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DIVERSIDADE MICROBIANA NA ILHA REI GEORGE (PENÍNSULA ANTÁRTICA) E PROSPECÇÃO DE ENZIMAS QUERATINOLÍTICAS

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"O homem se torna civilizado, não proporcionalmente à sua vontade de acreditar,

mas proporcionalmente à sua capacidade de duvidar."

Henry Louis Mencken

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SUMÁRIO

Diversidade microbiana na Ilha Rei George (Península Antártica) e prospecção de enzimas queratinolíticas

Autor: Igor Stelmach Pessi Orientador: Alexandre José Macedo

O estudo da diversidade microbiana no continente antártico é fundamental para entender os processos que moldam a distribuição microbiana em ambientes naturais, e contribui para entender os efeitos decorrentes de mudanças climáticas na biodiversidade do planeta. Em adição, microrganismos adaptados a baixas temperaturas apresentam enorme potencial para aplicação em diversos processos industriais. Este trabalho teve como objetivos avaliar a diversidade funcional e genética de comunidades microbianas de solos da Ilha Rei George (Península Antártica), e realizar a prospecção de enzimas queratinolíticas ativas em baixas temperaturas. A análise da diversidade funcional mostrou que as comunidades microbianas são formadas por uma alta diversidade de grupos funcionais (*H'* = 2.14-3.30), distribuídas heterogeneamente no ambiente. Similarmente, a análise da diversidade genética mostrou a presença de comunidades microbianas altamente diversas (*H'* = 5.33-6.24), formadas majoritariamente por membros dos filos Proteobacteria, Bacteroidetes e Euryarchaeota. Finalmente, em um pequeno rastreamento de 120 isolados, cinco apresentaram atividade queratinolítica, dentre eles uma possível nova espécie de *Pedobacter*, mostrando o alto potencial biotecnológico de microrganismos antárticos.

Palavras-chave: Península Antártica, diversidade genética, diversidade funcional, queratinase.

Microbial diversity in King George Island (Antarctic Peninsula) and prospecting for keratinolytic enzymes

Author: Igor Stelmach Pessi Advisor: Dr. Alexandre José Macedo

The study of microbial diversity in the Antarctic continent is vital to understand the processes which shape microbial distribution in natural environments, and contributes to understand the impacts of climate change on Earth's biodiversity. Furthermore, cold-adapted microorganisms present high potential for application in several industrial processes. The objectives of this study were to evaluate the functional and genetic diversity of soil microbial communities of King George Island, Antarctic Peninsula, and prospect for cold-active keratinolytic enzymes. The analysis of functional diversity showed that microbial communities are composed of a high diversity of functional groups (*H'* = 2.14-3.30), heterogeneously distributed in the environment. Similarly, the analysis of genetic diversity showed the presence of highly diverse microbial communities (*H'* = 5.33-6.24), composed mostly by members of the Proteobacteria, Bacteroidetes and Euryarchaeota phyla. Finally, through a small screening of 120 isolates, five showed keratinolytic activity, among them a putative novel species of *Pedobacter*, showing the high biotechnological potential of Antarctic microorganisms.

Keywords: Antarctic Peninsula, genetic diversity, functional diversity, keratinase.

1 INTRODUÇÃO

1.1 O continente antártico

O continente antártico (Figura 1) é considerado um dos ambientes mais extremos do planeta, pois apresenta diversas condições consideradas desafiadoras para a sobrevivência de seres vivos, como baixas temperaturas, pouca disponibilidade de água líquida e nutrientes, alta incidência de luz ultravioleta durante o verão e frequentes eventos de congelamento-descongelamento (CAMPBELL & CLARIDGE, 1987). Entretanto, as condições climáticas não são homogêneas no continente como um todo, que apresenta

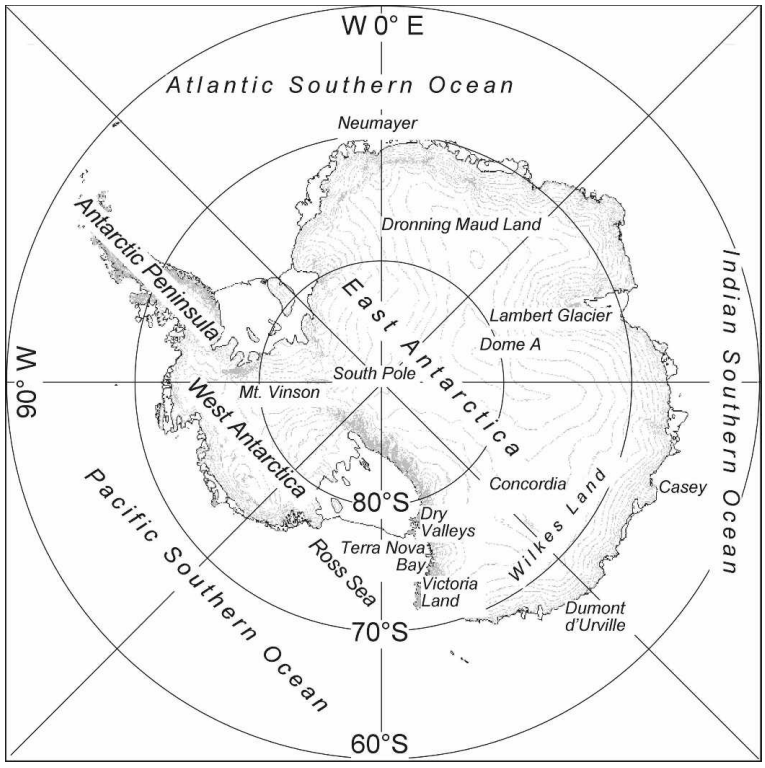

Figura 1. Mapa do continente Antártico (retirado de MASSON-DELMOTTE *et al*., 2008).

várias regiões climáticas diferentes (COWAN *et al*., 2010). Embora a maior parte do continente seja coberta por calotas de gelo glaciais, porções de terra sem gelo, compreendendo cerca de 0,4% da área total, estão distribuídas descontinuamente principalmente na margem costeira (CARY *et al*., 2010).

A Antártica também é o continente mais remoto da Terra, estando isolado do resto do mundo pelo Oceano Antártico e pela Corrente Circumpolar Antártica. A diferença de temperatura entre as correntes de ar de altas e baixas latitudes e a grande extensão do Oceano Antártico – cerca de 1.000 km na sua porção mais estreita, entre a América do Sul e o norte da Península Antártica (Passagem de Drake) – servem como importantes barreiras para a dispersão de colonizadores (PEARCE *et al*., 2009). A combinação de isolamento geográfico, condições ambientais extremas e acessibilidade limitada têm restringido as atividades humanas neste continente, sendo o primeiro relato de presença humana datado do ano de 1820, quando o capitão John Davis e seu grupo de pescadores desembarcaram na Antártica. Desde então, a colonização humana neste continente tem seguido um crescimento pseudoexponencial, após uma transição de exploração econômica (pesca e caça de focas e baleias) e política para investigação científica e turismo (COWAN *et al*., 2011). A vulnerabilidade de ecossistemas antárticos aos impactos causados pela exploração humana tem sido reconhecida a pouco mais de meio século, de modo que, em 1959, 12 nações assinaram o Tratado Antártico, que regulamenta as atividades humanas (políticas, econômicas e científicas) na Antártica (ARONSON *et al*., 2011).

Em geral, solos antárticos apresentam temperaturas de até -60°C, baixa umidade (conteúdo de água entre 1 e 10%), baixos níveis de nutrientes e alto pH,

devido à acumulação de sais provenientes de precipitações atmosféricas e intemperismo, que, por sua vez, contribuem para a baixa biodisponibilidade de água (CAMPBELL & CLARIDGE, 1987; MARGESIN & MITEVA, 2011). Entretanto, apesar das baixas temperaturas atmosféricas encontradas na Antártica, é sabido que a temperatura do solo durante o verão, no qual ocorrem períodos de alta incidência de radiação solar, pode chegar a 15°C (MÖLLER & DREYFUSS, 1996), e rochas podem ser aquecidas a até 10°C acima da temperatura do ar (DE LA TORRE *et al*., 2003).

Na Antártica encontra-se a maioria dos lagos subglaciais da Terra, ou seja, lagos permanente ou sazonalmente cobertos por uma extensa camada de gelo. Dos 174 lagos existentes na Antártica, mais de 150 são subglaciais, dos quais a maioria é interconectada por rios (MARGESIN & MITEVA, 2011). No caso do Lago Vostok, a cobertura de gelo possui cerca de 3.750 m de profundidade, o que representa cerca de 14 milhões de anos de isolamento (BULAT *et al*., 2011). Através de análises do gelo de acreção – formado pelo congelamento da água na interface água-camada de gelo – foi inferido que este lago é extremamente oligotrófico e altamente oxigenado (MARGESIN & MITEVA, 2011).

Em 1985, pesquisadores da Pesquisa Antártica Britânica documentaram uma depleção na camada de ozônio estratosférico sobre a Antártica, relacionada à concentração atmosférica de clorofluorcarbonetos (CFC's) (FARMAN *et al*., 1985). Apesar da proibição do uso de CFC's estabelecida pelo Protocolo de Montreal em 1987, o buraco na camada de ozônio sobre o continente persiste até os dias de hoje, podendo se estender a mais de 50% da área total durante a primavera (COWAN *et al*., 2010; ARONSON *et al*., 2011). Com a depleção da

camada de ozônio ocorre um aumento na irradiação de luz UV-B que, aliada aos longos períodos de incidência de luz solar durante o verão austral, geram graves ameaças ecológicas, como danos ao DNA e fotoinibição do fitoplâncton marinho e consequente alterações na dinâmica do ecossistema (SMITH *et al*., 1992). O estudo da diversidade microbiana na Antártica é de suma importância para prever o impacto das mudanças climáticas em todo o planeta, visto que seus efeitos – principalmente aumento da temperatura e acidificação do oceano – têm um maior e mais rápido impacto em ambientes polares (ARONSON *et al*., 2011).

1.1.1 Península Antártica e Ilha Rei George

A Península Antártica (Figura 2) é uma região dinâmica e variável do continente antártico, que apresenta características ambientais ligeiramente diferentes do resto do continente, pois recebe material diretamente do mar assim como de aves e mamíferos (TEIXEIRA *et al*., 2010). Apesar das baixas taxas de precipitação, apresenta solos com alto teor de umidade, que fornecem condições favoráveis para o crescimento microbiano (CAMPBELL & CLARIDGE, 1987).

Desde o início das atividades humanas no extremo sul do planeta, a Ilha Rei George, localizada no noroeste da Península Antártica, tem sido uma das regiões mais visitadas e mais densamente povoadas da Antártica. A primeira exploração científica nesta ilha data de 1908, com a chegada do navio “Pourquoi Pas?”, durante a segunda Expedição Antártica Francesa (SICIŃSKI *et al*., 2011). Atualmente, existem nove estações de pesquisa nesta ilha, somando uma população de pelo menos 85 pessoas durante o inverno, podendo chegar a 500

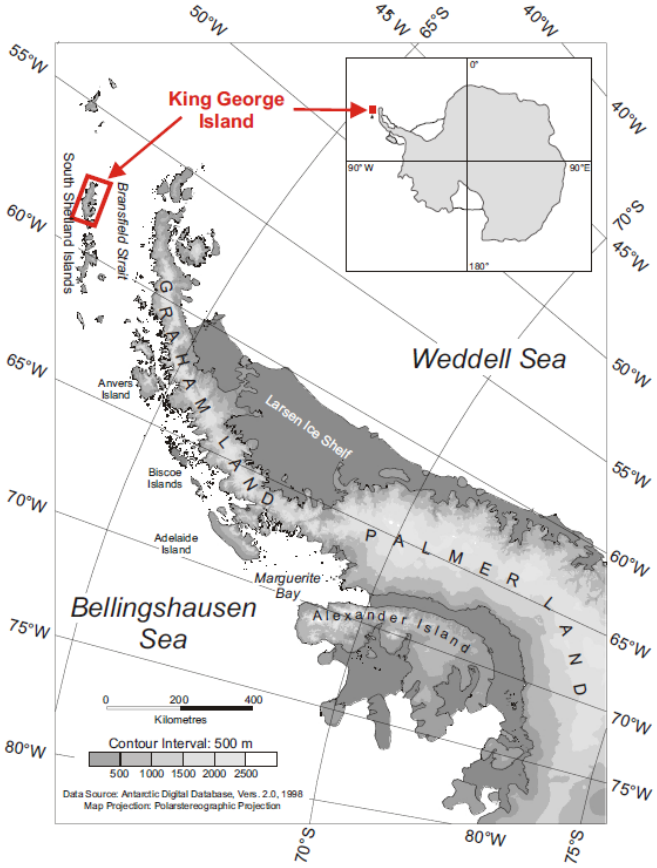

Figura 2. Mapa da Península Antártica, mostrando a localização da Ilha Rei George (retirado de BRAUN et al., 2001).

no verão (BRAUN *et al*., 2001). A Estação Antártica Brasileira Comandante Ferraz, estação de médio porte estabelecida em 1984, possui, atualmente, uma população de 10 a 15 pessoas durante o inverno e cerca de 50 pessoas durante o verão (NAKAYAMA *et al*., 2011). Também estão localizadas nesta ilha as estações Henryk Arctowski (Polônia), Machu Picchu (Peru), Jubani (Argentina), General Artigas (Uruguai), Presidente Eduardo Frei Montalva (Chile), Great Wall (China), Bellinghausen (Rússia) e King Sejong (Coréia do Sul). Atividades científicas nessa região têm acumulado extensiva informação biológica e oceanográfica por mais de 30 anos, sendo que alguns dos estudos realizados na

Ilha Rei George estão entre as pesquisas mais extensas realizadas em toda região antártica (SICIŃSKI *et al*., 2011).

Junto com o noroeste norte-americano e o platô siberiano, o oeste da Península Antártica é uma das áreas que apresentou a mais alta taxa de aquecimento nas últimas cinco décadas (VAUGHAN *et al*., 2003), acompanhado por mudanças no regime de precipitações (TURNER *et al*., 1997). Apesar de ainda não ser consenso na comunidade científica, acredita-se que o aumento da temperatura e umidade do solo pode resultar em um acréscimo significativo na emissão de gases de efeito estufa (como CO_2 e CH_4), devido ao aumento da decomposição de matéria orgânica por comunidades microbianas (DAVIDSON & JANSSENS, 2006). Nesse sentido, o estudo e monitoramento da diversidade microbiana em solos de regiões de altas latitudes, especialmente do oeste da Península Antártica, é essencial para avaliar o impacto do aquecimento global no clima do planeta.

1.2 Diversidade microbiana na Antártica

A atual situação do censo microbiano na Antártica é preocupante, devido a pouca quantidade de dados obtidos até o momento. No início dos estudos de microbiologia na Antártica, a natureza extrema do ambiente, assim como a completa ausência de estruturas biológicas em muitas regiões, levou a comunidade científica a concluir precipitadamente que muitas partes do continente seriam estéreis (COWAN *et al*., 2010). De fato, o conceito de condições extremas geralmente é enquadrado dentro de um contexto

antropocêntrico, sendo definido como condições fora do limite no qual humanos e a maioria dos eucariotos podem sobreviver (SHERIDAN *et al*., 2000). Entretanto, não obstante a escassez de dados, atualmente é sabido que diversos microrganismos têm colonizado ambientes antárticos por dezenas de milhões de anos, e diversos estudos têm mostrado que a diversidade e biomassa microbiana neste continente são inclusive mais altas do que o esperado (COWAN *et al*., 2002; AISLABIE *et al*., 2006; SMITH *et al*., 2006; NIEDERBERGER *et al*., 2008; CHONG *et al*., 2009; POINTING *et al*., 2009; TEIXEIRA *et al*., 2010; CHONG *et al*., 2012).

Um ponto central no estudo da ecologia microbiana é como a heterogeneidade e limitações espaciais modelam a distribuição de táxons procarióticos no ambiente em escalas locais, regionais e continentais (MARGESIN & MITEVA, 2011). No caso da Antártica, é consenso que a distribuição de espécies microbianas ou grupos funcionais de microrganismos neste continente não é homogênea. Ecossistemas antárticos podem apresentar diversos microambientes com diferentes condições ambientais e geológicas, que servem como “hotspots” para a ocorrência de determinados microrganismos. De modo geral, a estrutura das comunidades microbianas é altamente dependente destas microcondições, o que resulta em uma distribuição altamente heterogênea de microrganismos em escala regional e continental (YERGEAU & KOWALCHUK, 2008; TEIXEIRA *et al*., 2010; GANZERT *et al*., 2011). Comparações da distribuição microbiana em regiões da Península Antártica e Dry Valleys mostram claramente uma diminuição na complexidade das comunidades microbianas com o aumento da latitude (BARRETT *et al*., 2006; YERGEAU *et al*., 2007). Por esses

motivos, acredita-se que o continente antártico deve apresentar altos níveis de isolamento geográfico e endemismo (LAWLEY *et al*. 2004).

