharing the same habitat with *M. hispida*. The microbiological examination of different sympatric species would allow us to evaluate if different vibrio species appear in different hosts, indicating a pattern of host-microbe evolution. *Mussismilia hispida* is one of the most important reef builders in the South Atlantic Ocean. It has the largest spatial distribution, occurring along the coast of Brazil, from Rio Grande do Norte to Santa Catarina State (*c.* 5000 km) (Leão *et al.* 2003; Leão and Kikuchi 2005). Recent studies suggest that the genus *Mussismilia* is in danger of extinction (Francini-Filho *et al.* 2008). On the other hand, the genus *Palythoa* is widespread around the world, most notably at rocky shores and coral reefs. *Palythoa caribaeorum* may cover up to 50% of the total area of rocky shores in some locations at the coast of Brazil (Oigman-Pszczol *et al.* 2004). This organism is well known for its ability to produce a very potent nonproteinaceous toxin (called palitoxin) and copious amounts of mucus. These features may allow it to occupy areas of periodic desiccation and intense sunlight stress. We screened the diversity of heterotrophic bacteria associated with the cnidarians species using 16S rDNA gene sequencing in three locations at the coast of São Sebastião city (São Paulo State). In each location, we obtained several specimens of different cnidarians species, normally distant 1 m from each other. The dominant groups of vibrios were analysed in more depth with *recA* and *pyrH* gene sequencing, two proven taxonomic markers, with complementary evolutionary stories (Thompson *et al.* 2005, 2007; Sawabe *et al.* 2007). According to previous studies, the *recA* marker unravels any fuzziness between sister-species (due to recombination and/or slower molecular clock), whereas the *pyrH* marker clearly discriminates sister-species of vibrios.

Materials and methods

In total 54 cnidarians, specimens of *M. hispida*, *P. caribaeorum*, *P. variabilis* and *Z. solanderi* were sampled at rocky shores. The specimens were collected on 03/02/2005 and 01/02/2006 at three sites: Grande (23°50′25″S; 045°24′59″W), Portinho (23°50′25″S; 045°24′22″W) and Preta (23°49′10″S; 045°24′37″W) beaches located near the Marine Biology Center of USP (São Sebastião Channel, São Paulo, Brazil) by SCUBA diving between depths of 3–7 m. The beaches Grande, Portinho and Preta are about 2 km apart from each other, the

latter being on the continental side of the Channel and the first two in the opposite facing CEBIMAR-USP (Fig. S1). In the sampling performed in 2006, the mucus of each specimen was collected using individual sterile 50ml syringes and placed in 50ml sterile tubes *in situ* on board. The mucus samples were kept at *c.* 10°C for 6h prior microbiological examination. Whereas intact colonies of *M. hispida* and fragments of zoanthids were placed in sterile plastic bags and kept at *c.* 10°C for 6h prior microbiological examination in the 2005 sampling.

Isolation and preservation of strains

Bacterial isolation, purification and preservation were performed at University of Campinas. Aliquots of 1 ml of mucus samples were transferred to sterile tubes and diluted in 10 ml in sterile saline solution (3% NaCl, SSS). The mucus was resuspended by vigorous vortex for 3 min. Representative aliquots of 1 ml of diluted mucus samples were 10fold diluted in SSS and plated onto the culture medium Thiosulfate-citrate-bile salt-sucrose agar (Oxoid). Plates were incubated at 28°C for 48 h. Five representative colony morphotypes were picked in the highest dilution (10^5) for further purification. The pure cultures are maintained in vials with 20% glycerol at −80°C.

Taxonomic characterization

The preliminary characterization of all 151 vibrio isolates was obtained by partial 16S rDNA gene sequences (430 nt) as described previously with minor modifications (Thompson *et al.* 2001). Almost complete 16S rDNA sequences (>1300 nt) were performed for representative strains of putative new taxa. The total DNA from each culture was extracted with 50 μl sterile lysis buffer (10% SDS, 2·5 ml; 1 N NaOH, 5 ml and MilliQ water 92 ml), diluted in 150 μl of sterile MilliQ water and boiled for 15 min. The PCR reactions were composed of 37·5 μl sterile MilliQ water, 5·0 μl PCR buffer (10X), 1·5 μl Mg_2Cl (1·5 mmol l^{-1}), 0·4 μl dNTP's (0·2 mmol l^{-1} each), 1 μl forward primer (p27f – 5′AGA GTT TGA TCM TGG CTC AG3′, 20 μmol l^{-1}), 1 μl reverse primer (1401r – 5′CGG TGT GTA CAA GGC CCG GGA ACG3′, 20 μmol l^{-1}), 0·4 μl Ampli*Taq* DNA Polymerase (2 U μl^{-1}) and 1 μl template DNA (0·02 μg μl^{-1}). The thermal programme consisted of (i) 2 min at 95°C, (ii) 30 cycles of 1 min at 94°C + 1 min at 55°C and 3 min at 72°C, and (iii) 3 min at 72°C. PCR was performed using an Eppendorf thermocycler. The PCR products were purified using a solution of PEG8000 (20%)/2 mol l^{-1} NaCl. Purified PCR products were eluted in 50 μl sterile MilliQ water. Subsequently, 5·0 μl of purified PCR product was mixed with 4·0 μl ET Terminator™ Mix (GE Health Care), 0·2 μl sequencing primers (782r – 5′ACC AGG GTA TCT AAT CCT GT3′, 20 μmol l^{-1}), (10f – 5′GAG TTT GAT CCT GGC TCA G3′), and 0·8 μl MilliQ water. The thermal program consisted of 30 cycles of 20 s at 95°C + 15 s at 50°C + 1 min at 60°C. The purification of the sequencing products was obtained by mixing 1 μl ammonium acetate (7·5 mol l^{-1}) and 27·5 μl absolute ethanol, followed by incubation in dark for 30 min and subsequent centrifugation at 20 800 ***g*** for 75 min at 4°C, after this the supernates were removed and 100 μl of 70% ethanol was added, a new centrifugation was performed at 3700 rev min^{-1} for 45 min at 4°C. Separation of the DNA fragments was obtained in a MegaBace 1000 system (GE Health Care). Voltage and time of injection were 3 kV and 80 s. Running was performed at 9 kV for 100 min at 44°C.

Vibrios were further characterized by partial sequencing of *pyrH* (440 nt) and *recA* (600 nt) as described previously (Thompson *et al.* 2005, 2007). Briefly, PCR was composed of 38·2 μl sterile MilliQ water, 1·5 μl $MgCl_2$ (1·5 mmol l^{-1}), 5·0 μl PCR buffer (10X), 0·4 μl dNTP's (0·2 mmol l^{-1} each), 1·2 μl forward primer (pyrH80F – 5′GAT CGT ATG GCT CAA GAA G3′, 20 μmol l^{-1}), 1·2 μl reverse primer (pyrH530R – 5′TAG GCA TTT TGT GGT CAC G3′, 20 μmol l^{-1}), 0·4 μl Ampli*Taq* DNA Polymerase (2 U μl^{-1}) and 2·0 μl template DNA (0·05 μg μl^{-1}). The thermal programme consisted of (i) 5 min at 95°C, (ii) three cycles of 1 min at 95°C + 2 min 15 s at 55°C and 1 min 15 s at 72°C, (iii) 30 cycles of 30 s at 95°C + 1 min 15 s at 55°C and 1 min 15 s 72°C, and (iv) a final 7 min at 72°C. *pyrH* PCR products were purified with the enzyme Exosap according to the instructions of the manufacturer (GE Health Care). Sequencing was performed as described above using 0·6 μl of each PCR primer (pyrH80F and pyrH530R, 20 μmol l^{-1}) and an annealing temperature of 50°C. Sequencing of the *recA* gene was performed using the forward primers (recA1F – 5′TGA RAA RCA RTT YGG TAA AGG3′ and recA130F – 5′GTC TAC CAA TGG GTC GTA TC3′; 20 μmol l^{-1}) and reverse primers (recA2R – 5′TCR CCN TTR TAG CTR TACC3′ and recA720R – 5′GCC ATT GTA GCT GTA CCA AG3′; 20 μmol l^{-1}). Purification of the *recA* PCR products and sequencing was performed as described above with an annealing temperature of 55°C.

Raw sequence data were transferred to the Gene Builder module within Kodon package 2.03 (Applied Maths, Belgium) and ChromasPro ver. 1.34 (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. The sequences were aligned using ClustalW. Similarity matrices and phylogenetic trees were constructed using the software mega ver. 4.0 (Tamura *et al.* 2007). Trees were drawn using the

Neighbour-Joining method (Saitou and Nei 1987). The robustness of each topology was checked by 1000 bootstrap replications. The gene sequence data obtained in this study are also available through our website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The gene sequences are deposited in the GenBank under the accession nos EU716656–EU717075.

Representative vibrio isolates were characterized by rep-PCR analysis as described previously (Gomez-Gil *et al.* 2004, 2007). Briefly, genomic DNA was extracted with the Promega Wizard DNA extraction kit (Promega) according to the manufacture's instructions. The DNA obtained was adjusted to 50 ng ml^{-1} spectrophotometrically. DNA fingerprinting of all the strains was performed with rep-PCR using the $(GTG)_5$ primer. Rep-PCR products were amplified with the Ampli*Taq*® DNA polymerase enzyme (Applied-Biosystems, USA) and the products underwent electrophoresis in 2·25% 20 × 20 cm agarose gels for 18 h at 55 V and 4–8°C. The gels were stained with ethidium bromide and visualized after integration in a gel documentation system (UVP). TIFF files obtained were analysed with the GelCompar II software (ver. 4.5; Applied-Maths), a similarity matrix was calculated with the Jaccard coefficient and the dendrogram constructed with Ward (position tolerance of 0·59%). Type and references strains of all valid species of vibrios were incorporated into the analysis, as well as many strains from other origins, allowing clear species identification.

Results

We analysed the diversity of 151 vibrio isolates originating in the mucus of *M. hispida* and the sympatric zoanthids (*P. caribaeorum*, *P. variabilis* and *Z. solanderi*). The species and the numbers of isolates obtained from different cnidarians are summarized in the Table 1. According to the preliminary screening based on partial 16S rDNA sequences, most of the isolates were allocated in to the vibrio core group (Fig. S2, Table S1). To identify unambiguously the species of vibrios, we sequenced segments of the taxonomic markers *recA* and *pyrH*. We defined groups on the basis of the monophyletic groupings using concatenated sequences

Table 1 Strain list

Species name	No. isolates	Source	Date	Place of isolation
Allivibrio fischeri	1	*Mussismilia hispida*	2005	G
Photobacterium L3 sp. nov	7	*Palythoa caribaeorum*	2005	G, P
Photobacterium rosenbergii	1	*Mussismilia hispida*	2006	G
Vibrio alginolyticus	8	*Mussismilia hispida*	2005–2006	G, P, Pr
	1	*Palythoa caribaeorum*	2006	P
	1	*Palythoa variabilis*	2005	P
	3	*Zoanthus solanderi*	2005	Pr
Vibrio campbellii	9	*Mussismilia hispida*	2005–2006	G, P, Pr
	2	*Palythoa caribaeorum*	2006	P
Vibrio chagasii	9	*Mussismilia hispida*	2005–2006	G, P, Pr
Vibrio cincinnatiensis	1	*Mussismilia hispida*	2006	P
Vibrio diabolicus	1	*Mussismilia hispida*	2005	G
Vibrio fortis	1	*Mussismilia hispida*	2005	Pr
Vibrio harveyi	3	*Mussismilia hispida*	2005–2006	G, P, Pr
	11	*Palythoa caribaeorum*	2005–2006	G, P, Pr
Vibrio mediterranei	12	*Mussismilia hispida*	2005–2006	G, P, Pr
	3	*Palythoa caribaeorum*	2005	G
Vibrio parahaemolyticus	1	*Mussismilia hispida*	2005	G
Vibrio pelagius	1	*Mussismilia hispida*	2006	P
Vibrio ponticus	1	*Mussismilia hispida*	2006	G
Vibrio rotiferianus	4	*Mussismilia hispida*	2005–2006	G, P
	2	*Palythoa caribaeorum*	2005	G
Vibrio sinaloensis	6	*Mussismilia hispida*	2006	P, Pr
Vibrio tubiashii	6	*Mussismilia hispida*	2006	Pr
	5	*Palythoa caribaeorum*	2005–2006	G, P, Pr
Vibrio L1 sp. nov	43	*Mussismilia hispida*	2005–2006	G, P, Pr
	2	*Palythoa caribaeorum*	2006	Pr
	1	*Palythoa variabilis*	2005	P
Vibrio L2 sp. nov	2	*Mussismilia hispida*	2006	G
	3	*Palythoa caribaeorum*	2005	P, Pr

G, beach Grande; P, beach Portinho; Pr, beach Preta.

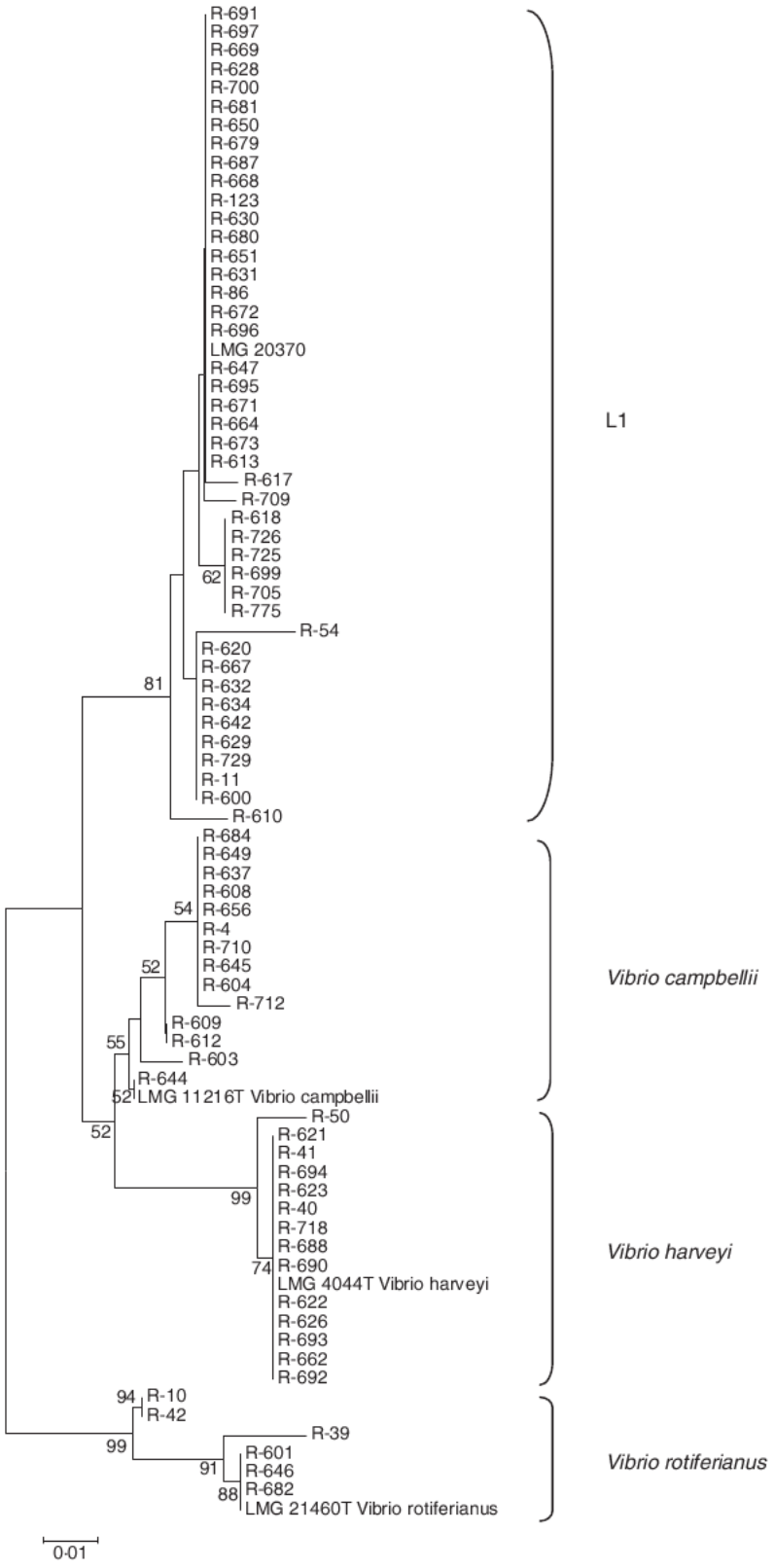


Figure 1 Phylogenetic tree based on *pyrH* sequences (440 bp) of 77 vibrios of the core group using the Neighbour-Joining method and Kimura-2P. Boostrap after 1000 repetitions are shown. Scale bar corresponds to 1% sequence divergence. Bootstrap values of <50% are not shown.

of *recA*, *pyrH* and 16S rDNA (Fig. 1). Isolates within the same group had more than 97% concatenated sequence similarity. Most of the vibrio isolates fell into known species with an apparent dominance of certain taxa. The vibrio core group (*Vibrio alginolyticus*, *Vibrio rotiferianus*, *Vibrio harveyi* and *Vibrio campbelli)*, *Vibrio mediterranei* (including its latter synonymous *Vibrio shilonii*), *Vibrio tubiashii*, *Vibrio sinaloensis* and *Vibrio chagasii* were found in 2005 and 2006. *Vibrio campbellii*, *V. rotiferianus* and *V. harveyi* isolates were associated with *M. hispida* and *P. caribaeorum* in 2 years (2005 and 2006) and in three different sites. Isolates identified into the species *V. tubiashii* were found mainly in 2006. Several isolates obtained in 2005 and 2006 were identified into the species *V. mediterranei*. The *V. chagasii* isolates were found only in association with *M. hispida* in 2005 and 2006. Most of the vibrio species were found in the three different places (Beach Grande, Portinho and Preta) (Fig. S4).

The concatenated sequences did not provide enough taxonomic resolution for the differentiation of the vibrio core group (Fig. S2). However, the analysis based only on *pyrH* and rep-PCR revealed that the vibrio core group isolates form distinct groups corresponding to the species *V. harveyi*, *V. campbellii* and *V. rotiferianus* (Fig. 1). The *recA* alone did not allow the clear-cut differentiation of isolates of the vibrio core group. We observed a rather low intraspecies sequence variation (<0·5%) in the concatenated segments of *pyrH*, *recA* and 16S rDNA in the various taxa detected in this study (Fig. S2). The *recA* gene appeared to show more intra-species variation, while the *pyrH* gene clearly discriminated closely related sister-species of vibrios (Fig. 1).

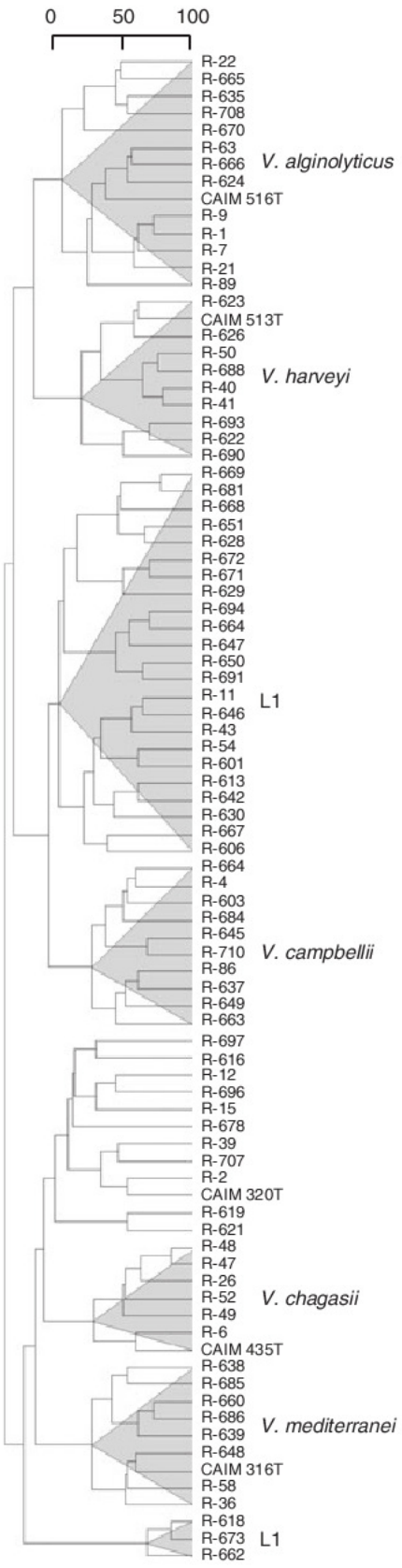
Fifty-seven isolates distributed in three groups (L1–L3) appeared to belong to new taxa as they formed separate groups on the basis of concatenated sequences and showed differences in the complete 16S rDNA gene sequences (1%) with their closest neighbours. The group L1 ($N = 46$) had <97% concatenated sequence similarity towards *V. harveyi*. Isolates of this group clustered with the strain LMG 20370. Group L2 comprised five isolates originating in *M. hispida* and *Palythoa*, represented for R-616, R-619 (Fig. S2) and R-77, R-78, and R-91 with the *pyrH* and 16S rDNA sequence obtained (Table S1). Group L3 comprised seven *Photobacterium* isolates of *P. caribaeorum* (R-15, R-89, R-13, R-16, R-17, R-34 and R90; Table S1, Fig. S2).

The rep-PCR analyses showed that the vibrio isolates formed cohesive species clusters clearly separated from each other on the basis of rep-PCR analysis. The rep-PCR analysis was reassuring and confirmed the grouping obtained by *pyrH*, *recA* and 16S rDNA gene sequences (Figs 2 and S2). For instance, the *V. harveyi* isolates R-647 and R-691 had *c.* 45% rep-PCR pattern similarity and 96% concatenated sequence similarity. Whereas the *V. harveyi* R-54 and R-601 had 55% rep-PCR pattern similarity, and 97% concatenated sequence similarity. Overall, the rep-PCR analysis provided a finer discrimination between isolates than the *pyrH* gene sequences. A negative correlation was found between the nucleotide substitutions in the concatenated sequences and the rep-PCR (Fig. S3). In general, the higher the rep-PCR similarity values, the lower the nucleotide substitutions (Spearman correlation = −0·324, $P < 0{\cdot}0001$, $n = 3486$). The correlation became more evident when only *pyrH* sequences were compared with rep-PCR. It became evident that most of the isolates within each species generated a different rep-PCR pattern (<85% band pattern similarity).

Discussion

The vibrio core group appears to be a dominant group among the vibrios of the microbiota of the four species Brazilian cnidarians. The overrepresentation of these vibrios may reflect as yet unknown ecological functions in the coral holobiont. *Vibrio alginolyticus* has been considered a water-column resident previously (Ritchie 2006), but we showed in this study that this species is present in the mucus of different cnidarians species. *Vibrio alginolyticus* is a coral pathogen (Cervino *et al.*, 2004, 2008), but other studies suggest that it could belong to the normal coral microbiota (Lampert *et al.* 2006). In Nitrogen fixing, *V. alginolyticus* strains might have a beneficial effect on *M. hispida*. *Vibrio mediterranei* was frequently found in association with the coral *M. hispida* and the sympatric zoanthids (i.e. *P. caribaeorum*, *P. variabilis* and *Z. solanderi*). A recent study suggested that vibrios, including *V. mediterranei*, no longer can be detected in mucus of health and bleached *Oculina patagonica*. This fact may be related to shortcomings of the FISH analysis used in this study (Ainsworth *et al.* 2008).

The species *V. chagassi* was proposed to encompass isolates originated of rotifer and fish cultures (Thompson *et al.* 2003), but we now expand its occurrence. This species was found in 2005 and 2006 associated only with *M. hispida*. However, we have no evidence to suggest that different cnidarians species select different vibrio species as is the case of the squid *Euprymna scolopes* and *Aliivibrio fischeri*. If a host-microbe interaction exists involving vibrios and corals as argued by Rohwer *et al.* (2001, 2002), we hypothesize that it would involve the selection of strains rather than species. Additional work would be needed to prove that specific clones of vibrios are picked up and maintained by the cnidarian hosts, with the examination of large co-occurring populations of dominant vibrios, such as the vibrio core group.



The analysis of the concatenated sequences of the genes *pyrH*, *recA* and 16S rDNA did not provide enough taxonomic resolution for the differentiation of the vibrio core group (Fig. S2). Although *recA* was suggested to be an identification marker for vibrios (Thompson *et al.* 2003), our current results show that this locus alone does not allow the differentiation of fresh environmental isolates of the vibrio core group. However, the analysis based only on *pyrH* and rep-PCR revealed that the vibrio core group isolates form distinct groups corresponding to the species *V. harveyi*, *V. campbellii* and *V. rotiferianus* (Fig. 1). The taxonomic resolution provided by *pyrH* appears to be enough for identifying species of vibrios and for disclosing putative new taxa. Several isolates allocated in the clusters L1, L2 and L3 appear to belong to new taxa as they do not cluster with any known vibrio species. Isolates of L1 group clustered with the strain LMG 20370. This strain was originally allocated within *V. harveyi* because it had 71% DDH similarity with the type strain of *V. harveyi*, but its AFLP patterns were clearly different from the type and reference strains of *V. harveyi* (Thompson *et al.* 2001). The results presented in this study enforce that L1 represents a new species. A polyphasic taxonomic approach will be undertaken to describe formally these putative new taxa, including the sequencing of additional genetic loci (i.e. *topA*, *ftsZ*, *mreB* and *atpA*) and phenotypic characterization.

We performed rep-PCR analysis to check if there was a clonal spread of successful vibrio genotypes among different cnidarian hosts. This analysis would give us information on intraspecific genome-wide variation of the most abundant taxa. Our results clearly show a tremendous genomic variation among the collection of co-occurring vibrio populations. Also, it was evident that isolates of different hosts and places had high mutual similarity, indicating no biogeographical effect on the distribution of vibrio populations at the scale of metres and kilometres. Genome size variation was shown in *V. splendidus* in a study of the vibrioplankton of the North Atlantic Ocean (Thompson *et al.* 2003). The factors leading to the genomic variation observed in this study are unknown at present, but might involve both intracellular processes (such as rearrangements and point mutations) and horizontal gene transfer processes within the coral mucus. Another

Figure 2 rep-PCR dendrogram of 83 representative vibrios isolates of Thiosulfate-citrate-bile salt-sucrose agar from cnidarians mucus (*Mussismilia hispida*, *Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*). Dendrogram generated with the Jaccard coefficient (similarity matrix) and constructed with Ward (optimization 0·35, position tolerance of 0·59%) from electrophoretic band patterns obtained with rep-PCR, (GTG)5 primer. Scale, per cent similarity of dendrogram.

interesting aspect of vibrio diversification disclosed in this study, is the seemingly absence of species specificity of the vibrios in different hosts.

The rep-PCR analysis confirmed the grouping obtained by *pyrH*, *recA* and 16S rDNA gene sequences. However, it is evident that strain identification within the vibrio core group will require the use of a multigene approach. Overall, the rep-PCR analysis provided a finer discrimination between isolates than the *pyrH* gene sequences. A negative correlation founded between the nucleotide substitutions in the *pyrH* sequences and the rep-PCR suggests that *pyrH* gene sequences can be used to predict whole genome similarity among vibrios. In a collection of papers on the taxonomy of Lactic Acid Bacteria, Naser *et al.* (2005, 2007) have shown the reliability of using single locus for species identification. The loci used by these authors (i.e. *atpA*, *pheS* and *rpoA*) were carefully chosen for providing species differentiation. pheS alone provided the discrimination of closely related species. In this study, we show that the taxonomic resolution provided by *pyrH* (but not *recA*) appears to be enough for identifying species of vibrios and for disclosing putative new taxa. The simplified version of the MLSA used in this study correlates well with rep-PCR analysis, but it was clear that the use of *recA* and 16SrDNA decreased the taxonomic resolution of our analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Map of the sampling region and photos of the cnidarians. (a) Map of Brazil highlighting the coast of São Sebastião in the São Paulo State. (b) São Sebastião channel. The beaches Preta, Portinho and Grande located near CEBIMAR-USP are showed. (c–f) The coral *Mussismilia hispida* and the zoanthids *Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*.

Figure S2 Phylogenetic tree based on *pyrH*, *recA*, and 16S rDNA sequences (1463 bp) of 140 vibrio isolates using the Neighbour-Joining method and Kimura-2P. Boostrap after 1000 repetitions are shown. Scale bar corresponds to 10% sequence divergence. Bootstrap values of <50% are not shown.

Figure S3 A regression plot of the similarity values of the pair-wise similarity of concatenated gene sequences and the rep-PCR band patterns of 87 representative vibrio isolates. The similarity matrix for the concatenated sequences was calculated with the Jukes–Cantor method, with all positions containing alignment gaps and missing data being eliminated (pairwise deletion option). The rep-PCR similarity matrix was calculated with the Jaccard band based coefficient (band position tolerance of 0·8%).

Figure S4 Scheme of distribution of vibrio populations in the three different places studied (Beaches Preta, Portinho and Grande).

Table S1 Detailed strain list.

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Figure 1S

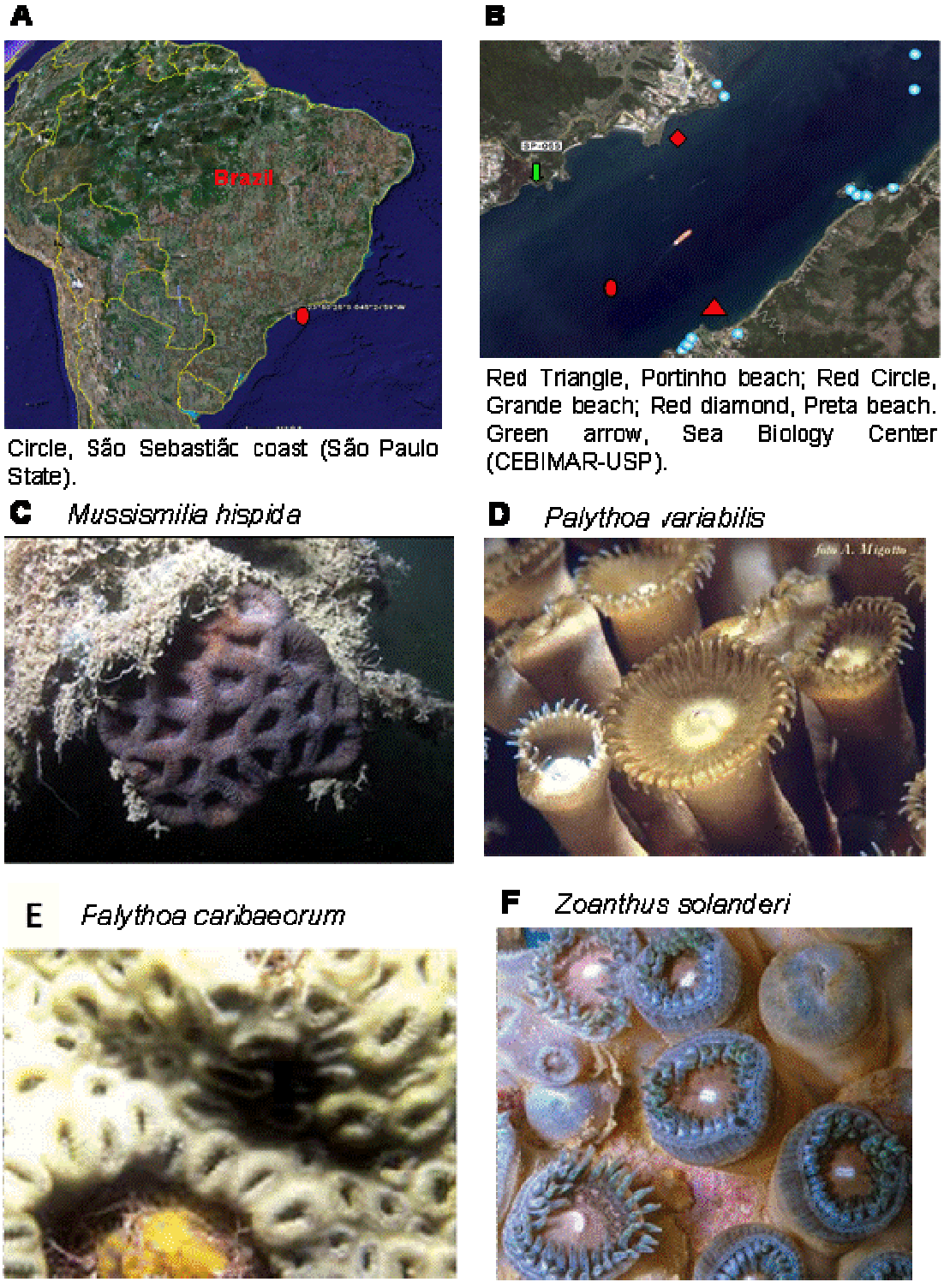

Figure S1. Map of the sampling region and photos of the cnidarians. (a) Map of Brazil highlighting the coast of São Sebastião in the São Paulo State. (b) São Sebastião channel. The beaches Preta, Portinho and Grande located near CEBIMAR-USP are showed. (c–f) The coral *Mussismilia hispida* and the zoanthids *Palythoa caribaeorum, Palythoa variabilis* and *Zoanthus solanderi*.

