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PROSPECÇÃO DE BACTÉRIAS EM REJEITOS DE UMA INDÚSTRIA DE AÇO

DULCECLEIDE BEZERRA DE FREITAS

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BELO HORIZONTE

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DULCECLEIDE BEZERRA DE FREITAS

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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética.

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BELO HORIZONTE

UFMG - 2008

“Se fechas a porta a todos os erros, deixarás fora à verdade”.

Rabindranath Tagore

Ao meu filho Lucas,
Aos meus pais,
com todo meu amor,
dedico

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LISTA DE ABREVIATURAS

ACE - Abundance-based coverage estimator
API - Analytical Profile Index
$AgNO_3$ - Nitrato de prata
ARDRA - *Amplified rDNA restriction analysis*/"análise de restrição do DNA ribossômico amplificado"
ATCC - American Type Culture Collection
Au - Ouro
BAC - *Bacterial artificial chromosome*/"cromossomo artificial bacteriano"
BFS - Blast furnace sludge/lama de alto forno
BOX - BOX element of *Streptococcus pneumoniae*
$CdCl_2$ - Cloreto de cádmio
$CoCl_2$ - Cloreto de cobalto
UFC - Unidade formadora de colônia
Cr - Cromo
$CuSO_4$ - Sulfato de cobre
DGGE - *Denaturing gradient gel electrophoresis/*"eletroforese em gel de gradiente de desnaturante"
DNA - Ácido desoxirribonucléico
dNTP - Desoxinucleotídeo trifosfato
EDTA - Ácido etilenodiaminotetracético
ERIC - Enterobacterial Repetitive Intergenic Consensus
ETS - Effluents-treatment station/estação de tratamento de efluentes
HCl - Ácido clorídrico
$HgCl_2$ - Bicloreto de mercúrio
HGT - Transferência gênica horizontal
HMPT - Hot metal pre-treatment/pré-tratamento de gusa
ITS - *Intergenic 16S-23S transcribed spacer*/"Região espaçadora intergênica 16S - 23S rDNA"
K - Potássio
Kg - Kilograma
m - Metro

M - Molar
mg - Miligrama (10^{-3}g)
MIC - Minimun inhibitory concentration
min - Minuto
mL - Mililitro (10^{-3}g)
µL - Microlitro (10^{-6}g)
mM - Milimolar
µM - Micromolar
MEE - *Multilocus enzyme electrophoresis*/"eletroforese de enzimas multilocus"
MLST - *Multilocus sequence typing* /"tipagem de seqüência multilocus"
mRNA - Ácido ribonucléico mensageiro
MRPL - Metal refining process with lance/"processo de refinamento de metal com lança"
ng - Nanograma (10^{-9}g)
NaCl - Cloreto de sódio
$NiCl_2$ - Cloreto de niquel
NPW - Newly-produced waste/"rejeitos recém produzidos"
OTU - *Operational taxonomic unit*/"unidade taxonômica operacional"
P - Fósforo
$Pb(NO_3)_2$ - Nitrato de chumbo
pb - Pares de base
PCR - *Polymerase Chain Reaction* / "Reação em Cadeia da Polimerase"
PFGE - *Pulse-field gel electrophoresis*/"gel de eletroforese de campo pulsado"
PTSS - Pre-treated steelmaking slag/"escória de aciaria"
rDNA - Ácido desoxirribonucléico ribossômico
RAPD - Random amplified polymorphic DNA
rep-PCR - repetitive extragenic palindromic PCR
RDP - Ribosomal Database Project II
RFLP - *Restriction Fragment Lenght Polimorphism*/"polimorfismo de comprimento de fragmento de restrição"
rRNA - Ácido ribonucléico ribossômico
S - Enxofre
WD - Waste deposited/"rejeitos depositados"

TBE - Tampão Tris borato de EDTA
tDNA - Ácido desoxirribonucléico transportador
TE - Tris-EDTA
TGGE - Temperature gradient gel electrophoresis
T-RFLP - Teminal gradient gel electrophoresis
tRNA - Ácido ribonucléico transportador
TSA - Ágar triptona de soja
U - Unidade
UPGMA - *Unweighted pair group methods with arthmatic mean*
$ZnSO_4$ - Sulfato de zinco