Entretanto, questões envolvendo endemismo versus distribuição cosmopolita em ambientes antárticos permanecem ainda não respondidas. Apesar das barreiras físicas impostas pelo Oceano Antártico e pela Corrente Circumpolar Antártica – esta última servindo como uma barreira fisiológica importante para qualquer organismo não adaptado a baixas temperaturas (ARONSON *et al*., 2011) –, é sabido que processos eólicos de alta altitude podem transportar microrganismos entre a Antártica e outros ambientes, especialmente a Patagônia (PEARCE *et al*., 2009; HUGHES & CONVEY, 2010). A questão que permanece ainda é se microrganismos transportados para a Antártica a partir de outros locais seriam capazes de se desenvolver e contribuir significantemente para os processos locais.

De modo geral, a diversidade de bactérias cultiváveis em solos antárticos é relativamente baixa, o que suporta a hipótese de que ambientes antárticos apresentam comunidades microbianas relativamente simples, se comparados com regiões temperadas (NEGOITA *et al*., 2001; SMITH *et al*., 2006; GILICHINSKY *et al*., 2007). Entretanto, apesar de técnicas cultivo-dependentes serem ainda continuamente utilizadas em estudos de ecologia microbiana, é atualmente aceito que tais métodos não permitem uma avaliação realística da diversidade microbiana (HANDELSMAN, 2004). É, portanto, razoável concluir que estudos mais antigos empregando análises baseadas em cultivo representam uma subestimativa grosseira da diversidade e abundância microbiana neste continente. De fato, abordagens cultivo-independentes geralmente revelam um

cenário diferente, indicando a ocorrência de comunidades microbianas altamente diversificadas na Antártica (AISLABIE *et al*., 2006; SMITH *et al*., 2006; BABALOLA *et al*., 2009; TEIXEIRA *et al*., 2010). O resultado mais surpreendente e consistente obtido nestes estudos é que uma alta proporção dos filotipos apresentam baixa homologia com sequências de 16S rRNA provenientes de microrganismos cultivados, o que sugere a ocorrência de um alto número de espécies microbianas desconhecidas – e possivelmente endêmicas – neste ambiente.

As geleiras são consideradas o componente mais severo da criosfera, por apresentarem temperaturas extremamente baixas (podendo chegar a -56°C), alta pressão hidrostática, assim como completa escuridão. Por esse motivo, geleiras são ambientes muito interessantes do ponto de vista paleoecológico, por conterem um enorme reservatório de vida microbiana, preservado em camadas cronológicas por milhares de anos. Células microbianas originadas de lugares distantes e geograficamente isolados são depositadas juntamente com a neve e embebidas gradualmente nas camadas de gelo, servindo como um registro da diversidade microbiana de épocas passadas (MARGESIN & MITEVA, 2011). Comparando-se o registro geoquímico de climas passados presente em testemunhos de gelo com o arquivo cronológico da diversidade microbiana, é possível inferir o efeito do clima global terrestre na abundância, origem e composição de comunidades microbianas (YAO *et al*., 2006; YAO *et al*., 2008; MITEVA *et al*., 2009).

Lagos antárticos subglaciais são considerados oásis nos desertos polares, abrigando uma alta diversidade e abundância de microrganismos (LAYBOURN-

PARRY & PEARCE, 2007; MOSIER *et al*., 2007; STINGL *et al*., 2008). A maioria destes lagos apresenta uma estratificação estável, abrigando populações de alta densidade e com baixa complexidade, como é o caso do Lago Ace, dominado por bactérias verdes sulfurosas (NG *et al*., 2010). O Lago Vostok – o maior lago subglacial da Terra, compreendendo 250 km de comprimento, 50 km de largura e mais de 1.200 m de profundidade (BULAT *et al*., 2011) – tem sido extensivamente estudado devido a sua importância em diversos campos, desde a origem e evolução da vida no nosso planeta até a busca de vida extraterrestre. Devido a preocupações relacionadas à introdução de espécies não nativas neste ambiente, que permanece isolado há milhões de anos, amostras de água do Lago Vostok ainda não foram obtidas, e todos os estudos até o momento têm sido realizados com amostras obtidas da cobertura de gelo ou do gelo de acreção (COWAN *et al*., 2011). Apesar do baixo número de células presente no gelo de acreção (~4 x 10^2 células ml^{-1}), diversas espécies microbianas têm sido detectadas, pertencentes a todos os grandes grupos filogenéticos (BULAT *et al*., 2004; CHRISTNER *et al*., 2001; D'ELIA *et al*., 2008).

A questão relativa à introdução de contaminantes microbianos na Antártica devido à ação antropogênica vem sendo considerada há bastante tempo, principalmente devido ao aumento da exploração científica neste ambiente. Entretanto, a grande maioria dos estudos tem focado na avaliação da contaminação de solos e corpos aquáticos próximos a estações científicas (COWAN *et al*., 2011), com diversos trabalhos demonstrando a presença de coliformes e outras bactérias fecais humanas nestes locais (SJÖLING & COWAN, 2000; HUGHES & THOMPSON, 2004). Mais recentemente, devido ao aumento

do acesso de turistas na Antártica, a preocupação com a disseminação antropogênica de microrganismos em ecossistemas terrestres antárticos vem crescendo (HUGHES e CONVEY, 2010; ARONSON *et al*., 2011). Apesar de ter sido concluído que a introdução de microrganismos não nativos neste ambiente provavelmente não levaria a uma alteração significativa na estrutura e função de comunidades microbianas, esforços são necessários para proteger estes ecossistemas da contaminação e homogeneização microbiana e genética. Em adição, acredita-se que o aumento da temperatura originado das mudanças climáticas facilitaria a invasão de ambientes antárticos por microrganismos não nativos, podendo resultar em um aumento no fluxo de energia e riqueza de espécies (COWAN *et al*., 2011). Entretanto, como grande parte da diversidade microbiana em diversos ambientes antárticos permanece ainda desconhecida, estudos se fazem necessários para elucidar a estrutura das comunidades microbianas que habitam este continente, de modo a avaliar com mais precisão os possíveis impactos das mudanças climáticas neste ambiente.

1.3 Microrganismos adaptados a baixas temperaturas

Cerca de 85% da biosfera se encontra permanentemente exposta a temperaturas abaixo de 5°C, em ambientes como fundo de oceanos, neve, solos “permafrost”, gelo marinho, geleiras, lagos e desertos polares. Esses ambientes têm sido colonizados com sucesso por muitos organismos, incluindo bactérias, arquéias, leveduras, algas unicelulares e fungos filamentosos (MARGESIN & MITEVA, 2011). O primeiro relato de vida microbiana em um ambiente de baixa

temperatura data do quarto século A.C., quando Aristóteles fez observações a respeito do que viria a ser conhecida como uma alga fotossintética que dá coloração vermelha à neve. Mais de dois milênios depois, em 1887, o cientista alemão Forster descreveu a habilidade de uma bactéria bioluminescente, obtida de um peixe conservado no frio, de se desenvolver a 0°C (DEMING, 2009).

Microrganismos adaptados a baixas temperaturas são únicos porque, apesar dos efeitos negativos que baixas temperaturas e ciclos de congelamento-descongelamento têm em reações bioquímicas e na integridade das estruturas celulares, são capazes de crescer e se multiplicar a taxas similares a espécies relacionadas de temperaturas mais amenas (GERDAY *et al*., 2000). De acordo com a faixa de temperatura na qual podem se desenvolver, microrganismos adaptados ao frio podem ser divididos em duas categorias, os psicrotolerantes, que crescem em temperaturas próximas a 0°C, mas possuem seu ótimo de crescimento acima de 20°C, e os psicrófilos, que crescem mais rápido a 15°C ou menos, sendo incapazes de se desenvolver acima de 20°C (CAVICCHIOLI *et al*., 2002)[1].

De modo geral, bactérias Gram-negativas são as mais numerosas nesses ambientes, incluindo espécies dos gêneros *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Cytophaga*, *Cellulophaga*, *Aeromonas*, *Vibrio*, *Serratia*, *Escherichia*, *Proteus*, *Psychroflexus*, *Psychromonas* e *Psychrobacter*. Dentre as bactérias Gram-positivas, predominam membros dos filos Firmicutes e

[1]Microrganismos psicrotolerantes também são conhecidos como “psicrotróficos”; entretanto, este termo – de origem grega, sendo traduzido como “que se alimentam de frio” – apesar de ser encontrado na literatura, é considerado errôneo e não será utilizado ao longo desse texto. Em adição, microrganismos psicrófilos e psicrotolerantes compartilham muitas características e podem ser aplicados de maneira semelhante em diversos processos; portanto, nesta seção, o termo psicrófilo aplica-se a ambas as classes de microrganismos.

Actinobacteria (bactérias Gram-positivas de baixo e alto conteúdo G + C, respectivamente), como *Bacillus*, *Clostridium*, *Arthrobacter* e *Micrococcus* (CANGANELLA & WIEGEL, 2011). Microrganismos capazes de se desenvolver em temperaturas muito abaixo do ponto de congelamento da água têm sido reportados nas últimas décadas, sendo o recorde de crescimento em baixas temperaturas atribuído às bactérias *Psychromonas ingrahamii* e *Colwellia psychrerythraea*, capazes de crescer a -12°C (DEMING, 2009).

Baixas temperaturas e congelamento influenciam o crescimento microbiano de várias maneiras, como, por exemplo, através de redução da taxa de reações bioquímicas, aumento na viscosidade do meio, mudanças na fluidez da membrana e na conformação de proteínas, alteração na disponibilidade de nutrientes e necessidade de proteção contra congelamento. Como água na forma líquida é essencial para todas as formas de vida, o limite inferior de temperatura para a viabilidade de organismos vivos é comumente definido como o ponto de congelamento da água existente dentro das células. Uma característica principal de muitos microrganismos psicrófilos é a síntese de compostos crioprotetores, que diminuem significativamente o ponto de congelamento da água celular. Outras adaptações relacionadas ao manejo de água líquida incluem ainda um aumento na fluidez da membrana – por meio do aumento da proporção de ácidos graxos polinsaturados e ramificados –, controle do equilíbrio osmótico transmembrana, biossíntese de compostos que protegem contra danos decorrentes de dessecação por congelamento, e desenvolvimento de sistemas biológicos de nucleação de gelo (MARGESIN *et al*., 2007). Outras adaptações comuns a microrganismos psicrófilos incluem a produção de proteínas de choque

térmico, redução/eliminação de reações metabólicas que geram oxigênio reativo ou aumento da produção de enzimas antioxidantes – devido à maior solubilidade do oxigênio em baixas temperaturas e, portanto, maior dano oxidativo à célula (PODAR & REYSENBACH, 2006). De fato, diversos estudos genômicos e proteômicos têm demonstrado que as principais funções que se encontram supereguladas durante o crescimento em baixas temperaturas são (i) a síntese proteica, enovelamento de RNA e proteínas (adaptação da estrutura molecular, garantindo uma maior flexibilidade); (ii) manutenção da fluidez da membrana; (iii) produção e captação de compostos crioprotetores; (iv) atividades antioxidantes; e (v) regulação de rotas metabólicas específicas (SAUNDERS *et al*., 2003; MÉDIGUE *et al*., 2005; METHÉ *et al*., 2005; RILEY *et al*., 2008; NG *et al*., 2010).

1.3.1 Enzimas de microrganismos psicrófilos

Uma das características mais interessantes de microrganismos psicrófilos do ponto de vista biotecnológico é a produção de enzimas ativas a baixas temperaturas, que podem apresentar atividade até dez vezes maior do que enzimas mesófilas. Em adição, estas enzimas são extremamente termolábeis, sendo frequentemente inativadas em temperaturas amenas. Apesar de apresentarem conformações e estruturas tridimensionais semelhantes a enzimas mesófilas, enzimas de microrganismos psicrófilos são extremamente flexíveis, especialmente na região do sítio ativo (D'AMICO *et al*., 2006; FELLER, 2007).

Para compensar a baixa energia cinética em baixas temperaturas, as enzimas de microrganismos psicrófilos apresentam uma série de adaptações

estruturais – redução do efeito hidrofóbico, diminuição nas interações iônicas e eletrostáticas, aumento na carga dos resíduos superficiais, loops superficiais adicionais, substituição de resíduos de prolina por glicina em loops superficiais, diminuição da razão arginina/lisina, diminuição nas interações entre domínios e subunidades, diminuição de interações aromáticas, entre outras – que tornam o sítio ativo e regiões adjacentes mais flexíveis. Esta maior flexibilidade aumenta a complementaridade com o substrato com um baixo custo energético, resultando em uma maior atividade em baixas temperaturas acompanhada de uma alta termolabilidade (CAVICCHIOLI *et al*., 2002).

As características estruturais e funcionais, que são únicas de enzimas de microrganismos psicrófilos, as tornam interessantes para diversas aplicações industriais e biotecnológicas, como, por exemplo, aditivos em detergentes, nas indústrias têxteis, de alimentos e de química fina, biorremediação, novas ferramentas em biologia molecular, etc. (MARX *et al*., 2007). Em adição, podem ser utilizadas diretamente ou como fonte de conhecimento para modificação de enzimas derivadas de organismos mesófilos (ROTHSCHILD & MANCINELLI, 2001). O interesse em enzimas que apresentam alta atividade em baixas temperaturas é óbvio, enquanto que uma termolabilidade relativamente alta possibilita a rápida inativação dessas enzimas sem a necessidade de utilização de tratamentos com altas temperaturas (MARX *et al*., 2007). Em adição, dispensam etapas de aquecimento dispendiosas, funcionam em ambientes frios e durante o inverno, geram altos rendimentos, acomodam um alto grau de especificidade estérica e minimizam reações químicas indesejáveis que podem ocorrer em altas temperaturas. Não obstante seu grande potencial biotecnológico,

poucas enzimas adaptadas ao frio estão em uso comercial, em comparação com enzimas termoestáveis (CAVICCHIOLI *et al*., 2002).

Proteases (EC 3.4) são enzimas hidrolíticas que possuem papel importante em diversos processos celulares da nutrição, fisiologia e patogênese microbianas. Em microrganismos, o papel mais simples destas enzimas é no processo de captação de nutrientes, atuando extracelularmente na degradação de proteínas insolúveis em peptídeos menores e aminoácidos, que são então captados pela célula. Outras funções compreendem a regulação da expressão gênica – por meio da modificação de proteínas repressoras, RNA polimerases, etc. –, modificação de enzimas, degradação de proteínas intracelulares, esporulação e patogênese (WARD *et al*., 2009). Na indústria, proteases são comumente utilizadas como aditivos em detergentes, processamento de comida (amaciamento de carnes, fabricação de cerveja, queijo, ração animal, etc.) e couro e produção de medicamentos (GERDAY et al., 2000). Detergentes contendo proteases de microrganismos psicrófilos são particularmente interessantes por possibilitarem o uso em baixas temperaturas e/ou durante o período do inverno, sem ser necessário gasto de energia adicional (MARX *et al*., 2007). Na indústria alimentícia, após o término do processamento enzimático, geralmente se faz necessário um aumento da temperatura de modo a inativar a enzima. Também neste caso, proteases psicrófilas são interessantes, pois são facilmente inativadas em temperaturas amenas, evitando o gasto adicional de energia e a degradação e/ou modificação de compostos sensíveis ao calor, preservando as qualidades organolépticas do produto (CAVICCHIOLI et al., 2002). O mercado de enzimas proteolíticas representa cerca de 60% da venda

total de enzimas, um mercado que gira 2,3 bilhões de dólares todo ano (WARD *et al*., 2009).

Dentro do grupo das proteases, encontra-se uma classe de enzimas conhecidas coletivamente como queratinases. Enzimas queratinolíticas são, portanto, proteases capazes de hidrolisar a queratina, componente principal de estruturas biológicas como pelos, penas e unhas. Devido à presença de pontes dissulfeto, a queratina é um composto altamente recalcitrante, não sendo degradada por proteases como tripsina, pepsina e papaína (Papadopoulos, 1986). Devido ao acúmulo de grandes quantidades de resíduos queratinosos, derivados principalmente da indústria de frangos, a reciclagem deste material altamente nutritivo tem se tornando alvo de diversos estudos de bioprospecção. Resíduos queratinosos representam uma fonte valiosa de proteínas e aminoácidos que podem ser utilizados como aditivos na alimentação animal ou fonte de nitrogênio para plantas (GUSHTEROVA *et al*., 2005). Em adição, queratinases também são comumente utilizadas em processos para amaciamento de couro, representando alternativas interessantes do ponto de vista ecológico e financeiro ao tratamento químico (GUPTA & RAMNANI, 2006). Portanto, estudos de bioprospecção, purificação e produção em larga escala de enzimas queratinolíticas se fazem necessários para suprir a demanda mundial, especialmente trabalhos envolvendo o estudo de microrganismos de ambientes extremos, que podem dar origem a novos compostos com propriedades interessantes.