Figure 2S

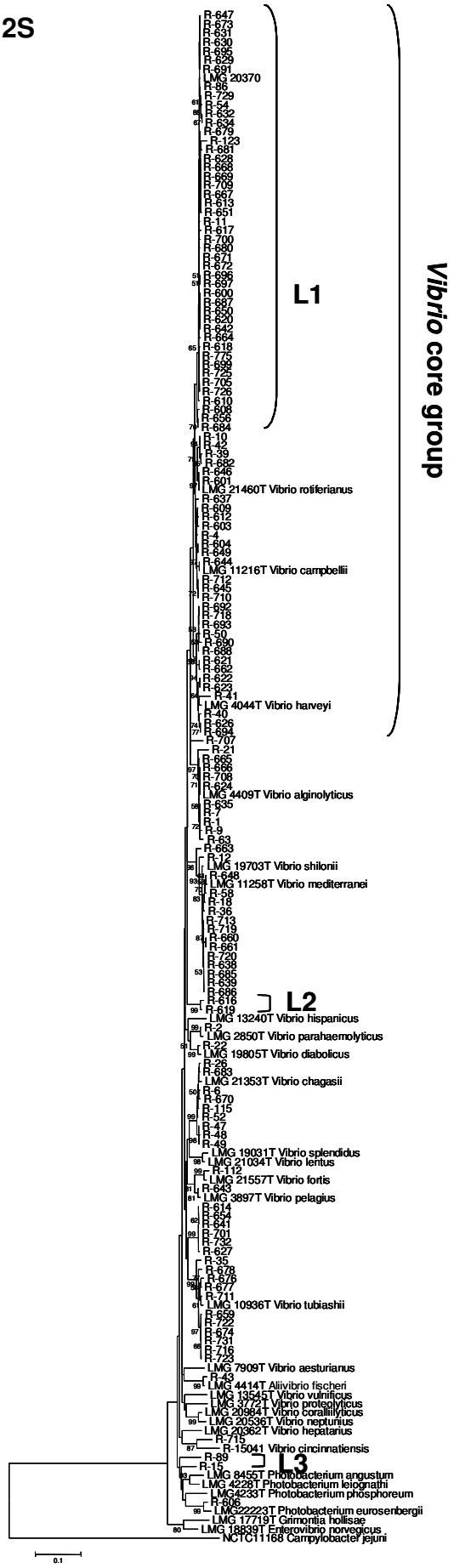

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Figure 3S

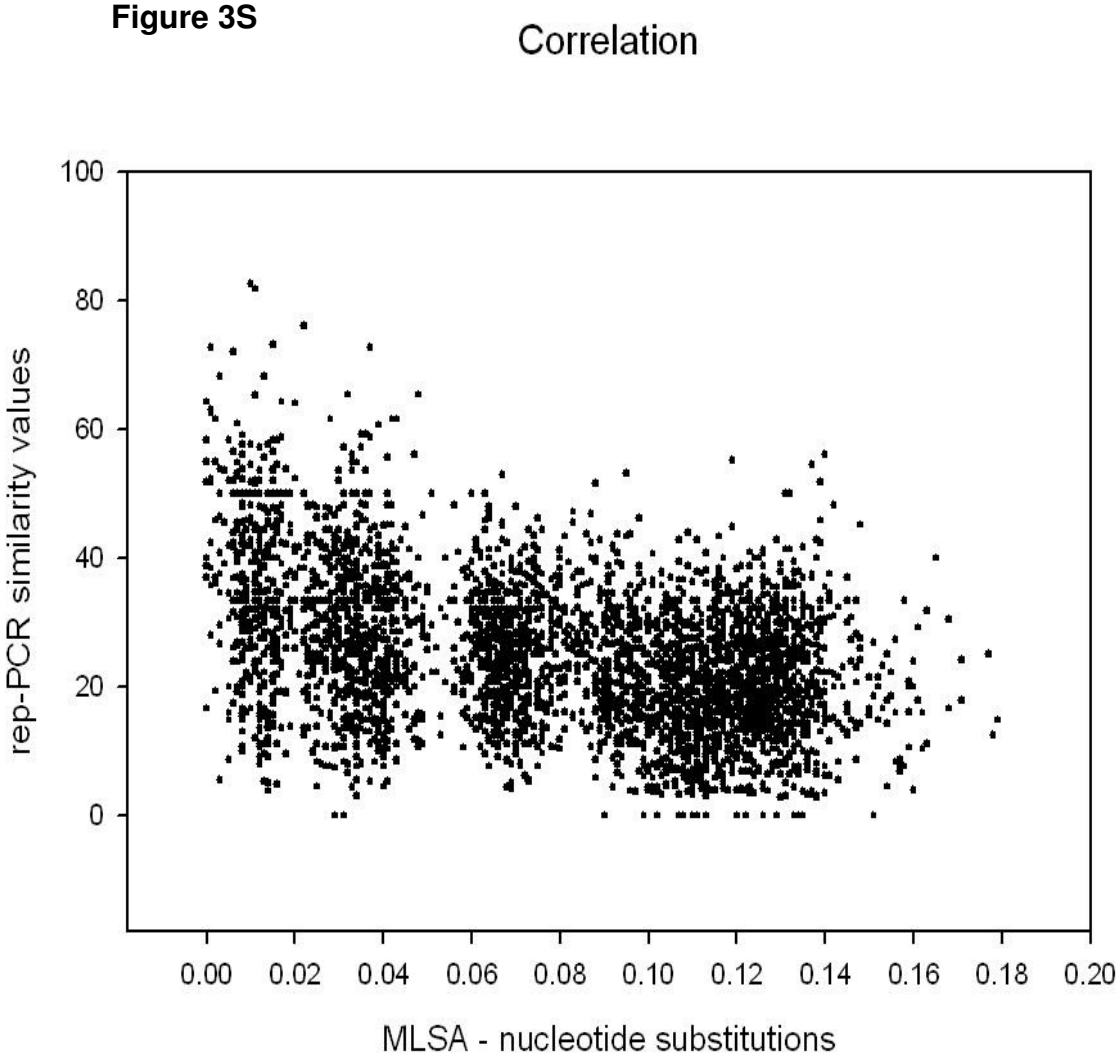

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Figure 4S. SUPPLEMENTARY ONLINE MATERIAL

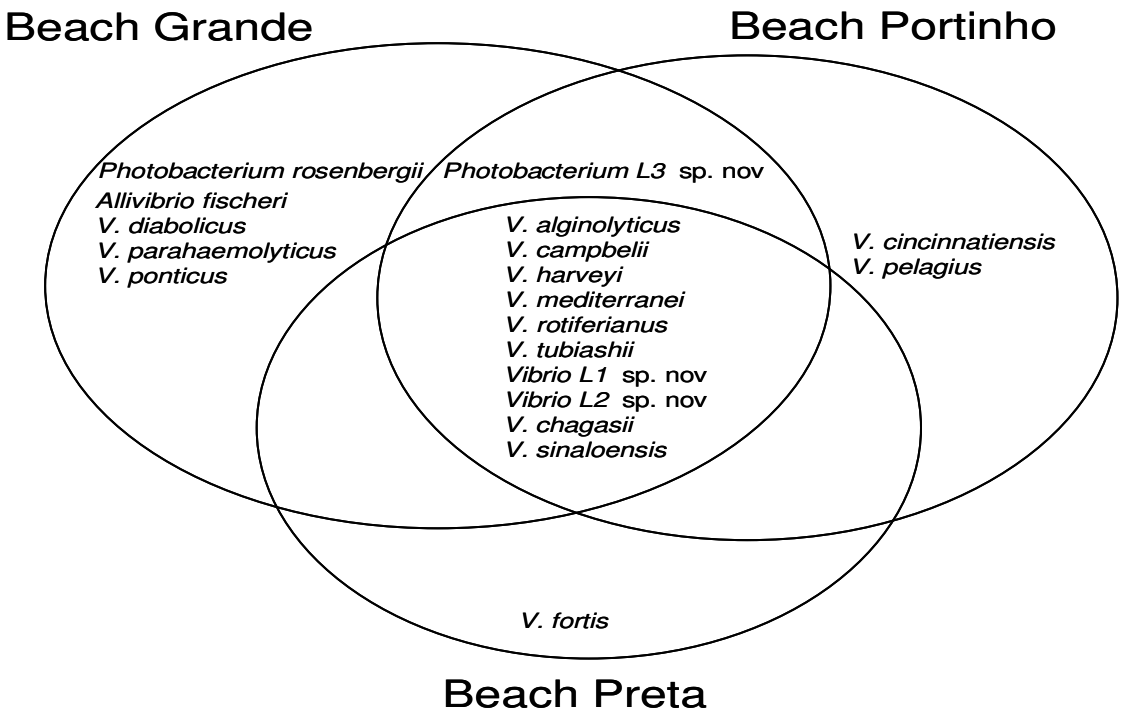

Figure S4. Scheme of distribution of vibrio populations in the three different places studied (Beaches Preta, Portinho and Grande).

Table 1S. Detailed strain list. SUPPLEMENTARY ONLINE MATERIAL

Species name	Strain nº	Morphotype	Source	Place	Date
V. alginolyticus	R-1	1.1.a	*Mussismilia hispida*	Beach Grande	3/2/2005
V. alginolyticus	R-21	1.10.a	*Mussismilia hispida*	Beach Grande	3/2/2005
V. alginolyticus	R-624	9.b.1	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. alginolyticus	R-635	19.d.	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. alginolyticus	R-665	5.b.2	*Mussismilia hispida*	Beach Preta	1/2/2006
V. alginolyticus	R-666	9.b.2	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. alginolyticus	R-7	1.2.c	*Mussismilia hispida*	Beach Grande	3/2/2005
V. alginolyticus	R-708	S.1.	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. alginolyticus	R-9	1.4.b	*Mussismilia hispida*	Beach Grande	3/2/2005
V. alginolyticus	R-63	2.1.b	*Palythoa variabilis*	Beach Portinho	3/2/2005
V. alginolyticus	R-80	3.2.a	*Zoanthus solanderi*	Beach Preta	3/2/2005
V. alginolyticus	R-81	3.2.b	*Zoanthus solanderi*	Beach Preta	3/2/2005
V. alginolyticus	R-104	3.3.a	*Zoanthus solanderi*	Beach Preta	3/2/2005
V. campbellii	R-612	5.a	*Mussismilia hispida*	Beach Preta	1/2/2006

V. campbellii	R-637	19.e	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. campbellii	R-4	1.1.d	*Mussismilia hispida*	Beach Grande	3/2/2005
V. campbellii	R-603	7f	*Mussismilia hispida*	Beach Grande	1/2/2006
V. campbellii	R-604	7g	*Mussismilia hispida*	Beach Grande	1/2/2006
V. campbellii	R-609	5f	*Mussismilia hispida*	Beach Preta	1/2/2006
V. campbellii	R-644	10g	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. campbellii	R-645	10h1	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. campbellii	R-649	1d	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. campbellii	R-710	Q1.c	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. campbellii	R-712	Q1.a	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. chagasii	R-26	1.11.c	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-47	1.15.e	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-48	1.16.a	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-49	1.16.c	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-6	1.2.b	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-115	3.4.d	*Mussismilia hispida*	Beach Preta	3/2/2005
V. chagasii	R-52	1.16.f	*Mussismilia hispida*	Beach Grande	3/2/2005
V. chagasii	R-670	3g	*Mussismilia hispida*	Beach Grande	1/2/2006
V. chagasii	R-683	6f	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. cincinnatiensis	R-715	10c2	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. diabolicus	R-22	1.10.b	*Mussismilia hispida*	Beach Grande	3/2/2005
Allivibrio fischeri	R-43	1.15.a	*Mussismilia hispida*	Beach Grande	3/2/2005
V. fortis	R-112	3.4.a	*Mussismilia hispida*	Beach Preta	3/2/2005
V. harveyi	R-40	1.5.e	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. harveyi	R-41	1.3.a	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. harveyi	R-50	1.16.d	*Mussismilia hispida*	Beach Grande	3/2/2005
V. harveyi	R-621	M1	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. harveyi	R-622	O1	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-623	O2	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-626	2f	*Mussismilia hispida*	Beach Preta	1/2/2006
V. harveyi	R-662	M2	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. harveyi	R-688	S2	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-690	9d	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. harveyi	R-692	P3	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-693	P2	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-694	P1	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. harveyi	R-718	N3	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. mediterranei	R-18	1.9.a	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. mediterranei	R-58	1.14.f	*Mussismilia hispida*	Beach Grande	3/2/2005
V. mediterranei	R-12	1.7.a	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. mediterranei	R-36	1.5.a	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. mediterranei	R-638	4c	*Mussismilia hispida*	Beach Grande	1/2/2006
V. mediterranei	R-639	1a	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. mediterranei	R-648	7e	*Mussismilia hispida*	Beach Grande	1/2/2006
V. mediterranei	R-660	3j2	*Mussismilia hispida*	Beach Grande	1/2/2006
V. mediterranei	R-661	3j1	*Mussismilia hispida*	Beach Grande	1/2/2006
V. mediterranei	R-663	3c2	*Mussismilia hispida*	Beach Grande	1/2/2006
V. mediterranei	R-685	6d3	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. mediterranei	R-686	6c1	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. mediterranei	R-713	2a	*Mussismilia hispida*	Beach Preta	1/2/2006

V. mediterranei	R-719	2.e.2	*Mussismilia hispida*	Beach Preta	1/2/2006
V. mediterranei	R-720	10j	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. parahaemolyticus	R-2	1.1.b	*Mussismilia hispida*	Beach Grande	3/2/2005
V. pelagius	R-643	10d	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. ponticus	R-707	4b	*Mussismilia hispida*	Beach Grande	1/2/2006
V. rotiferianus	R-10	1.4.c	*Mussismilia hispida*	Beach Grande	3/2/2005
V. rotiferianus	R-39	1.5.d	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. rotiferianus	R-42	1.3.b	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. rotiferianus	R-601	9f	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. rotiferianus	R-646	10i1	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. rotiferianus	R-682	6g	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. sinaloensis	R-614	17a	*Mussismilia hispida*	Beach Preta	1/2/2006
V. sinaloensis	R-627	19a	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. sinaloensis	R-641	1c	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. sinaloensis	R-654	10h2	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. sinaloensis	R-701	10i3	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. sinaloensis	R-732	10h3	*Mussismilia hispida*	Beach Portinho	1/2/2006
V. tubiashii	R-35	1.8.e	*Palythoa caribaeorum*	Beach Grande	3/2/2005
V. tubiashii	R-659	L5	*Mussismilia hispida*	Beach Preta	1/2/2006
V. tubiashii	R-676	16a2	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. tubiashii	R-677	16a1	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. tubiashii	R-678	16b2	*Palythoa caribaeorum*	Beach Preta	1/2/2006
V. tubiashii	R-711	Q1.b	*Palythoa caribaeorum*	Beach Portinho	1/2/2006
V. tubiashii	R-722	8a1	*Mussismilia hispida*	Beach Preta	1/2/2006
V. tubiashii	R-723	8a2	*Mussismilia hispida*	Beach Preta	1/2/2006
V. tubiashii	R-731	8c1	*Mussismilia hispida*	Beach Preta	1/2/2006
V. tubiashii	R-674	8d	*Mussismilia hispida*	Beach Preta	1/2/2006
V. tubiashii	R-716	8c2	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-600	9e	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-608	7a1	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-610	2c	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-613	5b1	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-617	6j	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-618	10b1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-620	17d	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-628	6h2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-629	6 e1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-630	10a1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-631	6d2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-632	19c3	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-634	19c1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-642	10c1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-647	6a	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-650	3c1	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-651	3b	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-656	17f	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-664	5c	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-667	3i	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-668	3h2	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-669	3h1	*Mussismilia hispida*	Beach Grande	1/2/2006

Vibrio L1 sp. nov	R-671	7b2	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-672	7b1	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-673	2b	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-680	10b2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-681	6h1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-684	6 e2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-687	6b1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-695	6d1	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-696	2i2	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-697	2i1	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-699	3a2	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-700	6c2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-705	10a2	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-709	17c	*Mussismilia hispida*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-725	3e	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L1 sp. nov	R-726	9g	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-729	10e	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-775	10a3	*Mussismilia hispida*	Beach Portinho	1/2/2006
Vibrio L1 sp. nov	R-11	1.6.a	*Mussismilia hispida*	Beach Grande	3/2/2005
Vibrio L1 sp. nov	R-86	2.9.b	*Palythoa variabilis*	Beach Portinho	3/2/2005
Vibrio L1 sp. nov	R-54	1.14.b	*Mussismilia hispida*	Beach Grande	3/2/2005
Vibrio L1 sp. nov	R-679	N2	*Palythoa caribaeorum*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-691	N1	*Palythoa caribaeorum*	Beach Preta	1/2/2006
Vibrio L1 sp. nov	R-123	1.16.b	*Mussismilia hispida*	Beach Grande	3/2/2005
Vibrio L2 sp. nov.	R-619	4a	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L2 sp. nov.	R-78	3.6.b	*Palythoa caribaeorum*	Beach Preta	3/2/2005
Vibrio L2 sp. nov.	R-91	2.3.e	*Palythoa caribaeorum*	Beach Portinho	3/2/2005
Vibrio L2 sp. nov.	R-616	3d	*Mussismilia hispida*	Beach Grande	1/2/2006
Vibrio L2 sp. nov.	R-77	3.6.a	*Palythoa caribaeorum*	Beach Preta	3/2/2005
Photobacterium L3 sp. nov.	R-13	1.7.b	*Palythoa caribaeorum*	Beach Grande	3/2/2005
Photobacterium L3 sp. nov.	R-15	1.7.d	*Palythoa caribaeorum*	Beach Grande	3/2/2005
Photobacterium L3 sp. nov.	R-16	1.7.f	*Palythoa caribaeorum*	Beach Grande	3/2/2005
Photobacterium L3 sp. nov.	R-17	1.7.g	*Palythoa caribaeorum*	Beach Grande	3/2/2005
Photobacterium L3 sp. nov.	R-34	1.8.d	*Palythoa caribaeorum*	Beach Grande	3/2/2005
Photobacterium L3 sp. nov.	R-89	2.3.c	*Palythoa caribaeorum*	Beach Portinho	3/2/2005
Photobacterium L3 sp. nov.	R-90	2.3.d	*Palythoa caribaeorum*	Beach Portinho	3/2/2005
Photobacterium rosenbergii	R-606	7i2	*Mussismilia hispida*	Beach Grande	1/2/2006

IV.4. *Photobacterium jeanii* sp. nov. isolated from corals and zoanthids.

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Running Title:

Photobacterium jeanii sp. nov.

Subject Category: New taxa, *Proteobacteria*

Footnote: The GenBank/EMBL accession numbers for the 16S rRNA, *recA, topA, ftsZ, mreB* and *rpoA* gene sequences of *Photobacterium jeanii* sp. nov. are GU065209 - GU065227 and AJ842701 listed in the Supplementary **Table S1**.

Summary

Four novel isolates (R-40508^T, R-40507, R-40903 and R-21419) were obtained from different cnidarian species (*Phyllogorgia dilatata, Merulina ampliata,* and *Palythoa caribaeorum*) from different places in Brazil and Australia. The novel isolates formed a tight phylogenetic group based on 16S rRNA, *recA, topA, ftsZ, mreB* and *rpoA*. Their closest phylogenetic neighbours were *Photobacterium leiognathi*, *P. rosenbergii* and *P. halotolerans*, sharing 97.1 to 97.5 % 16S rRNA gene sequence similarity. DNA–DNA hybridization (DDH) between a representative strain (R-40508^T) and the type strains of these *Photobacterium* species revealed less than 20 % DDH similarity, proving that the novel isolates belong to a new species. Several phenotypic features allow the differentiation of the new species from its closest phylogenetic neighbours. It has gelatinase and lipase activity, and can utilize melibiose, but it can not grow on 6 % NaCl. In addition, the novel species has the fatty acid $C_{16:0}$ iso, but it lacks the fatty acids $C_{17:0}$, $C_{17:0}$ cyclo, $C_{17:0}$ iso, $C_{17:1}$ ω8*c*, and $C_{17:1}$ ω9*c* iso. The name *Photobacterium jeanii* sp. nov. is proposed for this taxon. The G+C content of the type strain R-40508^T (= LMG 25436^T = CAIM 1817^T) is 45.5 mol%.

There is a growing interest in coral microbiology in the last years, particularly because of the increasing awareness related to the role of corals in the marine environment and their extinction (Rohwer *et al*., 2001; Dinsdale *et al*., 2008; Sussman *et al*., 2009; Shnit-Orland & Kushmaro, 2009). One of the main causes of coral extinction seems to be related to infectious diseases, mainly caused by vibrios (Rosenberg *et al*., 2007). Coral bacteria may also have a positive effect on the coral holobiont. Bacteria living in the coral mucus and tissue may be a first line of defence for their holobiont hosts, protecting them against diseases (Ritchie, 2006). *Photobacterium* species associated with corals were able to produce antibiotics that would protect the coral holobiont or would allow a competitive advantage (Ritchie, 2006). Other beneficial effects on the coral holobiont may include nitrogen fixation (Chimetto *et al.,* 2008), food resource (Kooperman *et al*., 2007), and chitin decomposition (Ducklow & Mitchell, 1979). *Photobacterium rosenbergii* was one of the most recently described species associated with healthy and diseased corals in

Australia (Thompson *et al*., 2005a). This species has also been found in association with healthy and diseased corals in Brazil (Chimetto *et al*., 2009).

In the present study, a detailed polyphasic taxonomic analysis was performed on four novel isolates obtained in different studies in order to determine their exact taxonomic position (**Table 1**). They appeared to be related to *Photobacterium* species on the basis of 16S rRNA analysis (Alves *et al*., in press; Chimetto *et al.*, 2009; Thompson *et al*., 2005b).

Table 1. Strains of *P. jeanii* sp. nov.

Isolate	Source	Locality	Isolation year
R-40507 (LMG 25437)	mucus of apparently healthy *Palythoa caribaeorum*	Portinho beach in São Sebastião Channel, São Paulo, Brazil.	2006
R-40508^T (LMG 25436^T; CAIM 1817^T)	mucus of apparently healthy *Palythoa caribaeorum*	Preta beach in São Sebastião Channel, São Paulo, Brazil.	2006
R-40903	mucus of apparently healthy *Phyllogorgia dilatata*	Abrolhos Bank, Bahia, Brazil.	2007
R-21419	apparently healthy *Merulina ampliata*	Magnetic Island, Australia	2002

All isolates were obtained using Marine Agar medium at 28 °C after 48h incubation. Gene sequences of 16S rRNA, recombination repair protein (*recA*), topoisomerase I (*topA*), cell division protein (*ftsZ*), actin-like cytoskeleton protein (*mreB*) and RNA polymerase alpha subunit (*rpoA*) were obtained as described previously (Chimetto *et al.,* 2008, 2009). Primers used for gene amplification and sequencing are described in Thompson *et al*. (2001a, 2005b) and Sawabe *et al.* (2007). Raw sequence data were transferred to ChromasPro ver. 1.34 (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. Pairwise similarities of these sequences with sequences from the EMBL database were calculated with the BioNumerics 4.5 software package (Applied Maths) using an open gap penalty of 100 % and a unit gap penalty of 0 %. Sequences were aligned using ClustalW. Similarity matrices and phylogenetic trees were constructed using the MEGA ver. 4.0 software (Tamura *et al*., 2007) and BioNumerics 4.5 software (Applied Maths). Trees were drawn using the Neighbour-Joining (Saitou & Nei, 1987) and Maximum Parsimony method (Eck *et al.,* 1966). The

robustness of each topology was checked by 1000 bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are available through the open access website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The GenBank accession numbers for the 16S rRNA, *ftsZ, mreB, recA, rpoA* and *topA* gene sequences are listed in **Supplementary Table S1**. DNA-DNA hybridization (DDH) experiments were performed using Ezaki´s microplate method as described in detail previously (Ezaki *et al*., 1989; Willems *et al*., 2001). The hybridization temperature was 40 °C in the presence of 50 % formamide. Reciprocal reactions were performed for every DNA pair and their variation was within the limits of this method (Goris *et al.,* 1998). The G+C content of the DNAs was determined using HPLC as described previously (Mesbah *et al*., 1989). Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). For fatty acid analysis, cells were grown on TSA (DIFCO) supplemented with 2 % NaCl for 24 h at 28 °C. Phenotypic characterization was performed as described previously (Thompson *et al.*, 2001b, 2006). Type strains of known *Photobacterium* species were included in these analyses as positive controls.

16S rRNA gene sequence analysis revealed that the four isolates formed a tight monophyletic group affiliated to the genus *Photobacterium* (**Figure 1**). The four novel isolates formed a tight cluster with more than 99 % 16S rRNA gene sequence similarity. The closest neighbours of the novel isolates were *Photobacterium leiognathi* (97.4 % sequence similarity), *P. rosenbergii* and *P. halotolerans* (both 97.1 % sequence similarity), *P. lutimaris* and *P. angustum* (both 97.0 % similarity). The 16S rRNA gene sequence similarity towards other validly named *Photobacterium* species was below 96.5 %. The phylogenetic analysis based on 16S rRNA, *recA, topA, ftsZ, mreB* and *rpoA* gene sequences (4,949 nt in total) confirmed that the isolates form a tight group related to *P. rosenbergii* (**Figure 2)**. The novel isolates shared less than 95 % concatenated gene sequence similarity with their closest neighbour, indicating clearly that they belong to a new *Photobacterium* species.

DDH experiments were performed with all four isolates and the type strains of the closest phylogenetic neighbours. DDH similarity between the novel isolates varied between 84 and 97 %, showing that they belong to a single species. The novel isolate R-40508^{T}

(LMG 25436^T) had only 19, 15, 11, and 9 % DDH similarity with *P. lutimaris* LMG 25278^T, *P. rosenbergii* LMG 22223^T, *P. leiognathi* LMG 4228^T and *P. halotolerans* LMG 22194^T, respectively. Standard deviations of all hybridization experiments were below 10 %. Clearly, the DDH data demonstrate that the four isolates represent a novel species of the genus *Photobacterium.*

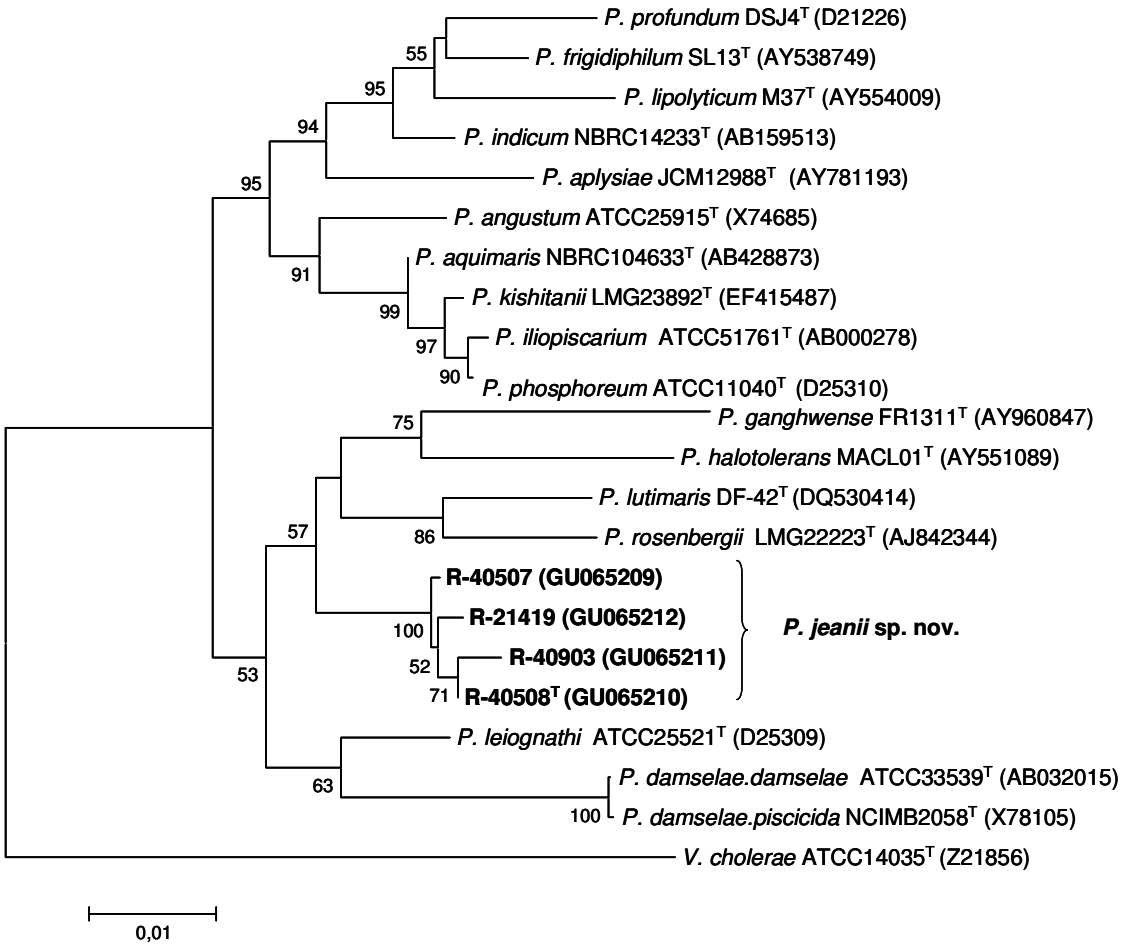

Figure 1. Neighbour-joining phylogenetic tree of *Photobacterium* species based on 16S rRNA gene sequences (1466 nt) showing the position of *P. jeanii* sp. nov. The optimal tree with the sum of branch length = 0.30919266 is shown. The evolutionary distances were computed using the Jukes-Cantor method. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) based on 1000 resamplings are shown. *V. cholerae* was used as outgroup. Bar, 1 % estimated sequence divergence.

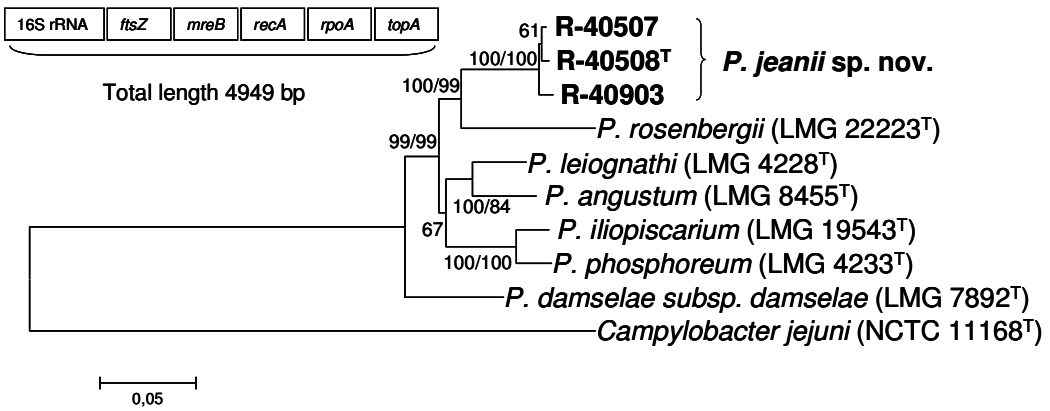

Figure 2. Neighbour-joining phylogenetic tree based on concatenated 16S rRNA, *ftsZ, mreB, recA, rpoA* and *topA* gene sequences (4.949 nt) showing the position of *P. jeanii* sp. nov. The evolutionary distances were computed using the Jukes-Cantor method. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) based on 1000 resamplings are shown. *C. jejuni* was used as outgroup. Bar, 5 % estimated sequence divergence.

Several phenotypic features can be used to differentiate the novel species from its closest phylogenetic neighbors. The novel species has gelatinase and lipase activity, and it can ferment melibiose, but it cannot grow on 6 % NaCl (**Table 2**). In addition, the novel species lacks the fatty acids $C_{17:0}$, $C_{17:0}$ cyclo, $C_{17:0}$ iso, $C_{17:1}$ ω8*c*, and $C_{17:1}$ ω9*c* iso, which are commonly found in *Photobacterium* and *Vibrio* species (**Table 3**). For instance, $C_{17:0}$ iso is found in *P. leiognathi, P. rosenbergii* and *P. lutimaris*, whereas $C_{17:0}$ is found in *P. leiognathi* and *P. halotolerans*. Phenotypic and chemotaxonomic variation was observed among the novel species, suggesting a good representation of the phenotype of the novel group (**Supplementary Table S2**). Based on the polyphasic analysis presented in this study, we propose to classify the four isolates in the new species, *Photobacterium jeanii* sp. nov.

Table 2. Phenotypic differences between *P. jeanii* sp. nov. and related *Photobacterium* species

Species: **1,** ***P. jeanii*** sp. nov. (four strains); **2,** ***P. leiognathi*** LMG 4228T (Baumann & Baumann, 1984; Nogi *et al.,* 1998); **3,** ***P. rosenbergii*** LMG 22223^T (Thompson *et al*., 2005a); **4,** ***P. lutimaris*** LMG 25278^T (Jung *et al.,* 2007); **5**, ***P. halotolerans*** LMG 22194^T (Rivas *et al*., 2006); **6**, ***P. ganghwense*** FR 1311^T (Park *et al*., 2006); **7**, ***P. lipolyticum*** KCTC 10562^T (Yoon *et al*., 2005); **8,** ***P. phosphoreum*** LMG 4233^T (Nogi *et al*., 1998; Ast *et al*., 2007; Yoshizawa *et al*., 2009), **9**, ***P. angustum*** CIP 75.10^T (Baumann & Baumann, 1984; Nogi *et al*., 1998; Yoshizawa *et al.,* 2009); **10**, ***P. aquimaris*** LC2-065T (Yoshizawa *et al*., 2009); **11**, ***P. indicum*** LMG 22857^T (Xie & Yokota, 2004; Ast *et al*., 2007) and **12,** ***P. profundum*** (Nogi *et al.,* 1998). +, Positive; -, negative; w, weak; v, variable; ND, not available. All taxa are negative for Gram stain, lysine- and ornithine- decarboxilase. Data in parentheses are for the type strains.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth at:												
6 % (w/v)NaCl	-	+	+	+	+	+	-	-	+	-	ND	ND
4ºC	-	-	-	+	+	+	+	+	-	-	+	+
Optimum growth temperature (ºC)	20-35	(26)	20-30	25-30	28	35	25-28	18	25	10-25	25	10
Catalase	+	(-)	+*	w	+	+	+	+	-	+	-	+
Oxidase	+	(+)	+	+	+	+	+	+	-	-	-	+
API ZYM:												
Esterase (C 4)	+	+	+	+	-	+	+	w	w	-	ND	ND
Esterase lipase (C8)	+	+ *	+	+	-	+	+	-	w	-	ND	ND
Lipase (C14)	+	-	(+)	-	+*	-	-	-[a]	-[a]	-	ND	+[a]
Valine arylamidase	w	-	+	-	-	-	-	w	-	-	ND	ND
Trypsin	+	w *	-	-	-	-	-	ND	ND	-	ND	ND
Naphthol-AS-BI phosphohydrolase	+	+ *	+	+	-	+	+	ND	ND	+	ND	ND
α-galactosidase	-	-	(+)	-	-*	-	-	-	-	w	ND	ND
α-glucosidase	+	- *	(+)	-	+*	+	-	ND	ND	-	ND	ND
API 20E:												
Arginine dihydrolase	+	+	+	+	-	+	-	-	-	-	+	+
Citrate	-	-	+	+	+	+	-	-	+	-	-	-
Tryptophane deaminase	-	- *	-	-	w	-	ND	-	ND	-	w	ND
Indole production	-	(-)	-	+	-	+	+	-	-	ND	+	+
Acetoin production	(w)	+	-	-*	-	-	-	-	+	ND	-	+
Gelatinase	+	-	-	-	+	+	-	-	v	-	-	ND
Inositol	-	-	(+)	+	-	+	-	-	-	ND	-	-
Melibiose	+	-	+	-	-	-	-	-	-	ND	-	-
Amygdalin	-	- *	+	+*	-	+	ND	-	ND	ND	-	ND
Arabinose	-	-	-	-	+	-	-	-	-	ND	-	-
DNA G+C content (mol%)	45.1-45.5	40-44	47.6-47.9	48.3	49.8	44	47	39.1	40-42	42.9	40	42

Data obtained in this study, [a] Seo *et al*. (2005).

Table 3. Cellular fatty acids content of *Photobacterium jeanii* sp. nov. and related taxa of the genus *Photobacterium*.