RESUMO

O Brasil possui uma importante reserva mineral e destaca-se internacionalmente como o maior exportador de minério de ferro do mundo. Em 2007, as indústrias siderúrgicas produziram 34 milhões de toneladas de aço. Cada tonelada de aço produzida gera 700 Kg de rejeitos. Esses rejeitos têm grande quantidade de cálcio, manganês, cromo, zinco, chumbo e fósforo, dificultando alguns deles sua reciclagem. Devido à escassez de conhecimento sobre comunidades bacterianas cultiváveis e não cultiváveis presentes nesses rejeitos, este trabalho propôs estudá-las usando abordagens clássica e molecular. Para isso, amostras de rejeitos, recém produzidos (NPW), com diferentes composições químicas foram coletadas na indústria siderúrgica Acesita S/A (Timóteo, Minas Gerais): lama de alto forno (BFS); pré-tratamento de gusa (HMPT); processo de refinamento de metal com lance (MRPL); escória de aciaria (PTSS) e estação de tratamento de efluentes (ETS). Oitenta e nove isolados bacterianos foram identificados pela análise da sequência do gene de rRNA 16S como pertencentes aos gêneros: *Bacillus* (45), *Pseudomonas* (18), *Micrococcus* (11) *Acinetobacter* (6) *Dietzia* (4) *Kocuria* (2), *Diaphorobacter* (1) *Staphylococcus* (1) e *Brevibacillus* (1), com predominância dos três primeiros. ITS e tDNA-PCR foram usados, sem sucesso, para tentar classificar os isolados de *Bacillus* em nível de espécie. Além disso, a diversidade genética e fenotípica dessas bactérias foram avaliadas por técnicas de *fingerprinting* genômico rep-PCR, (BOX, ERIC e $(GTG)_5$) e testes fisiológicos e bioquímicos. De um modo geral, demonstrou-se por estes métodos uma alta heterogeneidade genotípica e fenotípica nas comunidades bacterianas. Usou-se abordagem metagenômica para análise da diversidade bacteriana dos rejeitos recém produzidos (NPW) e de rejeitos depositados (WD), há quatro anos, em área adjacente à industria. Um total de 212 seqüências parciais do gene de rRNA 16S revelou a presença de 123 unidades taxonômicas operacionais (OTUs), determinadas pelo programa DOTUR. A análise filogenética das seqüências permitiu associá-las com 10 filos bacterianos: *Proteobacteria*, *Firmicutes*, *Bacteroidetes, Nitrospira, Chloroflexi, Actinobacteria, Planctomycetes, Acidobacteria, Deinococcus-Thermus, Gemmatimonadetes* e duas canditadas a divisões OP10 e OD1. Em ambas as bibliotecas o filo *Proteobacteria* foi altamente dominante com preponderância da classe *Gammaproteobacteria*. Seis OTUs, previamente ainda não classificadas podem representar um novo táxon. Baseado nos índices de diversidade (Simpson, Shannon-Weaver, Chao1 e ACE) a biblioteca WD apresentou maior diversidade do que NPW: o algoritmo LIBSHUFF mostrou que a composição delas foi significativamente diferente. Os resultados indicam que as comunidades bacterianas nos rejeitos de aço são complexas e apresentam grande diversidade genética e fenotípica, requerendo estudos posteriores.

Palavras-chave: Gene de rRNA 16S, rejeitos siderúrgicos, *fingerprinting* genômico, metagenômica, diversidade bacteriana