2 OBJETIVOS

O presente trabalho teve como objetivo geral:

- Avaliar a diversidade microbiana no ambiente antártico, por meio do estudo de comunidades microbianas da Ilha Rei George (Península Antártica).

Os objetivos específicos foram:

- Avaliar a diversidade funcional e genética das comunidades microbianas de solo;
- Avaliar a diversidade de microrganismos queratinolíticos e realizar a prospecção de enzimas queratinolíticas ativas em baixas temperaturas.

Este trabalho foi dividido em três capítulos, sendo o primeiro referente à avaliação da diversidade funcional, o segundo à análise da diversidade genética e o terceiro referente à prospecção de enzimas queratinolíticas.

CAPÍTULO 1: ANÁLISE DA DIVERSIDADE FUNCIONAL

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Short communication

Functional diversity of microbial communities in soils in the vicinity of Wanda Glacier, Antarctic Peninsula

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Running headline: *Microbial Diversity in Wanda Glacier*

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Abstract

Microbial functional diversity in four soils sampled in the vicinity of Wanda Glacier, Antarctic Peninsula, was determined using Biolog EcoPlates at 5°C and 25°C. Comparisons of the patterns of substrate utilization and the diversity index showed differences in community composition, reflecting the heterogeneous distribution of microorganisms in this environment. Differences in microbial diversity may be related to soil chemical properties. Higher incubation temperature influenced the overall microbial diversity, reducing richness due to the selection of psychrotrophic microorganisms. To our knowledge, this is the first study with microbial communities from Wanda Glacier and contributes to understanding the microbial diversity of Antarctic environments.

Key words: King George Island, Biolog EcoPlates, community-level physiological profile, extremophiles.

Antarctica presents difficult conditions for microorganism survival, such as low temperatures and low nutrient and water availability; however, this extreme environment has been successfully colonized by many microorganisms, including bacteria, yeasts, and unicellular algae, and recent data has shown that this environment harbors a unique diversity of microorganisms (18). Although several studies have addressed the microbial diversity in Antarctica, little is known about microbial functional diversity in the Antarctic Peninsula. Since many ecosystems found in Antarctica are composed almost exclusively of microorganisms, studies concerning microbial functions in Antarctica could contribute to understanding

ecological processes and predict the impact of climate change on this environment. In this study we used Biolog EcoPlates to evaluate the functional diversity of microbial communities in soils in the vicinity of Wanda Glacier at two incubation temperatures.

Wanda Glacier is located on the eastern coast of Admiralty Bay, King George Island, Antarctic Peninsula (Fig. 1A). It comprises an area of 1.63 km^2 and is linked to Admiralty Bay by a proglacial lake (14). Soil samples were collected aseptically in the vicinity of Wanda Glacier (Fig. 1B) in January 2010, during the 28^{th} Brazilian Antarctic Expedition in the austral summer season, and maintained at 4°C until processing. To evaluate the metabolic potential of whole microbial communities, samples were inoculated in Biolog EcoPlates (Biolog, Hayward, CA, USA), microtiter plates which contain, in triplicate, 31 ecologically relevant carbon substrates with a redox-sensitive tetrazolium indicator of microbial respiration (8). Prior to inoculation, samples were diluted 10-fold and centrifuged at 500 rpm for 20 minutes. Two plates were inoculated with each sample (100 µL in each well) and incubated in the dark, one at 5°C and the other at 25°C. Color formation was measured at 590 nm at regular intervals for 40 days, using a SpectraMax M_2^e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The turnover of each carbon was determined by subtracting the mean optical density (OD) of the three control wells from the average OD among the three replicates in each plate. Any corrected OD value higher than 0.006, the detection limit of the spectrophotometer, was considered a positive result for substrate utilization. In order to overcome the problems associated with fixed time comparisons (11), we chose to perform the analysis using data corresponding to the reading time when

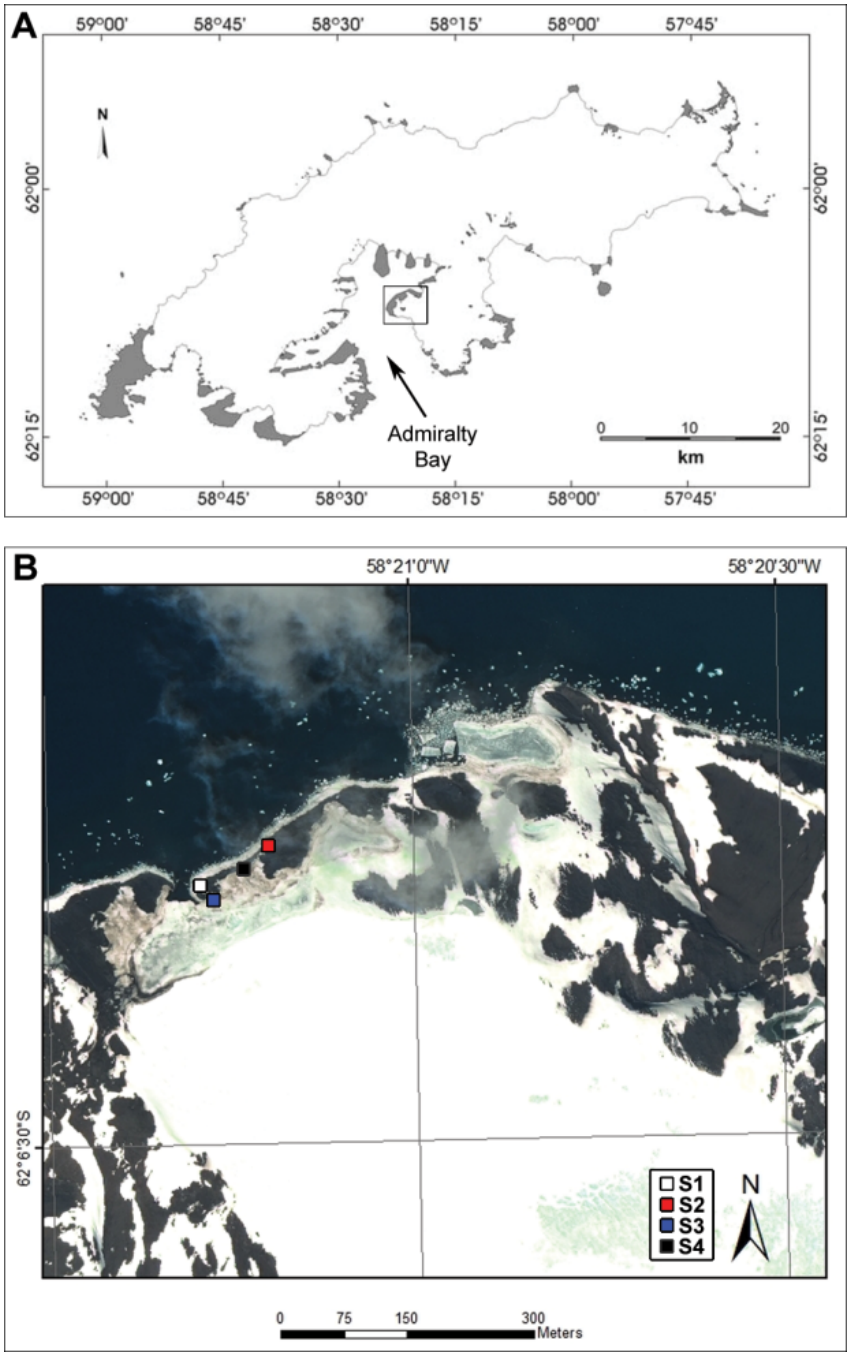

Fig. 1. A: Map of King George Island, Antarctic Peninsula, showing the location of Wanda Glacier [modified from Simões *et al.* (17)]. White and grey areas demarcate regions permanently or perennially covered with ice, respectively; B: satellite map of Wanda Glacier showing the sampling sites used in this study (Quickbird image kindly provided by Laboratório de Monitoramento da Criosfera, FURG, Rio Grande, RS, Brazil).

the average well color development (AWCD, 6) of all plates had reached the lag phase, meaning that no significant increase in the AWCD was observed, namely, 27 and 11 days for incubation at 5°C and 25°C, respectively. The Shannon diversity index (*H'*) and Shannon evenness (*E*) were calculated for each community according to Derry *et al.* (4). Soil chemical composition was analyzed according to Claessen (3) and water content and pH according to Newsham *et al*. (10).

Fig. 2 shows the patterns of carbon substrate utilization by each community at both temperatures. All carbon sources were metabolized at least by one community at each temperature. D-xylose, pyruvic acid methyl ester, putrescine, 2-hydroxy benzoic acid, Tween 40, and Tween 80 were metabolized by all communities at both temperatures. Of these, D-xylose and 2-hydroxy benzoic acid have been reported as constituents of root exudates, as well as other compounds extensively metabolized by the communities, such as D-malic acid, L-phenylalanine, L-asparagine, L-serine, L-threonine, and L-arginine (1). Moreover, high utilization of Tween 40 and Tween 80 has also been described for microbial communities from both Antarctic (16) and Arctic seawaters (15). Polyols, such as Tween 40 and Tween 80, are suggested to be accumulated by Antarctic algae and fungi in order to grow at low temperatures and protect against freeze-thaw events (13, 15).

One important observation is that the four soil communities showed distinct patterns of sole carbon source utilization (Fig. 2), even though samples were collected relatively near each other (Fig. 1B). This result illustrates the heterogeneous distribution of microorganisms in natural environments,

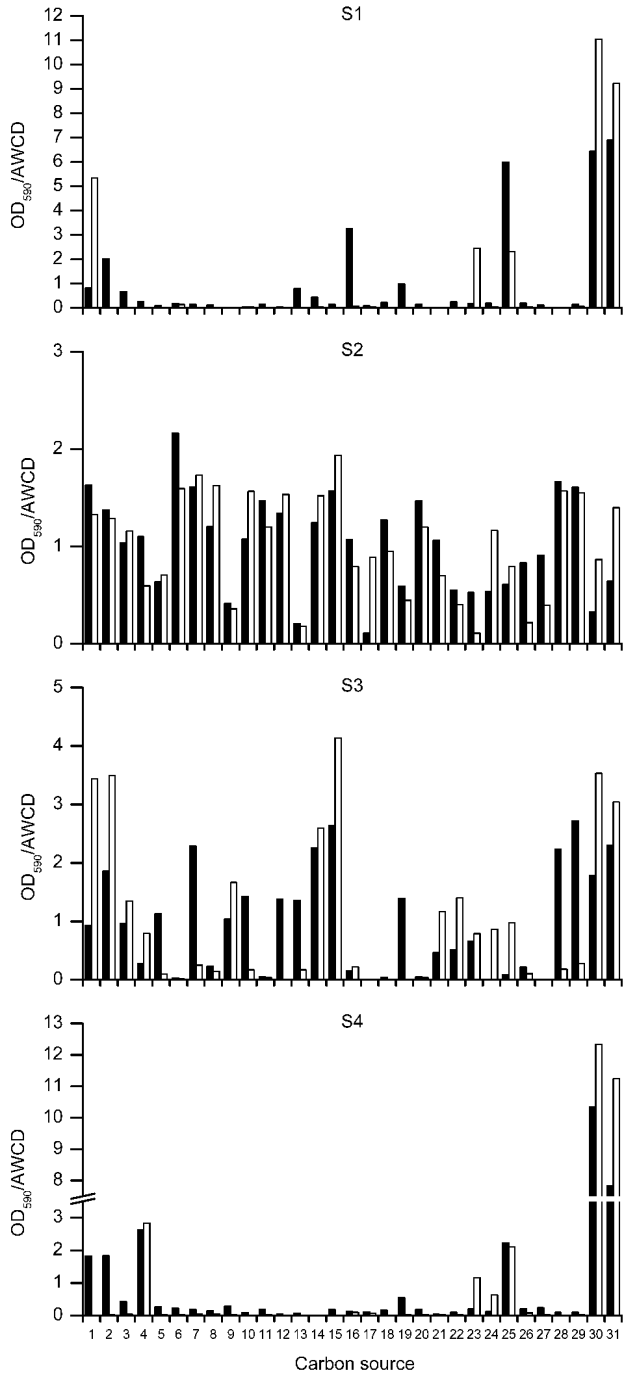

Fig. 2. Carbon source utilization by the communities at day 27 (5°C, filled bars) and 11 (25°C, empty bars). Data represent OD divided by the AWCD (6). Amino acids: 1, L-arginine; 2, L-asparagine; 3, glycyl-Lglutamic acid; 4, L-phenylalanine; 5, L-serine; 6, L-threonine. Carbohydrates: 7, D-cellobiose; 8, I-erythritol; 9, D-galactonic acid γ-lactone; 10, N-acetyl-D-glucosamine; 11, glucose-1-phosphate; 12, β-methyl-D-glucoside; 13, D,L-α-glycerol phosphate; 14, α-D-lactose; 15, D-mannitol; 16, D-xylose. Carboxylic acids: 17, γ -hydroxybutyric acid; 18, α-keto butyric acid; 19, D-galacturonic acid; 20, D-glucosaminic acid; 21, itaconic acid; 22, D-malic acid; 23, pyruvic acid methyl ester. Amines: 24, phenylethylamine; 25, putrescine. Phenols: 26, 2-hydroxy benzoic acid; 27, 4-hydroxy benzoic acid. Polymers: 28, α-cyclodextrin; 29, glycogen; 30, Tween 40; 31, Tween 80. Graphs are presented on different scales to better visualize substrates metabolized at low rates.

commonplace in studies of microbial diversity, especially in extreme environments (5, 20). Moreover, communities S1 and S4 showed a different pattern of substrate utilization from samples S2 and S3, metabolizing a few compounds such as Tween 40 and Tween 80 at a much higher rate than the others. Analysis of soil composition showed that the samples differed greatly in their chemical content, and it was observed that samples S1 and S4, as well as samples S2 and S3, have very similar chemical composition, especially in regard to the abundance of organic C (Table 1). It is well known that the availability of organic compounds and minerals directly influences the composition of soil microbial communities (5), and therefore the similar pattern of carbon source utilization observed among communities S1/S4 and S2/S3 may be related to similarities in the soil chemical composition.

Table 1. Water content, pH, and chemical composition of the soil samples.

Parameter	*Sample*			
	S1	*S2*	*S3*	*S4*
Water content (%)	17.91	13.45	13.66	7.51
pH	8.90	9.86	8.59	8.18
Organic C (%)	0.09	0.34	0.26	0.09
N (%)	<0.01	0.02	0.02	<0.01
Na (%)[a]	0.21	0.15	0.37	0.30
P (mg kg^{-1})[a]	219	229	229	266
K (mg kg^{-1})[a]	534	322	736	548
Al ($cmol_c$ kg^{-1})[b]	0.00	0.00	0.00	0.00
Ca ($cmol_c$ kg^{-1})[b]	2.00	6.70	2.80	1.90
Mg ($cmol_c$ kg^{-1})[b]	2.50	4.00	3.50	2.30
Mn (mg kg^{-1})[b]	3.00	2.00	1.00	2.00

[a] Extractable; [b] exchangeable.

Another important and striking finding is the different community response to the increase in incubation temperature. While for communities S2 and S3 the temperature increase basically changed the rate at which substrates were metabolized (Fig. 2), for communities S1 and S4, the temperature increase highly influenced the number of metabolized substrates and therefore the diversity of functional groups (Table 2). Whether incubation temperature influences the rate and/or the pattern of substrate utilization in BIOLOG experiments is a contradictory issue. So far, no definitive conclusion has been reached, with several studies supporting both scenarios (2, 4).

The majority of communities metabolized more substrates and showed higher diversity when incubated at 5°C than at 25°C, except community S2, which metabolized the same number of carbon sources at both temperatures (Table 2). A similar result was found by Teklay *et al*. (19) in a study on soils associated with the hybrid poplar in Canada, in which higher diversity was found at 5°C than at 15°C. In our study, although diversity was lower at 25°C, as shown by the Shannon diversity index, evenness, *i.e.* the distribution of species within the

Table 2. Number of positive results, diversity, and evenness for each community at days 27 (5°C) and 11 (25°C).

Sample	*Positive results*[a]		*Shannon diversity index (H')*		*Shannon evenness (E)*	
	5°C	*25°C*	*5°C*	*25°C*	*5°C*	*25°C*
S1	28	12	2.29	1.56	0.69	0.63
S2	31	31	3.30	3.28	0.96	0.96
S3	28	25	2.99	2.69	0.90	0.84
S4	29	11	2.14	1.49	0.64	0.62

[a] Numbers of metabolized substrates (optical density >0.006).

community, remained slightly unaltered (Table 2); therefore, it could be concluded that the lower diversity observed at 25°C is essentially explained by lower richness, *i.e.* fewer functional groups of microorganisms. Since higher temperatures constrain the growth of psychrophilic (cold-adapted) microorganisms, which are unable to grow above 20°C, at 25°C only the psychrotrophic (cold-tolerant) fraction of the community was active, resulting in lower diversity. Furthermore, since the maximum decrease in diversity observed at 25°C was 31.9% (community S1), it can be concluded that the communities studied here are composed mainly of psychrotrophic microorganisms, which seems to be the rule for Antarctic soil communities (12, 20). The prevalence of psychrotrophic microorganisms in Antarctic environments may be related to the increase in soil temperature through solar radiation, especially in the summer season, in which soil temperature may rise above 15°C (9). In addition, it may be that the lower diversity index obtained for communities S1 and S4 is also related to the soil chemical properties (Table 1). Since the major difference among the two types of soil resides in the organic C content, the lower diversity index obtained for communities S1 and S4 may be related to lower organic C availability, in agreement to the study by Gomez *et al.* (7).