Taxa: 1, *P. jeanii* (range profile of R-40508^T (LMG 25436^T), R-40507, R-40903 and R-21419); 2, *P. leiognathi* LMG 4228^T; 3, *P. rosenbergii* LMG 22223^T; 4, *P. lutimaris* LMG 25278^T; 5, *P. halotolerans* LMG 22194^T. Summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE. Summed feature 3 comprises $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*. Data are expressed as percentages of total fatty acids. Fatty acids representing <1 % are not shown. Data obtained in this study.

Fatty acid	1	2	3	4	5
$C_{12:0}$	2.8-3.6	3.9	3.2	2.5	5.5
$C_{12:0}$ 3-OH	2.5-3.9	2.6	3.5	2.3	6.5
$C_{14:0}$	3.4-4.5	4.2	3	2.5	-
$C_{15:0}$ iso	0-2	1	2	1.6	-
$C_{16:0}$	19.5-21.9	26	12.5	17.5	18.5
$C_{16:0\ iso}$	1.9-3.5	-	-	-	2
$C_{17:0}$	-	1.6	-	-	1.5
$C_{17:0}$ cyclo	-	10.6	-	-	-
$C_{17:0}$ iso	-	1.2	6	1.8	-
$C_{17:1}$ω8*c*	-	-	-	-	1.6
$C_{17:1}$ω9*c* iso	-	-	1.5	-	-
$C_{18:1}$ω6*c*	0-10.9	-	9.3	5	-
$C_{18:1}$ω7*c*	8.5-15	10	8.5	16	22.5
Summed feature 2	2.4-3.5	3.2	3.5	2.6	4.5
Summed feature 3	37.2-45	30.5	39	42	27

Description of *Photobacterium jeanii* sp. nov.

Photobacterium jeanii (jea´nii N.L. gen. n. jeanii of Jean, after the Belgian microbiologist Jean Swings).

Colonies are convex, round (1 mm in diameter), beige and opaque with entire and smooth margins after 2 days at 28 °C on MA. Cells are small cocco-bacilli Gram-negative, motile and catalase and oxidase-positive. Cells are 2-2.5 µm long and 1-2 µm wide after 1 day at 28 °C in MA. Green colonies with poor growth appear on the selective medium TCBS.

Prolific growth occurs between 20 and 35 °C and at NaCl concentrations (w/v) of 0,5-2 % in TSA. No growth appears at 4, 7 or 42 °C, or in 0 or 6 % NaCl. Positive for alkaline phosphatase, esterase (C 4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase (weak reaction), tripsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase (except R-40903), α-glucosidase, N-acetyl-β-glucosaminidase (except R-21419 and R-40508^T), arginine dihydrolase, acetoin production (weak for R-40508^T and R-40903) and gelatinase. Cells ferment glucose, melibiose, sucrose (except R-40507 and R-40903), and reduce nitrate to nitrite. Cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophane deaminase activities are absent. Citrate is not utilized, and H_2S and indole are not produced. Mannitol (except R-40903), inositol, sorbitol, rhamnose, amygdalin and arabinose are not fermented. The G+C content of the type strain (R-40508^T) is 44.5 mol%. The most abundant cellular fatty acids are summed feature 3 ($C_{15:0}$ iso 2-0H and/or $C_{16:1}$ ω7*c*), $C_{16:0}$, $C_{18:1}$ ω7*c*, and $C_{18:1}$ ω6*c*. The following fatty acids are detected in small amounts: $C_{14:0}$, $C_{16:0}$ iso, $C_{12:0}$, $C_{12:0}$ 3-OH, summed feature 2 ($C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE) and $C_{15:0}$ iso. The type strain is R-40508^T (= LMG 25436^T = CAIM 1817^T). It was isolated from the mucus of *Palythoa caribaeorum* in São Sebastião channel, Brazil.

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Supplementary data

Supplementary Table S1. GenBank accession numbers of the 16S rRNA gene and housekeeping gene sequences of the four strains of *Photobacterium jeanii* sp. nov. ND, no data available.

Strains	16S rRNA	*ftsZ*	*mreB*	*recA*	*rpoA*	*topA*
R-40508^T	GU065210	GU065217	GU065220	GU065223	GU065226	GU065214
R-40507	GU065209	GU065216	GU065219	GU065222	GU065225	GU065213
R-40903	GU065211	GU065218	GU065221	GU065224	GU065227	GU065215
R-21419	GU065212	ND	ND	ND	AJ842701	ND

Supplementary Table S2. Phenotypic variability amongst the strains of *P. jeanii* sp. nov.

Test of	R-40507	R-40508^T	R-40903	R-21419
N-acetyl-β-glucosaminidase	w	-	+	-
ONPG (β-galactosidase)	+	+	-	+
Acetoin production (VP)	+	w	w	+
Fermentation of:				
Mannitol	-	-	+	-
Sucrose	-	+	-	+

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IV.5. *Photobacterium swingsii* sp. nov. isolated from marine organisms.

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Footnote: GenBank accession numbers for the 16S rRNA gene are GQ386816 to GQ386818 and GQ386820 to GQ386822.

Summary

Six strains were isolated from oysters (*Crassostreae gigas*) and from the haemolymph of the spider crab (*Maja brachydactyla*) from Mexico and Spain, respectively. All isolated grew as small green colonies in TCBS agar, were Gram negative cocco-bacilli, had a facultative metabolism, were oxidase positive, and sensitive to the vibriostatic agent 0/129. Rep-PCR analysis of the isolates permitted to observe a high degree of genomic homogeneity among the seven strains analyzed. Several phenotypic traits differentiate these strains from other closely related species. DNA hybridization values between two representative strains and the type strains of the closest phylogenetic species, by 16S rRNA sequence similarities, CAIM 20^T (*P. frigidiphilum*) and CAIM 14^T (*P. aplysiae*) were between 53.85 and 44.01 %. These results permitted us to propose a new *Photobacterium* species, named *P. swingsii* with CAIM 1393^T (= CECT 7576^T) as the type strain.

Photobacterium is one of the oldest genera of the Vibrionaceae family of the gammaproteobacteria. The first species described in 1878 by F. Cohn was *Micrococcus phosphoreus*, latter renamed as *P. phosphoreum* by M. W. Beijerinck (1889). Currently, this genus contains 17 species (www.vibriobiology.net) with a worldwide distribution. Most of them show luminescence and are able to grow in general media but also in selective media like the widely used medium for vibrios thiosulphate-citrate-bile salts-sucrose (TCBS). Some strains are pathogenic for aquatic animals (*P. damselae* subsp. *piscicida*) but for most, no pathogenic traits have been described and are common inhabitants of marine ecosystems and also found living inside marine animals.

During a survey of the vibrios present in cultured oysters in Mexico and those found in the haemolymph of the spider crab in Spain, six isolates clustered closely by a genomic characterization methodology (rep-PCR) and apart from all other isolates analysed. The isolates were obtained from oyster (*Crassostreae gigas*) homogenates cultured in three different localities (**Table 1**) and also from the haemolymph of spider crabs (*Maja brachydactyla*) collected from the wild in the Canary Islands. Another two strains were isolated from corals in Brazil but died before they were characterized and only the 16S rRNA sequence of one (R13) was obtained. The strains were deposited at the Collection of Aquatic Important Microorganisms (CAIM, www.ciad.mx/caim) and two strains at the Colección Española de Cultivos Tipo (CECT, www.cect.org).

Table 1. Strains of *P. swingsii* sp. nov.

Isolate	**Source**	**Locality**	**Isolation date**	**Coordinates**
CAIM 1439	Oyster (*C. gigas*) homogenates	Manuela lagoon, Mulegé, Baja California, México	7 Nov. 2003	28° 01' 23'' N 114° 06' 22'' W
CAIM 1119	Oyster (*C. gigas*) homogenates	El Cardón estuary, Baja California Sur, México	7 Nov. 2003	26° 47' 36'' N 108° 50' 53'' W
CAIM 1206	Oyster (*C. gigas*) homogenates	La Cruz, Sinaloa, México	13 Feb. 2004	24° 05' 44'' N 108° 50' 46'' W
CAIM 1393^T CAIM 1394 CAIM 1395	Haemolymph of the spider crab (*Maja brachydactyla*)	Taliarte bay, Gran Canaria, Canary Islands	30 Nov. 2006	27° 59' N 15° 22' W

The isolates were collected in TCBS agar as small green round colonies 2-3 mm in diameter, non-luminescent with smooth borders. In Marine agar, after 24 h, they are also small colonies (2-3 mm), whitish, smooth, and non-luminiscent. CAIM 1119 and CAIM 1206 show weak agarolytic activity in Marine agar.

The phenotypic characterization of the strains was done with standard methods as described elsewhere (Macian *et al.*, 2001) and with the API 20E (Bio-Meriux, France). The strains were grown in TSA + 2.0 % NaCl and a suspension was prepared to a bacterial density that equals a 0.5 MacFarland standard in 2.5% sterile saline solution. Tests included the determination of temperature and salinity growth ranges, diverse biochemical responses, and the use of 49 substrates as sole carbon and energy sources (Macian, Ludwig, Aznar, Grimont, Schleifer, Garay & Pujalte, 2001). Results of the phenotypic test are presented in the species description. Several phenotypic tests were found to be able to differentiate these strains from closely related *Photobacterium* species (**Table 2**) and also some tests were found to differ between the strains (**Table 3**).

The 16S rRNA was amplified with universal primers (V16S-9F, AGA GTT TGA TCA TGG CTC AG; V16S-1491R, AGC GCT ACC TTG TTA CGA CTT) to obtain an almost complete sequence (>1450 bp). Amplification program was one cycle at 94° C for 2 min, 35 cycles at 94 °C for 1 min, 56 °C for 1min, 72 °C for 1min; and one final cycle at 72 °C for 5 min. Sequencing and PCR purification was done at Macrogen (Korea) The 16S rRNA sequences placed these isolates as belonging to the *Photobacterium* genus with *P. frigidiphilum* (96.8-95.6 % similarity, Jukes-Cantor correction, based on six sequences) and *P. aplysiae* (96.7-95.5 %) as their closest phylogenetic species (**Fig. 1**). The maximum difference found between the six strains of this new species analyzed was 98.6 %. GenBank accession numbers are GQ386816 to GQ386818 and GQ386820 to GQ386822

Table 2. Phenotypic differences between *P. swingsii* sp. nov. and its closest phylogenetic neighbours.

Data obtained in this study, except those taken from the cited authors (in parenthesis). Species: 1, *P. swingsii* sp. nov. (six strains); 2, *P. frigidiphilum* (CAIM 20^T); 3, *P. aplysiae* (Seo *et al.*, 2005); 4, *P. profundum* (Nogi *et al.*, 1998); 5, *P. lipolyticum* (Yoon *et al.*, 2005); 6, *P. phosphoreum* (CAIM 328^T); 7, *P. iliopiscarium* (CAIM 909^T).

Test	1	2	3	4	5	6	7
Arginine dihydrolase	+	-	+	+	-	NA	NA
Lysine decarboxylase	-	+	NA	-	-	NA	NA
Citrate	-	+	NA	NA	NA	NA	NA
Indole production	-	-	-	+	+	-	NA
Hydrolisis of gelatin	(+)	+	+	NA	-	NA	NA
Growth at							
6% NaCl	+	+	-	NA	-	NA	NA
4ºC	(+)	-	-	+	+	NA	NA
30ºC	+	+	+	-	+	NA	NA
Utilization of							
D-cellobiose	+	+	+	-	-	+	+
D-fructose	+	+	+	-	+	-	+
D-galactose	+	+	+	+	-	-	-
D-glucuronate	-	-	NA	NA	NA	-	+
D-maltose	+	+	+	+	+	-	+
D-mannose	+	+	+	+	-	-	+
D-ribose	+	+	NA	NA	NA	-	+
D-salicin	+	-	NA	NA	NA	-	-
D-trehalose	+	+	+	+	-	-	+
Fumarate	+	+	NA	NA	NA	-	+
Glycine	+	+	NA	NA	NA	+	+
Ketoglutarate	+	+	+	NA	NA	-	+
L-alanine	+	+	+	NA	NA	-	+
L-arabinose	+	+	-	-	-	+	+
L-aspartate	+	+	+	NA	NA	-	+
L-glutamate	+	+	+	NA	NA	-	+
L-glutamine	+	+	NA	NA	NA	-	+
L-threonine	+	+	+	NA	NA	-	-
Methanol	+	+	NA	NA	-	+	+
Piruvate	+	+	NA	NA	+	-	+
Propionate	+	+	NA	NA	NA	-	+
Sucrose	-	+	+	-	+	+	+

+, positive; -, negative; (+), most positive; (-), most negative; NA, not available.

Table 3. Phenotypic variability within the strains of *P. swingsii* sp. nov.

Test	CAIM 1119	CAIM 1206	CAIM 1439	CAIM 1394	CAIM 1395	CAIM 1393^T
Utilization of						
D, L-lactate	+	+	-	-	-	+
Putrescine	-	-	+	-	+	-
D-mannitol	-	-	+	-	+	-
Growth at 35 ºC	-	-	-	-	+	+
ONPG (β-galactosidase)	-	-	-	+	+	+

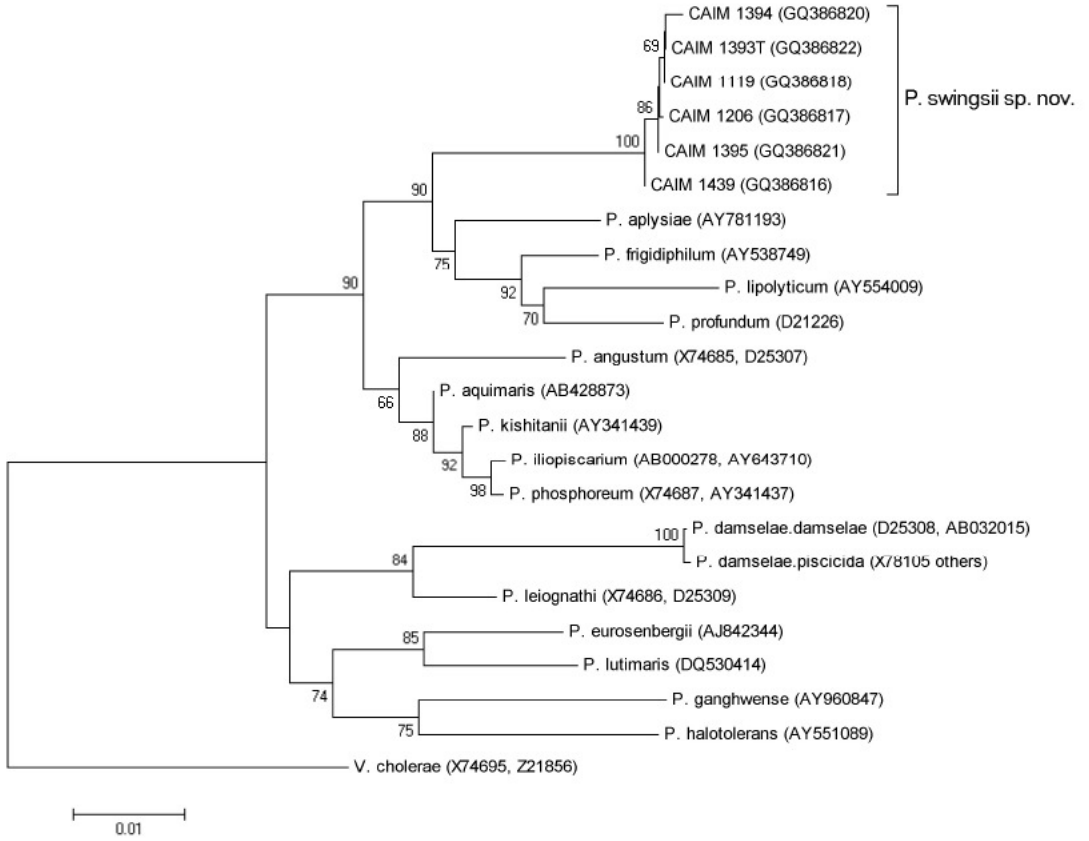

Figure 1. Dendrogram of *Photobacterium* species based on the almost complete 16S rRNA sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 0.31183234 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes *et al.*, 1969) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1336 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Kumar *et al.*, 2001).

To determine the genomic similarity of the strains, they were fingerprinted by rep-PCR as described previously (Cabanillas-Beltran *et al.*, 2006). Briefly, DNA of the strains was extracted with a commercial kit (Wizard Genomic DNA purification, Promega, USA) according to the manufacture's instructions. The DNA was adjusted to 50 ng μl^{-1} and amplified with the GTG_5 primer (GTG GTG GTG GTG), the amplification products were electrophoreticaly resolved (1.2 % 20 x 20 cm agarose gel for 140 min at 90 V in 1X TAE buffer at room temperature) and the resulting bands were analyzed with the GelCompar II software (ver. 4.5, Applied-Maths). The similarity matrix was calculated with the Jaccard coefficient (Kosman & Leonard, 2005) with a band position tolerance of 0.59 % (optimization of 0.34 %); the dendrogram was constructed with the Ward algorithm. Results showed strain genomic homogeneity among the six strains analyzed (**Figure 2**), but clearly different than the type strains of closely related species of *Photobacterium*.

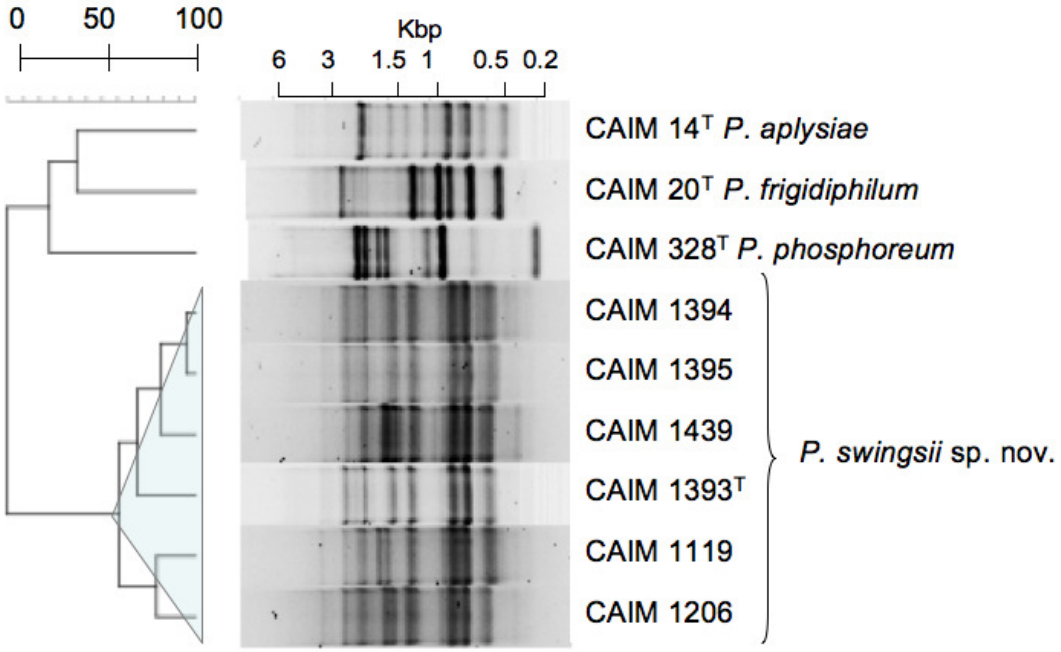

Figure 2. Genomic comparison of *P. swingsii* sp. nov. based on rep-PCR analysis. Similarities (scale) made with the Jaccard coefficient and the dendrogram generated with the Ward algorithm (optimization 0.34 % and band position tolerance of 0.59 %).

The % G+C was determined by quantitative PCR as proposed by Gonzalez & Saiz-Jimenez (2002) with some modifications. The strains were grown in 50 ml of TSB+2.0 % NaCl under constant agitation, they were centrifuged at 4,000 rpm for 30 min at 4 °C, washed with TAE 1X buffer and resuspended in 467 µl of TAE 1X gently with the pipette. 50 µl of 10 % SDS and 4 µl of 20 mg ml-1 of proteinase-K were added and incubated at 37 °C for 45 min and then at 65 °C for 15 min. 550 µl of phenol-choloform-isoamilic alcohol (24:24:1, Sigma) was added, mixed, and centrifuged (14,000 rpm 5 min at room temperature). The upper aqueous phase was transferred to a Phase Lock Gel (5 Prime) tube and one volume of phenol-choloform-isoamilic alcohol was added, and centrifuged again. The supernatant was transferred to a new tube and 4 µl of RNAase (Promega) were added and incubated at 37 °C for 30 min. A volume of chloroform-isoamyl alcohol (Sigma) was added, mixed and transferred to a Phase Lock Gel tube, centrifuged and the liquid passed on to a new tube. 1/10 volume of cold sodium acetate and 0.6 volumes of cold isopropanol were added, mixed until de DNA precipitates, and collected by spinning it with a heat-sealed Pasteur pipette. The DNA concentration was measured spectrophotometrically and adjusted to 1 µg μl^{-1}.

For the G+C mol% determination, a solution was prepared with 7.5 µl SYBR Green I (Molecular probes) at 0.1 µl ml^{-1}, 7.5 µl SSC 1X, 54.0 µl H_2O, and 6 µl DNA at 1.0 µg μl^{-1}, and dispensed in triplicate in a 96-well microtiter plate (25 µl in each well). The thermal denaturation was done in an iCycler real time thermocycler (ver. 3.0, Bio-Rad) with the following protocol: one step at 25 °C for 15 min, followed by 0.2 °C increments for 12 s until 100 °C was reached, and a final step of 60 °C for 30 min. Fluorimetric detections were done every 0.2 °C during the ramp. The melting temperature (T_m) was calculated with the negative first derivative of the temperature versus fluorescence (-dF/dT). The G+C mol% was calculated with a formula obtained with the thermal denaturation analyses of five strains whose genome is fully sequenced and therefore their %GC is known. The strains employed where *Bacillus subtilis* CECT 461 (43.5 %G+C), *Lactobacillus lactis* CECT 4433 (5.3 %GC), *Escherichia coli* CECT 433 (50.8 %G+C), *Pseudomonas aeruginosa* CECT 4122 (66.6 %G+C), *Vibrio campbellii* ATCC-BAA 1116 (45.4 %G+C), and *Corynebacterium glutamicum* CECT 77 (53.8 %G+C). The

formula obtained was %GC = 2.002 T_m – 111.5 (R^2 = 0.97). %G+C values for CAIM 1393^T was 46.7 % (T_m = 79.0 °C); for CAIM 1439, 47.5 % (T_m = 79.4 °C); and for CAIM 1394, 48.7 % (T_m = 80.0 °C).

Genomic DNA for DNA-DNA hybridizations was extract with the Dneasy® Blood & Tissue Kit (QIAGEN). DNA-DNA hybridization experiments were undertaken by the hidroxyapatite/microtitre plate method (Ziemke *et al.*, 1998), with a hybridization temperature of 60 ºC. Levels of DNA-DNA relatedness were determined between strain CAIM 1393^T and CAIM 1439 against the type strains of *Photobacterium aplysiae* CAIM 14^T and *P. frigidiphilum* CAIM 20^T. Reciprocal reactions were also performed and the DNA homology percentages reported are the means of a minimum of three hybridizations. CAIM 1393^T and *P. aplysiae* had a DNA-DNA value of 51.44% (reciprocal value 53.8 %); CAIM 1393^T and *P. frigidiphilum*, 44.01% (reciprocal value 50.0 %); and CAIM 1393^T and CAIM 1439, 86.5 % (reciprocal 89.6%).

These values and all the other presented data clearly define these strains as pertaining to a new *Photobacterium* species, for which the name of *P. swingsii* is proposed.

Description of *Photobacterium swingsii* sp. nov.

P. swingsii [swin.gs'i.i. N.L. gen. n. *swingsii* of Swings, in honour of the Belgian microbiologist Jean Swings].

Gram-negative small coccobacilli, oxidase-positive and facultatively anaerobic. Arginine dehydrolase positive and lysine and ornithine decarboxylase negative. Positive for Voges-Proskauer test, nitrate reduction, gelatine hydrolysis (except CAIM 1119), catalase (except CAIM 1395). Negative for citrate, H_2S production, indol production, triptophan deaminase (except CAIM 1395), and urease. Sensitive for the vibriostatic agent 0/129 at 10 and 150 μg. Able to grow in the presence of 3 and 6 % of NaCl, but not in 0 and 10 %; only CAIM 1395 could grow in 8 % NaCl. They could grow at 4 (except CAIM 1395), 20, and 30 °C, but not at 35 (except CAIM 1393^T and CAIM 1395) or 40 °C.

The strains tested utilized as sole source of carbon of acetate, amygdaline (except CAIM 1119), citrate, D-cellobiose, D-fructose, D-galactose, D-glucosamine, D-glucose, D-

maltose, D-mannose, D-melibiose (except CAIM 1395), D-ribose, D-salicin, D-trehalose, fumarate, glycerol, glycine, ketoglutarate, L-alanine, L-arabinose, L-aspartate, L-glutamate, L-glutamine, L-ornithine, L-threonine, methanol, piruvate, propionate, and succinate. None of the strain could utilize D-galacturonate, D-gluconate (except CAIM 1395), D-glucuronate, D-sorbitol, D-xylose, inositol, lactose (except CAIM 1395), L-histidine (except CAIM 1395), L-lisine, L-leucine (except CAIM 1395), L-ramnose, malate, M-inositol, p-hydroxybenzoate, sucrose, and γ-aminobutyrate (except CAIM 1395). Production of acid phosphatase, alkaline phosphatase, esterase lipase (C 8), lipase (C 14), leucine arylamidase, naphthol-AS-BI7 phosphohydrolase, valine arylamidase (except CAIM 1119); weak production of esterease (C 4) (CAIM 1119 negative). Negative production of α-chymotrypsin, α- fucosidase, α-galactosidase, α-mannosidase, β-galactosidase, β-glucosidase, β- glucuronidase, and α-glucosidase (except CAIM 1119 weakly positive). Variable results were obtained between the strains for eight tests (**Table 3**).

The type strain is CAIM 1393^T (=CECT 7576^T), the type strain and reference strains CAIM 1394 and CAIM 1395 were isolated from the haemolymph of wild spider crab (*Maja brachydactyla*) collected in the Canary Islands, Spain. The reference strains CAIM 1439, CAIM 1206, and CAIM 1119 were isolated from oyster (*Crassostrea gigas*) homogenates in north-western Mexico.

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IV.6. *Marinobacterium coralli* sp. nov. isolated from coral mucus (*Mussismilia hispida*).

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***Marinobacterium coralli* sp. nov., isolated from coral mucus (*Mussismilia hispida*)**

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Running Title:

Marinobacterium coralli sp. nov.

Subject Category: New taxa, *Proteobacteria*

Footnote: The GenBank/EMBL accession number for the 16S rRNA gene sequence of strain R-40509^T is GU183820.

Abstract

A Gram-negative, aerobic bacterium, designated R-40509^T, was isolated from mucus of the reef builder coral *Mussismilia hispida,* located in the São Sebastião Channel, São Paulo, Brazil. The strain is oxidase positive and catalase negative, and requires Na^+ for growth. Its phylogenetic position is in the genus *Marinobacterium,* and .the closest related species are *Marinobacterium sediminicola*, *Marinobacterium maritimum* and *Marinobacterium stanieri,* with 97.5 to 98.0 % 16S rRNA gene sequence similarity. The 16S rRNA gene sequence similarity.towards the other type strains of the *Marinobacterium* species are below 96 %. DNA-DNA hybridizations of R-40509^T against the type strains of the phylogenetically closest species of *Marinobacterium* revealed less than 70 % DNA-DNA relatedness, supporting novel species status of the strain. Phenotypic characterization revealed that the strain was able to grow between 15 and 42 °C, and in medium containing up to 9 % NaCl. The strain can be differentiated from the phenotypically related species by several features, including its ability to utilize D-alanine, L-alanine, bromo succinic acid, β-hydroxy butyric acid and α-ketovaleric acid but not acetate or L-arabinose. It produces acetoin (Voges Proskauer) but has not esterase lipase (C8) or catalase activities. It possesses C18:1 ω7*c* (35 %), summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c,* 25 %) and $C_{16:0}$ (22 %) as major cellular fatty acids. The G+C content of its DNA is 58.5 mol%. The name *Marinobacterium coralli* sp. nov. is proposed for this new taxon. The type strain is R-40509^T (= LMG 25435^T = CAIM 1449^T).

Coral reefs are the most diverse marine biomes on Earth. They are constructed by a variety of invertebrates (e.g. corals, algae, and sponges), serving as nurseries for marine life. These corals may secrete copious amounts of mucopolysaccharide material that contributes to the suspended organic matter in reefs (Wild *et al.,* 2004). Coral mucus plays an important role as a carrier of energy and nutrients to a range of planktonic and

benthic consumers (Ducklow & Mitchel, 1979). Moreover, it represents an important resource for microbial growth in reef ecosystems (Brown & Bythell, 2005). Corals harbour diverse microbial communities, and are better seen as holobionts (i.e. coral host+zooxanthellae+microbes) (Brown & Bythell, 2005, Rosenberg *et al.,* 2007). In these holobionts, mutualistic interactions such as microbial nitrogen and carbon fixation are key processes that allow bioproductivity in the reefs. The key role of bacteria in coral health is well-documented by various examples of symbiotic and pathogenic relationships (Rosenberg *et al.,* 2007). Bacteria living in coral mucus or tissue may act as a first line of defence for their holobiont hosts, and protect them against diseases (Ritchie, 2006, Reshef *et al.*, 2006, Shnit-Orland & Kushmaro, 2009).

In the present study a Gram-negative, aerobic bacterium R-40509^T, isolated from mucus of the coral *Mussismilia hispida,* one of the main reef builders of the South Atlantic Ocean, was investigated using a polyphasic taxonomic approach. Strain R-40509^T was isolated in the summer of 2006 from apparently healthy coral located in the São Sebastião Channel at Preta beach (coordinate 23°49´10´´S; 045°24´37´´W), São Paulo, Brazil. It was obtained from Marine Agar medium (MA, Difco) after incubation for 48h at 28 °C as described previously by Chimetto *et al.* (2008, 2009). It was tentatively allocated to the genus *Marinobacterium* which comprises 11 species from different sources and locations. *M. georgiense* originated from marine pulp mill effluent enrichment cultures (González *et al*., 1997). *M. stanieri* (Bauman *et al.*, 1983, Satomi *et al.,* 2002) and *M. jannaschii* (Bowditch *et al*., 1984, Satomi *et al.,* 2002) were isolated from coastal sea water, and *M. litorale* and *M. marisflavi* (Kim *et al*., 2007, 2009a) from sea water of the Yellow sea. *M. halophilum* (Chang *et al*., 2007) and *M. lutimaris* (Kim *et al.,* 2009b) were isolated from tidal flats (Getbol), while *M. rhizophilum* originated from roots of plants inhabiting a coastal tidal flat (Kim *et al*., 2008). *M. nitratireducens* and *M. sediminicola* were isolated from sea sediment (Huo *et al*., 2009), while *M. maritimum* was isolated from Artic marine sediment (Kim *et al*., 2009c).

Bacterial genomic DNA and 16S rRNA gene sequences were obtained as described previously (Thompson *et al*., 2001, Chimetto *et al*., 2008, 2009). Raw sequence data were transferred to the ChromasPro ver. 1.34 software (Technelysium Pty. Ltd, Tewantin,

Australia), where consensus sequences were determined. Sequences were aligned using the ClustalW software (Chenna *et al*., 2003). Pairwise similarities were calculated with the BioNumerics 4.5 software (Applied Maths, Belgium), using an open gap penalty of 100 % and a unit gap penalty of 0 %. Similarity matrices and phylogenetic trees were constructed using the MEGA ver. 4.0 software (Tamura *et al*., 2007) and the BioNumerics 4.5 software (Applied Maths, Belgium). Trees were drawn using the Neighbour-Joining (Saitou & Nei, 1987), Maximum Parsimony (Eck & Dayhoff, 1966) and Minimum Evolution methods (Rzhetsky & Nei, 1992). The robustness of the topologies of the trees was checked by 1000 bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/).

DNA-DNA hybridization (DDH) experiments were performed using the microplate method described by Ezaki *et al*. (1989), with minor modifications (Willems *et al*., 2001). Hybridizations were performed at 46 °C in the presence of 50 % formamide in four replicates. Reciprocal reactions were performed for every DNA pair in four replicates and their variation was within the limits of this method (Goris *et al.,* 1998). DNA G+C contents were determined by HPLC as described previously (Mesbah *et al*., 1989). Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). For fatty acid analysis, cells of the novel strain and the type strains of the closest known species (*M. sediminicola, M. Maritimum,* and *M. stanieri*) were grown on MA (Difco) for 24 h at 28 °C under aerobic conditions. Phenotypic characterization was performed using the API ZYM, API 20E and API 20NE kits (bioMérieux, France), and the Biolog GN2 microwell plates (Biolog Inc., USA), according to the manufacturer's instructions with minor modifications. Namely, cell suspensions for inoculation of the API tests were prepared in a 3 % (w/v) NaCl solution, and those for the Biolog GN2 microwell plates showed a turbidity (transmission) of 20 %. Cells for the suspensions were grown on Biolog medium for 24 h at 28 °C under aerobic conditions. Tests were read after 24 to 48 h of incubation at 28 °C. Growth at different temperatures (4–45 °C) and salt concentrations (0–14 % NaCl) was determined by incubation on TSA (Difco) at 28 °C for 72h. Catalase activity was determined by adding young cells to a drop of a 3 % H_2O_2

solution and observation of 0_2 production. Oxidase activity was tested using 1 % N,N,N',N'-tetramethyl *p*-phenylenediamine (Kovacs, 1956).

Based on 16S rRNA gene sequence analyses, the phylogenetic position of the novel isolate R-40509^T (16S rRNA gene sequence of 1349 nt), was in the genus *Marinobacterium*, and more precisely in a robust phylogenetic subcluster containing the species *M. sediminicola, M. maritimum and M. stanieri* (**Fig. 1**). The 16S rRNA gene sequence similarity of R-40509^T to these species ranged from 98.0 to 97.5 %, while it was below 96 % to the other known *Marinobacterium* species.