ABSTRACT

Brazil possess an important mineral reserve and is the biggest exporter of iron ore in the world. In 2007 the siderurgic industries produced 34 million tons of steel. The manufacture of each ton of steel produces 700 kg of wastes. The wastes contain high quantities of calcium, lead, manganese, chrome, zinc and phosphorus that dificult the recycling of these wastes. Due to the lack of knowledge about the culturable and non culturable bacterial communities found in these wastes the present work was proposed in order to study these bacterial communities using classic and molecular approaches. Newly-produced waste samples (NPW) with different chemical compositions were collected at the siderurgic industry Acesita S/A (Timóteo, Minas Gerais): blast furnace sludge (BFS); hot metal pre-treatment (HMPT); metal refining process with lance (MRPL); pre-treated steelmaking slag (PTSS) and effluents-treatment station (ETS). Eighty-nine bacterial isolates were identified by sequencing analysis of the 16S rRNA gene and the following genera were found: *Bacillus* (45), *Pseudomonas* (18), *Micrococcus* (11) *Acinetobacter* (6) *Dietzia* (4) *Kocuria* (2), *Diaphorobacter* (1) *Staphylococcus* (1) e *Brevibacillus* (1) with predominance of the first three genera. ITS and tDNA-PCR were used without success to classify the *Bacillus* isolates to the species level. The genetic and phenotypic diversities of all isolates were analysed by genomic fingerprinting techniques such as rep-PCR (BOX, ERIC e $(GTG)_5$) and by physiologic and biochemical tests. The use of these methods revealed high genotypic and phenotypic heterogeneity of the bacterial communities. The metagenomic approach was used to analyze the bacterial diversity of newly-produced steel waste (NPW) and steel waste deposited from four years ago (WD). A total of 212 partial 16S rRNA gene sequences revealed the presence of 123 taxonomic units (OTUs) determined by the program DOTUR. The phylogenetic analysis of those sequences allowed to associate them with 10 bacterial phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes, Nitrospira, Chloroflexi, Actinobacteria, Planctomycetes, Acidobacteria, Deinococcus-Thermus, Gemmatimonadetes* and two candidate divisions (OP10 and OD1). In both libraries the phylum *Proteobacteria* was highly dominant with predominance of the class *Gammaproteobacteria*. Six OTUs were not previously identified and could represent a new taxon. According to the calculated diversity indexes (Simpson, Shannon-Weaver, Chao1 e ACE) the WD library has higher diversity than the NPW library, and the LIBSHUFF algorithm showed that their composition is significantly different. The results indicate that bacterial communities found in iron ore wastes are complex and present high genetic and phenotypic diversities, suggesting the need for further studies.

Keywords: 16S rRNA gene; steelmaking waste; genomic fingerprinting; metagenomic bacterial diversity

ESTRUTURA DA TESE

Esta tese apresenta a seguinte organização: uma introdução geral com uma breve revisão bibliográfica, abordando os principais temas propostos no trabalho, e os objetivos gerais e específicos. A seguir, os artigos submetidos nas formas de capítulos: I "Molecular characterization of early colonizer bacteria from wastes in a steel plant", II "Genotypic and phenotypic diversity of *Bacillus* spp. isolated from Steel Plant wastes in Brazil" e III "Molecular bacterial diversity and distribution in waste from a steel plant". Finalmente, discussão geral, conclusão geral e referências bibliográficas.

I) INTRODUÇÃO

I. 1) Considerações gerais

A mineração é uma atividade básica que utiliza recursos naturais e é fundamental para o desenvolvimento socioeconômico de muitos países (Dias, 2001). Entretanto, sabe-se, há mais de quatro séculos, que as atividades de mineração resultam em impactos ambientais imediatos. Os principais problemas oriundos da mineração são: poluição da água, do ar e geração de áreas degradadas (Machado, 1989; Bitar, 1997; Dias, 2001). A ação de recuperação da área, cuja intensidade depende do grau de interferência ocorrido, pode ser realizada por meio de métodos edáfico – medidas de sistematização de terreno – e vegetativo – restabelecimento da cobertura vegetal (Dias, 2001).

O Brasil possui uma ampla reserva mineral decorrente de sua diversidade geológica e de suas extensão territorial, destacando-se no cenário internacional como o maior exportador de minério de ferro e ligas de nióbio (Barreto, 2001). A mineração é responsável por um terço da produção industrial brasileira, com destaque para o minério de ferro, seguido pelo calcário, ouro, manganês, diamante, pedras preciosas, feldspato, granito, quartzo, nióbio, fosfato, zinco e outras substâncias (Dias, 2001).

O minério de ferro é o mineral de maior produção e consumo no planeta sendo que a maioria das jazidas encontradas em poucos países, China, Brasil, Austrália e Índia detêm 77% dessas reservas. O Brasil possui a quinta maior reserva do mundo, equivalente a 17 bilhões de toneladas (8,3%). Além disso, as reservas de minério de ferro do Brasil e da Austrália apresentam o maior teor de ferro – 60%; entretanto, contém impurezas, como óxido de silício (SiO_2), óxido de alumínio (Al_2O_3), fósforo (P), dentre outros (Rosière *et al.,* 1997;). Os rejeitos gerados pela mineração constituem uma fonte potencial de contaminação ambiental (Ledin & Pedersen, 1996). Esses rejeitos incluem gases, soluções de pó, barro, material mineral, como: rejeitos de mina, processamento de minério, e rejeitos lixiviado (Ledin & Pedersen, 1996).