This is one of the few works concerning microbial functional diversity in the Antarctic Peninsula, and the first to study microbial communities associated with Wanda Glacier. This study contributes to the knowledge about the complex and particular microbial diversity found in extreme environments, showing the heterogeneous distribution of microbial functional groups within this ecosystem. Moreover, this study also endorses the view that soil chemical properties directly

affect the composition of microbial communities, which are by far the main mediators of nutrient cycling in ecosystems. As we document more information about the microbial functional diversity of extreme environments, we may be able to predict with more accuracy the effects of global climate change on the stability of these fragile environments.

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CAPÍTULO 2: ANÁLISE DA DIVERSIDADE GENÉTICA

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Unexpected high microbial diversity and co-predominance of Archaea and Bacteria in soils of King George Island, Antarctic Peninsula

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Running title: Microbial diversity in King George Island

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Abstract

Antarctica, the colder and dryer environment on Earth, harbors a unique diversity of microorganisms which have evolved for millions of years under harsh conditions. Although several studies have showed that microbial communities thriving in Antarctic environments are highly diverse, it is likely that due to methodological issues the true microbial diversity has been largely underestimated. In this study we applied the high-throughput pyrosequencing methodology to study the composition of soil microbial communities of King George Island, Antarctic Peninsula. Taxonomic classification of >40 000 sequences of the V5-V6 hypervariable region of the 16S rRNA gene showed that Archaea constitutes an important fraction of the communities studied, with the majority of the sequences being assigned to the Euryarchaeota phylum. Within the Bacteria domain, communities are composed mainly by Proteobacteria and Bacteroidetes. Taxonomic classification of the most abundant sequences at species level showed that communities are dominated by different species of the Gammaproteobacteria class, and uncharacterized species belonging to an unknown family within the Thermoplasmatales class (Archaea domain). The calculated richness and diversity indexes were astonishingly high for all communities (*H'* = 5.33-6.24), indicating that Antarctic microbial communities are much more diverse than what have been estimated. Altogether, these findings suggest that Antarctic microbial communities are highly diverse and composed of many unknown microbial species.

Key words: Antarctica / Microbial diversity / Pyrosequencing / Wanda Glacier

Introduction

The knowledge on composition and functions of microbial communities inhabiting less intervened extreme environments has proven to be extremely valuable for science from basic and applied standpoints. Particularly, organisms thriving on xerophilic or psychrophilic habitats are of key interest as their descriptions help us to define the kind of selections taking place and to recover new interesting activities that are needed from biomedical and environmental point of views. Antarctica, the colder and dryer continent on Earth, presents habitats on which such selecting pressures had been exerted extensively over millions of years. Thus, samples retrieved from this continent are excellent models to study such effects on the composition of microbial communities.

Increasing attention has been given to the effects of climate change on Earth's biodiversity, since perceptible atmospheric changes have been observed in the past decades (Aronson et al, 2011). The Antarctic Peninsula is among the regions which showed the fastest warming rate in the last five decades, accompanied by significant changes in the precipitation regimes (Turner et al, 1997; Vaughan et al, 2003). Since Antarctic environments are composed almost exclusively of microbial communities, the study and monitoring of the Antarctic microbial diversity is vital for predicting the impact of climate change on this continent as well as other environments of the planet, since its effects have a higher and more rapid impact in polar environments (Aronson et al, 2011).

There is much we still do not know about Antarctic microbial diversity. A large number of studies concerning this issue is found on the literature to date, but the overall majority has been carried out using either culture-based methods or

molecular techniques relying on a very low number of sequences, which have been proven to underestimate substantially the true diversity found on Antarctic environments (Handelsman, 2004; Roesch et al, 2007). Analysis of microbial diversity through pyrosequencing of 16S rRNA gene fragments provides a more precise picture of the composition of microbial communities inhabiting a given environment, since this technology allows the sequencing of a large number of amplicons in a single four-hour reaction. Furthermore, the use of barcoded primers allows the analysis of sequences obtained from distinct samples on the same sequencing run, which can be later sorted into their original source due to the presence of a unique tag sequence (Binladen et al, 2007; Bohorquez et al, 2012). Nonetheless, the associated labor and sequencing costs are still beyond the scope of most laboratories, and few studies applying the pyrosequencing technology to the study of Antarctic microbial diversity have been carried out to date (Teixeira et al, 2010; Bates et al, 2011; Lee et al, 2011; Yergeau et al, 2011). In order to provide a more consistent picture of the microbial diversity found in Antarctic environments, we used the pyrosequencing technology to assess the structure of soil microbial communities from King George Island, Antarctic Peninsula.

Material and methods

Soil sampling and DNA extraction

King George Island, the largest of the South Shetland Islands archipelago, Antarctic Peninsula, harbors nine permanent scientific bases, including the

Brazilian research station Estação Antártica Comandante Ferraz (EACF), located in Martel Inlet, Admiralty Bay (62°04'S, 58°21'W). In January 2010, during the 28th Brazilian Antarctic Expedition in the austral summer season, surface soil samples were collected in the vicinity of Wanda Glacier (Figure 1A), an unexplored area located in the oriental coast of Admiralty Bay. The sampling sites (Figure 1B, Table 1) are distributed at short distance intervals in the shoreline of Wanda Glacier, an area with no records of significant anthropogenic acute disturbances and no reports of terrestrial vertebrate settlements. Partial chemical composition of the soil samples – as previously described by Pessi et al (2011) – is depicted in Table 1.

Total metagenomic DNA was extracted from 5 g of each soil sample using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to instructions from the manufacturer, with few modifications. Since the kit is designed to recover DNA from small amounts of soil, we added 5 g of each soil to a sterile 15 ml falcon tube and carried out the extraction using 10 times the amount of each reagent, resulting in a final volume of 500 µl. Following extraction, DNA was quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Amplification of the 16S rRNA gene

In order to describe the microbial community composition, we applied the strategy previously developed and reported by Bohorquez et al (2012), due to the excellent taxonomical discrimination given by the V5-V6 hypervariable region of the 16S rRNA gene (Huse et al, 2008), which is able to cover up to 99.8% of the

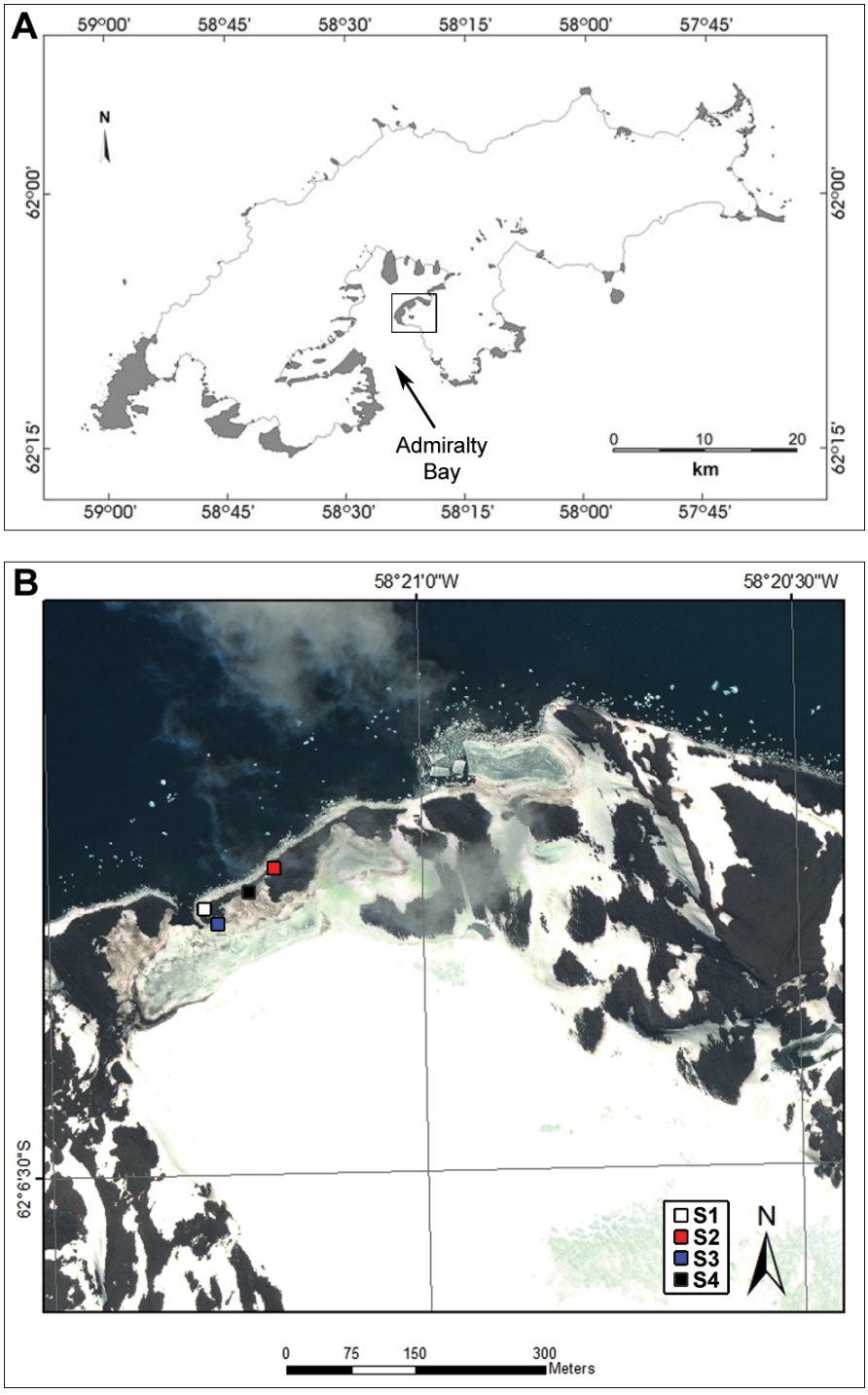

Figure 1. (A) Map of King George Island, Antarctic Peninsula, showing the location of Wanda Glacier. White and grey areas demarcate regions permanently or perennially covered with ice, respectively; (B) Satellite map of Wanda glacier showing the sampling sites used in this study. Figure taken from Pessi et al (2011), under permission from the publisher.

Table 1. Geographic coordinates and partial chemical composition of the soil samples used in this study.

Sample	*Coordinates*	*Soil T (°C)*	*Water Content (%)**	*pH**	C_{org} *(%)**	*N (%)**	C_{org}*/N ratio*
S1	62°06'20.0" S 58°21'19.9" W	2.3	17.91	8.90	0.09	<0.01	>9
S2	62°06'19.5" S 58°21'13.5" W	2.4	13.45	9.86	0.34	0.02	17
S3	62°06'20.7" S 58°21'18.7" W	4.6	13.66	8.59	0.26	0.02	13
S4	62°06'19.0" S 58°21'15.2" W	5.0	7.51	8.18	0.09	<0.01	>9

* Data from Pessi et al (2011).

sequences deposited and categorized in the Ribosomal Database Project (RDP) database (Bohorquez et al, 2012; also, see http://rdp.cme.msu.edu/).

A PCR reaction was carried out to amplify the V5-V6 hypervariable regions of the prokaryotic (Eubacterial and Archaeal) 16S rRNA gene, using the universal primers 807f and 1050r (Table 2). For this, 1-2 ng of template DNA was added to a PCR mixture consisting of 1X PCR Buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 1 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and sterile Milli-Q water to a final volume of 50 µl. PCR conditions were as follows: 94°C for 2 min.; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min.; and 72°C for 5 min. Following amplification, PCR products were purified using the UltraClean PCR Clean-Up kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and quantified in NanoDrop. PCR products were then used as templates on a new PCR reaction, including long complementary primers to extent the unique barcode and 454 adaptor sequences to the amplicons generated (as previously reported by Bohorquez et al, 2012), in order to avoid the potential biases

Table 2. Sequence of the primers used in this study, as reported by Bohorquez et al (2012).

Name	*Sequence (5'-3')*
807f	GGATTAGATACCCBRGTAGTC
1050r	AGYTGDCGACRRCCRTGCA
807f-b16*	GCCTCCCTCGCGCCATCAGTA**ATCTGCTC** *GGATTAGATACCCBRGTAGTC*
1050r-b2*	GCCTTGCCAGCCCGCTCAGAG**AGATGC** *AGYTGDCGACRRCCRTGCA*
1050r-b3*	GCCTTGCCAGCCCGCTCAGAG**AGCAGC** *AGYTGDCGACRRCCRTGCA*
1050r-b4*	GCCTTGCCAGCCCGCTCAGAG**AGCATG** *AGYTGDCGACRRCCRTGCA*
1050r-b5*	GCCTTGCCAGCCCGCTCAGAG**ATCATC** *AGYTGDCGACRRCCRTGCA*

* Sequences of the pyrosequencing adapter (underlined) and barcode (bold) are separated by 2 bp; sequence of the 16S rRNA primer is italicized.

generated by direct long primer amplification over a long number of cycles (Berry et al, 2011). New PCR reactions were carried out using the primer 807f-b16 but different reverse primers for each sample (Table 2), at the same conditions of the first PCR, except for the number of cycles (five).

Sequencing and data processing, diversity and composition analysis

Sequencing of the four amplicon datasets was performed on a Genome Sequencer FLX System (454 Life Sciences, Branford, CT, USA) at Helixxa's facility (Campinas, SP, Brazil). Prior to sequencing, PCR products were purified using the Agencourt AMPure XP kit (Beckman Coulter Inc., Brea, CA, USA).

In order to separate each of the four amplicon datasets by origin of sample (distinct barcode), to discard or to trim sequences of low quality values defined in flowgrams and to perform the bundled bioinformatics analytical steps to obtain the

results described below, we used the open source software package Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al, 2010). For more accurate comparisons, we performed rarefaction and diversity analyses using normalized datasets, all randomized to unique resamplings of 7000 sequences per dataset. OTUs (operational taxonomical units) were defined at 0.03 distance, following recommendations described elsewhere (Huse et al., 2010). The taxonomic identity of the sequences was obtained using the Classifier tool from the RDP's website (http://rdp.cme.msu.edu/) (Cole et al, 2009). Consensus sequences from the OTUs accumulating more number of sequences were selected using the QIIME software, and identified using the SeqMatch tool from the RDP's website.

Results

DNA recovery, OTU accumulation and richness

From each soil sample, we obtained between 0.73 and 1.61 µg of metagenomic DNA per gram of soil. As it has been reported for a wide variety of soils, the majority of the DNA extracted is of bacterial origin. Thus, assuming an extraction efficiency of 80% and an average DNA content of 10 fg per bacterial cell, the approximate amount of bacterial cells per gram of soil ranged from 0.91 x 10^8 to 2.01 x 10^8.

For all the amplicons obtained from these samples the average read length was 306 bp, which is nearly the average expected value that can be calculated for the V5-V6 hypervariable regions of the 16S rRNA gene in complex environmental datasets of taxonomically homogenous composition. Cleaning algorithms for

sequence quality were applied using default values, restricting windows and minimum quality values down to 20 and minimum sequence length to 100 bp. This resulted in a total of 42 993 sequences for the four datasets, and the average sequence read length decreased to 249 bp. As showed in Figure 2, all samples showed a similar pattern of OTU accumulation after normalization.

From the same datasets used for rarefaction and sequence distribution in OTUs (normalized to unique resamplings of 7000 sequences per dataset), we calculated the equivalence and comparisons in richness between communities. The calculated richness were at the same order of magnitude for all communities, with ACE defining for community S2 the minimum estimated OTU richness of 4 738, while for community S3, the maximum of 8 135 (Table 3). Similarly, OTU diversity, as shown by the Shannon's index, ranged from 5.33 (community S2) to 6.24 (community S3).