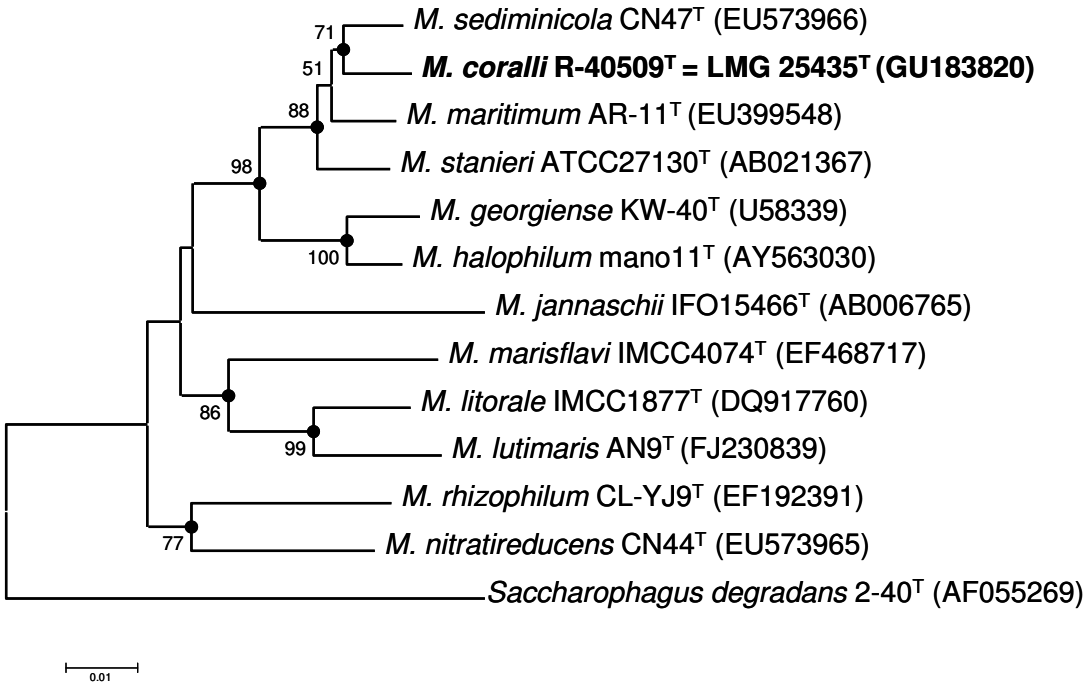

Figure 1. Neighbour-joining tree showing the phylogenetic position of *M. coralli* sp. nov. based on 16S rRNA gene sequences. The evolutionary distances were computed using the kimura 2-parameter method (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted using MEGA4 software. Bootstrap values (> 50 %) based on 1000 resamplings are shown. Solid circles indicate that bootstrap values > 60 % were recovered in the maximum-parsimony and the minimum evolution trees. *Saccharophagus degradans* was used as outgroup. Bar, 1 % estimated sequence divergence.

DNA-DNA hybridization (DDH) experiments were performed between the novel strain R-40509^T and the type strains of the closest phylogenetic neighbours (i.e. *Marinobacterium sediminicola*, *Marinobacterium maritimum* and *Marinobacterium stanieri*) (**Table 1**). The DNA-DNA relatedness values for R-40509^T against these species were 35 % (with *M. maritimum* LMG 25352^T), 29 % (with *M. sediminicola* LMG 25280^T) and 22 % (with *M. stanieri* LMG 6847^T). It can be concluded that strain R-40509^T represents a novel species in the genus *Marinobacterium* (Wayne *et al*., 1987; Stackebrandt & Ebers, 2006). The G+C content of the DNA of strain R-40509^T was 58.5 mol% (**Table 1**).

Table 1. DNA-DNA hybridization data, 16S rRNA gene sequence similarities and DNA G+C contents of *M. coralli* sp. nov. and related *Marinobacterium* species

Strain	**G+C content (mol%)**	**16S rRNA similarity**	**DNA-DNA relatedness values (%)**			
		1	**1**	**2**	**3**	**4**
1. *M. coralli* sp. nov. R-40509^T (LMG 25435^T)	58.5	100	100	29	30	20
2. *M. maritimum* LMG 25352^T	57.9[a]	97.8	42	100	39	24
3. *M. sediminicola* LMG 25280^T	56.3[b]	98.0	28	30	100	15
4. *M. stanieri* LMG 6847^T	56.0	97.5	24	26	22	100

[a] Kim *et al*., 2009c
[b] Huo *et al* ., 2009

Phenotypic characteristics were determined for the novel species and the type strains of the closest phylogenetic *Marinobacterium* species. The novel species can be differentiated from its closest phylogenetic neighbors by several phenotypic features (**Table 2**). The novel species grows in medium containing 9 % NaCl, utilizes D-alanine, L-alanine, bromo succinic acid, β-hydroxy butyric acid and α-ketovaleric acid but not acetate or L-arabinose. It produces acetoin (Voges Proskauer) but has not esterase lipase (C8) or catalase activities. The fatty acids $C_{10:0}$ and $C_{17:0}$ CYCLO, and the unknown fatty acid ECL 11.799 can also be used for differentiation, of the novel species from its closest phylogenetic neighbours (**Table 2**). The major cellular fatty acids of R-40509^T are $C_{18:1}$ ω7*c* (35 %), summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*) (25 %) and $C_{16:0}$ (22 %) (**Supplementary Table S1**).

Based on the polyphasic analysis presented in this study, we propose to classify strain R-40509^T in the new species *M. coralli* sp. nov.

Table 2. Phenotypic differences between *Marinobacterium coralli* sp. nov. and its closest phylogenetic neigbours.

Species: **1**, *M. coralli* sp. nov. R-40509^T (LMG 25435^T); **2**, *M. maritimum* LMG 25352^T; **3**, *M. sediminicola* LMG 25280^T and **4**, *M. stanieri* LMG 6847^T. Data for the reference species were obtained in this study.except when indicated. Abbreviations: +, positive; -, negative; w, weak, ND, not determined

Characteristic	1	2	3	4
Growth with NaCl (%w/v):				
0.5	+	+	+	-
9	+	-	-	+
10	-	-	-	+
Growth at (°C)				
7	-	+	-	-
42	+	-	+	-
Activity of				
Esterase lipase (C8)	-	w	-	+
Catalase	-	+	w	+
Production of:				
Acetoin (Voges Proskauer)	+	+	w	w
Hydrolysis of :				
Tween 80	w	-	-	+[a]
Utilization of:				
Acetate	-	-	+	-[a]
D-,L-lactic acid	+	w	w	-[a]
L-arabinose	-	w	w	-[b]
L-glutamic acid	+	w	-	+[b]
L-proline	+	w	w	+[b]
D-alanine	+	-	-	-[b]
L-alanine	+	-	-	+[b]
β-hydroxy butyric acid	+	-	-	+[b]
Bromo succinic acid	+	-	-	ND
α-ketovaleric acid	+	-	-	ND
FAME composition*				
$C_{10:0}$	3	4	3.5	-
$C_{16:0}$	22	18.5	16.8	17.6
$C_{17:0}$ CYCLO	-	1.5	-	-
Unknown fatty acid ECL 11.799	-	-	1.8	1

*Fatty acids data are expressed as percentages of the total amount of fatty acids. Fatty acids representing < 1 % are not shown. [a] Kim *et al*. (2009c). [b] Bauman *et a*l. (1983).

Description of *Marinobacterium coralli* sp. nov.

Marinobacterium coralli (co.ral'li. L. gen. n. *coralli* of coral, isolated from coral mucus).

Cells are Gram-negative, moderately halophylic, aerobic, motile, straight rods approximately 1 µm wide and 2–5 µm long. Catalase negative and oxidase positive. Colonies on MA are circular, slightly undulate, convex, smooth, beige translucent in colour and 0.8 mm in size after 1 day of incubation at 28 °C. Prolific growth occurs between 20 and 40 °C and at NaCl concentrations (w/v) ranging from 1 to 7 %. No growth is observed in 0 % NaCl or in ≥ 10 % NaCl, and at ≤ 7 °C or at ≥ 43 °C. The strain has alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase activity, and it produces acetoin (Voges Proskauer). It is capable to assimilate methyl pyruvate, monomethyl succinate, cis-aconitic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy phenylacetic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, DL-lactic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine , L-alanine, L-alanylglycine, L-glutamic acid, L-proline and phenyl ethylamine. Weak reactions were observed for hydrolyses of tween 40, tween 80, and for assimilation of N-acetyl-D-glucosamine, citric acid, alaninamide, L-asparagine and L-phenylalanine. It is negative for esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, tripsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase urease, gelatinase, and tryptophane deaminase activities, H_2S and indol production, nitrate and nitrate to N_2 gas reduction, glucose, manitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose fermentation, citrate, α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *m*-inusitol, α-lactose, α-D-lactose lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl D-glucoside, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, itaconic acid, malonic acid, propionic acid,

quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, putrescine, 2-amino ethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate, potassium gluconate, capric acid, adipic acid, malate, and trisodium citrate assimilation. The main cellular fatty acids are $C_{18:1}$ ω7*c*, summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*), $C_{16:0}$ corresponding to 82 % of the total FAME profile. The following fatty acids are present in small amounts: $C_{10:0}$ 3-OH (7 %), $C_{12:0}$ (6 %) and $C_{10:0}$ (3 %) (**Supplementary Table S1**). The phenotypical profile of *M. coralli* is at present based on one strain. As more strains of this species are isolated and tested, the profile may change slightly. The DNA G+C content of the type strain is 58.5 mol%. The type strain R-40509^T (= LMG 25435^T = CAIM 1449^T) was isolated from mucus of the endemic coral *Mussismilia hispida* located in the São Sebastião channel, SP, Brazil.

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Supplementary data

Supplementary Table S1. Cellular fatty acid contents of *Marinobacterium coralli* sp. nov. and related species of the genus *Marinobacterium*.

Taxa: 1, *M. coralli* R-40509^T (LMG 25435^T); 2, *M. maritimum* LMG 25352^T; 3, *M. sediminicola* LMG 25280^T; 4, *M. stanieri* LMG 6847^T. Summed feature 3 comprises C15:0 iso 2-OH and/or C16:1ω7*c*. Data are expressed as percentages of the total amount of fatty acids. Fatty acids representing < 1 % are not shown.

Fatty acid	1	2	3	4
$C_{10:0}$	3	4	3.5	-
$C_{10:0}$ 3-OH	7	9.5	8.5	6
$C_{12:0}$	6	4	2.3	4.4
$C_{16:0}$	22	18.5	16.8	17.6
$C_{17:0\ CYCLO}$	-	1.5	-	-
$C_{18:1}\omega7c$	35	30	36.5	43.5
Summed feature 3	25	29	30	26
Unknown fatty acid ECL 11.799	-	-	1.8	1

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IV.7. *Vibrio communis* sp. nov. isolated from marine animals (*Mussismilia hispida, Phyllogorgia dilatata, Palythoa caribaeorum, Palythoa variabilis* and *Litopenaeus vannamei*).

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***Vibrio communis* sp. nov., isolated from marine animals (*Mussismilia hispida, Phyllogorgia dilatata, Palythoa caribaeorum, Palythoa variabilis* and *Litopenaeus vannamei*)**

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Footnote: The GenBank/EMBL accession numbers for the sequences of *Vibrio communis* sp. nov. are GU078670 - GU078676 and AJ345066 (16S rRNA gene sequences), GU078680 - GU078684 (*gyrB* gene sequences), GU078693 - GU078696, EU717055 and EF596446 (*recA* gene sequences), GU078697 - GU078703 and AJ842625 (*rpoA* gene sequences), GU078704 - GU078710 (*topA* gene sequences), GU078691, GU078692, EU251617, EU251620, EU251630, EU251638, EU716913, EF596375 (*pyrH* gene sequences) and GU078685 - GU078690 (*mreB* gene sequences).

Abstract

Eight *Vibrio* isolates originating from *Mussismilia hispida, Phyllogorgia dilatata, Palythoa caribaeorum, Palythoa variabilis* in Brazil and *Litopenaeus vannamei* in Ecuador, were studied by means of a polyphasic approach. The novel isolates formed a tight monophyletic group in the genus *Vibrio*, and were closely related to species of the *Vibrio harveyi* group, to which they showed more than 99 % 16S rRNA gene sequence similarity. Analysis based on concatenated sequences of the following seven genes, i.e. 16S rRNA*, gyrB, recA, rpoA, topA, pyrH* and *mreB*; 5633 bp in length, showed a clear separation between the isolates and the species of the *V. harveyi* group. Amplified Fragment Length Polymorphism (AFLP) analysis, previously performed, revealed that a representative isolate of this group, i.e. LMG 20370, was clearly apart from the known *Vibrio* species (it belonged to the so called AFLP cluster A31; Thompson *et al*., 2001a). DNA-DNA hybridization (DDH) experiments with representatives isolates and the type strains of the *V. harveyi* species group, revealed high DDH similarity between the novel isolates (more than 74 %), and less than 70 % DDH similarity towards the related *Vibrio* species, proving the novel species status of the isolates. Phenotypically, the new species belong to the arginine dihydrolase (A) -negative, lysine (L) -positive and ornithine decarboxylase (O) -positives (A-/L+/O+) cluster, previously reported. Most species of the *V. harveyi* group (i.e. *V. rotiferianus, V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*) also belonged to this A-/L+/O+ cluster. However, several phenotypic features can be used for the identification of the new species. In contrast to its closest phylogenetic neighbours, the novel species has esterase (C4) and N-acetyl-β-glucosaminidase activity, but it does not produce acetoin, does not use citrate, α-ketoglutaric acid, propionic acid and does not ferment melibiose. The novel species can also be differentiated on the basis of the presence of the fatty acids $C_{17:0,}$ $C_{17:1}$ ω8c, $C_{17:0}$ iso and $C_{13:0}$ iso and the absence of the fatty acid $C_{18:0}$. The name *Vibrio communis* sp. nov., is proposed for this taxon. R-40496^T (= LMG 25430^T = CAIM 1816^T) is the type strain.

The Vibrionaceae family belongs to the *Gammaproteobacteria* and includes six genera: *Vibrio* (Baumann & Schubert, 1984), *Photobacterium* (Baumann & Baumann, 1984),

Salinivibrio (Mellado *et al.,* 1996), *Grimontia* (Thompson *et al.,* 2003a), *Enterovibrio* (Thompson *et al.,* 2002a) and *Aliivibrio* (Urbanczyk *et al.,* 2007). The family comprises over 115 formally described species (http://www.vibriobiology.net/). Vibrios are common inhabitants of aquatic environments, especially the ocean, and they are known to live either freely or associated as symbionts with aquatic animals in marine or estuarine waters, or as parasites of fish, crustaceans and molluscs (Thompson *et al*., 2004). Vibrios appear to have a key role in the health of corals (Rosenberg *et al*., 2007). They may provide protection against pathogens or contribute to nitrogen fixation within the holobiont (Ritchie 2006; Olson *et al*., 2009). *V. harveyi, V. campbellii, V. rotiferianus, V. alginolyticus, V. parahaemolyticus, V. natriegens* and *V. mytili* are members of *Vibrio* core group (Dorsch *et al.,* 1992) later called Harveyi clade (Sawabe *et al*., 2007b). Recently *V. azureus* (Yoshizawa *et al*., 2009) was included in this group. Bacteria of this clade have been found associated with coral disease, as in the yellow band disease, which is one of the most significant coral diseases of the tropics (Cervino *et al*., 2008). Members of *Vibrio* core group have been described as closely related species by 16S rRNA gene sequence and in some cases phenotypically indistinguishable by more than 100 phenotypic features. For instance several isolates phenotypically identified as *V. harveyi* were in fact *V. campbellii* by means of molecular identification and DDH (Gomez-Gil *et al*., 2004). Multilocus sequence analysis (MLSA) has become an important methodology for the taxonomy of *Vibrio*. It has greatly improved species identification and classification (Thompson *et al*., 2005, 2007; Sawabe *et al*., 2007b) and is now widely applied (Sawabe *et al*., 2007a; Gomez-Gil *et al*., 2008; Rameshkumar *et al*., 2008; Beaz Hidalgo *et al*., 2009; Yoshizawa *et al*., 2009; Xue-Wei *et al*., 2009; Yan *et al*., 2009).

In a recent study aiming at the taxonomic characterization of vibrios associated with corals in Brazil, several isolates belonging to potentially new species were obtained (Chimetto *et al.,* 2008*,* 2009; Alves *et al*., 2009). These isolates formed a large tight group (named L1 and/or *V. harveyi*–like) on the basis of *pyrH* gene sequences and were closely related with strain LMG 20370. This strain was reported in a previous study as a separated group, called AFLP cluster A31, within the *Vibrio* group (Thompson *et al*., 2001a). In the present study, a detailed polyphasic taxonomic analysis was performed in

order to determine the exact taxonomic position of a representative group of eight novel isolates, including LMG 20370 and 7 Brazilian isolates.

The eight isolates originated from different places and dates (**Table 1**).

Table 1. Isolates of *Vibrio communis* sp. nov.

Isolate	Source	Locality	Isolation year
R-40496^T (=LMG 25430^T = CAIM 1816^T)	mucus of apparently healthy *Mussismilia hispida*	Grande beach in São Sebastião Channel, São Paulo, Brazil.	2005
R-40498 (=LMG 25431)	mucus of apparently healthy *Mussismilia hispida*	Grande beach in São Sebastião Channel, São Paulo, Brazil.	2005
R-40501	mucus of apparently healthy *Palythoa caribaeorum*	Preta beach in São Sebastião Channel, São Paulo, Brazil.	2005
R-40504	mucus of apparently healthy *Palythoa variabilis*	Portinho beach in São Sebastião Channel, São Paulo, Brazil.	2005
R-40506 (=LMG 25432)	mucus of apparently healthy *Mussismilia hispida*	Portinho beach in São Sebastião Channel, São Paulo, Brazil.	2006
R-40900	mucus of apparently healthy *Mussismilia hispida*	Abrolhos Bank, Bahia, Brazil.	2007
R-40901 (=LMG 25433)	mucus of apparently healthy *Phyllogorgia dilatata*	Abrolhos Bank, Bahia, Brazil.	2007
LMG20370	Digestive gland of white shrimp(*Litopenaeus vannamei*)	CENAIM (Ecuador)	2000

They were obtained as described previously (Chimetto *et al.,* 2008*,* 2009). Gene sequences of the 16S rRNA, recombination repair protein (*recA*), topoisomerase I (*topA*), actin-like cytoskeleton protein (*mreB*), RNA polymerase alpha subunit gene (*rpoA*), DNA gyrase B subunit (*gyrB*) and urydilate kinase (pyrH), were obtained as described previously (Thompson *et al*., 2001a, 2007; Sawabe *et al.*, 2007b). Briefly, PCR products were purified with the enzyme Exosap according to the instructions of the manufacturer (GE Health Care). Subsequently, 5 µl of purified PCR product were mixed with 4 µl ET TerminatorTM Mix (GE Health Care), 0.6 µl of sequencing primers (20 µmol l^{-1}) and 0.4 µl of MilliQ water. The thermal program consisted of 30 cycles of 20 s at 95 °C, 15 s at 50 °C and 1 min at 60 °C. Purification of the sequencing products was done by adding 1 µl ammonium acetate (7.5 mol l^{-1}) and 27.5 µl absolute ethanol to each product,

incubation in the dark for 30 min and subsequent centrifugation at 20800 *g* for 75 min at 4 °C. After this the supernatant were removed and 100 µl of 70 % ethanol was added. A last centrifugation step was performed at 3700 rev min^{-1} for 45 min at 4 °C. Separation of the DNA fragments was performed using a MegaBace 1000 system (GE Health Care). Voltage and time of injection were 3 kV and 80 s. Running was performed at 9 kV for 100 min at 44 °C. Raw sequence data were transferred to ChromasPro ver. 1.34 (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. Sequences were aligned using ClustalW. Pairwise similarity was calculated with the BioNumerics 4.5 software package (Applied Maths), using an open gap penalty of 100 % and a unit gap penalty of 0 %. Similarity matrices and phylogenetic trees were constructed using the software MEGA ver. 4.0 software (Tamura *et al*., 2007). Trees were drawn using the Neighbour-Joining (Saitou & Nei, 1987) and Maximum Parsimony methods (Eck *et al.,* 1966). The robustness of each topology was checked by 1000 bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The GenBank accession numbers for the 16S rRNA*, gyrB, recA, rpoA, topA, pyrH* and *mreB* gene sequences are listed in **Supplementary Table S1**. DDH experiments were performed using Ezaki´s microplate method as described in detail previously (Ezaki *et al*., 1989; Willems *et al*., 2001).The hybridization temperature was 40 °C in the presence of 50 % formamide. Reciprocal reactions were performed for every DNA pair and their variation was within the limits of this method (Goris *et al.,* 1998). The DDH experiments were performed in four replicates. The G+C content of DNAs was determined by HPLC method as described previously (Mesbah *et al*., 1989). Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). For fatty acid analysis, cells were grown on TSA (DIFCO) supplemented with 1.5 % NaCl for 24 h at 28 °C. Catalase activity was determined adding yong cells to a drop of 3 % H_2O_2 solution and observation of O_2 production. Oxidase activity was tested using 1 % N,N,N'N'-tetramethyl *p*-phenylenediamine (Kovacs, 1956). Phenotypic characterization was performed using the API ZYM, API 20E (bioMérieux) and Biolog GN2 metabolic fingerprinting (Biolog Inc., USA) kits as described previously (Thompson *et al.*, 2001b, 2002b). Type strains of the phylogenetically closely related *Vibrio* species (i.e. *V.*

rotiferianus LMG 21460^T, *V. harveyi* LMG 4044^T, *V. parahaemolyticus* LMG2850T, *V. alginolyticus* LMG 4409^T, *V. campbellii* LMG 11216^T, *V. natriegens* LMG 10935^T and *V. azureus* LMG 25266^T) were included in the phenotypic analyses. The temperature range (0-42 °C) for growth was determined by incubation on tryptone soy agar (TSA; Oxoid) supplemented with 2 % NaCl (w/v). Growth at different NaCl concentrations (0-10 %, w/v) was determined by incubation on TSA (Oxoid) supplemented with NaCl for 72h at 28 °C. Phylogenetic analyses based on 16S rRNA gene sequence classified the eigth isolates in a tight monophyletic group in the genus *Vibrio* (**Figure 1**).

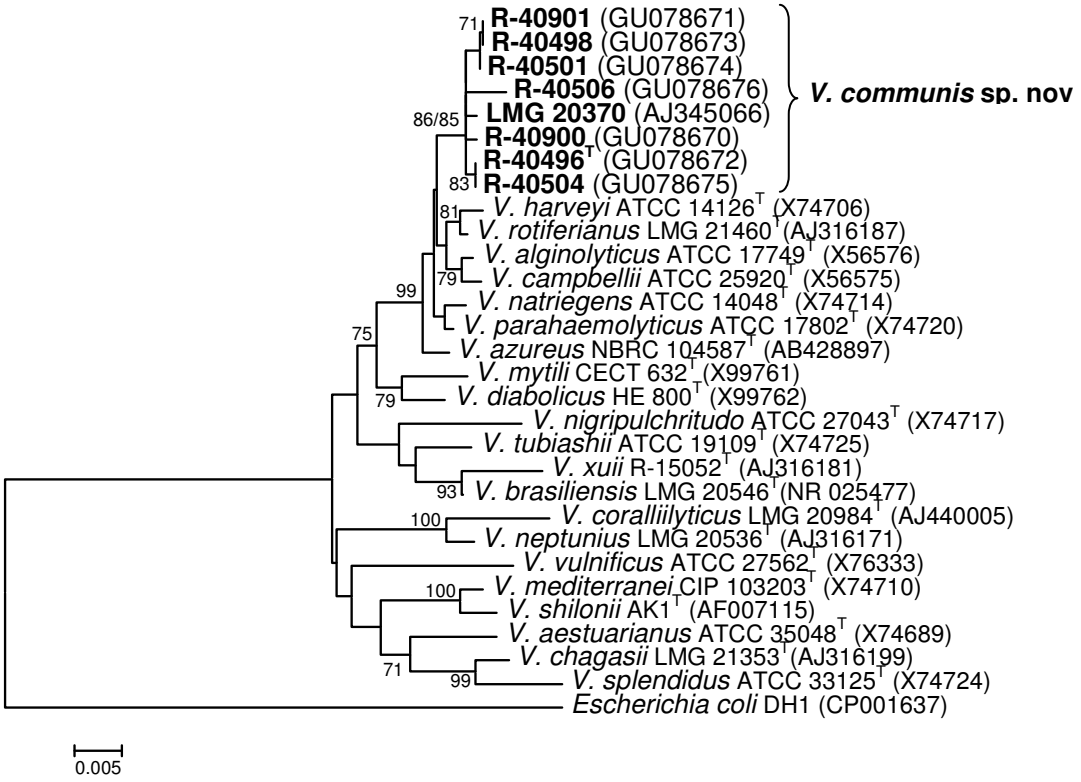

Figure 1. Neighbour-joining phylogenetic tree showing the phylogenetic position of *V. communis* sp. nov. based on 16S rRNA gene sequences (1470 bp). The optimal tree with the sum of branch length = 0.28450859 is shown. The evolutionary distances were computed using the Jukes-Cantor method. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Bootstrap values ($\geq$ 70 %) based on 1000 repetitions are shown. The bootstrap value for the highlighted branch was also computed by the maximum-parsimony methods. *Escherichia coli* was used as outgroup. Bar, 0.5 % estimated sequence divergence.

The eight isolates (R-40496^T, R-40498, R-40501, R-40504, R-40508, R-40900, R-40901 and LMG 20370) showed more than 99.8 % mutual 16S rRNA gene sequence similarity.

They were most closely related with species of the *Vibrio harveyi* group (Reichelt *et al*., 1976), also called the core group of the genus *Vibrio* (Dorsch *et al.,* 1992) or the *Harveyi* clade (Sawabe *et al.*, 2007b). Phylogenetic analysis with the maximum-parsimony method produced congruent results with the neighbour-joining method (**Supplementary Figure S1**). The 16S rRNA sequence similarity of the isolates towards its closest phylogenetic neighbours varied between 99.5 % for *V. rotiferianus* LMG 21460^T and 98.8 % for *V. mytili* CECT 632^T (**Supplementary Table S2**). Trees based on housekeping genes (i.e *gyrB, recA, rpoA, topA, pyrH* and *mreB*) confirmed the tight grouping of the novel isolates (**Supplementary Figures S2-S7**). Similarities between the novel isolates ranged from 97.4 % to 98.9 % (for *gyrB*), 98.3 % to 99.6 % (for *recA*), 99.4 % to 100% (for *rpoA*), 97.0 % to 99.5 % (for *topA*), 98.5 % to 99.8 % (for *pyrH*) and 95.5 % to 97.7 % (for *mreB*). Similarities between R-40496^T and type strains of the closest phylogenetic species of the *Vibrio harveyi* group ranged from 83.9 % to 91.7% (for *gyrB*), 89.0 % to 98.3 % (for *recA*), 95.8 % to 98.3 % (for *rpoA*), 80.7 % to 90.7 % (for *topA*), 87.5 % to 96.3 % (for *pyrH*) and 86.4 % to 96.5 % (for *mreB*) (**Supplementary Table S2**) . These data indicate that the isolates belong to a novel species in the *V. harveyi* group, since *gyrB, rpoA, topA* and *pyrH,* gene sequences have a high phylogenetic resolution for species identification in *Vibrio* (Thompson *et al.*, 2005, 2007). MLSA of concatenated 16S rRNA, *rpoA,* and *pyrH* gene sequences confirmed the tight grouping of the eight isolates (**Figure 2**) and a similar result was obtained by MLSA of concatenated 16S rRNA, *gyrB, recA, rpoA, topA, pyrH* and *mreB* gene sequences (**Supplementary Figure S8**). Clearly, the MLSA data confirmed that the eight isolates belong to a novel species within the genus *Vibrio*.

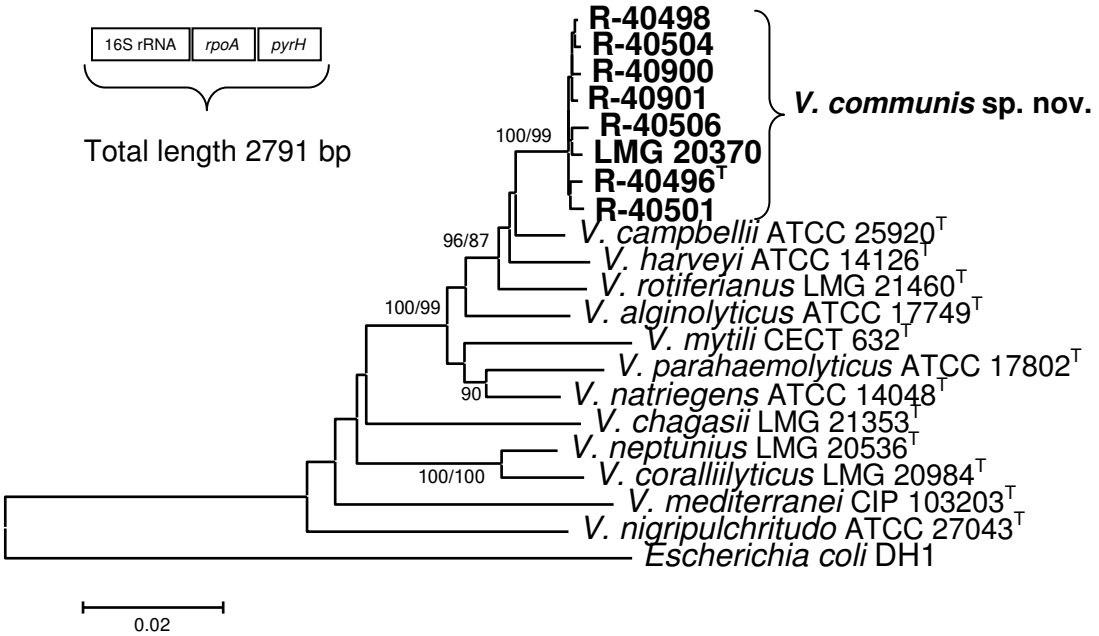

Figure 2. Neighbour-joining phylogenetic tree showing the phylogenetic position of *V. communis* sp. nov. based on concatenated 16S rRNA (1470 bp), *rpoA* (790 bp) *and pyrH* (531 bp) gene sequences. The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4.Bootstrap values (> 70%) based on 1000 repetitions are shown. The numbers at nodes denote the level of bootstrap values derived from the neighbor-joining and maximum-parsimony methods. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.

DDH experiments were performed with representative strains of the novel group and the type strains of the closest phylogenetic neighbours in order to prove that the isolates belong to a new species (**Supplementary Table S3**). The representative strains showed less than 70 % DDH similarity with *V. harveyi* LMG 4044^T, *V. campbellii* LMG 11216^T, *V. rotiferianus* LMG21460^T, *V. parahaemolyticus* LMG 2850^T, *V. alginolyticus* LMG 4409^T, *V. natriegens* LMG 10935^T, and *V. azureus* LMG 25266^T. The DDH similarity between the novel isolates varied bettween 74 % to 86 %. Similarity between LMG 20370 and *V. harveyi* LMG 4044^T was 66 %, whereas the DDH similarity between LMG 20370 and R-40496^T was 79 %. Clearly, DDH data proves that the new eight isolates represent a novel species.

Phenotypically, the eight isolates are assigned to the genus *Vibrio* (Alsina & Blanch, 1994), and belong to the arginine-dihydrolase (A) -negative, lysine (L) -positive and ornithine decarboxilase (O) -positive (A-/L+/O+) cluster (Noguerola & Blanch, 2008). Based on 16S rRNA gene sequences data and MLSA data most of the phylogenetically related species, i.e. *V. rotiferianus, V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* belong in the same phenotypic cluster (A-/L+/O+). However, *V. campbellii* and *V. natriegens* belong to the (A-/L-/O-) cluster and *V. mytili* belong to the (A+/L-/O-) cluster according to Noguerola & Blanch (2008). Several phenotypic features can be used to differentiate the novel species from its closest neighbours (**Table 2**).

In contrast to its closest neighbours, *V. communis* sp. nov. has esterase (C4), N-acetyl-β-glucosaminidase activity, but it does not produce acetoin, does not utilize citrate, α-ketoglutaric acid and propionic acid. It does also not ferment melibiose but it ferments amygdalin and has weak growth on 8 % NaCl. Yellow colonies are observed on thiosulfate-citrate-bile-sucrose (TCBS) agar and beige, translucent colonies on marine agar. Phenotypic variation was observed among the isolates (**Table S4**), suggesting a good representation of the phenotype of the new species. The novel isolates can also be differentiated from its neighbours on the basis of the presence of the fatty acids $C_{17:0,}$ $C_{17:1}$ ω8c, $C_{17:0}$ iso and $C_{13:0}$ iso, and the absence of the fatty acids $C_{18:0}$ which is normally present in other species of the *V. harveyi* group (**Supplementary Table S5**).

Based on the polyphasic analysis presented in this study, we propose to classify the eight isolates in the new species *V. communis* sp. nov. The novel species can be differentiated from its phylogenetic neighbours by means of AFLP (Thompson *et al*., 2001a), MLSA, DDH, and several phenotypic and chemotaxonomic tests.

Table 2. Differential phenotypic characteristics of *V. communis* sp. nov. and related species of the genus *Vibrio*.

Species: **1**, *V. communis* sp. nov. (eight strains); **2**, *V. rotiferianus* LMG 21460^T; **3**, *V. harveyi* LMG 4044^T; **4**, *V. parahaemolyticus* LMG2850^T; **5**, *V. alginolyticus* LMG 4409^T; **6**, *V. campbellii* LMG 11216^T; **7**, *V. natriegens* LMG 10935^T; and **8**, *V. azureus* LMG 25266^T. +, positive; -, negative; w, weak positive. Data for all these type strains were obtained in this study.

Characteristic	**(A-/L+/O+)***					**(A-/L-/O-)***		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Growth in NaCl (%w/v):								
8	w	-	-	+	+	w	+	-
10	-	-	-	-	+	-	-	-
Production of:								
Indol	+	+	+	+	+	+	-	-
Acetoin	+	-	-	-	+	-	-	+
Enzyme activities of:								
Esterase (C 4)	+	+	-	+	+	+	-	-
Lipase (C14)	+	-	+	-	-	+	+	-
β-galactosidase	+	-	+	-	-	-	+	-
N-acetyl-β-glucosaminidase	+	-	-	+	+	+	-	+
α-chymotrypsin	+	+	+	-	-	+	-	-
Tryptophane deaminase	-	+	-	-	-	-	+	-
Urea	-	+	-	-	-	-	+	-
Utilization of:								
L-arabinose	-	+	-	+	-	w	+	-
Citrate	-	-	+	-	-	-	+	-
Cis-aconitic acid	-	-	+	w	+	-	+	-
α-ketoglutaric acid	-	-	+	-	+	-	+	-
Propionic acid	-	-	+	-	+	-	+	-
L-glutamic acid	+	-	+	w	+	+	+	w
Saccharose	+	+	+	-	+	-	-	-
Glycerol	+	-	-	+	+	+	-	-
L-alanine	+	w	+	-	+	-	-	-
L-alanylglycine	+	-	+	-	+	+	-	w
D,L-α-glycerol phosphate	+	-	-	+	+	+	-	-
Fermention of:								
Amygdalin	+	+	-	-	-	+	-	+
Melibiose	-	+	+	-	-	-	+	-

*Phenotypic cluster according Noguerola & Blanch. (2008), arginine dihydrolase (A), lysine decarboxylase (L) and ornithine decarboxylase (O). Characteristics in bold are useful to differentiate A-,L+,O+ *Vibrio* species according Noguerola & Blanch. (2008).

Description of *Vibrio communis* sp. nov.

Vibrio communis (com.mu'nis. L. masc. adj. *communis*, common, widespread, frequent in the marine environment).