Nos últimos dez anos a produção siderúrgica brasileira apresentou bons índices de produtividade, em torno de 30 e 34 milhões de toneladas de aço. O aço é o material mais importante no mundo contemporâneo, tendo papel essencial no desenvolvimento de muitos países. As indústrias siderúrgicas geram 700 kg de rejeitos por tonelada de aço produzido e esses resíduos têm grande quantidade de Zn, P, K, S e Cr o que, conseqüentemente, dificulta

sua reciclagem. Portanto, a recuperação desses rejeitos deve ser considerada como prioridade nos processos de mineração e siderurgia (Almeida, 1992; Dias, 2001).

O tratamento biológico de rejeitos tem sido utilizado como método de diminuição da carga poluente nos rios, emitida por indústrias ou pela população em geral., A biolixiviação é atualmente aplicada em escala industrial para recuperação de alguns metais. Outra aplicação desse método seria na remoção do fósforo, uma vez que esse elemento químico tem valor econômico na agricultura e na indústria farmacêutica (França *et al.,* 2004). Os rejeitos industriais muitas vezes contêm metais valiosos, mas geralmente em baixa concentração, como por exemplo, cobre ou zinco. Portanto, o uso de microrganismos na detoxificação ou recuperação de alguns desses materiais é importante tanto sob o aspecto econômico como para minimizar a poluição ambiental.

O minério de ferro, normalmente, precisa ser submetido a processos físicos de diferentes formas (fragmentação, moagem, separação e outros) para separação do ferro. Tal situação ocorre no processamento de minério de ferro de alto teor e de alguns minerais industriais. Os minerais industriais podem ser usados diretamente ou podem passar por algum ajuste granulométrico para serem utilizados. Mas, a maioria dos minérios, inclusive alguns de ferro, precisa passar pela preparação (liberação do mineral de interesse) e por processos de separação do mineral de interesse (concentração). Tratar o minério nada mais é do que processá-lo. Esse processamento tem como objetivo preparar a matéria-prima para os processos químicos, térmicos e metalúrgicos.

O processo para a obtenção do ferro e conseqüentemente dos rejeitos inclui várias etapas. Primeiro, a matéria-prima é submetida ao processo de redução, sendo que os rejeitos gerados são retirados no processo, ou seja, durante o processo de redução da matéria-prima. Durante todo esse processo é feita à limpeza dos gases. Nesta limpeza utiliza-se água para facilitar a retirada dos rejeitos, formando uma lama que após ser decantada no espessador é levada a um filtro de aspiração, e o conteúdo obtido é chamado lama de alto forno/ blast furnace sludge (BFS). O sistema de exaustão do processo pré-tratamento de gusa visa basicamente à redução do teor de fósforo (P) e do enxofre de gusa produzido no processo de alto-forno. Existe um filtro que coleta o pó aspirado durante o tratamento e esse pó é enviado para o silo, onde se retira o rejeito pré-tratamento de gusa/ hot metal pre-treatment (HMPT). O processo de refino de metal com lança é um processo de produção de aço. Neste processo uma lança sopra oxigênio em alta pressão para o interior do forno, onde ocorre separação das impurezas, como gases e escória, gerando um rejeito chamado de processo de refino de metal com lança/metal refining process with lance (MRPL). A escória de aciaria/ pre-treated

steelmaking slag (PTSS), também é gerada durante a produção de aço inox, composta basicamente por óxidos básicos, sendo esse material resultante da agregação de diversos elementos que não são agregados ao aço. Os efluentes gerados pela laminação são bombeados até a estação de tratamento de efluentes e levado ao filtro, onde é gerado um outro rejeito chamado estação de tratamento de efluentes/ effluents-treatment station (ETS).

I. 2) Diversidade bacteriana

Os procariotos foram às primeiras formas de vida que surgiram no planeta. Estima-se que eles surgiram há cerca de 3,8 bilhões de anos. Portanto, a longa história evolutiva pode explicar as diversidades genética, morfológica e fisiológica desses microrganismos. As bactérias e arquéias – organismos procariontes – são estruturalmente simples, unicelulares e microscópicas e podem ser encontradas de forma isolada ou formando agregados celulares (Pace, 1997; Torvisk *et al.,* 2002; Schleifer, 2004).