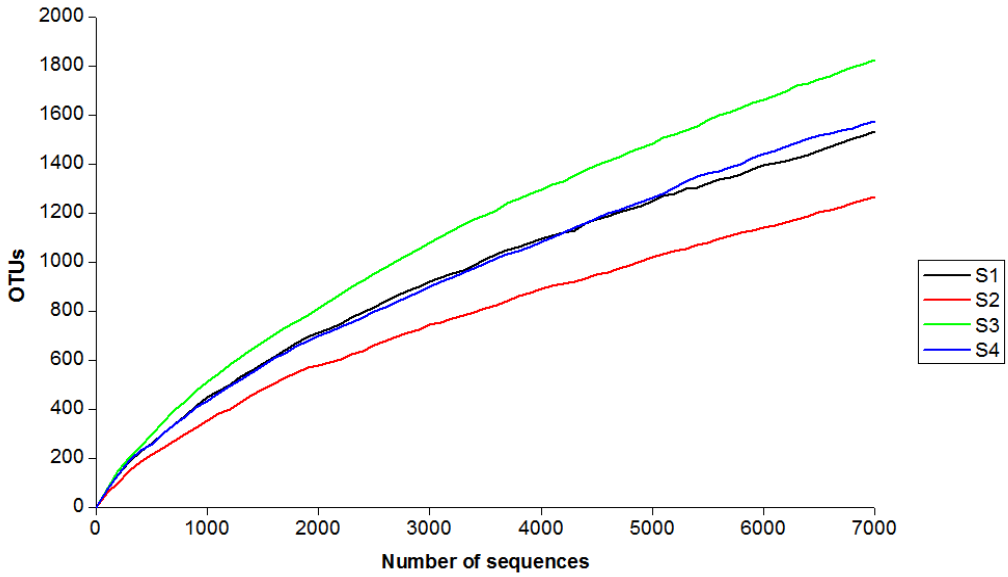

Figure 2. Rarefaction curves of normalized datasets of partial 16S rRNA gene sequences obtained from soil samples of Wanda Glacier.

Table 3. Richness and diversity values calculated for the communities.

Community	*Number of sequences*	*Estimated OTU richness*		H'*
		ACE	*CHAO*	
S1	10 999	5 485	3 555	5.99
S2	11 484	4 738	3 064	5.33
S3	7 053	8 135	4 668	6.24
S4	13 457	5 610	3 471	5.94

* Shannon's diversity index

Bacterial community composition

Taxonomic classification of the sequences revealed that the communities are composed of 17 highly divergent phyla, with a predominance of Proteobacteria, Bacteroidetes and Euryarchaeota, except for community S2, which showed low abundance of Euryarchaeota (Figure 3). Actinobacteria, Planctomycetes and Acidobacteria were also found in minor abundance. Moreover, all communities showed a large number of sequences of bacterial origin that cannot be classified into any phyla currently described in the RDP database.

Refining the classification of the two predominant phyla, namely Proteobacteria and Bacteroidetes, to class level (Figure 4), we can observe that, within the Proteobacteria phylum, communities are composed mainly of bacteria belonging to the Gamma- and Betaproteobacteria classes. Moreover, at this higher discriminatory level, a considerable amount of the sequences previously assigned to this phylum could not be assigned to a specific class. Regarding the Bacteroidetes phylum, the majority of the sequences were assigned to the Flavobacteria class, with the exception of community S2, in which Sphingobacteria represents the dominant class. However, it should be noted that, in this sample, the phylum Bacteroidetes was found in a higher proportion than the others (Figure

3). Once again, many sequences were not assigned to any class within this phylum.

Taxonomical classification of predominant members of the communities

By selecting representative consensus sequences from the OTUs which accumulated more than 10 times the average number of sequences per dataset, we were able to identify with a greater resolution the identity of the predominant members of the communities (Table S1). Applying the functions to select such consensus sequence out of all the OTUs implemented in QIIME, allowed us to

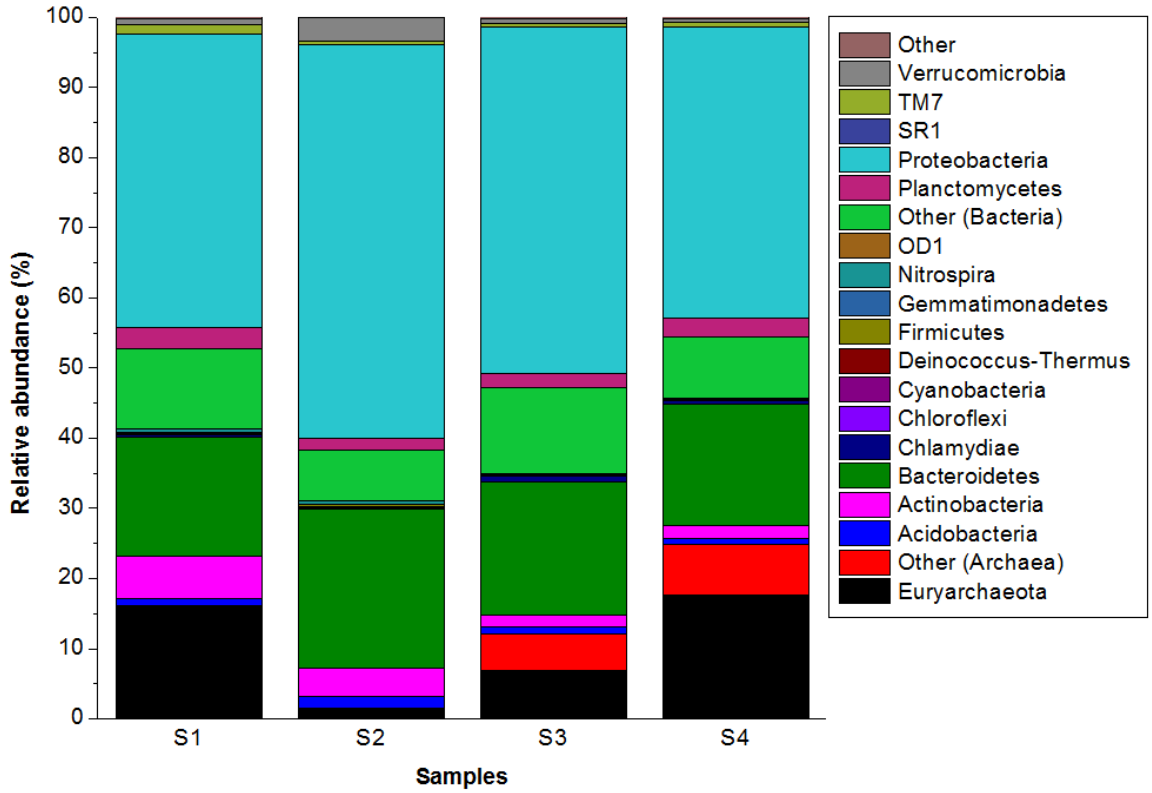

Figure 3. Taxonomical classification at phylum level (Naïve Bayesian Classifier – RDP) of partial 16S rRNA gene sequences obtained from soil samples of Wanda Glacier.

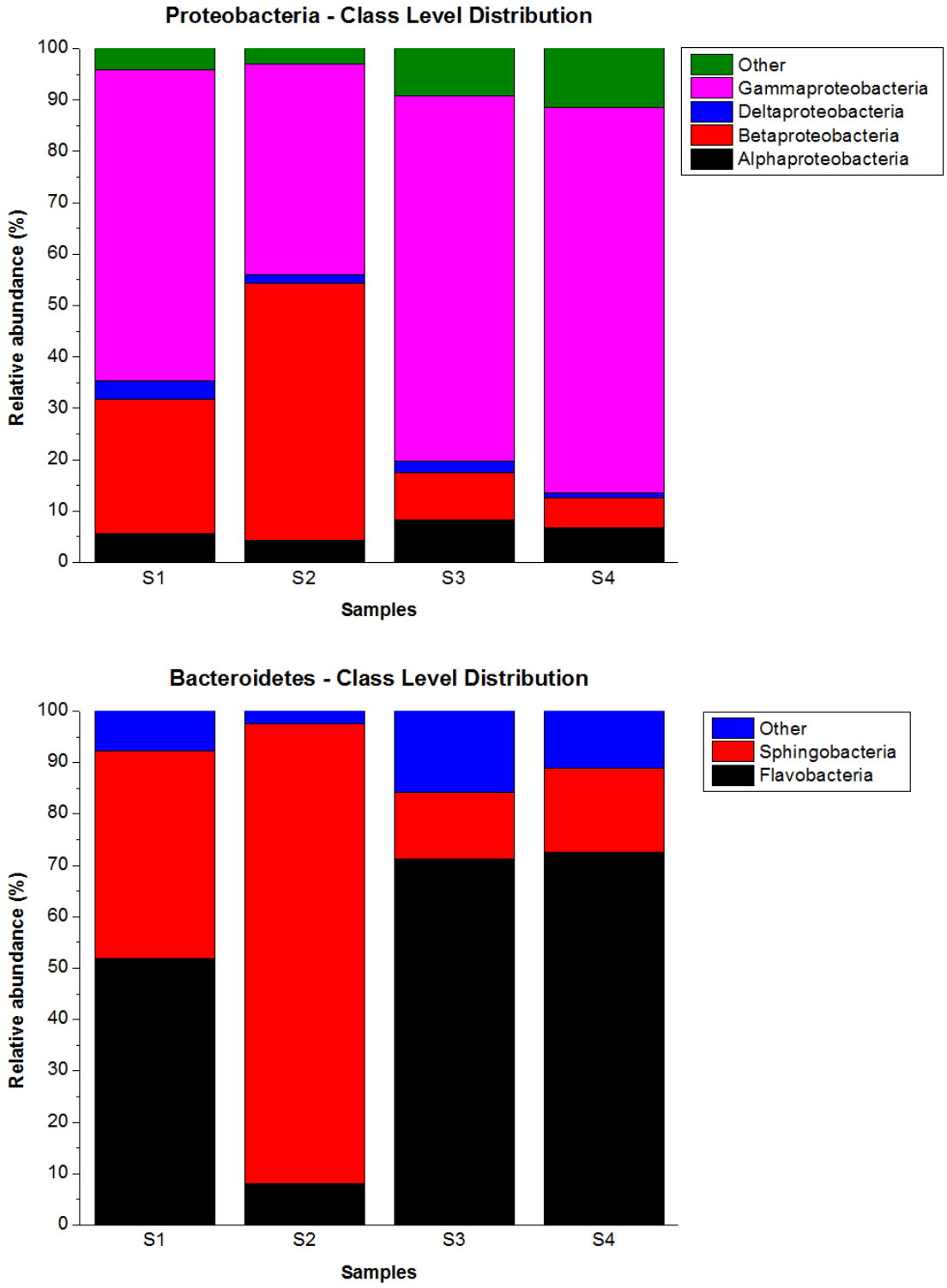

Figure 4. Class composition (Naïve Bayesian Classifier – RDP) of the phyla Proteobacteria and Bacteroidetes, based on partial 16S rRNA gene sequences obtained from soil samples of Wanda Glacier.

perform SeqMatch analysis finding the nearest neighbors classified in the database. This analysis revealed that the communities are dominated by an assemblage of Bacteria and Archaea (Table S1). Within the Archaea domain, predominant members were found to belong to an unknown family of the Euryarchaeota phylum. As for the Bacteria domain, predominant members were assigned to the Proteobacteria and Bacteroidetes phyla. The genera *Pseudomonas*, *Granulosicoccus*, *Thiobacillus*, *Ohtaekwangia*, *Terrimonas*, *Neptunomonas*, *Methylophaga*, *Ulvibacter* and *Hydrocarboniphaga* were among the dominant members of the communities. However, the similarity is in many cases low enough to consider that the sequences are not coming from described species inside the genera. Other phylotypes unresolved at the genus level were also found among the dominant members of the communities.

Discussion

The amount of metagenomic DNA recovered from the soil samples was relatively low (0.73-1.61 μg g^{-1}), if compared to DNA extracted from soil of tropical regions (Zhou et al, 1996). By calculating the approximate number of bacterial cells necessary to yield this amount of DNA, we found that the samples contained 0.91-2.01 x 10^8 cells g^{-1}, which is a similar number to what have been estimated by direct microscopic counts of bacterial cells in soil samples from nearby areas (Zdanowski & Weglenski, 2001; Xiao et al, 2007; Grzesiak et al, 2009). This observation, along with other reports for continental Antarctica (Cowan et al, 2002; Aislabie et al, 2006; Smith et al, 2006), shows that the microbial load of Antarctic environments is, in fact, much higher than it was formerly assumed.

Regarding sequence distribution and frequency in OTU accumulation, the results indicated that these communities follow the pattern coined as “rare biosphere” (Sogin et al, 2006), with some OTUs defined by just one single sequence, indicating the presence of very different phylogenetic groups in very low abundance. On the other hand, for all communities we observed a group of at least 8 OTUs which accumulated more than 10 times the average number of sequences (Table S1), representing the dominant members in the microbial communities analyzed. While evidently the description of the average number of sequences per OTU is still not saturated with the normalized 7000 sequences per sample, it is possible to define the OTUs accumulating more sequences, and thus, representing the predominant members of the microbial community. While its precise abundance can change if a deeper sequence sampling effort is applied, their relative significant contribution to the community is very likely to be similarly observed and maintained.

Perhaps the most striking finding of this study was the astonishingly high OTU richness and diversity observed. As can be seen in Table 3, the Shannon’s diversity index calculated for the communities ranged from 5.33 to 6.24, while the CHAO index ranged from 4 738 to 8 135. Both were surprisingly high given the extreme environment sampled, for which it was not expected to have such estimated richness of microorganisms, comparable to soil from temperate regions (Dunbar et al, 2000). These indexes show that the microbial diversity of Antarctic terrestrial environments is much higher than what have been previously reported (Niederberger et al, 2008; Chong et al, 2009; Pointing et al, 2009; Teixeira et al, 2010; Chong et al, 2012). According to Schloss & Handelsman (2005), the

richness detected in a sample is highly dependent of the number of sequences analyzed. Since the overwhelming majority of the studies of microbial communities in Antarctica deals with a much lower number of sequences, is plausible to conclude that the true microbial diversity of Antarctic environments has been highly underrated.

At this point, we wanted to clarify the identity (taxonomical classification) of the sequences that are composing these datasets. Phylum level classification shows that the communities are composed of phyla belonging to both Archaea and Bacteria domains (Figure 3). The high abundance of Archaea (mainly belonging to the Euryarchaeota phylum) in these communities was not expected, since it has been suggested that soil may be an inhospitable environment for these microorganisms (Borneman & Triplett, 1997; Ayton et al, 2010). On the other hand, Archaea have proven to be an important fraction of the Southern Ocean picoplankton (DeLong et al, 1994; Murray et al, 1999; Church et al, 2003), although the majority of the phylotypes have been assigned to the Crenarchaeota phylum. Controversially, Euryarchaeota seems to be more abundant in temperate seas, being scarcely found or absent in Antarctic waters (Murray et al, 1999; Massana et al, 2000; DeLong, 2003; Nakayama, 2011).

Along with Euryarchaeota, Proteobacteria and Bacteroidetes were the predominant phyla in the communities, with Actinobacteria, Planctomycetes and Acidobacteria also being found in minor abundance (Figure 3). Proteobacteria are ubiquitous in Antarctic terrestrial environments, and it has been suggested that members of this phylum might be more strongly selected for in high than in low productivity soils (Cowan et al, 2010). Nevertheless, as we report in this study,

consistently with the findings of Aislabie (2006), Proteobacteria are also abundant in soils with low organic content, and therefore, other environmental factors are more likely to shape its distribution. Bacteroidetes are also widespread in Antarctic environments, and are well-known for their ability to degrade a wide range of polymers. Hence, this catabolic versatility may confer an advantage to these microorganisms in such oligotrophic environments (Cowan et al, 2010).

Although Proteobacteria and Bacteroidetes are among the nine bacterial phyla most abundant in soils worldwide (Janssen, 2006), the pattern of phylum distribution differed greatly. The predominance of Proteobacteria and Bacteroidetes in the communities here studied is consistent with a few previous reports from soil, sediments and coastal waters of Antarctic Peninsula (Foong et al, 2010; Ghiglione & Murray, 2011; Srinivas et al, 2011). Nevertheless, several studies have shown that other phyla, such as Actinobacteria, *Deinococcus-Thermus*, Acidobacteria and Firmicutes, which were highly underrepresentated in these datasets, also make up a considerable proportion of Antarctic microbial communities (Aislabie et al, 2006; Smith et al, 2006; Niederberger, 2008).

Recently, Teixeira et al. (2010) have showed that rhizosphere bacterial communities from Admiralty Bay are dominated by Firmicutes, Actinobacteria and Proteobacteria. As proposed by the authors, the analysis of the structure of microbial communities inhabiting bulk soil is needed to corroborate their results. Although the present study was not carried out using soil sampled from the exact same location, our results demonstrate a clear differentiation among microbial communities inhabiting bulk and vegetation-covered soil, since Actinobacteria were found in minor abundance and Firmicutes were nearly absent in our

samples. Moreover, Archaea and Bacteroidetes, groups which constitute a large proportion of the microbial communities here studied, were nearly absent in the rhizosphere communities.

Other interesting finding was the relative similarity in phylum distribution observed among the four communities. It is clear that the analysis of sequences of the 16S rRNA gene does not reveal the metabolic diversity of active members from a community, and analysis of the functional diversity of microbial communities obtained from these same samples shows that each community is composed from a unique set of functional groups, even though samples were collected relatively near each other (Pessi et al, 2011). However, comparison of the results from both studies should be made with caution, due to major differences in the methodology employed.