Cells are 1 µm wide 2.5–3.5 µm long, Gram-negative, motile bacilli. Catalase- and oxidase-positive. It forms translucent, convex, smooth-rounded colonies with entire margins, beige in colour and 1 mm in size after 1 day incubation at 28 °C on TSA. Prolific growth occurs between 15 and 37 °C and at NaCl concentrations (w/v) of 0.5–6.0 %. Weak growth is observed in the presence of 8 % NaCl. No growth is observed in 0 or ≥ 9 % NaCl at ≤ 7 °C or ≥ 42 °C. Yellow colonies were able to grow on the selective medium TCBS. The isolates are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, tripsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, N-acetyl-β-glucosaminidase, α-glucosidase (except R-40498), lysine decarboxylase and ornithine decarboxylase (except R-40900) enzyme activities, indol and acetoin (Voges Proskauer) production, glucose, manitol, saccharose and amygdalin fermentation, gelatinase diffusion (except R-40504), nitrate reduction to NO_2, and weak positive for β-glucosidase activity (negative for R-40496^T, R-40498, R-40900 and R-40901). They are negative for cystine arylamidase, α-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, arginine dihydrolase, urease and tryptophane deaminase enzyme activities, citrate utilization (except R-40498), H_2S production, fermentation of inositol, sorbitol, rhamnose, melibiose, arabinose and reduction of nitrate to N_2 gas. All strains utilize α-cyclodextrin, dextrin, glycogen, tween 40, tween 80, N-acetyl-D-glucosamine, cellobiose, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, psicose, sucrose, D-trehalose, methyl pyruvate, D-gluconic acid, DL-lactic acid, alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-threonine, inosine, uridine, thymidine, glycerol, DL-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate. None of the strains utilizes N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, *i*-erythritol, L-fucose, *m*-inositol, α-lactose, α-D-lactose lactulose, D-melibiose, D-raffinose, L-rhamnose, turanose, xylitol, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid

lactone, D-galacturonic acid, D-glucosaminic acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy phenylacetic acid, itaconic acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, DL-carnitine, γ-aminobutyric acid, phenyl ethylamine, putrescine, 2-amino ethanol and 2,3-butanediol. The following phenotypic features are variable (the results in bracket are from type strain R-40496^T): D-galactose (-), gentiobiose (+), β-methyl D-glucoside (-), D-sorbitol (-), monomethyl succinate (-), α-hydroxy butyric acid (+), α-keto butyric acid (+), succinic acid (+), bromosuccinic acid (+), glucuronamide (+), D-alanine (-), hydroxy L-proline (-), L-proline(+) , D-serine (-), L-serine (+), urocanic acid (+) (**Supplementary Table S4**). The fatty acids profiles of the eight novel isolates were similar. The main cellular fatty acids are summed feature 3 ($C_{15:0}$ iso 2-0H and/or $C_{16:1}$ ω7c), $C_{18:1}$ω7c, $C_{16:0}$ and $C_{14:0}$ corresponding to 71.3 % of the total FAME profile (**Supplementary Table S5**). The following fatty acids are present in small amounts: summed feature 2 ($C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE), $C_{12:0}$ 3-OH, $C_{12:0}$, $C_{16:0}$ iso, $C_{17:0,}$ $C_{17:1}$ ω8c, $C_{17:0}$ iso and $C_{13:0}$ iso. The DNA G+C content of the type strain (R-40496^T) is 45.8 mol%, the DNA G+C content range of the novel species varies from 45.6-46.0 mol% The type strain R-40496^T (= LMG 25430^T = CAIM 1816^T) was isolated from the mucus of the endemic coral *Mussismilia hispida* in São Sebastião channel, SP, Brazil.

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Supplementary data

Supplementary Table S1. GenBank accession numbers of the housekeeping genes of the *Vibrio communis* sp. nov. strains, the type strains of the phylogenetically related *Vibrio* species and *E. coli* used in this study.

Strains	***gyrB***	***recA***	***rpoA***	***topA***	***pyrH***	***mreB***
***V. communis* R-40496^T**	GU078680	GU078693	GU078697	GU078704	EU251617	GU078686
***V. communis* R-40498**	GU078681	GU078694	GU078698	GU078705	EU251620	GU078687
***V. communis* R-40501**	GU078682	GU078695	GU078699	GU078706	EU251630	GU078688
***V. communis* R-40504**	GU078683	GU078696	GU078700	GU078707	EU251638	GU078689
***V. communis* R-40506**	GU078684	EU717055	GU078701	GU078708	EU716913	GU078690
***V. communis* R-40900**	ND	ND	GU078702	GU078709	GU078691	ND
***V. communis* R-40901**	ND	ND	GU078703	GU078710	GU078692	GU078685
***V. communis* LMG 20370**	ND	AJ842438	AJ842625	ND	EF596375	ND
V. rotiferianus	EU118210	AJ580868	AJ842688	DQ907515	EF596722	DQ907445
V. harveyi	AB298221	AJ842440	AJ842627	DQ907488	EU118238	DQ907422
V. campbellii	AB298205	EF596670	AJ842564	EF596698	EF596641	DQ907408
V. parahaemolyticus	AY527390	AJ580864	AJ842677	DQ907509	GU266286	DQ907440
V. alginolyticus	AB298202	AJ842373	AJ842558	DQ907472	GU266285	DQ907405
V. natriegens	AB298232	AJ842473	AJ842658	DQ907500	FM202573	DQ907432
V. mytili	AB298231	AJ842472	AJ842657	DQ907499	GU266287	DQ907431
V. chagasii	AM162568	AJ842385	AJ842572	DQ481649	EU118252	DQ481637
V. nigripulchritudo	AB298236	AJ842480	AJ842667	DQ907505	GU266290	DQ907437
V. mediterranei	EF380258	AJ842459	AJ842644	DQ907496	GU266288	DQ907428
V. neptunius	AB298234	AJ580861	AJ842663	DQ907503	GU266291	DQ907435
V. coralliilyticus	AB298210	AJ842402	AJ842587	EF114213	GU266292	DQ907412
***Escherichia coli* DH1**	CP001637	CP001637	CP001637	CP001637	CP001637	CP001637

ND = not determined

Supplementary Table S2. Sequence similarity values (%) between the 16S rRNA, *gyrB, recA, rpoA, topA, pyrH* and *mreB* gene sequences of *V. communis* sp. nov. (R-40496^T) and type strains of related *Vibrio* species.

Type strain *	**16S rRNA**	***gyrB***	***recA***	***rpoA***	***topA***	***pyrH***	***mreB***
Vibrio rotiferianus	99.5	91.7	98.3	97.8	90.7	92.0	91.2
Vibrio harveyi	99.3	91.6	96.0	97.9	90.4	93.8	93.6
Vibrio campbellii	99.3	91.6	96.8	98.3	90.4	96.3	96.5
Vibrio parahaemolyticus	99.2	86.3	90.3	97.0	85.8	89.1	91.5
Vibrio natriegens	99.2	84.8	92.6	96.0	84.2	88.9	89.1
Vibrio azureus	99.2	90.8	NA	NA	NA	89.0	80.8
Vibrio alginolyticus	99.1	85.2	94.7	99.1	81.3	87.5	90.8
Vibrio mytili	98.8	83.9	89.0	95.9	80.7	87.9	86.4

* Strains and accession numbers of the type strains used in MLSA analyses are available in the trees of each gene in supplementary material S2-S7. NA = Data not available

Supplementary Table S3. DDH values and DNA G+C contents of *Vibrio* strains examined in this study.

Strains: **1**, R-40496^{T}; **2**, R-40498; **3**, R-40501; **4**, R-40504; **5**, R-40506; **6**, R-40900; **7**, R-40901; **8**, LMG 20370; **9**, *V. rotiferianus* LMG 21460^{T}; **10**, *V. harveyi* LMG 4044^{T}; **11**, *V. campbellii* LMG 11216^{T}; **12**, *V. parahaemolyticus* LMG 2850^{T}; **13**, *V. natriegens* LMG 10935^{T}; **14**, *V. alginolyticus* LMG 4409^{T}; **15**, *V. azureus* LMG 25266^{T}; **16**, *V. diabolicus* LMG 19805^{T*}; **17**, *V. pelagius* LMG 3897^{T*}.

Strains	G+C (mol%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	45.8	100																
2	45.6	81	100															
3	45.7			100														
4	45.7				100													
5	45.8	84	74			100												
6	45.7	83				76	100											
7	46.0	80					86	100										
8	45.2	79	79			84			100									
9	44.5	42								100								
10	45.2	53				53	50		66[a]		100							
11	45.6	43								36	62[a]	100						
12	46-47[b]	30											100					
13	45.6	23								21	35[a]	22		100				
14	45.6	29								22	46[a]	22		24	100			
15	45.2	15								22		20		17	13	100		
16	45.6	35					27	30		36	33						100	
17	45.7	17								20		16		17	17	15	20	100

* Strains included as outgroup, [a] Data from Thompson. (2003b), [b] Data from Farmer *et al*. (2005).

Supplementary Table S4. Phenotypic variability among the strains of *V. communis* sp. nov.

Utilization of	R40496^T	LMG20370	R40498	R40501	R40504	R40506	R40900	R40901
D-galactose	-	+	-	-	-	+	+	+
Gentiobiose	+	+	-	+	+	+	+	+
β-methyl D-glucoside	-	-	-	-	+	+	-	-
D-sorbitol	-	w	-	-	-	w	-	+
Monomethyl succinate	-	-	-	w	-	+	-	-
α-hydroxy butyric acid	+	-	-	w	-	+	-	-
α-keto butyric acid	+	-	-	-	-	+	-	-
Succinic acid	+	-	-	+	-	+	+	+
Bromosuccinic acid	+	-	-	+	+	+	+	+
Glucuronamide	+	w	-	-	-	+	+	-
D-alanine	-	+	-	-	+	+	+	+
Hydroxy L-proline	-	-	-	-	-	+	+	+
L-proline	+	-	-	-	+	+	+	+
D-serine	-	-	-	-	-	+	-	+
L-serine	+	-	+	+	-	+	-	+
Urocanic acid	+	w	-	-	-	-	w	-

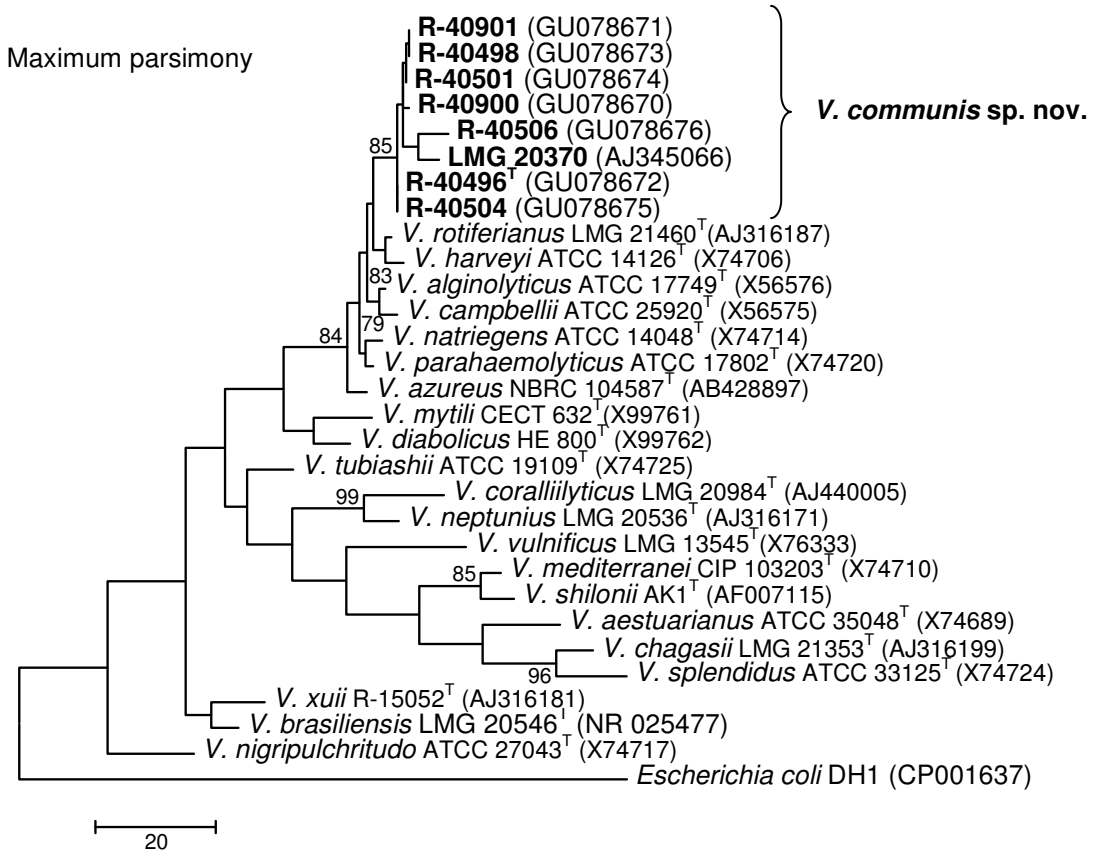
W = weak reaction

Supplementary Table S5. Cellular fatty acids contents of *Vibrio communis* sp. nov. and its closest phylogenetic neighbours.

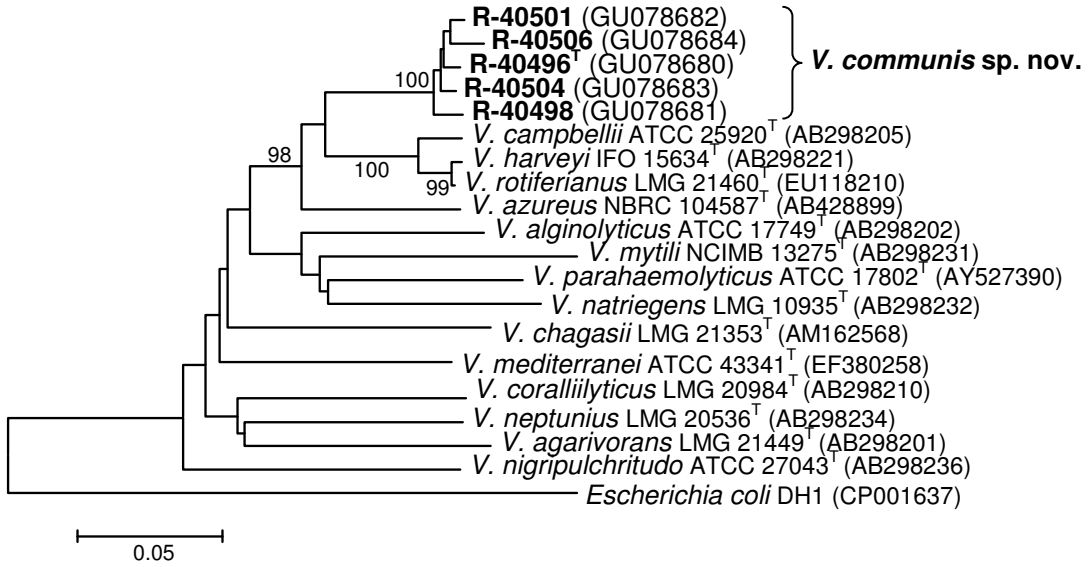
Taxa: 1, *V. communis* sp. nov. R-40496^T; 2, (average profile of R-40496^T, R-40498, R-40501, R-40504, R-40506, R-40900, R-40901 and LMG 20370); 3, *V. rotiferianus* LMG 21460^T; 4, *V. harveyi* LMG 4044^T; 5, *V. campbellii* LMG 11216^T; 6, *V. parahaemolyticus* LMG2850^T; 7, *V. natriegens* LMG 10935^T; 8, *V. alginolyticus* LMG 4409^T; 9, *V. azureus* LMG 25266^T.

Summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE. Summed feature 3 comprises C15:0 iso 2-OH and/or C16:1ω7*c*. Data are expressed as percentages of total fatty acids. Fatty acids representing <1 % are not shown. All data was obtained in this study.

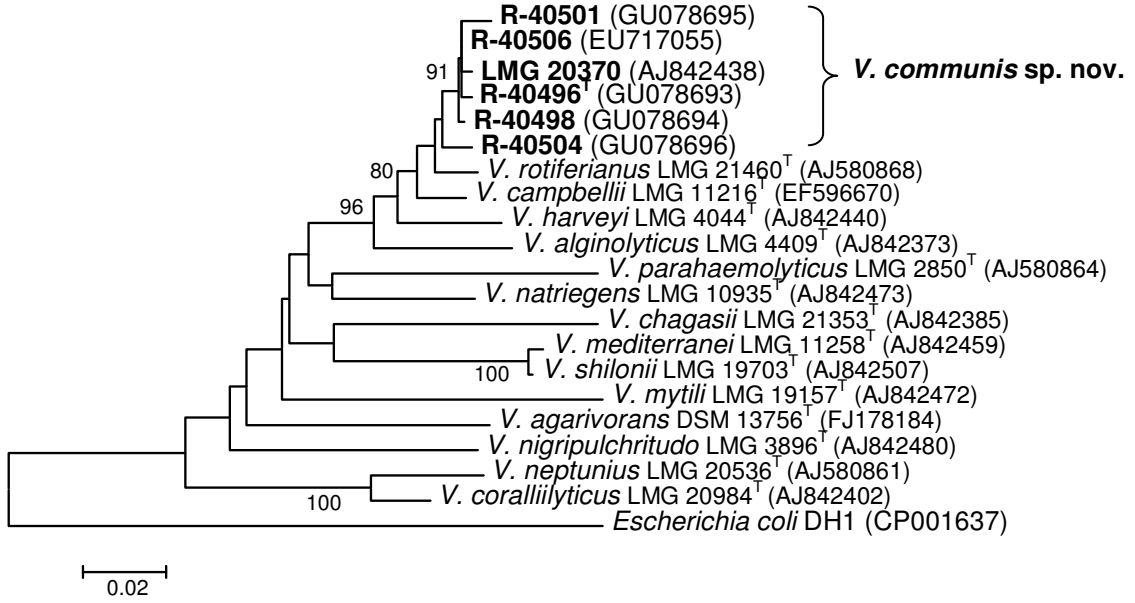
Fatty acid	1	2	3	4	5	6	7	8	9
C12:0	2.6	3.4±1.2	3	3.5	4.5	3.5	4	6	3
C12:0 3-OH	2.8	2.0±0.7	1.3	2.2	2	1.5	4.3	3.5	1.3
C13:0 iso	1	1.1±0.2	-	-	-	-	-	-	-
C14:0	5.8	5.8±1.1	7	4.7	10	10	-	6.5	6
C15:0 ANTEISO	-	-	-	-	-	-	-	-	1.7
C16:0	14.3	13.2±2.0	28.6	15.6	30	26	26.5	24	27.3
C16:0 iso	2.5	4.7±2.0	-	3.8	-	-	-	-	4
C17:0	2.3	1.5±0.4	-	-	-	-	-	-	1.4
C17:0 iso	1.4	1.4±0.6	-	-	-	-	-	-	1.5
C17:1ω8*c*	1.5	1.6±0.3	-	-	-	-	-	-	-
C18:0	-	-	1.2	1.6	1	-	-	-	1.5
C18:1ω7*c*	16.6	15.3±1.9	12.7	24	8	20	36.8	13	6.7
Summed feature 2	4.5	3.7±1.1	3.2	4.4	6.9	3.8	-	8	2.2



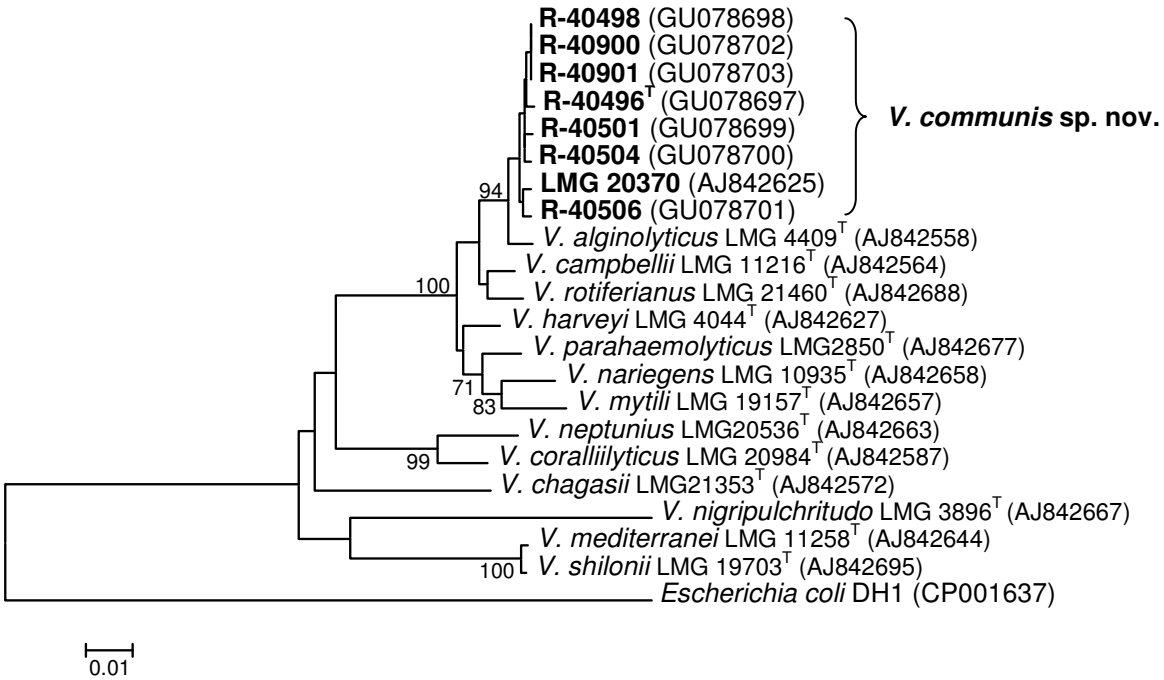
Supplementary - Figure S1. Phylogenetic tree based on the maximum-parsimony method, using partial 16S rRNA gene sequences (1470 bp). Bootstrap values are expressed as percentages of 1000 replications; only values >70 % are shown. *Escherichia coli* was used as an outgroup. Bar, 20 substitutions.



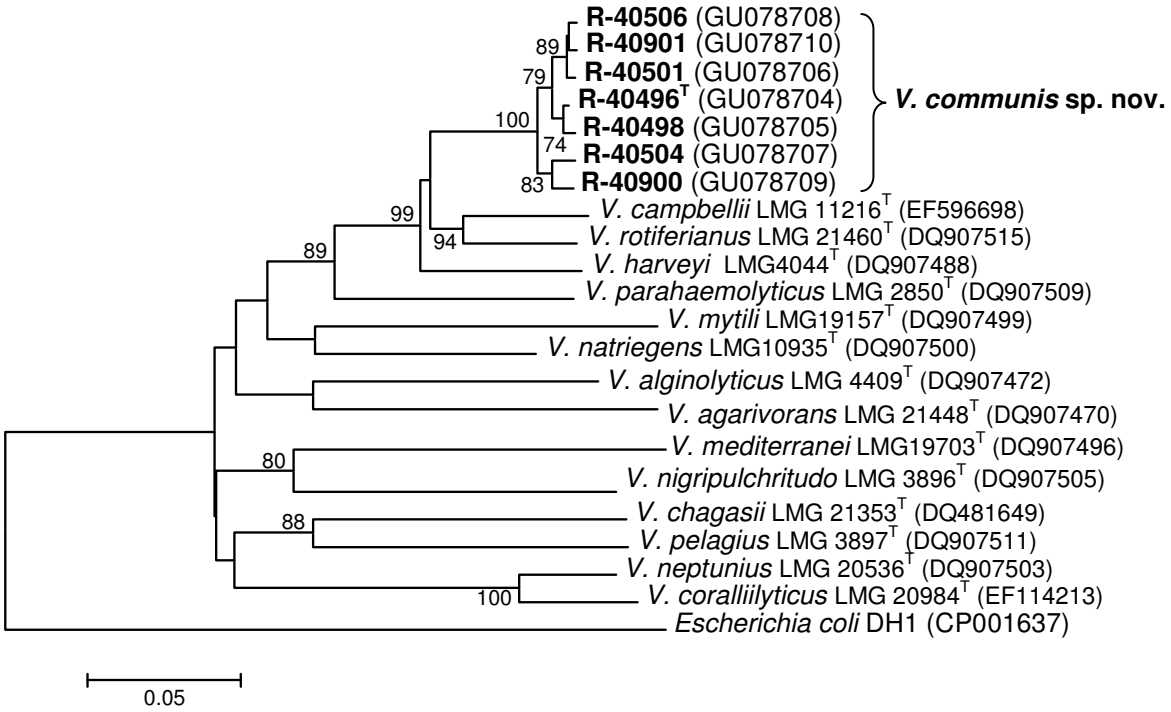
Supplementary - Figure S2. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *gyrB* gene sequences (775 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 5 % estimated sequence divergence.



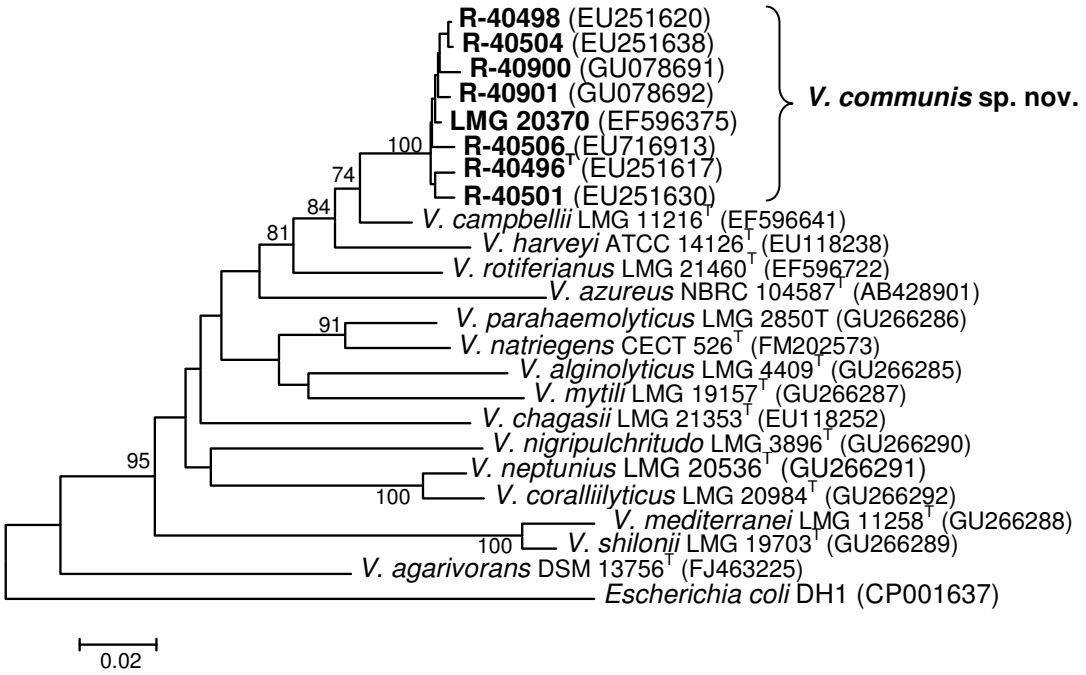
Supplementary - Figure S3. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *recA* gene sequences (556 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.



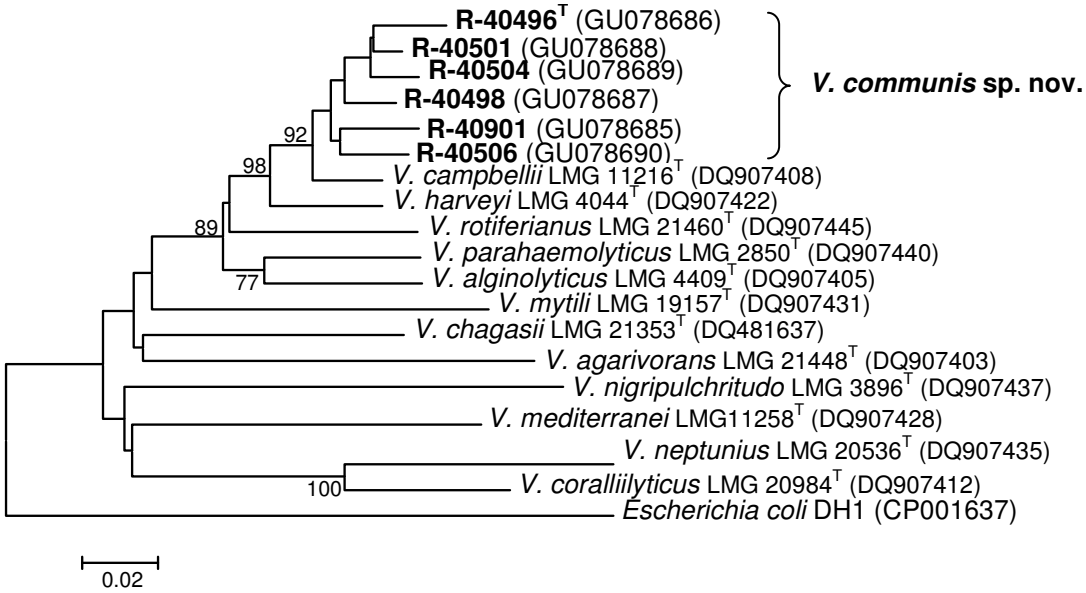
Supplementary - Figure S4. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *rpoA* gene sequences (790 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 1 % estimated sequence divergence.



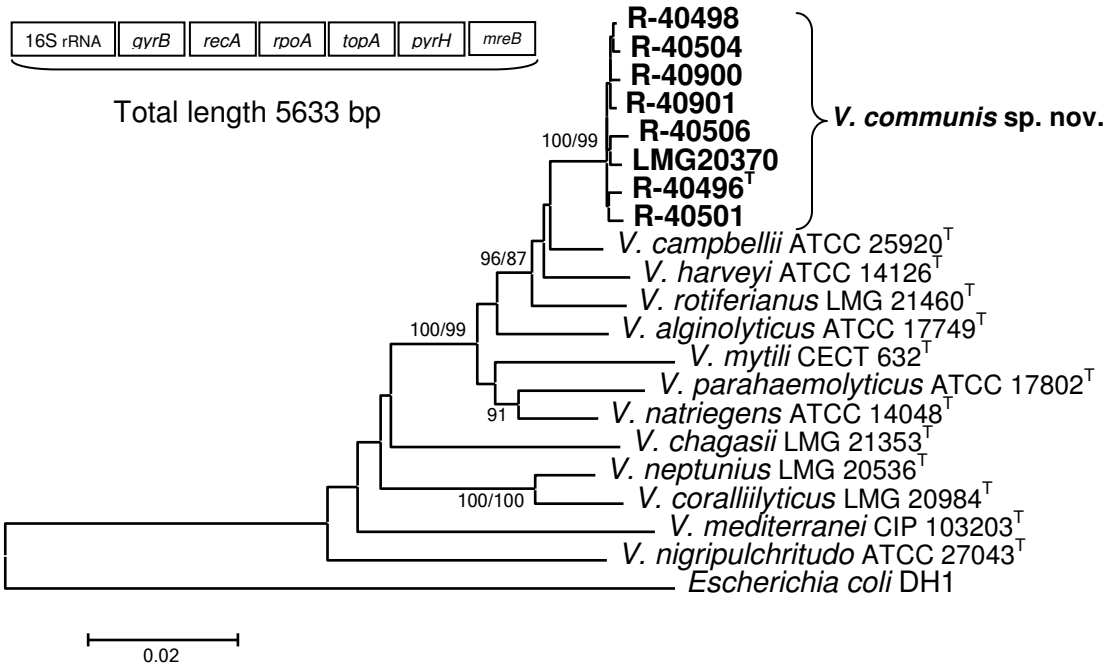
Supplementary - Figure S5. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *topA* gene sequences (654 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 5 % estimated sequence divergence.



Supplementary - Figure S6. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *pyrH* gene sequences (531 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.



Supplementary - Figure S7. Neighbour-joining phylogenetic tree showing the position of *V. communis* sp. nov. based on *mreB* gene sequences (857 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 70 %) are shown based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.



Supplementary Figure S8. Neighbour-joining phylogenetic tree showing the phylogenetic position of *V. communis* sp. nov. based on concatenated 16S rRNA (1470 bp), *gyrB* (775 bp), *recA* (556 bp), *rpoA* (790 bp), *topA* (654 bp), *pyrH* (531 bp) *and mreB* (857 bp) gene sequences. The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4.Bootstrap values (> 70 %) based on 1000 repetitions are shown. The numbers at nodes denote the level of bootstrap values derived from the neighbor-joining and maximum-parsimony methods. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.

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IV.8. *Vibrio variabilis* sp. nov. and *Vibrio marinum* sp. nov, isolated from *Palythoa caribaeorum.*

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Vibrio variabilis sp. nov. and _Vibrio marinum_ sp. nov., isolated from _Palythoa caribaeorum_

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Running Title:

Vibrio variabilis sp. nov. and *Vibrio marinum* sp. nov.

Subject Category: New taxa, *Proteobacteria*

Footnote: The GenBank/EMBL accession numbers for the sequences of *Vibrio variabilis* sp. nov. R-40492^T and *Vibrio marinum* sp. nov. R-40493^T are GU929924 and GU929925 (16S rRNA gene sequences), GU929926 and GU929927 (*ftsZ* gene sequences), GU929928 and GU929929 (*gyrB* gene sequences), GU929934 and GU929935 (*recA* gene sequences), GU929936 and GU929937 (*rpoA* gene sequences), GU929938 and

GU929939 (*topA* gene sequences), GU929932 and GU929933 (*pyrH* gene sequences) and GU929930 and GU929931 (*mreB* gene sequences) respectively.

Abstract

Two novel vibrio strains (R-40492^T and R-40493^T) originating from the zoanthid *Palythoa caribaeorum* in Brazil in 2005 were taxonomically characterized by means of a polyphasic approach, comprising Multilocus Sequence Analysis (MLSA), DNA–DNA hybridizations (DDH) and phenotypic characterization. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strains R-40492^T and R-40493^T fell within the genus *Vibrio*, and were most closely related to each other with 99 % similarity and to *V. neptunius,* LMG 20536^T, *V. coralliilyticus* LMG 20984^T, *V. nigripulchritudo* LMG 3896^T, *V. sinaloensis* LMG 25238^T and *V. brasiliensis* LMG 20546^T with 98.5 to 97.1 % similarity. DDH experiments showed that these strains had less than 15 % similarity to the phylogenetically closest *Vibrio* species. In addition, MLSA analysis of concatenated sequences (16S rRNA, *ftsZ, gyrB, recA, rpoA, topA, pyrH* and *mreB*; 6035 bp in length) showed that the two novel strains formed a separated branch with less than 92 % concatenated gene sequence similarity towards the known vibrios. Two novel species are proposed to accommodate these novel strains, namely *Vibrio variabilis* sp. nov. (R-40492^T = LMG 25438^T = CAIM 1454^T) and *Vibrio marinum* sp. nov. (R-40493^T = LMG25439^T = CAIM 1455^T). The major fatty acids of both *V. variabilis* and *V. marinum* are summed feature 3 (comprising $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*), $C_{18:1}$ ω7*c* and $C_{16:0}$ corresponding to 64 % and 70 % of the total FAME profile, respectively. The DNA G+C contents of these two novel species are 46.8 (R-40492^T) and 46.3 (R-40493^T) mol%. Phenotypic features such as the production of acetoin and activities of N-acetyl-β-glucosaminidase, β-galactosidase, trypsin and cystine arylamidase can be used to discriminate the two novel species from their phylogenetic closest neighbours. In addition, the fermentation of mannitol, inositol and amygdalin, the assimilation of D-glucuronic acid, glucose-1-phosphate and glucose-6-phosphate, as well as the fatty acids $C_{15:0}$ iso 3-OH, $C_{17:0}$ iso, and $C_{18:0}$ can contribute to the identification of the novel species.