Atualmente, estima-se que o número total de células procarióticas existentes na Terra é de aproximadamente $4 - 6 \times 10^{30}$, ocupando diferentes ambientes. Encontram-se distribuídas no oceano (1.2×10^{29}), no solo ($2,6 \times 10^{29}$) e na superfície terrestre ($0,25 - 2,4 \times 10^{30}$). As bactérias, integrantes essenciais da biota da Terra, são encontradas nos mais variados nichos ecológicos da Terra: dos trópicos ao Ártico e à Antártica; de minas subterrâneas e campos de óleo à estratosfera e o topo de grandes montanhas; de desertos ao Mar Morto; de fontes termais às águas hidrotémicas (Xu, 2006). Avalia-se que a diversidade microbiana exceda, em ordens de magnitude, à diversidade de plantas e animais (Whitman *et al*, 1998; McInerney *et al.,* 2002; Oren, 2004). Entretanto, atualmente, apenas uma ínfima fração dessa extraordinária diversidade da biosfera é conhecida. Estima-se, que mais de 99% das bactérias não são cultiváveis pelas técnicas de rotinas laboratoriais e, portanto, não estão acessíveis para a pesquisa básica e biotecnologia (Rodriguez-Valera, 2002; Streit & Schmitz, 2004; Sharma *et al.,* 2005).

As bactérias apresentam uma imensa diversidade genética e desempenham funções únicas e essenciais na manutenção de ecossistemas, como componentes fundamentais de cadeias alimentares, de ciclos biogeoquímicos (carbono, nitrogênio, fósforo e enxofre), em decomposição de material orgânico, manutenção da fertilidade e da estrutura do solo (Ledin & Pedersen, 1996; Myers, 1996). A capacidade que as bactérias têm de sobreviver e colonizar um ambiente depende, em parte, da sua plasticidade genômica - as bactérias podem mutar, adquirir e perder genes, formando um conjunto genômico adaptável a um determinado

habitat (Dobrindt & Hacker, 2001). A transferência gênica lateral – conjugação, transformação e transdução (Dobrindt, *et al*, 2003) é responsável pela troca de informação genética entre diversas bactérias e contribuem para a adaptação a novos ambientes e para a diversidade encontrada nas populações bacterianas (Davison, 1999; Lawrence, 1999).

I. 3) Gene de RNA ribossômico

Os RNAs ribossômicos (rRNA) são moléculas antigas e essenciais na fisiologia celular. Interagem de modo específico com as proteínas ribossômicas para a formação dos ribossomos, participando, assim, da síntese de proteínas (Rosselló-Mora & Amann, 2001; Robertson *et al.,* 2005). Os genes de rRNA são altamente conservados, sofrem uma lenta evolução e pouca transferência lateral, funcionando assim, como um relógio para a história evolutiva (Olsen *et al*, 1996; Gorab, 2001; Acinas *et al.,* 2004).

As bactérias apresentam três tipos de genes de rRNAs (5S, 16S e 23S) que estão organizados lado a lado e presentes em múltiplas cópias no genoma bacteriano, podendo variar de bactéria para bactéria. O número de operons em bactérias varia de um a 15, e pode apresentar heterogeneidade entre eles em um mesmo genoma (Fogel *et al.,* 1999; Klappenbach *et al.,* 2001; Acinas *et al.,* 2004). Esses operons contêm uma região espaçadora intergênica – ITS – localizada entre os genes de rRNA 16S e 23S. Essa região é altamente variável e pode ser usada para distinguir espécies bacterianas filogeneticamente relacionadas (Klappenbach *et al.,* 2001; Dahllöf, 2002; Acinas *et al.,* 2004; Osório *et al.,* 2005).

Entre as três moléculas, o gene de rRNA 16S (de aproximadamente 1600 nucleotídeos) tornou-se o mais utilizado para análises filogenéticas (Grahn *et al.,* 2003), por apresentar tamanho satisfatório, além de regiões altamente conservadas entre as espécies (Roselló-Mora & Amann, 2001). Inicialmente, o gene de rRNA 5S (de aproximadamente 120 nucleotídeos) foi utilizado para inferências filogenéticas, mas devido ao seu tamanho relativamente pequeno as informações contidas nesse gene são limitadas. O gene de rRNA 23S (de aproximadamente 2500 nucleotídeos) possui alto poder de resolução, pois apresenta maior número de sítios polimórficos. Contudo, estudos baseados nesse gene são restritos devido ao seu tamanho (Olsen *et al.,* 1996; Roselló-Mora & Amann, 2001).