By selecting representative consensus sequences from the OTUs accumulating more number of sequences and analyzing using the SeqMatch tool in the RDP's website, we were able to elucidate the identity of the dominant members of the communities, pinpointing only a range of 8-14 representative sequences to be analyzed in detail (constituting 21-37% of the total number of sequences). Using this approach, we found that the communities are dominated by microorganisms belonging to both prokaryotic domains (Table S1). Within the Archaea domain, dominant members were assigned to the Euryarchaeota class, related to sequences of uncultured Archaea from surface sediments from the Pacific Arctic Ocean and from temperate waters, hydrothermal vents and desert soil. Within the Bacteria domain, communities are dominated by members of the Gammaproteobacteria class. This result is compatible with patterns of phylum

composition obtained from the complete datasets run under Naïve Bayesian Classifier of RDP, showing the congruence and correctness of the approach we have taken to simplify and group the sequences for analyses of alpha and beta diversity. Moreover, similarly to the results of phylum distribution, the analysis of the predominant OTUs showed that identity of the dominant members did not differ substantially among the four communities.

As concluding remarks, the main findings can be summarized as: (i) the surprisingly high diversity of microorganisms found inhabit the soils sampled in this study shows that microbial communities thriving in Antarctic environments are much more diverse than what have been estimated; (ii) the detection of divergent OTUs with extremely low representation, reinforcing the concept of the rare biosphere; (iii) the unexpected high abundance of Archaea in the samples, mainly belonging to an uncharacterized family within this phyla; (iv) the peculiar phylum distribution, with the predominance of the Proteobacteria and Bacteroidetes phyla; (v) the high abundance of sequences that could not be assigned to any known phyla or classes, as well as the low identity with sequences of known microorganisms available in the RDP database observed among the predominant OTUs in these communities, indicating a high number of unknown microbial species – and possibly endemic – in this environment.

Acknowledgments

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Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
Sample S1	**1 472**	**21.03**	
S1_38056	300	4.29	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Xanthomonadales Family Sinobacteraceae Genus *Hydrocarboniphaga* S000652802 0.708 1377 *Panacagrimonas perspica*; Gsoil 142; AB257720 Genus *Nevskia* S000015378 0.701 1304 *Nevskia ramosa* (T); Soe1; AJ001010 Unclassified Sinobacteraceae S002046495 0.704 1420 uncultured bacterium; D13W_30; HM057752
S1_37254	229	3.27	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Pseudomonadales Family Pseudomonadaceae Genus *Pseudomonas* S000028020 0.886 0616 uncultured bacterium; BUG-62; AJ344196 S000129391 0.886 1439 *Pseudomonas* sp. 2N1-1; AJ419674 S000431617 0.886 1363 uncultured *Pseudomonas* sp.; FTL201; AF529093
S1_19521	191	2.73	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Chromatiales Family Granulosicoccaceae Genus *Granulosicoccus* S000859419 0.875 1399 *Granulosicoccus antarcticus* (T); IMCC3135; EF495228 S001249711 0.875 1390 uncultured bacterium; IHE3_169; AB476271 S002496835 0.879 1416 uncultured gammaproteobacterium; PET-031; JF344155

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S1_24002	176	2.51	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.799 0899 uncultured archaeon; BC-C; AY396002 S002490173 0.766 0891 uncultured archaeon; P-3_A2; HQ910241 Unclassified Euryarchaeota S000358560 0.784 0900 uncultured archaeon; BC-D; AY396003
S1_42525	161	2.30	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.790 0899 uncultured archaeon; BC-C; AY396002 S000358561 0.893 0894 uncultured archaeon; BC-E; AY396004 S000358562 0.717 0890 uncultured archaeon; BC-F; AY396005
S1_593	156	2.23	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Pseudomonadales Family Pseudomonadaceae Genus *Rhizobacter* S000850811 0.886 1340 uncultured betaproteobacterium; MVP-12; DQ676337 Unclassified Proteobacteria S001148524 0.901 0962 uncultured bacterium; LOXA-d02; EU869552 S001148618 0.901 0974 uncultured bacterium; UOXA-f12; EU869646

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S1_35578	140	2.00	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.865 0899 uncultured archaeon; BC-C; AY396002 S000358561 0.755 0894 uncultured archaeon; BC-E; AY396004 Unclassified Euryarchaeota S000358560 0.734 0900 uncultured archaeon; BC-D; AY396003
S1_27318	119	1.70	Domain Bacteria Phylum Proteobacteria Class Betaproteobacteria Order Hydrogenophilales Family Hydrogenophilaceae Genus *Thiobacillus* S000012826 0.894 1314 betaproteobacterium 5Z-C1; AJ224618 S000653817 0.894 1436 *Rhodocyclaceae bacterium* FTL9; DQ451825 S001039689 0.897 1403 uncultured bacterium; 2As60; EU735722
Sample S2	**2 604**	**37.21**	
S2_26167	552	7.89	Domain Bacteria Phylum Proteobacteria Class Betaproteobacteria Order Methylophilales Family Methylophilaceae Genus *Methylotenera* S000799526 0.880 1450 uncultured bacterium; Rap1_6A; EF192877 S001684320 0.880 1425 uncultured bacterium; MACA-EFT44; GQ500809 Unclassified Methylophilaceae S000397704 0.880 1403 betaproteobacterium BP-5; AY145571

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S2_3554	541	7.73	Domain Bacteria Phylum Bacteroidetes Class Bacteroidetes incertae sedis Genus *Ohtaekwangia* S000932259 0.775 1316 uncultured bacterium; FFCH11997; EU133665 S001252921 0.783 1398 uncultured bacterium; MABRDTU26; FJ529979 S001321744 0.779 1396 uncultured bacterium; Ge09; FJ710659
S2_25196	433	6.19	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Unclassified Gammaproteobacteria S001546181 0.820 0920 uncultured bacterium; UKCSB08; GQ176316 S001546193 0.820 0920 uncultured bacterium; UKCSB116; GQ176328 S001603083 0.820 0920 uncultured bacterium; UKCSB223; GQ443485
S2_33138	222	3.17	Domain Bacteria Phylum Bacteroidetes Class Sphingobacteria Order Sphingobacteriales Family Chitinophagaceae Genus *Terrimonas* S000613782 0.898 1307 uncultured bacterium; CJRA14; DQ202140 S000960899 0.898 1385 uncultured Bacteroidetes bacterium; AS56; EU283377 S001556583 0.875 1373 uncultured bacterium; ENR17; FJ536924
S2_22253	213	3.04	Domain Bacteria Phylum Bacteroidetes Class Bacteroidetes incertae sedis Genus *Ohtaekwangia* S000932259 0.720 1316 uncultured bacterium; FFCH11997; EU133665 S001252921 0.724 1398 uncultured bacterium; MABRDTU26; FJ529979 S001321744 0.720 1396 uncultured bacterium; Ge09; FJ710659

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S2_10050	169	2.41	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Pseudomonadales Family Pseudomonadaceae Genus *Pseudomonas* S000129391 0.905 1439 *Pseudomonas* sp. 2N1-1; AJ419674 S002424132 0.908 1409 uncultured bacterium; SW-Jan-47; HQ203863 S002425160 0.908 1409 uncultured bacterium; MF-Jan-47; HQ225125
S2_5631	153	2.19	Domain Bacteria Phylum Proteobacteria Class Betaproteobacteria Order Burkholderiales Family Comamonadaceae Genus *Variovorax* S001350554 0.875 1400 *Variovorax* sp. S23408; D84617 S002949816 0.875 1400 *Variovorax boronicumulans*; S32547-a; AB649023 Unclassified Comamonadaceae S001794485 0.875 1345 *Variovorax* sp. SGM1-15; GU181268
S2_28619	140	2.00	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Unclassified Gammaproteobacteria S001546181 0.804 0920 uncultured bacterium; UKCSB08; GQ176316 S001546193 0.804 0920 uncultured bacterium; UKCSB116; GQ176328 S001603083 0.804 0920 uncultured bacterium; UKCSB223; GQ443485
S2_41118	97	1.39	Domain Bacteria Phylum Proteobacteria Class Betaproteobacteria Unclassified Betaproteobacteria S001051569 0.861 1373 uncultured bacterium; M6A-625; AM991243 S001598359 0.835 1400 uncultured bacterium; AK1AB1_06D; GQ396827 S001598466 0.905 1398 uncultured bacterium; AK4AB2_06E; GQ396934

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S2_38156	84	1.20	Domain Bacteria Phylum Proteobacteria Class Betaproteobacteria Order Hydrogenophilales Family Hydrogenophilaceae Genus *Thiobacillus* S000922791 0.891 1421 uncultured bacterium; RS06101_B92; EU101281 S001598418 0.887 1429 uncultured bacterium; AK4AB1_03F; GQ396886 S001598618 0.887 1404 uncultured bacterium; AK4DE2_08C; GQ397086
Sample S3	**1772**	**25.32**	
S3_39672	334	4.77	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Oceanospirillales Family Oceanospirillaceae Genus *Neptunomonas* S000870482 0.911 1403 *Neptunomonas japonica* (T); JAMM 0745; AB288092 S000891059 0.911 1396 *Neptunomonas japonica*; JAMM 1380; AB291226 S000891060 0.911 1396 *Neptunomonas japonica*; JAMM 1475; AB291227
S3_29706	302	4.31	Domain Archaea Phylum Crenarchaeota Class Thermoprotei Unclassified Thermoprotei S001017884 0.885 0621 uncultured archaeon; YdcBA39; AB424726 Unclassified Archaea S001261070 0.885 1286 uncultured archaeon; S26-7a; FJ571902 S001261090 0.889 1286 uncultured archaeon; S26-27a; FJ571922

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S3_34351	144	2.06	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Pseudomonadales Family Pseudomonadaceae Genus *Pseudomonas* S000028020 0.897 0616 uncultured bacterium; BUG-62; AJ344196 S000129391 0.897 1439 *Pseudomonas* sp. 2N1-1; AJ419674 S000431617 0.897 1363 uncultured *Pseudomonas* sp.; FTL201; AF529093
S3_25991	123	1.76	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Oceanospirillales Family Oceanospirillaceae Genus *Neptunomonas* S000870482 0.868 1403 *Neptunomonas japonica* (T); JAMM 0745; AB288092 S000891059 0.868 1396 *Neptunomonas japonica*; JAMM 1380; AB291226 S002180865 0.871 1410 endosymbiont bacterium; Omu 16 c5904; FN773258
S3_37411	107	1.53	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Chromatiales Family Granulosicoccaceae Genus *Granulosicoccus* S000859419 0.923 1399 *Granulosicoccus antarcticus* (T); IMCC3135; EF495228 S001249711 0.923 1390 uncultured bacterium; IHE3_169; AB476271 S001417743 0.923 1430 *Granulosicoccus* sp. ZS4-22; FJ889674

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S3_37783	105	1.50	Domain Bacteria Phylum Bacteroidetes Class Flavobacteria Order Flavobacteriales Family Flavobacteriaceae Genus *Formosa* S001199262 0.891 1340 *Formosa spongicola* (T); A2; FJ348469 S001577364 0.891 1346 *Formosa* sp. EM151; GQ331112 Unclassified Flavobacteriaceae S000921191 0.914 1341 uncultured bacterium; SS1_B_08_12; EU050891
S3_14670	94	1.34	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Alteromonadales Family Alteromonadaceae Genus *Aestuariibacter* S001284051 0.750 0834 uncultured bacterium; Lc2z_ML_287; FJ355193 Genus *Glaciecola* S000824102 0.736 1357 *Glaciecola* sp. G20; AB295496 S000966624 0.739 1354 *Glaciecola lipolytica* (T); E3; EU183316
S3_41466	90	1.29	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Thiotrichales Family Piscirickettsiaceae Genus *Methylophaga* S000961678 0.908 1417 uncultured bacterium; S11-28; EU287211 S000961716 0.912 1414 uncultured bacterium; S11-66; EU287249 S001447480 0.908 1107 uncultured bacterium; CH3NH2_C10; FJ937710

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S3_31689	89	1.27	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358562 0.868 0890 uncultured archaeon; BC-F; AY396005 S000358564 0.783 0913 uncultured archaeon; BC-G'; AY396007 Unclassified Euryarchaeota S000358560 0.779 0900 uncultured archaeon; BC-D; AY396003
S3_6151	82	1.17	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Unclassified Gammaproteobacteria S001003745 0.813 1412 uncultured bacterium; P0X3b5B06; EU491383 S001003849 0.846 1415 uncultured bacterium; P0X4b2G11; EU491487 S002284749 0.813 1422 uncultured marine bacterium; 16_07_02G04; FR684049
S3_39981	80	1.14	Domain Bacteria Phylum Bacteroidetes Class Flavobacteria Order Flavobacteriales Family Flavobacteriaceae Genus *Ulvibacter* S000647653 0.876 1400 uncultured bacterium; Belgica2005/10-ZG-1; DQ351797 S001023122 0.876 1324 uncultured bacterium; Hg91A3; EU236346 S001682753 0.868 0983 uncultured Bacteroidetes bacterium; s44; GQ472787

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S3_41009	76	1.09	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Xanthomonadales Family Sinobacteraceae Genus *Hydrocarboniphaga* S000652802 0.754 1377 *Panacagrimonas perspica*; Gsoil 142; AB257720 S002295310 0.746 1262 uncultured bacterium; KA6190063; HM445555 S002745295 0.750 1260 uncultured bacterium; ncd2561c01c1; JF225362
S3_41984	74	1.06	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Unclassified_Gammaproteobacteria S000921095 0.874 1337 uncultured proteobacterium; SS1_B_01_48; EU050794 S001023211 0.877 1324 uncultured bacterium; Hg62E1; EU236435 S002480933 0.900 1420 uncultured sediment bacterium; WS152JS0307; HQ190984
S3_40934	72	1.03	Domain Bacteria Phylum Bacteroidetes Class Flavobacteria Order Flavobacteriales Family Flavobacteriaceae Unclassified Flavobacteriaceae S000805912 0.925 0778 bacterium b1cb3; EF207067 S000880214 0.909 1423 uncultured bacterium; N67e_105; EF646138 S002436140 0.909 1419 uncultured bacterium; SBS-RV-084; HQ326339

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
Sample S4	**1885**	**26.94**	
S4_4533	485	6.93	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.850 0899 uncultured archaeon; BC-C; AY396002 S000358561 0.744 0894 uncultured archaeon; BC-E; AY396004 Unclassified Euryarchaeota S000358560 0.813 0900 uncultured archaeon; BC-D; AY396003
S4_32957	221	3.16	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Pseudomonadales Family Pseudomonadales incertae sedis Genus *Dasania* S001003386 0.769 1417 uncultured bacterium; P7X3b1E08; EU491024 Unclassified Gammaproteobacteria S001003561 0.762 1421 uncultured bacterium; P9X2b7E10; EU491199 S002342015 0.762 1319 uncultured bacterium; AMSMV-0-B162; HQ588412
S4_28892	220	3.14	Domain Archaea Unclassified Archaea S001260954 0.922 1281 uncultured archaeon; R15-71a; FJ571786 S001261051 0.922 1284 uncultured archaeon; S16-80a; FJ571883 S001261100 0.926 1282 uncultured archaeon; S26-37a; FJ571932
S4_21471	202	2.89	Domain Archaea Unclassified Archaea S001260954 0.919 1281 uncultured archaeon; R15-71a; FJ571786 S001260967 0.919 1282 uncultured archaeon; R15-84a; FJ571799 S001261051 0.919 1284 uncultured archaeon; S16-80a; FJ571883

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S4_13367	189	2.70	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Oceanospirillales Family Oceanospirillaceae Genus Neptunomonas S002180811 0.849 1438 endosymbiont bacterium; Omu 6 c451; FN773204 S002180812 0.849 1437 endosymbiont bacterium; Omu 6 c503; FN773205 S002180813 0.849 1438 endosymbiont bacterium; Omu 6 c468; FN773206
S4_3030	138	1.97	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.832 0899 uncultured archaeon; BC-C; AY396002 S000358561 0.725 0894 uncultured archaeon; BC-E; AY396004 Unclassified Euryarchaeota S000358560 0.733 0900 uncultured archaeon; BC-D; AY396003
S4_36551	132	1.89	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Xanthomonadales Family Sinobacteraceae Genus *Hydrocarboniphaga* S000652802 0.774 1377 *Panacagrimonas perspica*; Gsoil 142; AB257720 S002295310 0.767 1262 uncultured bacterium; KA6190063; HM445555 S002745295 0.770 1260 uncultured bacterium; ncd2561c01c1; JF225362

Table S2. Taxonomic afiliation at species-level of the predominant sequences in each dataset.