The genus *Palythoa* is widespread around the world, most notably at rocky shores and coral reefs. *Palythoa caribaeorum* may cover up to 50 % of the total area of rocky shores in some locations at the coast of Brazil (Oigman-Pszczol *et al*., 2004). This organism is well known for its ability to produce a very potent nonproteinaceous toxin (called palitoxin) and copious amounts of mucus. These features may allow it to occupy areas of periodic desiccation and intense sunlight stress. *Palythoa caribaeorum* is sympatric with several coral species (*Mussismilia* spp.) in the Brazilian coast, sharing the same habitat with the main Brazilian reef builders. In a survey on the diversity of heterotrophic bacteria associated with *Palythoa caribaeorum* in São Paulo (Brazil), two novel vibrio strains, R-40492^T (LMG 25438^T) and R-40493^T (LMG25439^T), were obtained from mucus of apparently healthy *Palythoa caribaeorum,* located in two sites (Preta beach, coordinates 23° 49' 10" S; 045° 24' 37" W and Portinho beach, coordinates 23° 50' 25" S; 045° 24' 22" W) Chimetto *et al*. (2008, 2009). They were obtained on Thiosulfate-citrate-bile salt-sucrose agar (TCBS, Oxoid) after 48 h of incubation at 28 °C. The aim of the present study was to perform a detailed taxonomic characterization of the two novel strains based on a polyphasic approach.

Gene sequences of their 16S rRNA, cell division protein (*ftsZ*), DNA gyrase B subunit (*gyrB*), recombination repair protein (*recA*), RNA polymerase alpha subunit gene (*rpoA*), topoisomerase I (*topA*), urydilate kinase (*pyrH*), and actin-like cytoskeleton protein (*mreB*) were obtained as described previously (Sawabe *et al.*, 2007; Thompson *et al*., 2001a, 2007). Briefly, PCR products were purified with the enzyme Exosap according to the instructions of the manufacturer (GE Health Care). Subsequently, 5 µl of purified PCR product were mixed with 4 µl ET TerminatorTM Mix (GE Health Care), 0.6 µl of sequencing primers (20 µmol l^{-1}) and 0.4 µl of MilliQ water. The thermal program consisted of 30 cycles of 20 s at 95 °C, 15 s at 50 °C and 1 min at 60 °C. Purification of the sequencing products was done by adding 1 µl ammonium acetate (7.5 mol l^{-1}) and 27.5 µl absolute ethanol to each product, incubation in the dark for 30 min and subsequent centrifugation at 20800 *g* for 75 min at 4 °C. After this, the supernatant was removed and 100 µl of 70 % ethanol was added. A last centrifugation step was performed at 3700 rev min^{-1} for 45 min at 4 °C. Separation of the DNA fragments was performed using a MegaBace 1000 system (GE Health Care). Voltage and time of injection were 3

kV and 80 s. Running was performed at 9 kV for 100 min at 44 °C. Raw sequence data were transferred to ChromasPro ver. 1.34 (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. Sequences were aligned using ClustalW. Pairwise similarities were calculated with the BioNumerics 4.61 software (Applied Maths, Belgium) using an open gap penalty of 100 % and a unit gap penalty of 0 %, and with the Jalview 2.4.0.b2 software (Waterhouse *et al*., 2009). Similarity matrices and phylogenetic trees were constructed using the software MEGA ver. 4.0 (Tamura *et al*., 2007) and BioNumerics 4.61 software (Applied Maths, Belgium). Trees were drawn using the Neighbour-Joining (Saitou & Nei, 1987) and Maximum Parsimony methods (Eck *et al.,* 1966). The robustness of each topology was checked by 1000 bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The GenBank accession numbers for *ftsZ, gyrB, recA, rpoA, topA, pyrH* and *mreB* gene sequences used in this study are listed in **Supplementary Table S1**. DDH experiments were performed using Ezaki´s microplate method as described in detail previously (Ezaki *et al*., 1989; Willems *et al*., 2001). The hybridization temperature was 40 °C in the presence of 50 % formamide. Reciprocal reactions (e.g. AxB and BxA) were performed for every DNA pair and their variation was within the limits of this method (Goris *et al.,* 1998). The DDH experiments were performed in four replicates. DNA G+C contentss were determined by HPLC as described previously (Mesbah *et al*., 1989). Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). For fatty acid analysis, cells were grown on TSA (DIFCO) supplemented with 1.5 % NaCl for 24 h at 28 °C. Catalase activity was determined by adding young cells to a drop of 3 % H_2O_2 solution and observation of O_2 production. Oxidase activity was tested using 1 % N,N,N'N'-tetramethyl *p*-phenylenediamine (Kovacs, 1956). Phenotypic characterization was performed using the API ZYM, API 20E (bioMérieux) and Biolog GN2 metabolic fingerprinting kits as described previously (Thompson *et al.*, 2001b, 2002). Type strains of closely related *Vibrio* species (i.e. *V. neptunius* LMG 20536^T, *V. coralliilyticus* LMG 20984^T, *V. nigripulchritudo* LMG 3896^T, *V. sinaloensis* LMG 25238^T and *V. brasiliensis* LMG 20546^T) were included in these analyses. The temperature range (0-42 °C) for growth was determined by incubating the isolates on tryptone soy agar (TSA; Oxoid)

supplemented with 2 % NaCl (w/v). Growth at different NaCl concentrations (0-14 %, w/v) was determined on TSA (Oxoid) by incubation for 72h at 28 °C.

Phylogenetic analysis based on 16S rRNA gene sequences of R-40492^T (1328 bp) and R-40493^T (1507 bp) classified the novel strains in the genus *Vibrio* (**Figure 1**). The two novel strains were most closely related to each other with 99 % similarity, and to *V. neptunius* LMG 20536^T, *V. coralliilyticus* LMG 20984^T, *V. nigripulchritudo* LMG 3896^T, *V. sinaloensis* LMG 25238^T and *V. brasiliensis* LMG 20546^{T}with 98.5 to 97.1 % similarity (**Table 1**).

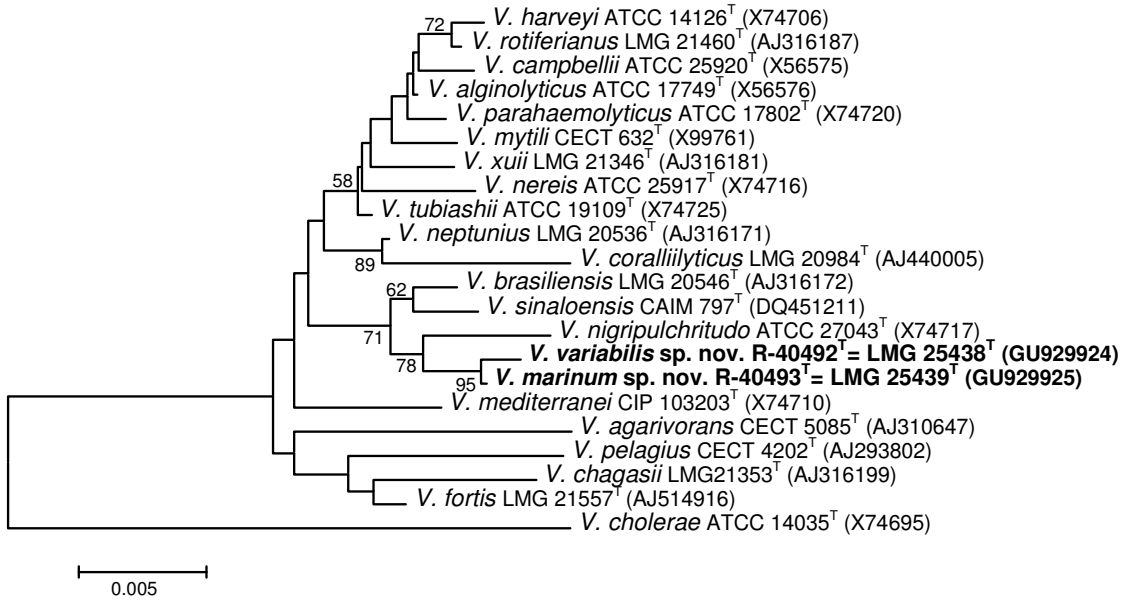

Figure 1. Neighbour-joining phylogenetic tree showing the phylogenetic position of *V. variabilis* sp. nov. and *V. marinum* sp. nov. based on 16S rRNA gene sequences. The evolutionary distances were computed using the Jukes-Cantor method. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) based on 1000 repetitions are shown. *Vibrio cholerae* was used as outgroup. Bar, 0.5 % estimated sequence divergence. Fragments of 1328 bp and 1507 bp were obtained for *V. variabilis* and *V. marinum*, respectively.

Table 1. DNA-DNA hybridization data and 16S rRNA gene sequence similarities of *V. variabilis* sp. nov., *V. marinum* sp. nov. and related *Vibrio* species.

Strain	16S rRNA similarity (%)		DNA-DNA hybridization values (%):						
	1	2	1	2	3	4	5	6	7
1. *V. variabilis* sp. nov. R-40492^T (= LMG 25438^T)	100		100	57	10	11	3	9	5
2. *V. marinum* sp. nov. R-40493^T (= LMG 25439^T)	99.0	100	55	100	13	11	2	7	8
3. *V. neptunius* LMG 20536^T	98.5	97.1	8	9	100	33	4	8	14
4. *V. coralliilyticus* LMG 20984^T	98.2	97.3	9	11	48	100	3	11	12
5. *V. nigripulchritudo* LMG 3896^T	98.2	98.5	11	12	13	21	100	8	8
6. *V. sinaloensis* LMG 25238^T	98.2	98.3	11	9	17	15	7	100	10
7. *V. brasiliensis* LMG 20546^T	98.1	98.4	15	13	24	22	6	14	100

Trees based on partial sequences of the housekeeping genes *ftsZ* (525 bp), *gyrB* (743 bp), *recA* (556 bp), *rpoA* (790 bp), *topA* (553 bp), *pyrH* (531 bp) and *mreB* (830 bp) confirmed their phylogenetic position in the genus *Vibrio*, and revealed they constituted a separate branch (**Supplementary Figures S1-S7**). Similarities between R-40492^T and R-40493^T were 94.5 % (for ftsZ), 95.4 % (for *gyrB*), 95.7 % (for *recA*), 98.5 % (for *rpoA*), 97.8 % (for *topA*), 96.7 % (for *pyrH*) and 95.6 % (for *mreB*), confirming their close relationship to each other. Gene sequence similarities among R-40492^T, R-40493^T and the type strains of the closest phylogenetic species, for which these housekeeping gene sequences were available through GenBank/EMBL data base or the website TAXVIBRIO (http://www.taxvibrio.lncc.br/) (i.e. *V. neptunius*, *V. coralliilyticus, V. nigripulchritudo* and *V. brasiliensis*, were less than 92 % (**Supplementary Table S2**). A neighbour-joining tree based on concatenated gene sequences of 16S rRNA, *ftsZ, gyrB, recA, rpoA, topA, pyrH* and *mreB* (6035 bp in length), confirmed the separate phylogenetic position of the novel strains R-40492^T and R-40493^T in the genus *Vibrio*, with support of high bootstrap values in both Neighbour-Joining and Maximum-Parsimony methods (**Figure 2**).

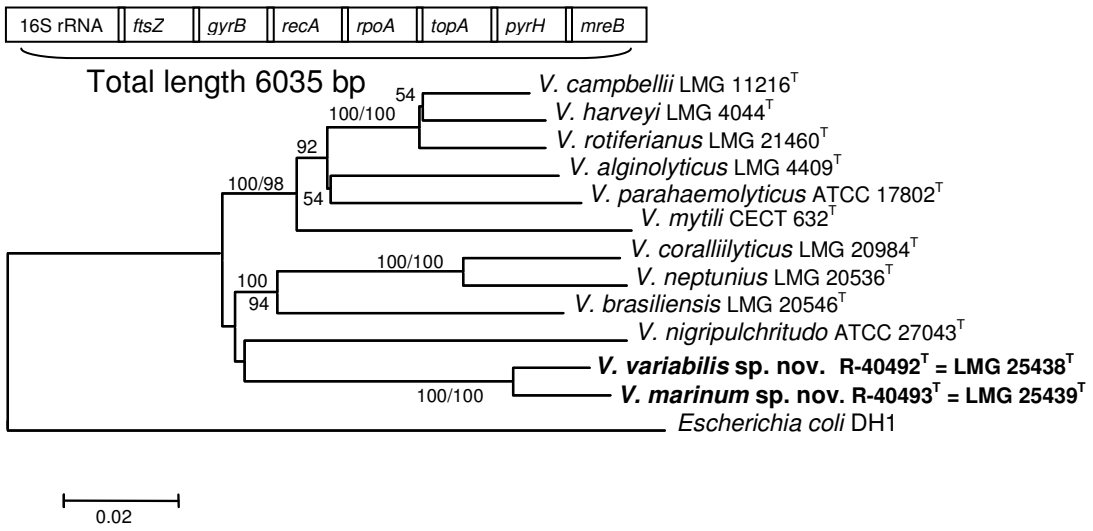

Figure 2. Neighbour-joining phylogenetic tree showing the phylogenetic position of *V. variabilis* sp. nov. and *V. marinum* sp. nov. based on concatenated 16S rRNA, *ftsZ, gyrB, recA, rpoA, topA, pyrH and mreB* gene sequences (6035 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) based on 1000 repetitions are shown. The numbers at nodes denote the level of bootstrap values derived from the neighbour-joining and maximum-parsimony methods. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.

DNA–DNA hybridization experiments were performed among strains R-40492^T, R-40493^T and the type strains of the closest phylogenetic neighbours (**Table 1**). The results showed that the novel strains R-40492^T and R-40493^T had 55-57 % mutual DDH similarity. This value is below the threshold for species delineation, considering the deviation of the methodology which is known to be around 7 %. The DDH similarity towards other species was 15 % (**Table 1**). DDH data clearly provide evidence that the two novel strains belong to two novel *Vibrio* species.

The two novel strains shared the main phenotypic and chemotaxonomic features of the genus *Vibrio* (Baumann & Schubert, 1984). They were Gram-negative, motile, oxidase- and catalase-positive and showed prolific growth on thiosulfate/citrate/bile salts/sucrose agar (TCBS). The major fatty acids were summed feature 3 (comprising $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*), $C_{18:1}$ ω7*c* and $C_{16:0}$ corresponding to 64 % (R-40492^T) and 70 % (R-

40493^T) of the total FAME profile (**Supplementary Table S1**). Prolific growth occurred in media containing 2.0 % (w/v) NaCl at 28 °C and no growth without NaCl.

The isolates were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, arginine dihydrolase and gelatinase activities, indol production, glucose, manitol, saccharose and amygdalin fermentation, nitrate reduction to nitrite and assimilation of dextrin, glycogen, tween 40, tween 80, N-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl D-glucoside, psicose, D-raffinose, D-sorbitol, sucrose, D-trehalose, turanose, methyl pyruvate, acetic acid, DL-lactic acid, succinic acid, alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-serine, L-threonine, inosine, uridine, thymidine, glycerol and DL-α-glycerol phosphate. They were negative for α-chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase and urease activities, H_2S and acetoin (Voges Proskauer) production, reduction of nitrate to N_2 gas, sorbitol, melibiose, and arabinose fermentation and assimilation of α-cyclodextrin, citrate, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, *i*-erythritol, L-fucose, *m*-inusitol, α-lactose, α-D-lactose lactulose, L-rhamnose, xylitol, monomethyl succinate, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, D-alanine, L-aspartic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, γ-aminobutyric acid, urocanic acid, phenyl ethylamine, putrescine, 2-amino ethanol and 2,3-butanediol.

Phenotypic features such as acetoin production, N-acetyl-β-glucosaminidase, β-galactosidase, trypsin Lipase (C14) and cystine arylamidase activities, mannitol, inositol, L-rhamnose and amygdalin fermentation and assimilation of D-glucuronic acid, glucose-1-phosphate and glucose-6-phosphate can be usedl to differentiate the two novel species

from the phylogenetically related and other known *Vibrio* species, and from each other (**Table 2 and Supplementary Table S3-S4**).

The phenotypic profiles of the two novel strains are based on one strain. However, five strains, isolated as described by Chimetto *et al*. (2009) (R-77, R-78, R-91, R-616, and R-619), have been reported (*Vibrio L2* sp. nov.) to be closely related with these two new taxa based on 16S rRNA and *pyrH* gene sequences. Unfortunately, all strains, except R-78 (=R-40492^T) and R-91 (=R-40493^T), lost viability. As more strains of these species are isolated and tested, the phenotypic profile may change slightly.

Based on the polyphasic analysis performed in this study, the creation of two new species, *V. variabilis* sp. nov (R-40492^T = LMG 25438^T = CAIM 1454^T) and *V. marinum* sp. nov (R-40493^T = LMG 25439^T = CAIM 1455^T) is proposed, to allocate the novel strains.

Table 2. Features that differentiate *V. variabilis* sp. nov. and *V. marinum* sp. nov. from its closest phylogenetic neigbours.

Species: **1**, *V. variabilis* sp. nov. R-40492^T (= LMG 25438^T); **2**, *V. marinum* sp. nov. R-40493^T (= LMG 25439^T); **3**, *V. neptunius* LMG 20536^T; **4**, *V. coralliilyticus* LMG 20984^T; **5**, *V. nigripulchritudo* LMG 3896^T; **6** *V. sinaloensis* LMG 25238^T; **7**, *V. brasiliensis* LMG 20546^T. +, Positive; -, negative; W, weak; ND, not determined. All data were obtained in this study except where indicated otherwise.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Growth with 8 NaCl (%w/v):	-	w	-	-	-	+	-
Growth at 40 °C	+	-	-	ND	-	+	+
Production of:							
Acetoin (Voges Proskauer)	-	-	+	+	-	-	-
Fermentation of:							
Mannitol	+	+	-	+	-	-	+
Inositol	-	+	-	-	-	-	-
L-rhamnose	+	-	-	-	-	-	-
Amygdalin	+	+	-	-	+	-	+
Melibiose	-	-	-	-	+	-	-
Enzyme activity:							
Lipase (C14)	-	+	+	w	-	-	+
Cystine arylamidase	+	-	-	-	-	-	-
Trypsin	+	-	+	+	-	-	-
N-acetyl-β-glucosaminidase	+	w	+	-	-	-	-
β-galactosidase	+	+	-	-	w	-	+
Tryptophan deaminase	-	-	-	+	-	+	-
Utilization of:							
D-glucuronic acid	-	+	-	-	ND	-	-
Propionic acid	-	-	-	w	ND	-	-
Glucose-1-phosphate	-	+	+	-	ND	-	-
Glucose-6-phosphate	-	+	+	+	ND	-	-
α-ketoglutaric acid	-	+	+	+	ND	-	-
G+C content (mol %)	46.8	46.3	46[a]	46.2	46-47[b]	45.8	45.9[a]
FAME composition*							
$C_{12:0}$	2.3	5	2	1.8	-	4.5	2.1
$C_{15:0}$ iso 3-OH	1.3	-	-	1	-	-	-
$C_{16:0}$	13	16	18.4	15.5	23	20	17
$C_{17:0}$	-	-	1.8	2	-	-	-
$C_{17:0}$ iso	9	3.9	-	2.2	-	-	-
$C_{18:0}$	-	1.4	1.8	1	2.4	-	1.3

*Fatty acids profiles of known *Vibrio* species (type strain) were obtained in this study under the same conditions. Data are expressed as percentages of total fatty acids. Fatty acids representing <1 % are not shown. [a] Thompson *et al*. (2003b); [b] Baumann & Schubert (1984).

Description of *Vibrio variabilis* sp. nov.

Vibrio variabilis (L. masc. adj. *variabilis*, changeable, variable, referring to the change of colour of the colonies.)

Cells are 0.9 µm wide 1.5–2.5 µm long, Gram-negative, motile rods. Forms translucent, convex, smooth-rounded colonies with entire margins, 1 mm in size after 1 day incubation at 28 °C on TSA. It is beige in colour, but colonies my turn black after some time under limited light conditions. Growth occurs between 15 and 40 °C and at NaCl concentrations (w/v) of 0.5–7.0 %. No growth is observed in 0 or ≥ 8 % NaCl or at ≤ 7 or ≥ 42 °C. Colonies are able to grown on the selective medium TCBS. The strain was positive for valine arylamidase, cystine arylamidase, tripsin, N-acetyl-β-glucosaminidase activities, assimilation of α-hydroxy butyric acid and rhamnose fermentation, but negative for D-glucuronic acid, α-keto glutaric acid, succinamic acid, glucuronamide, DL-carnitine, glucose-1-phosphate and glucose-6-phosphate assimilaton, lipase (C14) activity and inositol fermentation (**Supplementary Table S2**). The main cellular fatty acids are summed feature 3 (comprising $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c,* 31.5 %), $C_{18:1}$ ω7*c* (19.5 %) and $C_{16:0}$ (13 %). The following fatty acids are present in small amounts: $C_{17:0}$ iso (9 %), $C_{14:0}$ (5.7 %), $C_{15:0}$ iso (5.5 %), $C_{12:0}$ (2.3 %) summed feature 2 ($C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE, 2.1 %), $C_{13:0}$ iso, (1.9 %), $C_{12:0}$ 3-OH (1.6 %), $C_{15:0}$ iso 3-OH (1.3 %) (**Supplementary Table S1**). The DNA G+C content of strain R-40492^T is 46.8 mol%. The type strain (R-40492^T = LMG 25438^T = CAIM 1454^T) was isolated from the mucus of the zoanthid *Palythoa caribaeorum* in Preta Beach, São Sebastião channel, SP, Brazil.

Description of *Vibrio marinum* sp. nov.

Vibrio marinum (ma.ri'num. L. neut. adj. *marinum* of the sea, marine).

Cells are 1 µm wide 1.5–4.0 µm long, Gram-negative, motile bacilli. Forms translucent, convex, smooth-rounded colonies with entire margins, 1 mm in size and beige in colour after 1 day incubation at 28 °C on TSA. Growth occurs between 15 and 37 °C and at NaCl concentrations (w/v) of 0.5–8.0 %. Weak growth is observed in the presence of 8 % NaCl. No growth is observed in 0 or ≥ 9 % NaCl or at ≤ 7 or ≥ 40 °C. Colonies are able

to grown on the selective medium TCBS. The strain was positive D-glucuronic acid, α-keto glutaric acid, succinamic acid, glucuronamide, DL-carnitine, glucose-1-phosphate and glucose-6-phosphate assimilation, lipase (C14) activity and inositol fermentation. Weak reaction was observed for N-acetyl-β-glucosaminidase activity and α-hydroxy butyric acid assimilation. It was negative for valine arylamidase, cystine arylamidase, tripsin activities and rhamnose fermentation (**Supplementary Table S2**). The main cellular fatty acids are summed feature 3 (comprising $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c,* 30.5 %), $C_{18:1}$ ω7*c* (23.5 %) and $C_{16:0}$ (16 %). The following fatty acids are present in small amounts: $C_{14:0}$ (7.5 %), $C_{12:0}$ (5 %) $C_{17:0}$ iso (3.9 %), summed feature 2 ($C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE, 2.9 %), $C_{12:0}$ 3-OH (2.5 %), $C_{15:0}$ iso (2 %), $C_{18:0}$ (1.4 %) and $C_{13:0}$ iso, (1.2 %) (**Supplementary Table S1**). The DNA G+C content of strain R-40493^T is 46.3 mol%. The type strain (R-40493^T = LMG 25439^T = CAIM 1455^T) was isolated from the mucus of the zoanthid *Palythoa caribaeorum* in Portinho Beach, São Sebastião Channel, SP, Brazil.

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Supplementary data

Supplementary Table S1. GenBank accession numbers of the housekeeping genes sequences of *Vibrio variabilis* sp. nov., *Vibrio marinum* sp. nov., the type strains of the phylogenetically related *Vibrio* species and *Escherichia coli* DH1, used in this study.

Strains	*ftsZ*	*gyrB*	*recA*	*rpoA*	*topA*	*pyrH*	*mreB*
***V. variabilis sp.nov.* R-40492^T**	GU929926	GU929928	GU929934	GU929936	GU929938	GU929932	GU929930
***V. marinum sp.nov.* R-40493^T**	GU929927	GU929929	GU929935	GU929937	GU929939	GU929933	GU929931
V. campbellii	DQ907337	AB298205	EF596670	AJ842564	EF596698	EF596641	DQ907408
V. harveyi	DQ907350	AB298221	AJ842440	AJ842627	DQ907488	EU118238	DQ907422
V. rotiferianus	EF596703	EU118210	AJ580868	AJ842688	DQ907515	EF596722	DQ907445
V. alginolyticus	EF027344	AB298202	AJ842373	AJ842558	DQ907472	GU266285	DQ907405
V. parahaemolyticus	DQ907367	AY527390	AJ580864	AJ842677	DQ907509	GU266286	DQ907440
V. mytili	DQ907358	AB298231	AJ842472	AJ842657	DQ907499	GU266287	DQ907431
V. coralliilyticus	DQ907341	AB298210	AJ842402	AJ842587	EF114213	GU266292	DQ907412
V. neptunius	DQ907361	AB298234	AJ580861	AJ842663	DQ907503	GU266291	DQ907435
V. nigripulchritudo	EF027347	AB298236	AJ842480	AJ842667	DQ907505	GU266290	DQ907437
V. brasiliensis	DQ907335	AB298204	AJ842376	AJ842563	DQ907473	*	DQ907407
***E. coli* DH1**	CP001637	CP001637	CP001637	CP001637	CP001637	CP001637	CP001637

* Sequence used was retrieved from the database "The Taxonomy of Vibrios" (http://www.taxvibrio.lncc.br/)

Supplementary Table S2. Sequence similarity values (%) between the *ftsZ*, *gyrB, recA, rpoA, topA, pyrH* and *mreB* gene sequences of *V. variabilis* sp. nov. (R-40492^T), *V. marinum* sp. nov. (R-40493^T) and the type strains of the phylogenetically related *Vibrio* species.

Type strain *	***V. variabilis* sp. nov. R-40492^T = LMG 25438^T**						
	ftsZ	***gyrB***	***recA***	***rpoA***	***topA***	***pyrH***	***mreB***
Vibrio brasiliensis	79.9	84.5	86.9	90.9	79.2	84.4	84.3
Vibrio coralliilyticus	80.2	82.7	86.0	91.4	75.3	81.7	81.7
Vibrio neptunius	81.0	83.7	84.9	91.0	74.5	82.4	81.2
Vibrio nigripulchritudo	80.4	83.0	87.2	91.2	76.7	83.0	81.7
	***V. marinum* sp. nov. R-40493^T = LMG 25439^T**						
Vibrio brasiliensis	81.6	83.8	87.4	91.3	79.2	84.4	84.4
Vibrio coralliilyticus	82.1	83.6	86.6	91.8	75.0	84.0	81.8
Vibrio neptunius	81.6	84.1	86.1	90.9	74.5	83.7	80.7
Vibrio nigripulchritudo	82.5	83.9	87.5	91.3	76.3	83.6	81.4

* Strains and accession numbers of the type strains used in MLSA analyzes are available in the trees of each gene in the supplementary material S1-S7.

Supplementary Table S3. Cellular fatty acid contents of *V. variabilis* sp. nov., *V. marinum* sp. nov. and the phylogenetically related *Vibrio* species.

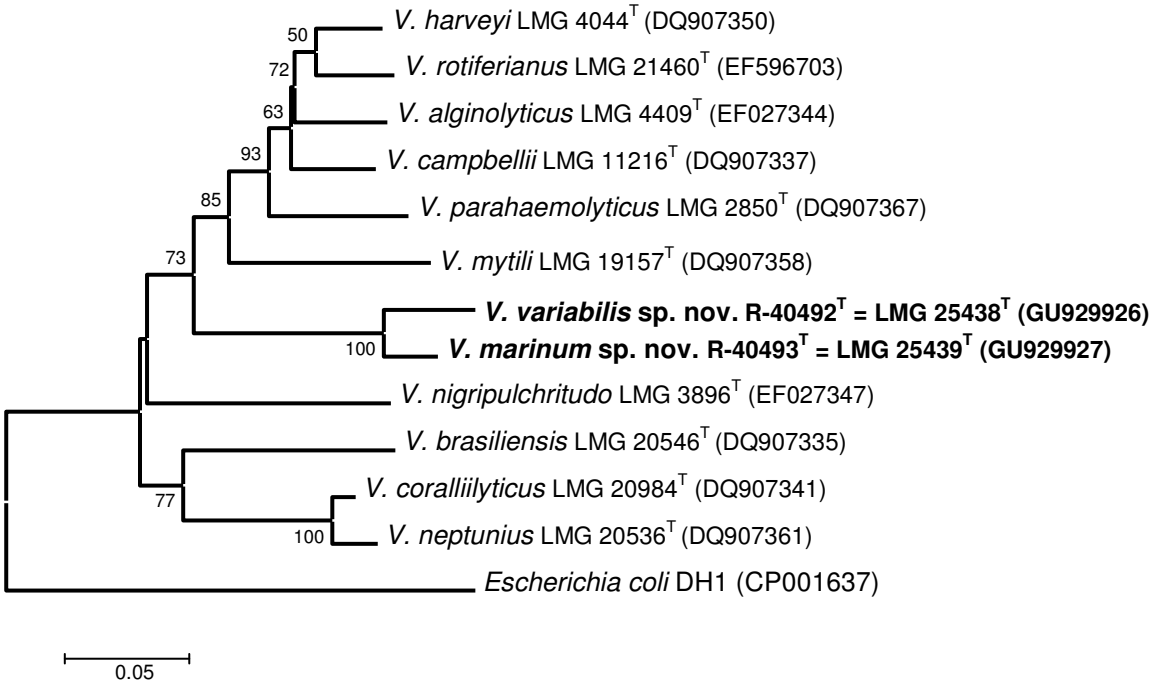
Taxa: **1,** *V. variabilis* R-40492^T; **2**, *V. marinum* R-40493^T; **3**, *V. neptunius* LMG 20536^T; **4**, *V. coralliilyticus* LMG 20984^T; **5**, *V. nigripulchritudo* LMG 3896^T; **6**, *V. sinaloensis* LMG 25238^T; **7**, *V. brasiliensis* LMG 20546^T. Summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ ALDE. Summed feature 3 comprises $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*. Data are expressed as percentages of total fatty acids. Fatty acids representing <1 % are not shown.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**
$C_{12:0}$	2.3	5	2	1.8	-	4.5	2.1
$C_{12:0}$ 3-OH	1.6	2.5	1.7	2.3	3	1.5	2.1
$C_{13:0}$ iso	1.9	1.2	-	1.7	-	-	-
$C_{14:0}$	5.7	7.5	4.7	5	6	12	5.6
$C_{15:0}$ iso	5.5	2	-	1.6	-	-	-
$C_{15:0}$ iso 3-OH	1.3	-	-	1	-	-	-
$C_{16:0}$	13	16	18.4	15.5	23	20	17
$C_{16:0}$ iso	-	-	-	1.2	-	-	1.5
$C_{16:1}$ ω9c	-	-	-	-	1.6	1.7	-
$C_{17:0}$	-	-	1.8	2	-	-	-
$C_{17:0}$ iso	9	3.9	-	2.2	-	-	-
$C_{17:1}$ ω6*c*	-	-	1.2	-	-	-	-
$C_{17:1}$ ω8*c*	-	-	1.3	1.9	-	-	-
$C_{18:0}$	-	1.4	1.8	1	2.4	-	1.3
$C_{18:1}$ ω7*c*	19.4	23.5	22.5	21.5	18.7	9.6	26.7
$C_{18:1}$ ω9*c*	-	-	-	-	1.2	-	-
Summed feature 2	2.1	2.9	2.3	3	6	7.8	2.2
Summed feature 3	31.5	30.5	35.8	32	38	40	34.9
Unknown 12.484	-	-	-	1	-	1.2	-

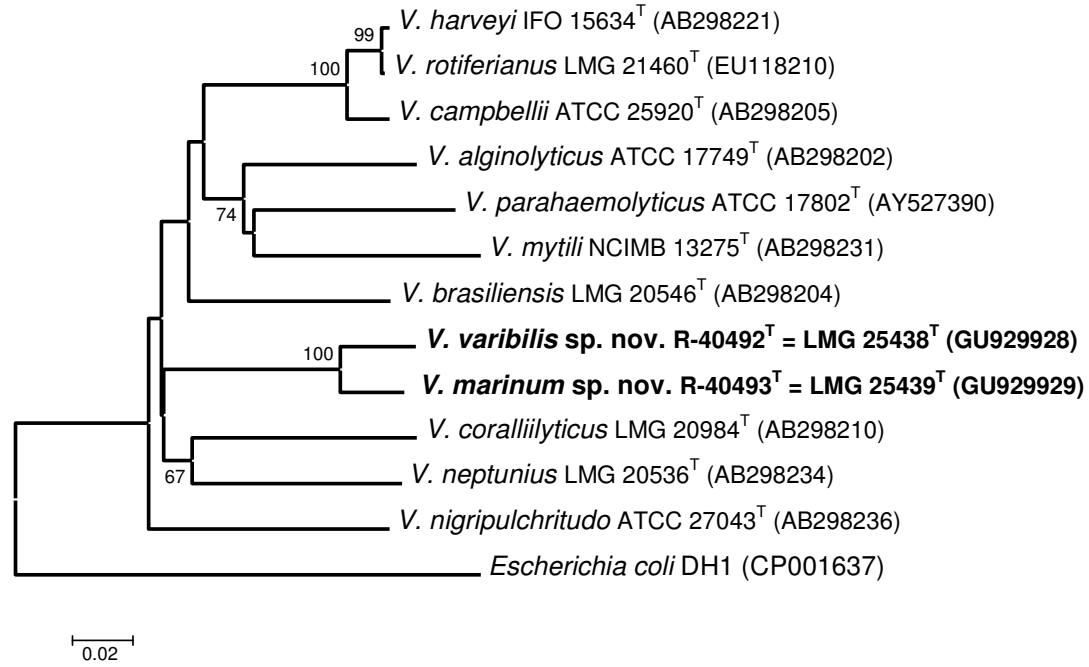
Supplementary Table S4. Phenotypic variability between *V. variabilis* sp. nov. (**R-40492^T**) and *V. marinum* sp. nov. **(R-40493^T).**