ReprSeqID	Seq/OTU	%Seq	Results
S4_40966	123	1.76	Domain Archaea Phylum Euryarchaeota Class Thermoplasmata Order Thermoplasmatales Unclassified Thermoplasmatales S000358559 0.875 0899 uncultured archaeon; BC-C; AY396002 S000358561 0.773 0894 uncultured archaeon; BC-E; AY396004 Unclassified Euryarchaeota S000358560 0.773 0900 uncultured archaeon; BC-D; AY396003
S4_39467	104	1.49	Domain Bacteria Phylum Proteobacteria Class Gammaproteobacteria Order Chromatiales Family Granulosicoccaceae Genus *Granulosicoccus* S000859419 0.923 1399 *Granulosicoccus antarcticus* (T); IMCC3135; EF495228 S001249711 0.923 1390 uncultured bacterium; IHE3_169; AB476271 S001417743 0.923 1430 *Granulosicoccus* sp. ZS4-22; FJ889674
S4_39500	71	1.01	Domain Bacteria Phylum Bacteroidetes Class Flavobacteria Order Flavobacteriales Family Flavobacteriaceae Unclassified Flavobacteriaceae S000805912 0.909 0778 bacterium b1cb3; EF207067 S000880214 0.891 1423 uncultured bacterium; N67e_105; EF646138 S002436140 0.891 1419 uncultured bacterium; SBS-RV-084; HQ326339

CAPÍTULO 3: BIOPROSPECÇÃO DE ENZIMAS QUERATINOLÍTICAS

Diversity of psychrophilic keratinolytic bacteria isolated from King George Island, Antarctic Peninsula

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Abstract

The Antarctic continent, although the colder and dryer environment on the planet, harbors an outstanding diversity of microorganisms which for several millions of years have adapted their metabolism to these harsh conditions. Bioprospection studies in this environment are increasing exponentially, since the use of cold-adapted enzymes in many industrial processes represents innumerous advantages, both from an economical as an ecological point of view. This holds true for processes involving the degradation of keratin, a vey recalcitrant compound accumulating in the environment mainly in the form of keratinous wastes from many industries. Antarctic still remains an untapped source of novel compounds, so this study was carried out to verify the ability of Antarctic microorganisms to produce keratinolytic enzymes. Five out of 120 bacteria isolated from soil, water and snow of King George Island, Antarctic Peninsula, belonging to the genera *Arthrobacter*, *Pseudomonas*, *Pedobacter* and *Janthinobacterium*, were able to grow using keratin as sole carbon source. This report shows that Antarctic bacteria are sources of cold-active keratinolytic enzymes, which may have application in industrial processes.

Keywords: Antarctica, keratinase, keratin azure

Introduction

Keratinases are proteases which are capable of hydrolyze keratin, a major component of biological structures such as hair, feathers and nails. Great attention has been given to studies concerning the bioprospection of keratinolytic enzymes

in the past few years, due to the increasing accumulation of keratinous wastes derived mainly from the poultry industry, and the lack of efficient alternatives for recycling this highly recalcitrant material. The presence of disulfide bonds and high contents of cross-linkage in keratin difficult its degradation by common proteases such as trypsin, pepsin, and papain (Papadopoulos 1986). Nevertheless, several microorganisms are known to efficiently degrade keratin.

The Antarctic Peninsula is a region of the Antarctic continent known to harbor many species of birds and mammals, specially penguins and seals. Due to its warmer temperature and higher moisture content, compared to mainland Antarctic, soils from the Antarctic Peninsula are favorable environments for the development of highly diversified microbial communities (Campbell and Claridge 1987), which are known to decompose several kinds of compounds at rates comparable to communities from environments with milder temperatures (Pessi et al. 2011). Enzymes from cold-adapted microorganisms (collectively known as psychrophiles) are becoming a major aim of scientific investigations, mainly due to its great potential for application in several industrial and biotechnological processes, but also given the lack of knowledge concerning this issue. Cold-active enzymes present higher activity at low temperatures than enzymes from mesophile microorganisms, and are commonly extremely thermosensible (Gerday et al. 2007). These two features renders these enzymes very interesting for several applications, since they work at room temperature and are easily inactivated with gentle heating, lowering the costs related to energy consumption (Marx et al. 2007). Nevertheless its high potential, few cold-adapted enzymes are available for commercial use, compared to thermostable enzymes (Cavicchioli et

al. 2002). This study was carried out to investigate the ability of bacteria isolated from King George Island, Antarctic Peninsula, to degrade keratin.

Materials and methods

Sampling and isolation of microorganisms

King George Island is the largest of the South Shetlands Islands archipelago, Antarctic Peninsula. Three water, four soil and five snow samples were taken in the vicinity of Wanda Glacier (Figure 1), located on the eastern coast of Admiralty Bay, King George Island. Samples were collected aseptically in polypropylene bottles and maintained at 4°C until processing.

For the isolation of microorganisms, samples were inoculated into five culture media (Luria-Bertani agar, plate count agar, R2A agar, nutrient agar and tryptic soy agar), and incubated at 5°C for 28 days. Prior to inoculation, 10 g of each soil sample were suspended in 90 mL of NaCl 0.5% and centrifuged at 500 rpm for 10 minutes. After incubation, morphologically different colonies were selected, and isolated in the same culture medium and temperature.

Screening and measuring of keratinolytic activity

To verify the production of keratinolytic enzymes by the isolates, first was carried out a qualitative screening of proteolytic activity. For this, isolates were inoculated into 1% skim milk agar and incubated at 5°C for 28 days. Colonies presenting a surrounding transparent halo after incubation, indicating the breakdown of casein, were considered positive for proteolytic activity. In order to

verify the ability to utilize keratin as sole carbon source, protease-producing microorganisms were then inoculated into a solid culture media containing 40% feather meal, and incubated at the same conditions of the previous screening. Microorganisms that were able to grow using keratin as sole carbon source were considered positive for keratinolytic activity.

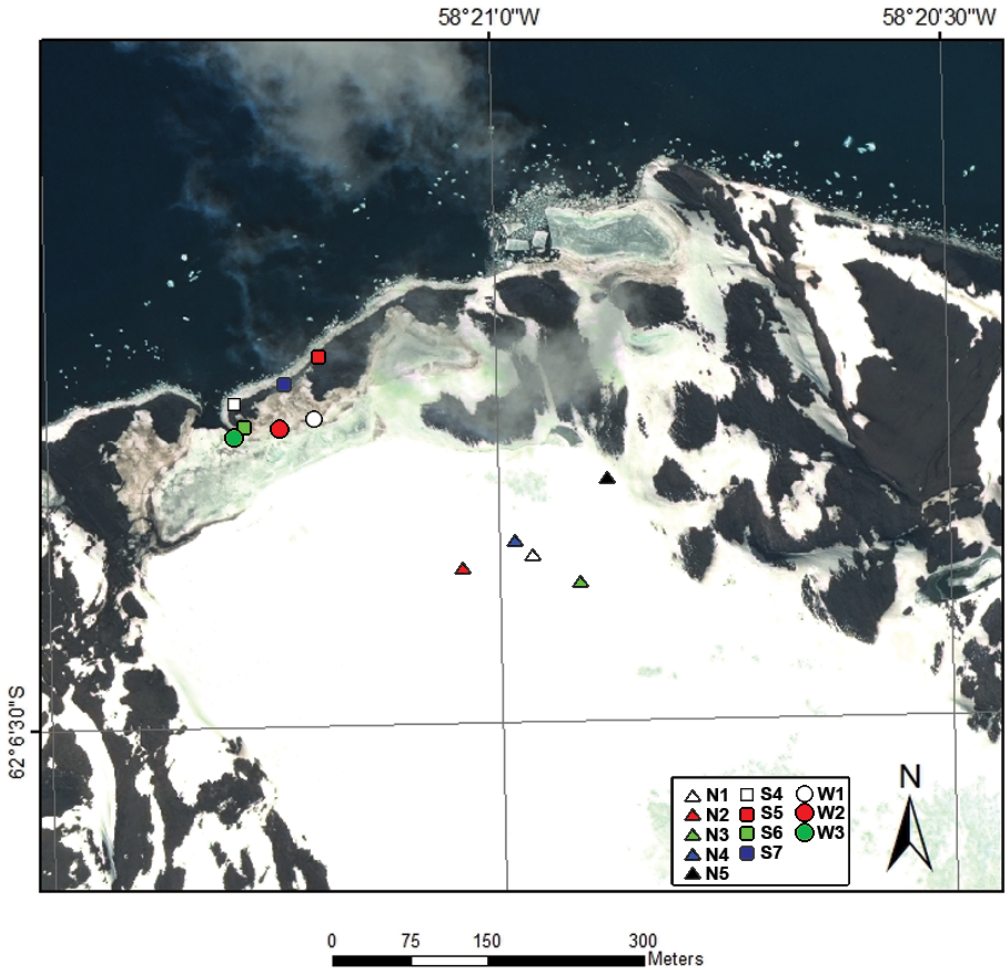

Figure 1. Satellite picture of Wanda Glacier (King George Island, Antarctic Peninsula) showing the sampling sites used in this study (Quickbird image kindly provided by Laboratório de Monitoramento da Criosfera, FURG, Rio Grande, RS, Brazil). Triangles, squares, and circles indicate snow, soil, and water samples, respectively.

In order to measure the extent of keratin degradation, keratinolytic microorganisms were inoculated into a liquid medium containing keratin azure. First, a inoculum was prepared by picking up a single colony of each isolate and suspending in NaCl 0.5%, and adjusting to an optical density (600 nm) of 0.1. Then, 100 µL of the bacterial suspension was inoculated into 10 mL of a liquid medium containing yeast extract 0.05% and keratin azure 1%, and incubated at 5°C for 28 days. Periodically, tubes were analyzed and release of dye into the culture medium was estimated visually in reference to an inoculated tube.

Amplification and sequencing of the 16S rRNA gene

DNA from the keratinolytic microorganisms was extracted according to general methods. A PCR reaction was carried out to amplify the 16S rRNA gene, using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'), in a PCR mixture consisting of 1X PCR Buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 1 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1-2 ng of template DNA and sterile Milli-Q water to a final volume of 50 µL. PCR conditions were as follows: 94°C for 5 min.; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min.; and 72°C for 5 min. Following amplification, PCR products were purified using the UltraClean PCR Clean-Up kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and submitted to sequencing. Sequencing of the amplicons was performed in an ABI Prism 3130xl System (Applied Biosystems, Carlsbad, CA, USA), using both reverse and forward primers separately.

Taxonomic classification and phylogenetic analysis

Contig sequences from the alignment of both amplicons were obtained using the BioEdit Sequence Alignment Editor (Hall 1999). The taxonomic identity of the 16S rRNA sequences was obtained using the BLAST tool from the NCBI's website (http://blast.ncbi.nlm.nih.gov/), and the three sequences showing the highest degree of identity were selected. Sequences alignment and phylogenetic analysis (neighbor-joining) were carried out using the Mega 5 software.

Results and discussion

After incubation at 5°C, 120 morphologically distinct isolates were obtained from the snow, water and soil samples. Of these, five were considered as keratinolytic after screening on feather meal agar (Table 1).

Taxonomic classification of partial 16S rRNA gene sequences showed that the five keratinolytic microorganisms belong to the Actinobacteria, Proteobacteria and Bacteroidetes phyla (Table 1). Sequence match analysis using the BLAST algorithm showed that the isolates are related to species of the genera *Arthrobacter* (isolates S15T3 and S25P1), *Pseudomonas* (S25T1), *Janthinobacterium* (S35T1) and *Pedobacter* (S25T2). Phylogenetic analysis indicated that, out of the five isolates, four did not formed a cluster with its nearest neighbor found in the NCBI database, although were grouped with bacteria of the same genera (Figure 2, Table 1).Isolate S25T2 formed an outline group among *Pedobacter* isolates. Given the low nucleotide identity with the top match in the NCBI database (only 86%), this isolate might belong to a different genera within the Sphingobacteria class, yet undescribed. Isolate S25T1, despite the high

Table 1. Taxonomic classification and keratinolytic activity of the isolates.

Phylogenetic affiliation	*Isolate*	*Top match, nucleotide identity, accession number*	*Keratinolytic activity*
Actinobacteria, Actinobacteria	S15T3	*Arthrobacter sulfonivorans* strain ALL, 97%, NR025084	+
	S25P1	*Arthrobacter oryzae* strain KV-651, 96%, NR041545.1	+
Proteobacteria, γ-proteobacteria	S25T1	*Pseudomonas mandelii* strain CIP 105273, 98%, R024902.1	+
Proteobacteria, β-proteobacteria	S35T1	*Janthinobacterium lividum* strain DSM 1522, 97%, NR026365.1	++
Bacteroidetes, Sphingobacteria	S25T2	*Pedobacter steynii* strain WB2.3-45,89%, NR042605.1	+

+ = low activity; ++ = high activity. Extent of keratin degradation was estimated visually as the amount of azure dye released into the culture medium.

nucleotide identity (98%) with a strain of *Pseudomonas mandelii*, was clustered with a different species of this genera. Isolates S15T3 and S25P1, although showing highest identity with different species of the *Arthrobacter* genus, formed a distinct group within this clade. Nevertheless, given the length of the branch, we can assume that they do not belong to the same species. Finally, isolate S35T1 was the only one which clustered with its closest neighbor found in the NCBI database (*Janthinobacterium lividum* strain DSM 1522), meaning that it is likely to belong to the same species.

The five isolates which were able to grow on feather meal agar plates, indicating that they possess the ability to utilize keratin as sole carbon source, were inoculated in a liquid medium containing keratin azure (sheep's wool keratin impregnated with azure dye) as substrate, in order to estimate the extent of keratin degradation. As keratin is degraded, the azure dye is released into the medium,

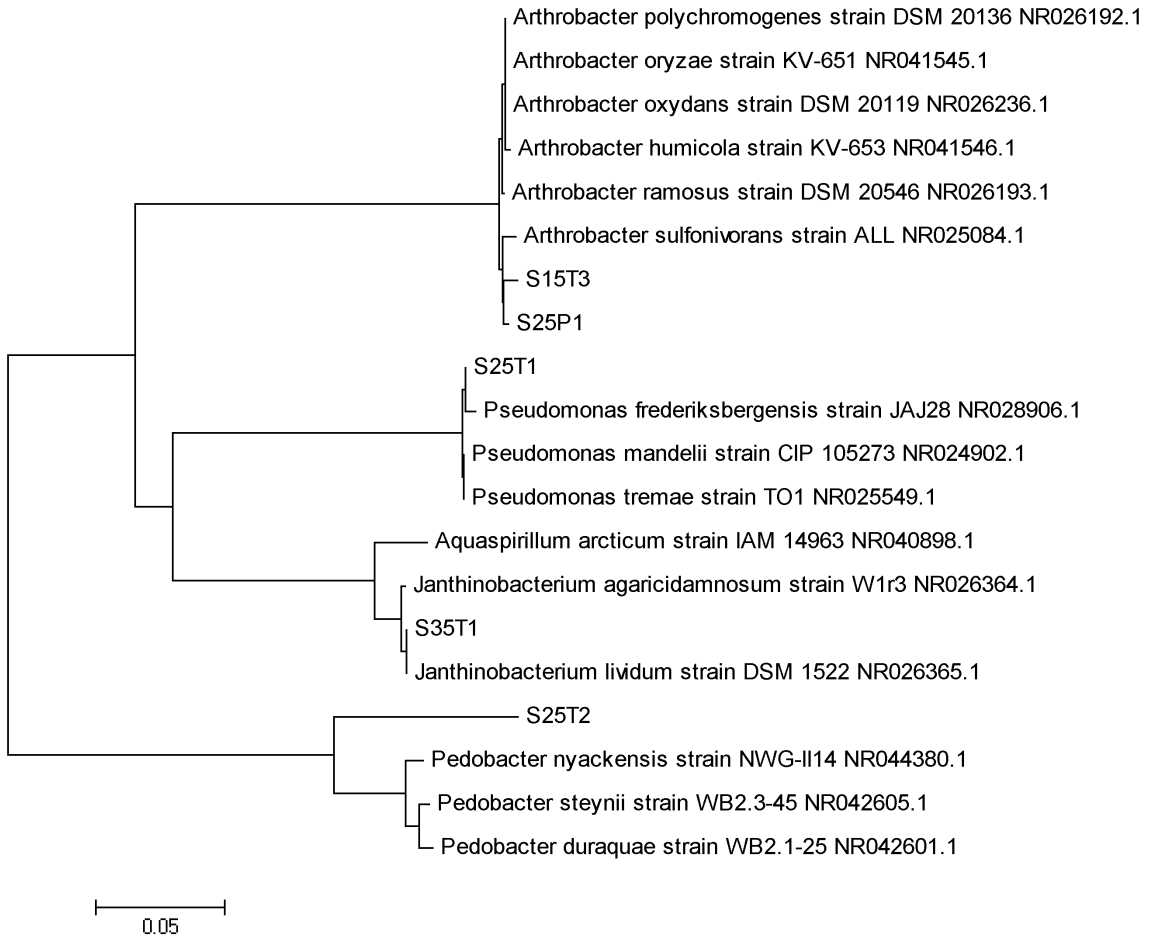

Figure 2. Phylogenetic tree (Neighbor-Joining method), derived from 16S rRNA gene sequence data, showing the phylogenetic relationship among the keratinolytic microorganisms and the three top matches in the NCBI database.

giving a visual estimate of the amount of keratin metabolized. Figure 3 shows that the isolates degraded keratin at distinct rates after 28 days of incubation at 5°C (Figure 3). Given that the wave lengths in which the azure dye and the microbial cells absorb the most are very similar (590 and 600 nm, respectively), we were not able to measure spectrophotometrically the amount of dye released. In addition, pelleting the cells by centrifuging for 5 min at 12 000 rpm turned out to be ineffective as well, since the optical density measured at 590 nm in the

supernatant did not correlate with visual estimates of dye release (data not shown). Therefore, we decided to base our results only in visual estimates of the amount of dye released into the medium. Isolate S35T1 showed the highest keratinolytic activity after 28 days. Isolate S25P1 showed moderate activity, and the other three isolates were able to degrade keratin in a much lower extent. Nevertheless, we must emphasize that this assay is only a visual estimate of the keratinolytic activity, and differences observed in keratin degradation by the different isolates may be due to distinct growth rates and nutritional necessities. Even so, density of growth did not seem to be related to the degree of keratinolytic activity, since the isolate S35T1 released the highest amount of dye into the medium in the absence of visible growth.

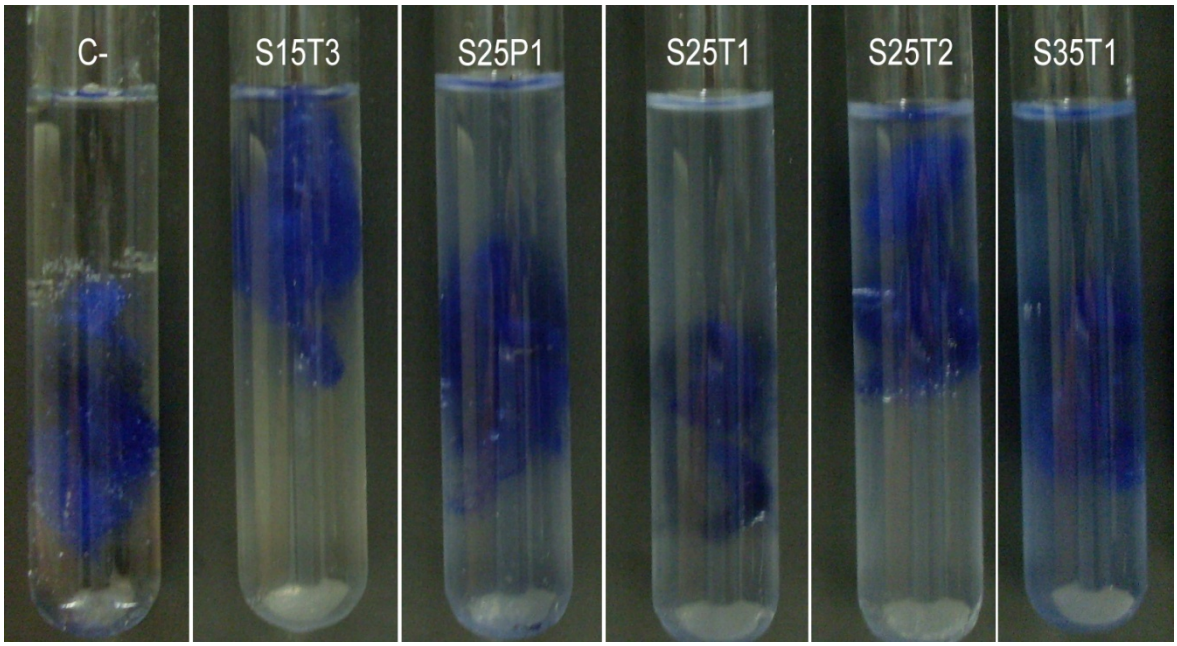

Figure 3. Extent of keratin degradation by the different isolates, as showed by the amount of azure dye released into the medium, after incubation at 5°C for 28 days.

The five isolates were classified within common phyla found in Antarctic soils, namely Actinobacteria, Proteobacteria and Bacteroidetes (Aislabie et al. 2006; Smith et al. 2006), being related to different species previously described to posses proteolytic activity, isolated from Antarctica and other cold environments (Margesin et al. 2003; Reddy et al. 2009; Yu et al. 2009). Interestingly, Reddy et al. (2009), in a bioprospection study for cold-active enzymes from bacteria obtained from an Arctic glacier, isolated microorganisms showing high nucleotide identity with the same strains found to be related to four of the five isolates reported in this study (*Arthrobacter sulfonivorans* ALL, *Arthrobacter oxydans* DSM 20119, *Janthinobacterium lividum* DSM 1522, *Pseudomonas frederiksbergensis* JAJ28 and *Pseudomonas mandelii* CIP 105273). This observation endorses the view that at least a fraction of the microbial diversity of Antarctic environments is composed of cosmopolitan species found in other cold environments around the globe, also agreeing with the old microbiological tenet “everything is everywhere, but the environment selects”, formulated by the Dutch microbiologist Lourens Baas Becking (De Wit and Bouvier 2006). Nevertheless, of the microorganisms found to be related with coincidental strains in both studies, only one isolate from the Arctic glacier, related to *Arthrobacter oxydans* DSM 20119, was found to posses proteolytic activity. This goes to show that, although bacteria belonging to the same or related species are found in both environments, they are likely to display distinct functions within the communities.

The search for novel keratinolytic enzymes has attracted much attention in the past few years, since keratinous wastes represent a valuable source of proteins and amino acids, but are very resistant to degradation by common

proteases. Moreover, the Antarctic continent has proven to harbor a largely unexplored diversity of microorganisms, which are likely to posses novel and interesting characteristics. To date, few studies have been carried out to investigate the ability of cold-adapted Antarctic bacteria to degrade keratin. In this work we report that keratinolytic bacteria thrive in Antarctic environments and are active at low temperatures. Further studies addressing this issue are likely to discover novel compounds that may represent interesting alternatives for the recycling of keratinous wastes.

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3 DISCUSSÃO GERAL

O continente Antártico é frequentemente citado como o ultimo ambiente inalterado do planeta, onde a combinação de isolamento geográfico, extremos ambientais e acessibilidade limitada tem restringido o impacto de atividades humanas (COWAN *et al*., 2011). Entretanto, esta visão é errônea, pois estudos têm mostrado que microrganismos não nativos são frequentemente transportados para a Antártica por processos eólicos de alta altitude (PEARCE *et al*., 2009; HUGHES & CONVEY, 2010), e que contaminantes (principalmente bactérias patogênicas humanas) têm sido introduzidos neste ambiente devido ao aumento das atividades científicas e turísticas (SJÖLING & COWAN, 2000; HUGHES & THOMPSON, 2004).

O estudo da diversidade microbiana no continente Antártico se faz necessário por muitos motivos. Devido à acessibilidade limitada muito pouco se sabe a respeito da estrutura das comunidades microbianas presentes neste ambiente. O conhecimento da diversidade de microrganismos que habitam o continente Antártico é fundamental para avaliar o impacto das mudanças climáticas em todo o planeta, visto que seus efeitos são muito mais pronunciados em ambientes polares (ARONSON *et al*., 2011), como evidenciado pela rápida mudança nas características atmosféricas de diversas regiões da Península Antártica (TURNER *et al*., 1997; VAUGHAN *et al*., 2003). Em adição, como as comunidades microbianas presentes neste ambiente têm evoluído durante milênios sob condições adversas de temperatura e disponibilidade de água, o continente Antártico é um potencial reservatório de microrganismos com

características únicas. Logo, o estudo da diversidade microbiana antártica pode dar origem a novos compostos com grande potencial de aplicação em diversos processos industriais.

A análise da diversidade funcional de comunidades microbianas de quatro solos obtidos da Ilha Rei George (Península Antártica) (capítulo 1) mostrou que as comunidades presentes neste ambiente são formadas por uma alta diversidade de grupos funcionais de microrganismos, capazes de utilizar uma ampla gama de substratos. Uma importante observação derivada deste estudo é que as comunidades presentes em cada uma das amostras diferem substancialmente entre si, apesar de terem sido coletadas em locais relativamente próximos. A análise da composição química das amostras mostrou que a diversidade de grupos funcionais parece estar relacionada com a quantidade de carbono orgânico presente no solo, como tem sido reportado em diversos estudos (GOMEZ *et al*., 2006; GANZERT *et al*., 2011). Comparando as análises realizadas a 5 e a 25°C, é possível observar que uma maior riqueza de grupos funcionais foi obtida em uma temperatura mais baixa. Temperaturas mais altas inibem o crescimento de microrganismos psicrófilos, resultando em um menor número de espécies capazes de se desenvolver. Novamente, a diferença na diversidade observada nas duas temperaturas de incubação variou significativamente entre as quatro comunidades, mostrando que cada comunidade apresenta uma composição única de grupos funcionais. Estes resultados apontam para o fato de que ambientes antárticos abrigam comunidades microbianas antárticas altamente diversas, que estão heterogeneamente distribuídas em micronichos com

diferentes condições ambientais, como já relatado para outras regiões da Antártica (YERGEAU & KOWALCHUK, 2008; GANZERT *et al*., 2011).

Entretanto, a análise da diversidade genética (capítulo 2) revelou um quadro diferente, mostrando que a composição taxonômica das comunidades microbianas presente em cada uma das amostras é relativamente similar. Entretanto, estes dois estudos foram realizados utilizando técnicas diferentes, o que dificulta a sobreposição dos resultados. Enquanto a diversidade funcional foi analisada utilizando placas Biolog Ecoplates, técnica que envolve o cultivo de microrganismos, a diversidade genética foi estimada por pirosequenciamento de fragmentos do gene da subunidade 16S do RNA ribossomal amplificados a partir de DNA metagenômico, método independente de cultivo. É consenso na comunidade científica que apenas uma pequena fração (<1%) das comunidades microbianas geralmente pode ser cultivada (SCHLOSS & HANDELSMAN, 2003), portanto estudos metagenômicos mais aprofundados e o desenvolvimento de novas técnicas são necessários, de modo a permitir a correlação entre a identidade dos microrganismos que compõem as comunidades com os processos que eles realizam no ambiente.

Como resultado da avaliação da diversidade genética de comunidades de solo da Península Antártica, foram observados índices extremamente altos de riqueza e diversidade de táxons, similares aos obtidos para comunidades de solo de regiões temperadas (DUNBAR *et al*., 2000). Apesar de já ter sido relatada uma alta diversidade microbiana em diversas regiões da Antártica (NIEDERBERGER *et al*., 2008; CHONG *et al*., 2009; POINTING *et al*., 2009; TEIXEIRA *et al*., 2010; CHONG *et al*., 2012), nossos resultados apontam para o fato de que

comunidades microbianas na Antártica são de fato muito mais diversas do que tem sido observado. Nosso estudo foi o primeiro dentre os realizados com comunidades microbianas na Antártica a analisar um grande número de sequências (mais de 40.000 incluindo as quatro comunidades). Como a riqueza de espécies calculada para uma determinada comunidade é altamente dependente do número de sequências analisadas (SCHLOSS & HANDELSMAN, 2005), nós acreditamos que novos estudos incluindo um número maior de sequências provavelmente corroborarão nossos resultados no que diz respeito aos altos índices de diversidade observados.

A classificação taxonômica das sequências em nível de filo e classe mostrou que as comunidades microbianas nas amostras analisadas são compostas por filos dos domínios procarióticos (Archaea e Bacteria), sendo os filos mais abundantes Proteobacteria, Bacteroidetes e Euryarchaeota. Apesar de estes filos estarem entre os mais abundantemente encontrados em solos (JANSSEN, 2006), o padrão de distribuição encontrado foi diferente do que tem sido relatado para outros ambientes da Antártica (AISLABIE *et al*., 2006; SMITH *et al*., 2006; NIEDERBERGER, 2008). Em adição, a análise das sequências encontradas em maior proporção revelou que as comunidades microbianas estudadas são dominadas por espécies pertencentes a ambos os domínios procarióticos. Uma sequência relacionada a uma família desconhecida dentro do domínio Archaea compõe uma grande parte da comunidade, junto com espécies de bactérias principalmente pertencentes à classe Gammaproteobacteria. Entretanto, mesmo no caso de sequências atribuídas com sucesso a determinado gênero, o baixo índice de identidade sugere que se trata de espécies ainda não

descritas. Esta observação, juntamente com o fato de uma grande parte das sequências não ter sido agrupada em filos ou classes conhecidos, indica a presença na Antártica de uma alta quantidade de espécies microbianas ainda não descritas.

Finalmente, realizando a prospecção de atividade queratinolítica em microrganismos isolados de amostras de solo, água e neve da Ilha Rei George (capítulo 3), obteve-se cinco isolados capazes de degradar queratina em baixas temperaturas, relacionados aos gêneros *Arthrobacter*, *Pseudomonas*, *Janthinobacterium* e *Pedobacter*. A busca por enzimas queratinolíticas tem sido alvo de muitas investigações científicas, devido ao grande problema originado do acúmulo de resíduos queratinosos no ambiente. Queratinases psicrófilas, em particular, são muito interessantes do ponto de vista econômico e ecológico. Por serem ativas em baixas temperaturas, diminuem consideravelmente o gasto de energia de processos industriais. Neste trabalho nós demonstramos que a Antártica é uma fonte em potencial de enzimas queratinolíticas psicrófilas.

Como conclusão, os principais resultados dos três trabalhos que compõem esta dissertação podem ser sumarizados como (i) a alta diversidade microbiana encontrada na Ilha Rei George aponta para o fato de que a diversidade de microrganismos na Antártica como um todo é mais alta do que tem sido relatada; (ii) as comunidades microbianas estudadas são formadas por grupos funcionais de microrganismos capazes de degradar uma ampla gama de substratos, entre eles a queratina; (iii) grande parte dos membros que compõem as comunidades estudadas não puderam ser classificados dentro de grupos conhecidos, indicando a presença em grande quantidade na Antártica de espécies não descritas e

possivelmente endêmicas; (iv) bactérias antárticas apresentam um alto potencial biotecnológico, considerando que, em um pequeno rastreamento, cinco espécies foram identificadas como capazes de produzir queratinases a baixa temperatura.

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APÊNDICE A – CARTA DE ACEITAÇÃO DE ARTIGO

Preview

From: sharuta@tmu.ac.jp

To: alexandre.macedo@ufrgs.br

CC: hohta@mx.ibaraki.ac.jp

Subject: Microbes and Environments - Decision on Manuscript ID ME11311.R1

Body: @@date to be populated upon sending@@

Dear Dr. Macedo:

It is a pleasure to accept your manuscript entitled "Functional diversity of microbial communities in soils at the vicinity of Wanda glacier, Antarctic Peninsula" in its current form for publication in the Microbes and Environments.

Thank you for your fine contribution. We look forward to your continued contributions to the Journal.

Shin Haruta
Senior Editor, Microbes and Environments

Date Sent: 03-Nov-2011

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APÊNDICE B – CURRICULUM VITAE RESUMIDO

PESSI, I. S.

1. DADOS PESSOAIS:

Nome: Igor Stelmach Pessi

Data de nascimento: 14/08/1987

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Início: março/2005 – Término: dezembro/2009

Mestre em Ciências:

Universidade Federal do Rio Grande do Sul

Programa de Pós-Graduação em Biologia Celular e Molecular

Início: março/2010 – Término: fevereiro/2012

3. ESTÁGIOS:

Bolsista de Iniciação Científica (CNPq):

Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde – Departamento de Microbiologia

Orientação: Dra. Sueli Teresinha Van der Sand

Atividade: pesquisa relacionada ao controle biológico do fungo fitopatógeno *Bipolaris sorokiniana*.

Início: janeiro/2006 – Término: agosto/2008

Estagiário (FDRH):

Fundação de Ciência e Tecnologia (CIENTEC)

Departamento de Alimentos – Laboratório de Microbiologia

Supervisão: Eliane Manara Rossoni

Atividade: análise e controle microbiológico de alimentos em geral

Início: setembro/2008 – Término: janeiro/2010

4. PRÊMIOS E DISTINÇÕES:

Best Oral Presentation Prize "Maria Therezinha Martins" – The American Society for Microbiology, XII Encontro Nacional de Microbiologia Ambiental, 2010.

5. ARTIGOS COMPLETOS PUBLICADOS:

PESSI, I. S.; ELIAS, S. O.; SIMÕES, F. L.; SIMÕES, J. C. & MACEDO, A. J. Functional diversity of microbial communities in soils in the vicinity of Wanda glacier, Antarctic Peninsula. *Microbes and Environments*, 2011(no prelo).

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POLONI, A.; MULLER, M. V. G.; PESSI, I. S. & VAN DER SAND, S. T. Análise da variabilidade morfológica e taxa de crescimento de culturas policonidiais e monoconidiais de *Bipolaris sorokiniana*. *Biociências (Porto Alegre)*, 16: 52-63, 2008.

6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

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