Utilization of	R-40492^T	R-40493^T
Lipase (C14)	-	+
Valine arylamidase	+	-
Cystine arylamidase	+	-
Tripsin	+	-
N-acetyl-β-glucosaminidase	+	w
Inositol	-	+
Rhamnose	+	-
D-glucuronic acid	-	+
α-hydroxy butyric acid	+	w
α-keto glutaric acid	-	+
Succinamic acid	-	+
Glucuronamide	-	+
DL-carnitine	-	+
Glucose-1-phosphate	-	+
Glucose-6-phosphate	-	+

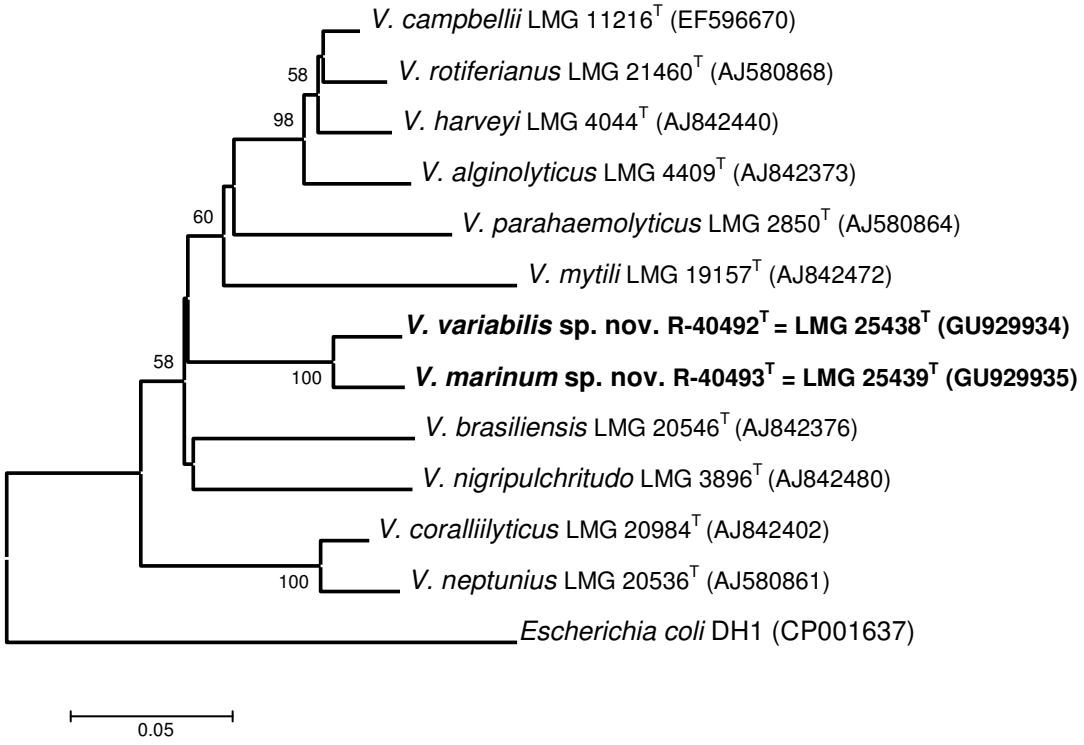
W = weak reaction



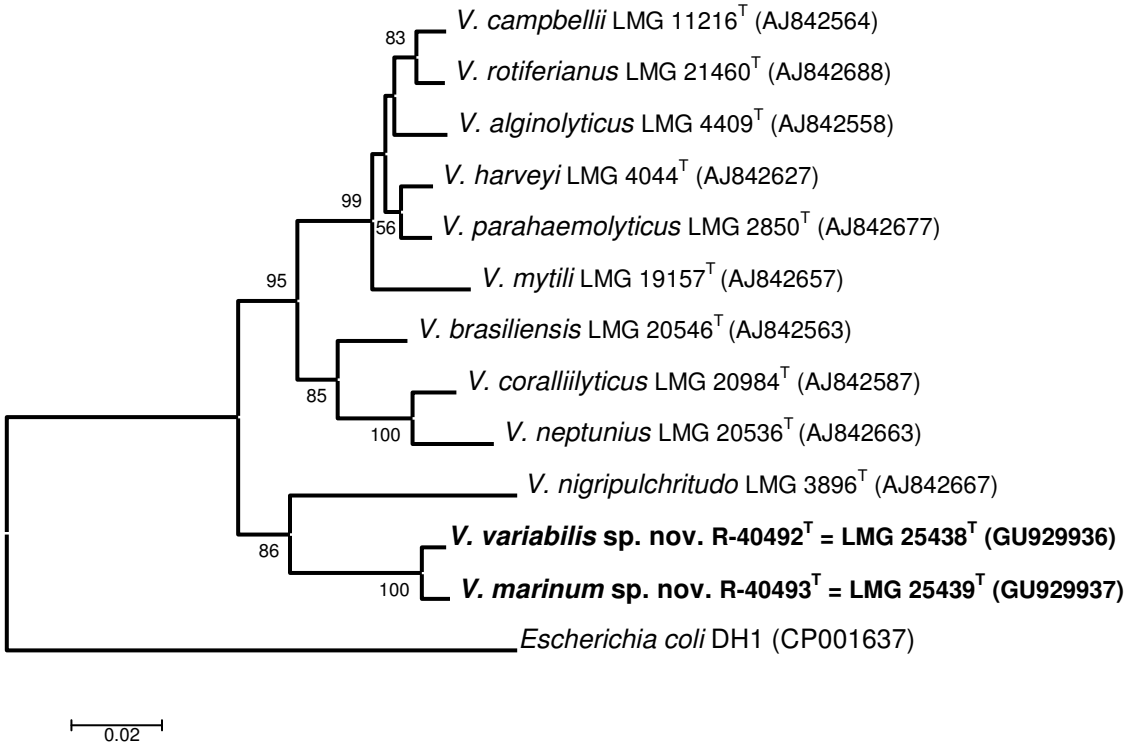
Supplementary - Figure S1. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *ftsZ* gene sequences (525 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) shown are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 5 % estimated sequence divergence.



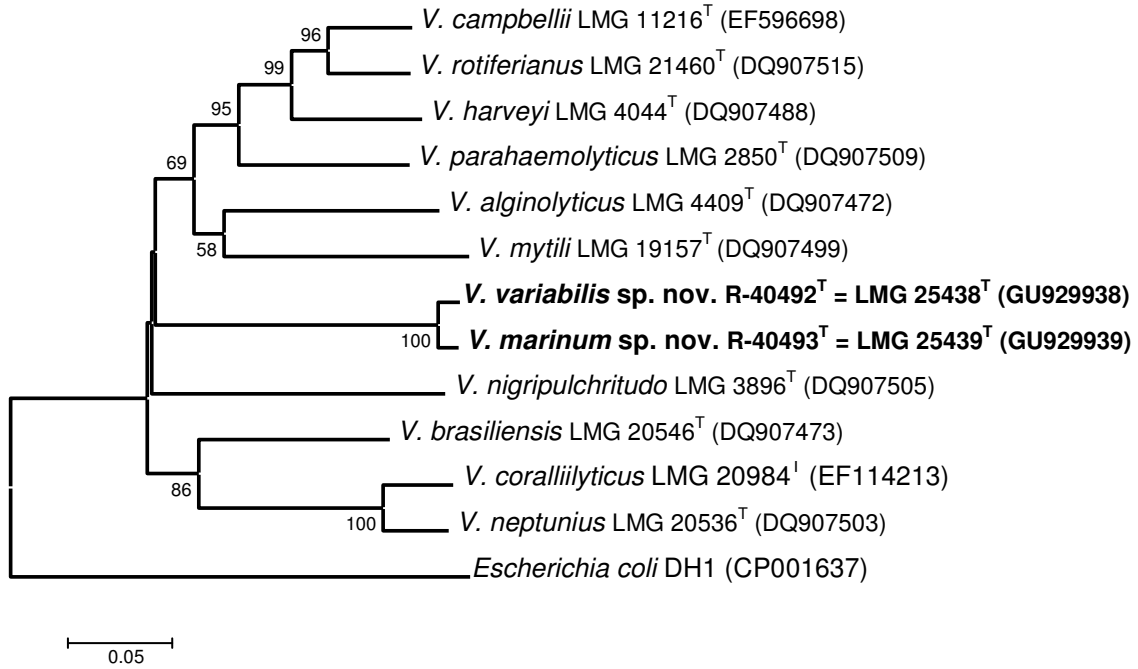
Supplementary - Figure S2. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *gyrB* gene sequences (743 bp).. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 2 % estimated sequence divergence.



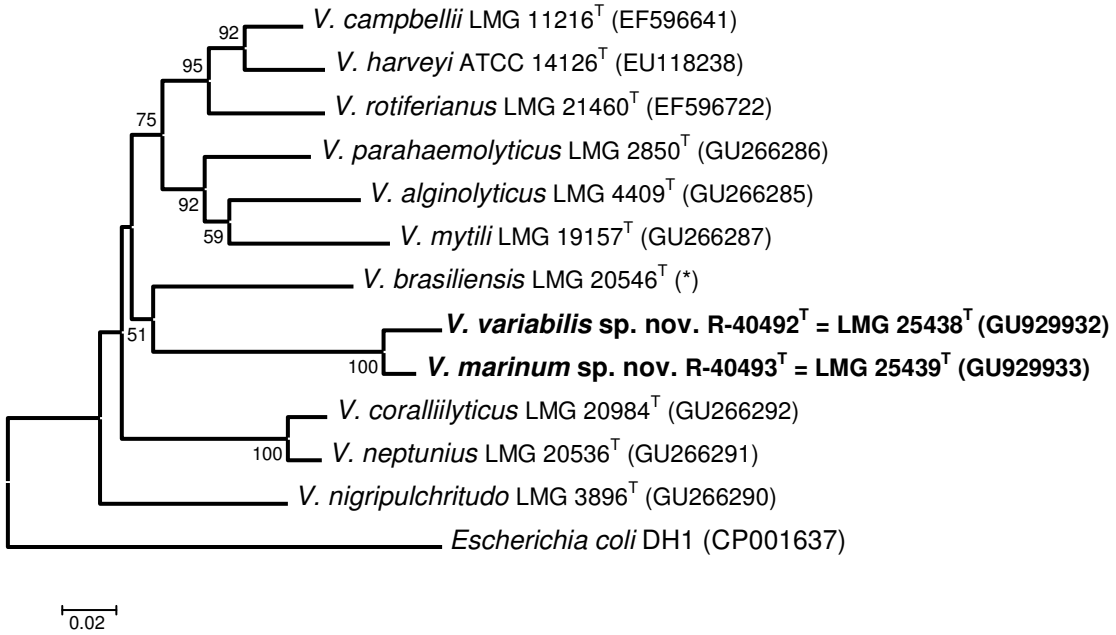
Supplementary - Figure S3. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *recA* gene sequences (556 bp). Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 5 % estimated sequence divergence.



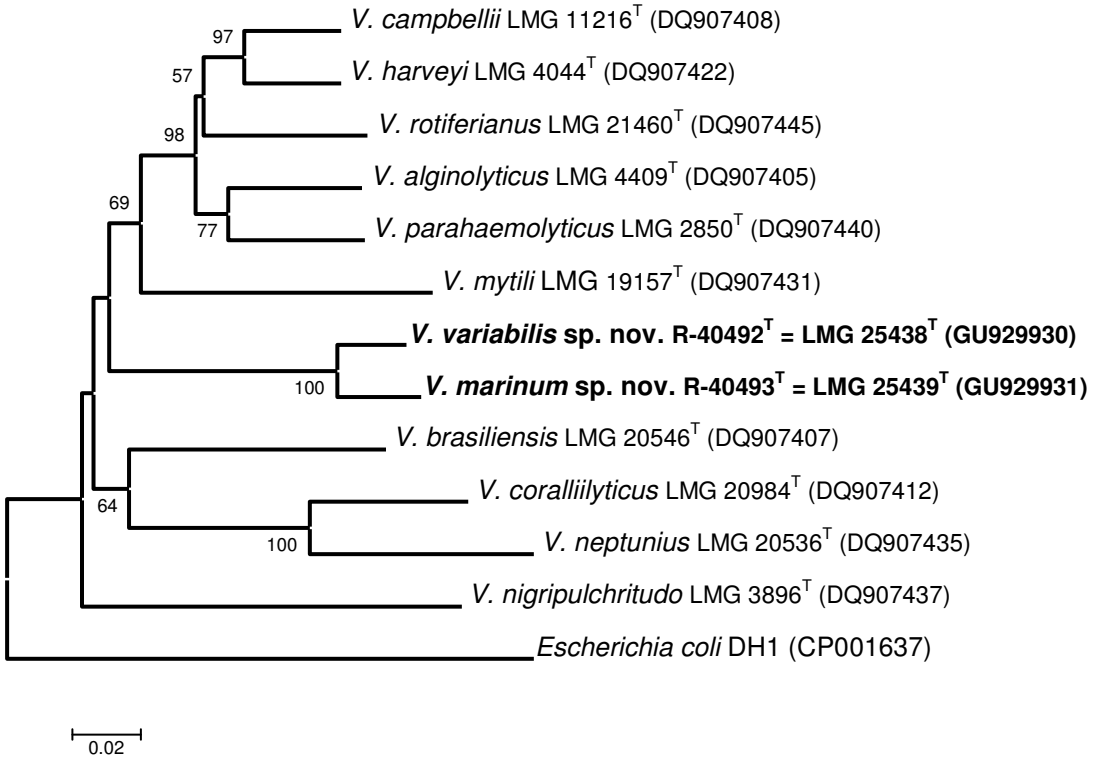
Supplementary - Figure S4. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *rpoA* gene sequences (790 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) shown are based on 1000 repetitions. *Escherichia coli* was used as outgroup. Bar, 2 % estimated sequence divergence.



Supplementary - Figure S5. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *topA* gene sequences (553 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 5 % estimated sequence divergence.



Supplementary - Figure S6. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *pyrH* gene sequences (531 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 2 % estimated sequence divergence. * Sequence used was retrieved from the database The Taxonomy of Vibrios (http://www.taxvibrio.lncc.br/).



Supplementary - Figure S7. Neighbour-joining phylogenetic tree showing the position of *V. variabilis* sp. nov. and *V. marinum* sp. nov based on *mreB* gene sequences (830 bp). The evolutionary distances were computed using the Jukes-Cantor method. Phylogenetic analyses were conducted in MEGA4. Bootstrap values (> 50 %) shown are based on 1000 repetitions. *E. coli* was used as outgroup. Bar, 2 % estimated sequence divergence.

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IV.9. *Marinomonas brasilensis* sp. nov. isolated from the coral *Mussismilia hispida* and reclassification of *Marinomonas basaltis* as a later synonym of *Marinomonas communis*.

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Marinomonas brasilensis sp. nov. isolated from the coral _Mussismilia hispida_ and reclassification of _Marinomonas basaltis_ as a later synonym of _Marinomonas communis_

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Running Title:

Marinomonas brasilensis sp. nov.

Subject Category: New taxa, _Proteobacteria_

Footnote: The GenBank/EMBL accession number for the 16S rRNA gene sequence of strain R-40503^T is GU929940.

Abstract

A Gram-negative, aerobic bacterium, designated R-40503^T was isolated from mucus of the reef builder coral, *Mussismilia hispida,* located in the São Sebastião Channel, São Paulo, Brazil. Phylogenetic analyses revealed that strain R-40503^T belongs to in the genus *Marinomonas*, and more precisely in the sublineage containing *Marinomonas vaga*, *Marinomonas basaltis*, *Marinomonas communis*, *Marinomonas aquimarina* and *Marinomonas ostreistagni*. The 16S rRNA gene sequence similarity of R-40503^T was above 97 % with the type strains of *Marinomonas vaga*, *Marinomonas basaltis*, *Marinomonas communis* and *Marinomonas pontica*, and below 97 % with the type strains of the other described *Marinomomas* species. Strain R-40503^T showed less than 35 % DDH relatedness with the type strains of the phylogenetically closest *Marinomonas* species, demonstrating it should be classified into a novel species. AFLP supported the latter. Several phenotypic features can be used to discriminate the novel species from its neighbours. For instance, oxidase and urease activity, utilization of L-asparagine, the presence of the fatty acid C12:1 3-OH, and absence of the fatty acids C10:0 and C12:0. The DNA G+C content of strain R-40503^T is 46.5 mol%. The name *Marinomonas brasilensis* sp. nov. (type strain is R-40503^T = LMG 25434^T = CAIM 1459^T) is proposed for this new taxon. Concurrently, a close relationship between *M. basaltis* and *M. communis* was observed. The type strains of these species showed 78 % DDH relatedness and 63 % AFLP pattern similarity. Their phenotypic features were very similar, and their DNA G+C content was identical (46.3 mol%). These data demonstrate synonymy of *Marinomonas basaltis* and *Marinomonas communis.*

Mussismilia hispida is one of the major reef-builders corals along the northeastern Brazilian coast, and it also has the widest geographic distribution among its genus (from Maranhão to Santa Catarina states, ca. 5000 km) (Leão & Kikuchi, 2005). The ability of *Mussismilia* to survive in different regions indicates its adaptation to wide environmental gradients, such as temperature, water turbidity and pollution. However, recent studies have revealed that *M. hispida* and *M. braziliensis* are threatened by extinction (Castro *et al.*, 2010; Francini-Filho *et al.*, 2008). Microorganisms appear to play a key role in coral

health. Microorganisms and a coral host make up a holobiont (Rosenberg *et al.,* 2007), and holobiont microbiota appear to protect their host by providing it nourishment and antibiotics (Raina *et al.,* 2009; Shnit-Orland & Kushmaro, 2009). It is also recognized that the holobiont harbours a vast microbial diversity. In the last 10 years a growing number of studies have focused on the characterization of the coral microbiota diversity and ecology (Alves *et al*., 2009; Dinsdale *et al*. 2008; Rohwer *et al*. 2001).

The genus *Marinomonas* was created in 1983 to accommodate two *Alteromonas* species, *Alteromonas communis* and *Alteromonas vaga* (Baumann *et al*., 1972), which were distinct from the other species of *Alteromonas* (Van Landschoot & De Ley, 1983). At present, the genus *Marinomonas* comprises 15 species, mainly originating from sea-water of different geographical locations. *M. communis* and *M. vaga* (Baumann *et al.,* 1972; Van Landschoot and De Ley, 1983) were isolated from the Pacific Ocean, *M. pontica* (Ivanova *et al.,* 2005) from the Black Sea, *M. dokdonensis* (Yoon *et al.,* 2005) from the East Sea of Korea, and *M. mediterranea* (Solano & Sanchez-Amat, 1999) and *M. aquimarina* (Macián *et al.,* 2005) from the Mediterranean Sea. *M. polaris* (Gupta *et al.,* 2006) and *M. ushuaiensis* (Prabagaran *et al.,* 2005) were isolated from the subantarctic region, while *M. primoryensis* (Romanenko *et al.,* 2003) and *M. arctica* (Zhang *et al*., 2008) were isolated from sea-ice. *M. ostreistagni* (Lau *et al.,* 2006) and some *M. aquimarina* strains (Macián *et al.,* 2005) were isolated from oysters. *M. basaltis* (Chang *et al*., 2008) and *M arenicola* (Romanenko *et al*., 2009) were isolated from marine sandy sediment, while *M. balearica* and *M. pollencensis* (Espinosa *et al*., 2009) were isolated from seagrass *Posidonia oceanica.*

In the present study, one isolate (R-40503^T), obtained during a survey of the heterotrophic bacterial diversity associated with cnidarians in São Paulo (Brazil) (Chimetto *et al*., 2008, 2009), was investigated using a polyphasic taxonomic approach. The strain was isolated using the nitrogen-free (NFb) selective medium supplemented with 3 % NaCl after 4 days of incubation at 28 °C and it was previously placed in the genus *Marinomonas* by means of 16S rRNA gene sequence analysis.

The 16S rRNA gene sequence of R-40503^T (1425 nt) was obtained as described previously (Thompson *et al*., 2001, Chimetto *et al*., 2008, 2009). The raw sequence data

were transferred to the ChromasPro ver. 1.34 software (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. The sequence was aligned with sequences from EMBL using the ClustalW software (Chenna *et al*., 2003). Pairwise similarities were calculated with the BioNumerics 4.61 software (Applied Maths, Sint-Martiens-Latem Belgium), using an open gap penalty of 100 % and a unit gap penalty of 0 %. Similarity matrices and phylogenetic trees were constructed using the MEGA ver. 4.0 (Tamura *et al*., 2007) and the BioNumerics 4.61 software (Applied Maths, Belgium). Trees were drawn using the Neighbour-Joining (Saitou & Nei, 1987) and Maximum Parsimony methods (Eck & Dayhoff, 1966). The robustness of the topologies of the trees were checked by bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/).

The phylogenetic position of strain R-40503^T based on 16S rRNA gene sequence analysis was in the genus *Marinomonas*, and more precisely in a phylogenetic cluster containing the species *M. vaga, M. basaltis, M. communis, M. aquimarina* and *M. ostreistagni* (**Fig. 1 and Supplementary Figure S1)**. The 16S rRNA gene sequence similarity of R-40503^T was above 97 % with the type strains of *M. vaga* (97.9 %), *M. basaltis*, *M. communis* and *M. pontica* (all three 97.2 %), and below 97 % with the type strains of the other known *Marinomonas* species.

DNA-DNA hybridizations were performed between strain R-40503^T and the type strains of the closest phylogenetic neighbours, i.e. *M. vaga*, *M. basaltis, M. communis* and *M. aquimarina* (**Table 1**), using the microplate method described by Ezaki *et al*. (1989) with minor modifications (Willems *et al*., 2001). Hybridizations were performed at 40.7 °C in the presence of 50 % formamide. Reciprocal reactions were performed for every DNA pair and their variation was within the limits of this method (Goris *et al.,* 1998). The DDH relatedness between R-40503^T and the tested type strains was below 70 % (Table 1), which demonstrates that strain R-40503^T represents a novel species in the genus *Marinomonas* (Wayne *et al*., 1987; Stackebrandt & Ebers, 2006).

The DDH relatedness between *Marinomonas basaltis* LMG 25279^T and *Marinomonas communis* LMG 2864^T was above 70 % (i.e. 78 %), which suggests these species are

synonymous. Chang *et al*. (2008) obtained 56.2 % DDH similarity between the same pair of type strains, but the additional data of the present study support the value of 78 %.

The authenticity of *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T were verified by means of their 16S rRNA sequences**.** The sequences of both type strains showed 100 % similarity with those deposited, indicating the authenticity of the LMG strains **(Figure 1)**. The 16S rRNA gene sequence similarity between *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T was 98.7 %.

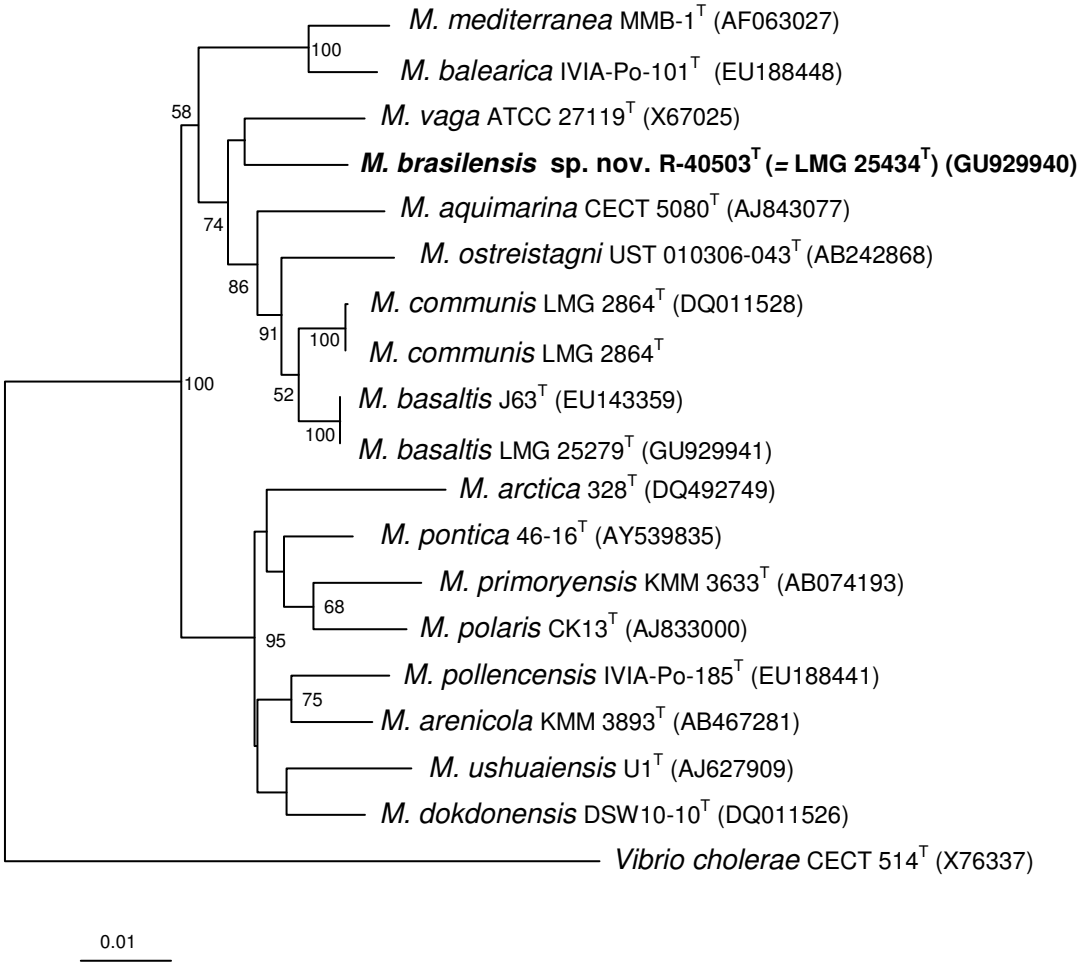

Figure 1. Neighbour-joining phylogenetic tree showing the phylogenetic position of *M. brasilensis* sp. nov. based on 16S rRNA gene sequences. The evolutionary distances were computed by BioNumerics 4.61 software (Applied Maths, Belgium). Bootstrap values (> 50 %) based on 1000 repetitions are shown. *Vibrio cholerae* was used as outgroup. Bar, 1 % estimated sequence divergence.

DNA G+C contents were determined for R-40503^T, *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T by HPLC as described previously (Mesbah *et al*., 1989). The DNA G+C content of strain R-40503^T was 46.5 mol% (**Table 1**), while LMG 25279^T and LMG 2864^T strains had the same value 46.3 mol%.

Table 1. DNA-DNA hybridization data, 16S rRNA gene sequence similarities and DNA G+C contents of *M. brasilensis* sp. nov. and phylogenetically related *Marinomonas* species

Strain	**G+C content (mol%)**	**16S rRNA Similarity (%)**	**DNA-DNA relatedness values (%) :**				
		1	**1**	**2**	**3**	**4**	**5**
1. *M. brasilensis* sp. nov. R-40503^T (= LMG 25434^T)	46.5	100	100				
2. *M. vaga* LMG 2845^T	47.5	97.9	34	100			
3. *M. basaltis* LMG25279^T	46.3	97.2	20	17	100		
4. *M. communis* LMG 2864^T	46.3	97.2	19	18	78	100	
5. *M. aquimarina* LMG25236^T	49	96.7	9	8	12	11	100

AFLP analysis was performed for strain R-40503^T, *M. basaltis* LMG 25279^T, *M. communis* LMG 2864^T, *M. vaga* LMG 2845^T and three *M. aquimarina* strains (**Figure 2**), basically as reported by Beaz Hidalgo *et al*. (2008). Briefly, 1 μg of DNA was digested with *Taq*I (5'TCGA3') and *Hind*III (5'AAGCTT3') (Amersham Pharmacia Biotech, Sweden), and subsequently ligated with double-stranded adaptors complementary to the ends of the restriction fragments, with T4 ligase (Amersham Pharmacia Biotech), to generate template DNA for PCR amplification. A selective PCR was then performed with the primers H01-6FAM (5'GACTGCGTACCAGCTTA3', labeled at the 5' end with the fluorescent dye 6-FAM) and T13 (5'GTTTCTTATGAGTCCTGACCGAG3'), using the conditions described by Thompson *et al*. (2001), in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). Separation of the selective PCR products was performed using a capillary ABI Prism 3130XL DNA sequencer (Applied Biosystems). The level of reproducibility was controlled by generating the AFLP pattern of *Marinomonas brasilensis* sp. nov. R-40305^T three times, starting from different subcultures. Normalization of the resulting electrophoretic patterns was performed using

the Gene Mapper 4.0 software (Applera Co., Norwalk, CT). For subsequent analysis fragments of 20 to 600 base pairs were transferred into the BioNumerics™ 4.61 software (Applied Maths, Belgium) algorithm. The similarity between the patterns of R-40503^T ranged from 93.0 to 94.4 %.

For numerical analysis, the zone from 40- and 580-bp was used. Similarity values were calculated using the Dice coefficient (tolerance value of 0.15 %), and a dendrogram was constructed using the UPGMA.

The similarity level chosen to delineate the AFLP clusters was 63 %, as previously proposed by Beaz Hidalgo *et al*. (2008). Strains with AFLP profiles showing more than 63 % similarity can be considered as members of the same species. The AFLP data supported the DDH data obtained in this study. R-40503^T showed at most 46 % pairwise band pattern similarity with its closest phylogenetic neighbours, being below the cut-off similarity level of 63 %, while the type strains of *M. basaltis* and *M. communis* constituted a distinguishable cluster with 69 % mutual AFLP pattern similarity **(Figure 2).** AFLP has been reported as a universally applicable technique with a high discriminatory power and a good reproducibility (Janssen *et al*., 1996; Savelkoul *et al*., 1999). It was proven to be useful for discrimination at the species level and below in *Aeromonas*, *Acinetobacter*, *Campylobacter*, *Xanthomonas* (Savelkoul *et al*., 1999), *Vibrionaceae* (Thompson *et al*., 2001), *Bradyrhizobium* (Willems *et al*., 2001), *Arcobacter* (On *et al*., 2003) and *Pantoea* (Brady *et al*., 2007). Phenotypic characteristics were determined in this study for the novel species and the type strains of the closest phylogenetic *Marinomonas* species i.e. *M. vaga*, *M. basaltis, M. communis* and *M. aquimarina.* Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). Cells for fatty acid analysis were grown on MA (Difco) for 24 h at 28 °C under aerobic conditions. Phenotypic characterization was performed using the API ZYM, API 20E and API 20NE kits (bioMérieux, France), and the Biolog GN2 microwell plates (Biolog Inc., USA), according to the manufacturer's instructions with minor modifications. Namely, cell suspensions for inoculation of the API tests were prepared in a 3 % (w/v) NaCl solution, and those for the Biolog GN2 microwell plates showed turbidity (transmission) of 20 %.

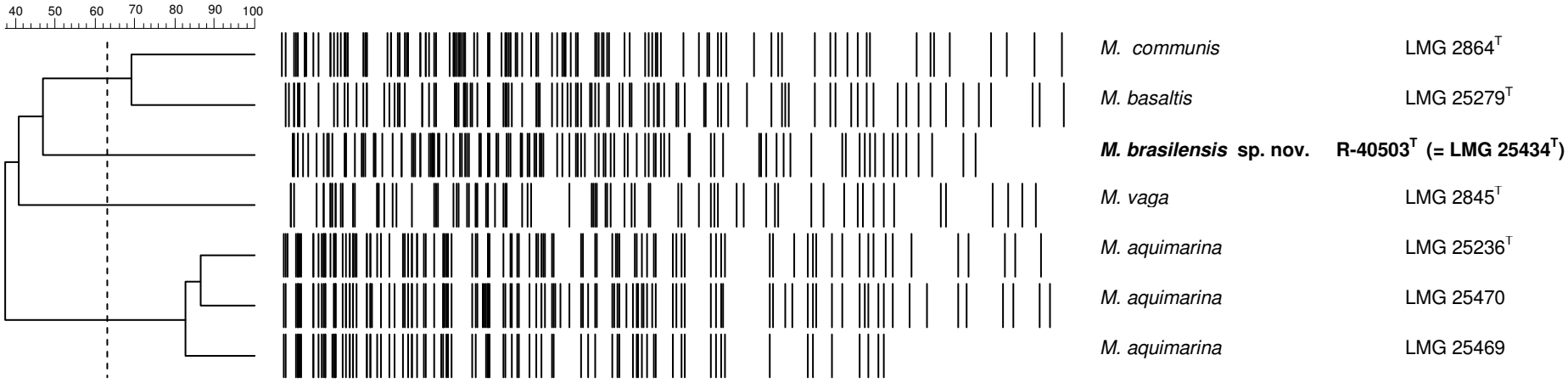

Figure 2. AFLP DNA fingerprints of *M. brasilensis* sp. nov. R-40503^T and strains of phylogenetically related *Marinomonas* species. The dendrogram was constructed with the UPGMA method after calculation of the band pattern similarity (%) using the DICE coefficient. The cut-off similarity level used to delineate AFLP clusters is 63 %. Strains with AFLP profiles showing more than 63 % similarity can be considered as members of the same species.

Cells for the suspensions were grown on Biolog medium for 24 h at 28 °C under aerobic conditions. The results of the tests were recorded after 24 to 48 h of incubation at 28 °C. Growth at different temperatures (4–42 °C) was determined by incubation on TSA (Difco) for 72 h. Growth at different salt concentrations (0–14 % NaCl) was determined by incubation on TSA (Difco) at 28 °C for 72h. Catalase activity was determined by adding young cells to a drop of a 3 % H_2O_2 solution and observation of 0_2 production. Oxidase activity was tested using 1 % N,N,N',N'-tetramethyl *p*-phenylenediamine (Kovacs, 1956). The novel species was differentiated from its closest phylogenetic neighbours by several phenotypic features (**Table 2**). Strain R-40503^T grew in medium containing 13 % NaCl, utilized tween 80, sucrose and L-asparagine but not α-ketoglutaric acid, L-aspartic acid, L-serine, L-ornithine and bromo succinic acid. It had oxidase activity, and was not able to grow at 40 ˚C (**Table 2**).

This novel species could be differentiated from its neighbours on the basis of the presence of the fatty acids $C_{12:1}$ 3-OH and the absence of the fatty acids $C_{10:0}$ and $C_{12:0.}$ The major cellular fatty acids of R-40503^T were $C_{18:1}$ ω7*c* (48.8 %), summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*) (19 %), $C_{16:0}$ (10.5 %) and $C_{10:0}$ 3-OH (8 %) (**Supplementary Table S1**).

Phenotypic features of *M. basaltis* and *M. communis* were very similar, except for some features, namely *M. communis* utilized saccharose, D-fructose, succinamic acid, urocanic acid and putrescine and had urease activity, whereas *M. basaltis* did not. Some results of the phenotype of *M. basaltis* obtained in this study are in contrast with those reported by Chang *et al*. (2008). They reported no growth in less than 1 % or more than 7 % NaCl, no esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase activities, but activities for trypsin and N-acetyl-β-glucosaminidase, and assimilation of L-arabinose, L-aspartic acid and glycerol. However, in this study, growth was observed at 0.5 – 11 % NaCl, as well as activities for esterase (C 4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Trypsin and N-acetyl-β-glucosaminidase activities, and assimilation of L-arabinose, L-aspartic acid and glycerol were not observed. In our hands, no significant phenotypic or genotypic differences were found between *M. communis* and *M. basaltis*.

Table 2. Phenotypic differences between *Marinomonas brasilensis* sp. nov. and its phylogenetic closest neighbours.

Species: **1**, *M. brasilensis* R-40503^T (= LMG 25434^T); **2**, *M. vaga* LMG2845^T; **3,** *M. basaltis* LMG 25279^T; **4**, *M. communis* LMG 2864^T; **5**, *M. aquimarina* LMG 25236^T. Data for the reference species were obtained in this study, except when indicated. Abbreviations: +, positive; -, negative; w, weak reaction, NA, not available. All data were obtained in this study (except some data of *M. aquimarina* LMG 25236^T) using the same laboratory conditions.

Characteristic	1	2	3	4	5
Growth with NaCl (%w/v):					
12	+	+	-	-	+
13	w	+	-	-	w
Growth at (°C)					
40	-	w	+	+	+
Activity of:					
Oxidase	+	-	+	+	+
Urease	+	+	-	+	+
Utilization of:					
Tween 80	+	w	-	-	-[a]
Sucrose	+	+	-	-	-[a]
α-D-glucose	+	w	+	+	NA
Alaninamide	+	+	-	-	NA
L-asparagine	+	-	+	+	NA
L-arabinose	w	-	-	-	-[a]
Cellobiose	w	-	w	w	-[a]
Glycerol	w	-	-	-	-[a]
Turanose	w	+	-	-	NA
α-hydroxy butyric acid	w	+	+	+	NA
α-ketobutyric acid	w	-	+	+	NA
Methyl pyruvate	-	-	w	+	+[a]
α-ketoglutaric acid	-	+	-	-	+[a]
L-aspartic acid	-	+	-	-	+[a]
L-serine	-	+	+	+	+[a]
L-ornithine	-	+	-	-	+[a]
Putrescine	-	w	-	+	-[a]
Bromo succinic acid	-	+	-	-	NA
Glycyl-L-aspartic acid	-	w	-	-	NA

[a] Data from Marcian *et al*. (2005).

Based on the phylogenetic, genotypic and phenotypic data, the new species *M. brasilensis* sp. nov. is proposed to encompass the strain R-40503^T (= LMG 25434^T = CAIM 1459^T).

The present study further revealed a synonymy between the species *M. basaltis* and *M. communis.* Following the taxonomic guidlines, *M. basaltis* (Chang *et al*., 2008) should be considered a later synonym of *M. communis* (Baumann *et al.,* 1972; Van Landschoot and De Ley, 1983).

Description of *Marinomonas brasilensis* sp. nov.

Marinomonas brasilensis (bra.si.len'sis. N.L. fem. adj. *brasilensis* of or belonging to Brazil).

Cells are Gram-negative, aerobic, halophilic, motile, straight rods approximately 1 µm wide and 1.5–3 µm long. Catalase- and oxidase- positive. Colonies on MA are circular, undulate, convex, smooth, beige in colour and 1 mm in size after 1 day of incubation at 28 °C. Prolific growth occurs between 20 and 35 °C and at NaCl concentrations (w/v) ranging from 1 to 11 %. No growth is observed in 0 % NaCl or in ≥ 14 % NaCl, and at ≤ 7 °C or at ≥ 40 °C. The strain has alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, urease and tryptophane deaminase enzyme activities, but it does not have lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and gelatinase activities. It produces acetoin (Voges Proskauer reaction), but no H_2S or indol. It does not ferment glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose. It is negative for nitrate reduction to nitrite or N_2 gas. It is capable to assimilate citrate, tween 40, tween 80, D-fructose, α-D-glucose, D-mannose, sucrose, monomethyl succinate, DL-lactic acid, D-saccharic acid, succinic acid, alaninamide, L-asparagine, L-glutamic acid, L-proline, inosine, uridine, and it is positive for hydrolysis of esculin. It has weak reaction for assimilation of α-cyclodextrin, L-arabinose, cellobiose, turanose, α-hydroxy butyric acid, α-keto butyric acid, urocanic acid and glycerol. It is negative for assimilation of dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, *i*-erythritol, L-fucose, D-galactose, gentiobiose, *m*-inositol, α-lactose, α-D-lactose lactulose, maltose, D-mannitol, D-melibiose, β-methyl D-glucoside,

psicose, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, xylitol, methyl pyruvate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy phenylacetic acid, itaconic acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-alanine, L-alanylglycine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, thymidine, phenyl ethylamine, putrescine, 2-amino ethanol, 2,3-butanediol, DL-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate, potassium gluconate, capric acid, adipic acid, malate, and trisodium citrate. The main cellular fatty acids are $C_{18:1}$ ω7*c*, summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*), $C_{16:0}$ and $C_{10:0}$ 3-OH corresponding to 86 % of the total FAME profile. The following fatty acids are present in small amounts: unknown fatty acid ECL 11.799 (5 %) $C_{12:1}$ 3-OH (3.6 %), $C_{18:0}$ (2.2 %) and $C_{14:0}$ (1.8 %) (**Supplementary Table S1**). The phenotypic profile of *M. brasilensis* sp. nov. is at present based on one strain. Five strains (R-237, R-236, R-249, R-256, and R-278) isolated at the time of collection as described in Chimetto *et al*. (2008) clustered together in this new taxa by 16S rRNA gene sequences, but unfortunately the viability of only one strain (R-278 = R-40503^T) was maintained. As more strains of this species are isolated and tested, the profile may change slightly. The DNA G+C content of the type strain is 46.5 mol%. The type strain R-40503^T (= LMG 25434^T = CAIM 1459^T) was isolated from mucus of the endemic coral *Mussismilia hispida* located in the São Sebastião channel, SP, Brazil.

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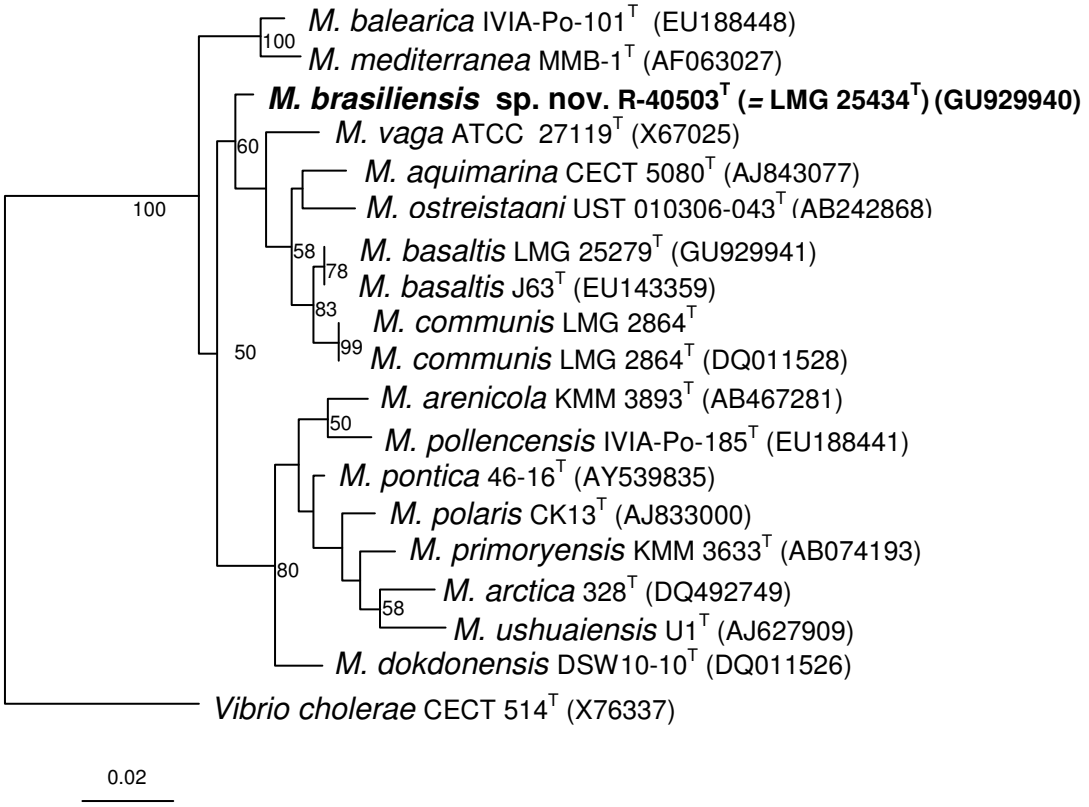
Supplementary data

Suplementary Table S1. Cellular fatty acid contents of *Marinomonas brasilensis* sp. nov. and phylogenetically related *Marinomonas* species.

Taxa: **1**, *M. brasilensis* R-40503^T (= LMG 25434^T); **2**, *M. vaga* LMG2845^T; **3,** *M. basaltis* LMG 25279^T; **4**, *M. communis* LMG 2864^T; **5**, *M. aquimarina* LMG 25236^T. Summed feature 3 comprises $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$. Data are expressed as percentages of total fatty acids. Fatty acids representing <1 % are not shown. All data were obtained in this study on the same laboratory conditions.

Fatty acid	1	2	3	4	5
$C_{10:0}$	-	2.9	4.3	2.6	3.4
$C_{10:0}$ 3-OH	8	14.2	13.9	14.3	7.6
$C_{12:0}$	-	2.5	5.4	5.4	3.5
$C_{12:1}$ 3-OH	3.6	-	-	-	-
$C_{14:0}$	1.8	2.1	2.5	2	1.6
$C_{16:0}$	10.5	10.5	8.5	7.5	11.4
$C_{18:0}$	2.2	1.7	-	1.3	1.4
$C_{18:1}\omega6c$	-	-	8.6	-	-
$C_{18:1}\omega7c$	48.8	45.8	27.6	42.3	47
Summed feature 3	19	18.7	26.4	23.9	22.5
Unknown 11.799	5	1.7	-	-	-



Suplementary Figure S1. Maximum Parsimony phylogenetic tree showing the phylogenetic position of *M. brasilensis* sp. nov. based on 16S rRNA gene sequences. The evolutionary distances were computed by BioNumerics 4.61 software (Applied Maths, Belgium). Bootstrap values ($\geq$ 50 %) based on 100 repetitions are shown. *Vibrio cholerae* was used as outgroup. Bar, 2 % estimated sequence divergence.

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DISCUSSÃO GERAL E CONCLUSÕES FINAIS

V.1. DISCUSSÃO GERAL

A importância de corais na manutenção da biodiversidade marinha é inestimável. Corais são um dos principais organismos construtores de recifes e componentes importantes de sistemas recifais, aumentando a complexidade de habitates em costões rochosos e estabelecendo uma complexa teia de associações e de eventos em sucessão (Castro, 1994). Recifes de corais são um dos biomas mais diversos do mundo e são considerados as florestas tropicais úmidas dos oceanos (Sebens, 1994; Bellwood *et al.,* 2004; Zorzetto & Falcão, 2004). Por outro lado, o meio marinho em geral, sobretudo, o meio costeiro (costões rochosos e recifes costeiros) vem sofrendo um processo severo de degradação. Estudos recentes demonstraram que *Mussismilia* spp. está em processo de extinção (Francini-Filho *et al.*, 2008). A situação no cenário internacional é crítica, com pelo menos um terço de todas as espécies de corais ameaçadas de extinção (Carpenter *et al*., 2008; Bourne *et al*., 2009). Diversos fatores parecem favorecer a degradação dos corais, especialmente, mudanças climáticas (Harvell *et al.*, 2002, Hoegh-Guldberg *et al*., 2008, Doney *et al*., 2009), impactos antropogênicos (Szmant, 2002; Feely *et al*, 2004; Newton *et al*., 2007) e doenças infecciosas (Rosenberg *et al*., 2007; Fancini-Filho *et al*., 2008; Bourne *et al*., 2009).

Microrganismos têm um papel fundamental na saúde de corais. O holobionte é um metaorganismo complexo e depende da interação de diferentes parceiros para sua homeostasia (Rowher *et al*., 2002, Rosenberg *et al* 2007, Bourne *et al*., 2009). Em um modelo simplificado, zooxantelas fornecem matéria orgânica ao coral, se abrigam em seus tecidos e utilizam deles metabólitos. Muitos nutrientes que não podem ser oferecidos pelas zooxantelas são fornecidos pelos demais seres associados, como por exemplo, bactérias que são responsáveis pela captura de fósforo e ferro do ambiente, podendo também fornecer mais de 50 % do nitrogênio fixado ao coral hospedeiro (Kushmaro & Kramarsky-Winter, 2004). Bactérias e outros microrganismos também podem servir como alimento para corais (Ducklow & Mitchell, 1979). Bactérias podem formar uma barreira de defesa do hospedeiro através de biofilmes, além de produzirem antimicrobianos que podem atuar contra patógenos de corais (Ritchie *et al*., 2006; Reshef

et al., 2006, Rosenberg *et al.*, 2007, Bourne *et al*., 2009; Nissimov *et al*., 2009; Rypien *et al*., 2009). Porém, a complexidade do funcionamento do holobionte é evidente. Uma mesma linhagem bacteriana pode ser patogênica ou mutualista, dependendo dos parâmetros ambientais, tais como temperatura e nutrientes dissolvidos na água do mar.

O melhor entendimento da composição microbiana de corais nos auxilia a compreender processos ecológicos complexos. A taxonomia é um dos ramos mais antigos da biologia, parecendo até trivial, porém ela fornece as primeiras evidências quanto à composição da microbiota de corais. O presente trabalho confirmou algumas premissas anteriores, tais como a abundância de novos taxa em corais (Rohwer *et al*., 2002). Entretanto, os resultados deste trabalho não mostraram fortes evidências de associações específicas entre determinadas espécies bacterianas e determinados hospedeiros, corais. A contribuição de vibrios na fixação de nitrogênio não foi quantificada, porém é plausível cogitar que estes microrganismos teriam um papel na nutrição do coral. Além disto, os vibrios estariam ligados à degradação de Dimetilsulfonoproprionato (DMSP) e dimetilsulfito (DMS), dois compostos-chave no ciclo do enxofre e na regulação do clima, com papel importante na formação de nuvens (Raina *et al*., 2009). *Vibrio shilloni* e *V. coralliilyticus* foram os primeiros vibrios, patógeno de corais, demonstrados pelos postulados de Koch (Rosenberg *et al.,* 2007). No entanto, o potencial patogênico de ambas as espécies parece estar restrito a algumas linhagens virulentas e condições ambientais propícias.

Atualmente, a caracterização de microrganismos é feita através da taxonomia polifásica que se baseia em um conjunto de dados que integra informações fenotípicas, genotípicas, filogenéticas e ecológicas. Dados baseados em sequências de genes têm revelado ser uma potente ferramenta da taxonomia, auxiliando o entendimento das relações filogenéticas, além de formar um crescente banco de dados para referências futuras. Sequências de genes que codificam proteínas essenciais do metabolismo celular bacteriano (*housekeeping*) mostram uma resolução taxonômica capaz de delinear espécies. Zeigler (2003) examinou a utilidade de genes amplamente distribuídos na determinação de linhagens e espécies bacterianas. Vários genes que codificam proteínas revelaram ser capazes de predizer com um alto grau de precisão e acurácia a relação de

todo o genoma. Entretanto, o melhor resultado parece ser obtido pela combinação de três genes que tem alta, moderada e fraca conservação genética. Similarmente, Konstantinidis *et al.* (2006b) concluiu que 3 é o número necessário de genes para diferenciação de espécies usando multilocus, pois desta forma, evento de TGL ou recombinação em um sinal filogenético conflitante com as outras duas filogenias será detectada. Adicionalmente, uma vez que os múltiplos genes estabeleçam a identificação de espécie dentro de um determinado taxa um único gene pode ser suficiente para designar uma linhagem adicional como membro desta espécie (Stackebrandt *et al.,* 2002; Zeigler, 2003).

Os estudos acerca da microbiota de corais tiveram seu início na década de 70 no âmbito internacional. No Brasil, os primeiros estudos em microbiologia de corais iniciaram com o presente trabalho. Os padrões de composição de vibrios e outros heterótrofos observados neste trabalho foram avaliados em estudos paralelos em Abrolhos (Alves *et al*., 2009).

V.1.1. DIVERSIDADE DE BACTÉRIAS HETEROTRÓFICAS CULTIVÁVEIS

Neste estudo foram encontradas, associadas ao muco do coral *M. hispida* e do zoantídeo simpátrico *P. caribaeorum*, bactérias representantes das classes *Gammaproteobacteria* (92 %), *Epsilonproteobacteria, Alphaproteobacteria* e *Actinobacteria,* com predominância de *Vibrio* (54 %) e *Pseudoalteromonas* (23 %), embora, *Photobacterium, Shewanella*, *Marinomonas, Erythrobacter, Arcobacter, Thalassolituus, Ferrimonas, Marinobacterium* também estivessem presentes. Grupos que também já foram documentados em outros estudos de diversidade bacteriana em corais (Koren & Rosenberg, 2006; Lampert *et al*., 2006, Littman *et al.,* 2009). No estudo de Reis *et al*. (2009) os autores encontraram por método independente de cultivo grande diversidade bacteriana associada ao muco de espécimes saudáveis e doentes de *M. braziliensis* do Banco de Abrolhos, direcionadas a *Proteobacteria* (*Alfa* e *Gamma*) e *Cyanobacteria*. Além de uma imensa diversidade microbiana ainda desconhecida taxonomicamente. Nossos dados estão de acordo com Rohwer *et al.*, (2001), onde a maioria das bactérias cultivadas isoladas de *Montastraea franksi* e caracterizadas pelo

sequenciamento do rRNA 16S foram relacionadas a *Gammaproteobacteria* (83 %), sendo mais comumente identificadas como *Pseudoalteromonas* (39 %) e *Vibrio* (38 %). *Proteobacteria* (*Gamma* e *Alfa*) incluindo *Vibrio*, *Pseudoalteromonas, Erythrobacter, Shewanella* e *Actinobacteria* isoladas do muco do coral *Fungia Scutaria* foram as bactérias heterotróficas mais frequentemente encontradas por métodos dependente de cultivo no estudo de Lampert *et al*. (2006). Estes autores sugeriram que estas bactérias fariam parte da microbiota normal do hospedeiro. Eles também salientaram que mais de 30 % dos isolados representavam espécies novas. Mais de 12 % dos isolados obtidos neste trabalho representavam espécies novas.

Vibrio spp., *Pseudoalteromonas* spp. e *Photobacterium* spp. foram as bactérias heterotróficas mais frequentemente encontradas associadas a *M. hispida* e *P. caribaeorum* nos dois anos de coleta. *Alteromonas* sp. também foi detectada nesses dois hospedeiros em 2005, embora tivesse sido isoladas em NFb. Todos os demais grupos microbianos foram encontrados associados apenas com *M. hispida*, porém a diversidade encontrada em 2005 mostrou-se diferente da encontrada em 2006. Não foi obtido um claro padrão de distribuição de espécies entre os diferentes pontos de coleta e os diferentes hospedeiros associados, exceto pelo fato de que no ano de 2006 houve uma maior diversidade de grupos microbianos associados a *M. hispida* isolados especialmente na praia preta. Apesar do trabalho pioneiro de Rohwer ter sugerido que algumas espécies de corais abrigam uma microbiota específica independente de tempo e localização geográfica (Rohwer *et al*., 2002), estudos recentes questionam este paradigma. Littman *et al*. (2009) mostraram que a posição geográfica determina os tipos de gêneros dominantes em Acroporídeos. O presente trabalho mostrou que uma mesma espécie de *Vibrio* pode ocorrer em diferentes espécies simpátricas de corais, reforçando o trabalho de Littman. É importante salientar que os dados obtidos por métodos independentes de cultivo e por isolamento e caracterização taxonômica, métodos dependentes de cultivo, são complementares, porque esses métodos recuperam grupos microbianos distintos, mas não são comparáveis, pois determinados grupos microbianos são altamente adaptados ao crescimento em meio de cultura, enquanto que outros grupos raramente crescem em meio de cultura.

V.1.2. DIVERSIDADE, ABUNDÂNCIA E TAXONOMIA DE VIBRIOS

A taxonomia de vibrios vem sendo refinada nos últimos anos (Thompson *et al*. (2001a,b, 2004, 2005b, 2007, 2009). A contribuição do presente trabalho na taxonomia de vibrios se deve (i) a identificação de espécies e populações encontradas em corais, (ii) validação do *pyrH* como um marcador taxonômico de alta resolução, e (iii) as descrições de espécies novas por meio de uma abordagem polifásica.

Com base no sucesso do uso de MLSA (Multilocus Sequence Analysis) na caracterização de espécies de vibrios, nossos dados sugerem que sequências do gene *pyrH* têm maior poder de resolução taxonômica. Portanto, este gene é uma ferramenta importante no estudo de vastas coleções de isolados ambientais. Sequências do gene *pyrH* foram também capazes de discriminar espécies-irmãs, como por exemplo *V. harveyi* e *V. campbellii* e revelar taxa novos. O rep-PCR também confirmou os achados por MLSA, e mostrou ser uma potente ferramenta para avaliar a variação intra-específica nas espécies mais abundantes encontradas neste estudo. Estes dados mostram claramente uma grande diversidade genômica entre a coleção de populações de *Vibrio* que co-ocorrem nos hospedeiros, indicando quase nenhuma clonalidade. Isto significa que há uma grande variação em nível de nucleotídeos e alelos nas linhagens do muco destes cnidários, que poderiam estar relacionadas com processos intracelulares (mutação de ponto e rearranjos gênicos) ou estar associada com processos de transferência gênica lateral. MLSA e rep-PCR mostraram evidências de que isolados obtidos de diferentes hospedeiros e lugares tiveram mútua similaridade, indicando ausência de efeito biogeográfico na distribuição de populações de *Vibrio* na escala de metros e Kilômetros. Nossos dados indicaram também a expansão da ocorrência de *V. chagasii,* descrito em 2003 associados com rotíferos e cultura de peixes, e relatado agora por este estudo associado ao coral *M. hispida*.

Sequências do gene 16S rRNA, hibridização DNA-DNA e AFLP (Amplified Fragment Length Polymorphism) eram ferramentas proeminentes anteriormente. Porém, mais atenção é dada agora a análises de sequências de genes *housekeeping*, através da metodologia de MLSA. O essencial no uso de MLSA é que os loci evoluam mais rapidamente do que o gene 16S rRNA, aumentando desta forma a acurácia filogenética

(Hanage *et al*., 2006). Dentro de um gênero em particular, o uso de sequências parciais de genes é suficiente para predizer com exatidão a relação genômica em nível de espécie. Como um único gene pode estar sujeito a influência de recombinação ou transferência gênica lateral o uso de vários genes que codificam diferentes proteínas torna-se vantajoso para diminuir estes possíveis efeitos (Zeigler, 2003; Konstantinidis *et al.,* 2006b).

O uso de sequências gênicas de 16S rRNA, *ftsZ, gyrB, recA, rpoA, topA, pyrH* e *mreB* para a caracterização de novas espécies de vibrios (*V. communis, V. marinun, V. variabilis* e *P. jeanii*) superou o poder discriminatório da HDD. A potencialidade do MLSA havia sido ressaltada por Zeigler (2003). MLSA tornou-se uma ferramenta extremamente importante na taxonomia de *Vibrio*, uma vez que toda a informação de identificação pode tornar-se disponível na internet, com a construção de bases de dados de acesso público, incorporando novos dados constantemente através de recursos de análise computacional. Os resultados do presente trabalho foram disponibilizados neste site, (http://www.taxvibrio.lncc.br/), que é mantido pelo nosso grupo. Embora, todas essas ferramentas de biologia molecular auxiliem enormemente a taxonomia microbiana atual, a integração dos dados fenotípicos possibilita um melhor entendimento de como essa variabilidade genotípica é expressa. Os dados deste trabalho revelam que linhagens de uma mesma espécie mostram grande variação fenotípica, como por ex. em *V. communis* sp. nov. e em *P. jeanii* sp. nov. A utilização de diferentes compostos como fonte de carbono assim como atividades enzimáticas variaram entre os isolados de cada uma dessas espécies mostrando que o fenótipo auxilia a visualizar a variabilidade intra-específica das espécies e o potencial que cada uma apresenta na degradação ou utilização de compostos sob diferentes condições de crescimento. Marcadores quimiotaxonômicos como análises do perfil de ácidos graxos, FAME (Fatty acids methyl ester), são úteis para caracterizar as espécies. No entanto, a identificação de espécies por meio de caracteres fenotípicos é, no mínimo, problemática (Thompson *et al*., 2004). Além disso, em alguns casos espécies diferentes como *V. campbellii*, *V. harveyi* e *V. rotiferianus* podem compartilhar mais de 100 características fenotípicas (Gomez-Gil *et al*., 2003, 2004, Thompson *et al*., 2004). O presente trabalho mostra que esquemas de classificação e identificação de espécies de vibrios devem ser baseados em dados genômicos.

Sequências de vibrios são frequentemente encontradas em bibliotecas de 16S rRNA de muco e tecido de corais. O muco de *Oculina patagonica* contém aprox. 68 % de sequências de vibrios (Koren & Rosenberg, 2006). Em *Acropora millepora*, 17,4 % das sequências de 16S rRNA pertencem ao grupo dos vibrios (Litmann *et al*., 2009). Muco de espécimes saudáveis e doentes de *Pocillopora damicornis* contém 6 x 10^6 vibrios/cm^3 e 2 x 10^7 vibrios/cm^3 respectivamente (Luna *et al*., 2007). A abundância de vibrios no muco de *M. hispida* 4,4 x 10^6 UFC/mL (± 1,3 x 10^6) foi duas ordens de grandeza maior do que na água do mar circundante (Alves *et al*., 2009). Vibrios tendem a dominar a microbiota do hospedeiro em ambientes com altos níveis de nutrientes e altas temperaturas (Eilers *et al*., 2000). Portanto, a abundância de vibrios em corais pode ser influenciada por fatores ambientais que geram estresse no hospedeiro (como elevação da temperatura da água do mar e acúmulo de nutrientes). Estas bactérias apresentam um tempo de duplicação de aproximadamente 15 minutos, podendo desta forma se proliferar rapidamente. Sob condições favoráveis, o crescimento de linhagens potencialmente patogênicas poderia resultar em doença nos corais (Rosenberg *et al*., 2007; Thurber *et al*., 2009).

A espécie nova *V. communis* parece ser um dos vibrios mais abundantes nos corais. Esta espécie é vizinha de *V. harveyi* (Sawabe *et al*., 2007b). *V. communis* e outras espécies de vibrios (*V. alginolyticus, V. harveyi*, *V. campbellii, V. parahaemolyticus*) foram capazes de fixar nitrogênio. Outras espécies novas de vibrios (*V. communis*, *V. variabilis* e *V. marinun*) além de outras duas espécies de *Photobacterium* (*P. jeanii* e *P. swingsii*) aparecem em menor frequência. Não há evidências para sugerir que diferentes cnidários selecionem determinadas espécies de vibrios como ocorre com a sépia *Euprymna scolopes* e o vibrio *Allivibrio fischeri*.

V. alginolyticus é comumente encontrado em associação com diversos animais marinhos. Um dos primeiros relatos desta espécie associada ao muco de corais sugeriu que eles tivessem um papel positivo na degradação de componentes do muco, de forma a possibilitar que seus constituintes pudessem ser utilizados por organismos que se alimentam de detritos do recife, assim como servir de alimento para o próprio coral (Ducklow & Mitchell, 1979; Lampert *et al*., 2006). Recentemente, estudos têm relatado papéis patogênicos para esta espécie em moluscos, rotíferos, peixes, crustáceos, corais e

até mesmo em humanos (Lee *et al*., 1997, Liu *et al*., 2004, Li *et al*., 2009). Proteases extracelulares, como a alcalina serina protease, uma toxina extracelular, parecem ser um dos seus principais fatores de virulência (Lee *et al*., 1997; Balebona *et al*., 1998). Estudos de patogenicidade usando algumas espécies de *Vibrio* (*V. campbellii, chagasii, V. nereis e V. xuii*) e *P. rosenbergii*, isolados de coral escleractíneo branqueado, mostraram que eles causaram baixa ou nenhuma mortalidade nos testes com cobaias, enquanto as espécies *V. corralliilyticus, V. rotiferianus* e *V. tubiashii* induziram mortalidade (Austin *et al*., 2005). Recentemente, Cervino *et al*. (2008) investigaram isolados do *Vibrio* core grupo potencialmente patogênicos. Estes autores infectaram corais saudáveis usando 2 diferentes linhagens de *V. alginolyticus,* isoladas de lesões causadas pela doença da banda amarela em corais do Caribe e Indo-pacífico, e observou que quando as linhagens eram inoculadas sozinhas não havia o surgimento da doença, mas quando as 2 linhagens eram inoculadas concomitantemente os sinais da doença tornavam-se presentes. Embora, a infecção ocorresse mesmo durante temperatura de ambiente normal (26-28 °C) uma vez o coral infectado a elevação da temperatura causou aumento na virulência da doença mostrando que a expansão das lesões rapidamente aumentava com elevação da temperatura da água do mar (29-30 °C). A literatura mostra que diferentes linhagens apresentam diferentes potenciais patogênicos e, portanto, nem todas as linhangens seriam patogênicas (Alves Jr *et al*., 2009). Estes autores também relataram que as espécies mais abundantes encontradas tanto em corais aparentemente saudáveis quanto em corais doentes pertencem ao *Vibrio core* grupo (*V. alginolyticus* e *V. harveyi*-like), além de *V. coralliilyticus,* reconhecido patógeno de corais (Bem-Haim *et al*., 2002).

A presença das espécies do *Vibrio core* grupo mostra-se desta forma amplamente distribuída tanto em corais do Banco de Abrolhos (BA) quanto em São Sebastião (SP). A capacidade de fixar nitrogênio poderia lhes conferir vantagem na competição com outras espécies presentes no hospedeiro. No entanto, quando o coral for exposto a situações de estresse causadas por mudanças nas condições ambientais, como aumento da temperatura da água do mar ou altos níveis de nutrientes (alto nível de amônia dissolvida, fosfato e matéria orgânica), poderiam atuar como patógenos oportunistas, passando a expressar genes de virulência, como ocorre, por exemplo, com *V. coralliilyticus* e *V. shilonii* (Bem-Haim *et al*., 2003, Rosenberg *et al*., 2007). Recentemente, Tait *et al*. (2010) sugeriram

que Quorum Sensing (QS) pode ter um importante papel na dinâmica dos vibrios no holobionte. Seus dados revelaram que *V. harveyi,* isolado do muco do coral *Acropora millepora,* parece ter um efeito inibitório na sinalização QS em alta temperatura (30 °C). Essa inibição poderia ser usada como uma vantagem para dominar o nicho no holobionte sobre as demais espécies incluindo outros vibrios, o que permitiria aumento da sua população em períodos de alta temperatura.

V. 2. CONCLUSÕES FINAIS

- Este trabalho serve como base para estudos futuros sobre ecologia do holobionte a partir da coleção bacteriana caracterizada.

- MLSA é uma ferramenta potente para a taxonomia de vibrios, superando o poder de HDD na classificação de novas espécies.

- Vibrios são abundantes no muco de cnidários. *V. communis, V. alginolyticus*, e *V. harveyi* são as espécies mais frequentemente encontradas. Estas espécies apresentam potencial de fixação de nitrogênio e podem também estar envolvidas em outros processos ecológicos no holobionte. A maioria dos taxa encontrados em 2005 e 2006 provavelmente compõem a microbiota normal do muco dos cnidários.

- Há uma grande diversidade de linhagens, cada linhagem representando uma população distinta, associadas aos cnidários estudados. Com exceção de *V. chagasii*, espécies de vibrios não foram específicas para uma determinada espécie de cnidário.

- Algumas das espécies novas parecem ter ampla distribuição geográfica (como por ex. *V. communis* sp. nov.), enquanto que outras (*V. marinum* sp. nov.) estão restritas aos locais analisados neste estudo. O muco de cnidários é uma fonte para a descoberta de novos taxa.

V.3. PERSPECTIVAS FUTURAS DE ESTUDO

- **Influência dos vibrios na saúde de corais.** Por meio da realização de bioensaios em aquários e experimentos de infecção em campo pretendemos comprovar se as

linhagens isoladas neste trabalho são realmente patogênicas. Paralelamente, serão caracterizados genes de virulência por meio de mutação sítio-dirigida.

- **Metagenômica.** Análise metagenômica de sistemas recifais para determinar a composição total da microbiota, tendo uma visão ampla acerca da diversidade.

- **Corais de ambientes de difícil acesso.** Estudo do coral *M. hispida* em ambientes remotos como as ilhas oceânicas, para confrontar dados sobre especificidade bactéria-hospedeiro e distribuição biogeográfica desta microbiota, além de determinar a saúde dos holobiontes.

VI. 1. REFERÊNCIAS BIBLIOGRÁFICAS

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VI.2. ABREVIAÇÕES

AFLP	Amplified Fragment Length Polymorphism
ARA	Acetylene Reduction Assay
ARDRA	Amplified Ribosomal DNA Restriction Analysis
API	Analytical Profile Index
BCCM	Belgium Coordinated Collection of Microorganisms
BFN	Bactérias fixadoras de nitrogênio
bp	Base pair
DDH	DNA–DNA hybridization
EMBL	European Molecular Biology Laboratory
FAME	Fatty acids methyl ester
FBN	Fixação biológica de nitrogênio
FN	Fixação do nitrogênio
ftsZ	Cell division protein gene
GenBank	Genetic sequence database
gyrB	DNA gyrase B subunit gene
HDD	Hibridização DNA-DNA
LMG	Laboratory of Microbiology, Ghent University
MA	Marine agar
MLSA	Multilocus Sequence Analysis
mreB	Actin-like cytoskeleton protein gene
NFb	Nitrogen-free medium
nif	nitrogen fixing gene
PCR	Polymerase chain reaction
pyrH	Urydilate kinase gene
RAPD	Random Amplified polymorphic DNA
rep-PCR	Repetitive extragenic palindromic - PCR
recA	Recombination repair protein gene
rpoA	RNA polymerase alpha subunit gene
rRNA	ribossomal RNA
TCBS	Thiosulfate-citrate-bile salt-sucrose agar
TGL	Transferência Gênica Lateral
topA	Topoisomerase I gene
sp. nov.	Species nova
VLPs	Virus like particles
UFC	Unidades formadoras de colônia