Além disso, o gene de rRNA 16S apresenta ampla utilização em pesquisas de estudos taxonômicos, devido ao fácil acesso nos bancos de dados, como GenBank e Ribosomal Database Project (RDPII – *online*). Assim, esse gene tornou-se uma ferramenta extremamente comum e utilizada em inferências filogenéticas (Woese, 1987; Dunbar *et al.,*

1999; Roselló-Mora & Amann, 2001; Dahlöf, 2002). Um exemplo da utilização desse gene ocorreu em 1970, quando Woese mostrou que a vida era dividida em três domínios – Bacteria, Archaea e Eukarya – (Woese, 1987; Woese *et al.,* 1990), baseado no seqüenciamento, dos genes de rRNA 16S, para procariotos, e de 18S, para eucariotos, de 500 organismos cultiváveis.

Do ponto de vista da diversidade, o número de divisões existentes dentro do domínio Bacteria proposto por Woese et al., (1987) era de 12 divisões, todas descritas com base principalmente em seqüências de rDNA 16S de organismos cultivados. Segundo Hugenholtz et al., (1998), o domínio Bacteria compreendia pelo menos, 36 divisões, incluindo, além das 12 divisões descritas por Woese et al., (1987), 12 novas divisões possíveis sendo que, dessas 36 divisões, 13 eram caracterizadas apenas por seqüências de rDNA 16S isoladas diretamente do meio ambiente, ou seja, sem representante cultivado, sendo denominadas “divisões candidatas”. Atualmente, estima-se que existem mais de 53 divisões (ou filos) dentro do domínio Bacteria, identificadas principalmente pelas análises filogenéticas a partir de métodos independentes de cultivo de amostras ambientais utilizando o gene de rRNA 16S, sendo que aproximadamente 50% dessas divisões não possuem representantes cultivados (Rappé & Giovannoni, 2003). A partir de então, esse sistema filogenético baseado na análise do gene de rRNA 16S, tornou-se modelo para estudos evolutivos e também para o estabelecimento da relação entre as mais diversas espécies existentes (McInerney *et al.,* 2002).

Estudo baseado em cultivos bacterianos indica que representantes de algumas dessas divisões constituem um grupo de microrganismos de ocorrência cosmopolita, encontrados nos mais diversos habitats, enquanto que outros se restringem há certos habitats mais específicos, como; geotérmico, solo, marinho, água doce, poluente associado, metal ácido lixiviado, sub-superfície, e como simbiontes e comensais (Schlegel *et al.*, 1992). Como ilustra a Figura 1, eram 12 as divisões (ou filos) bacterianas reconhecidas em 1987 (*Firmicutes, Actinobacteria, Proteobacteria, Cyanobacteria*, *Thermotogae, Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Chlamydiae*, *Chlorobi*, *Spirochaetes* e *Deinococcus-Thermus*), mais 14 filos com representantes cultiváveis (*Aquificae, Thermodesulfobacteria, Dictyoglomus, Coprothermobacter*, *Caldithrix*, *Desulfurobacterium, Acidobacteria*, *Nitrospira, Verrumicrobia*, *Fusubacteria*, *Gemmatimonadetes*, *Fibrobacteres*, *Deferribacteres* e *Synergistes*). Por último, 26 filos que são considerados divisões candidatas e são identificados de acordo com a fonte original ou clone de seqüências que define o grupo bacteriano. Dentre essas divisões candidatas OP11 é o mais bem representado pelas

seqüências encontradas nas amostras ambientais e constitui um dos maiores grupos bacterianos (Figura 1).

Atualmente, existem diferentes estratégias empregadas para o estudo da diversidade genética microbiana em amostras ambientais. Essas estratégias se baseiam em técnicas moleculares que consistem em isolar amostras da comunidade e submetê-las a análises diretas de seu DNA (Dahlöf, 2002; Rodriguez-Valera, 2002). O DNA total pode ser obtido por meio de extração, e os genes de interesse podem ser amplificados pela técnica de *polymerase chain reaction*/"reação em cadeia da polimerase"(PCR) e avaliados por diversas metodologias, como: clonagem, seqüenciamento, hibridização *in situ*, *denaturing gradient gel electrophoresis*/ eletroforese em gel de gradiente de desnaturante (DGGE), *terminal gradient gel electrophoresis*/"polimorfismo do fragmento de restrição terminal" (T-RFLP), bibliotecas metagenômicas e DNA microarrays (*microchip* de DNA). Assim, demonstrou-se que os organismos cultiváveis de diferente ambientes representam apenas uma pequena fração da comunidade microbiana, e passou-se a compreender a importância dos microrganismos não cultiváveis (Torsvik *et al.,* 2002; Keller & Zengler, 2004; Kemp & Aller, 2004; Rodriguez-Valera, 2004; Sharma *et al.,*2005).

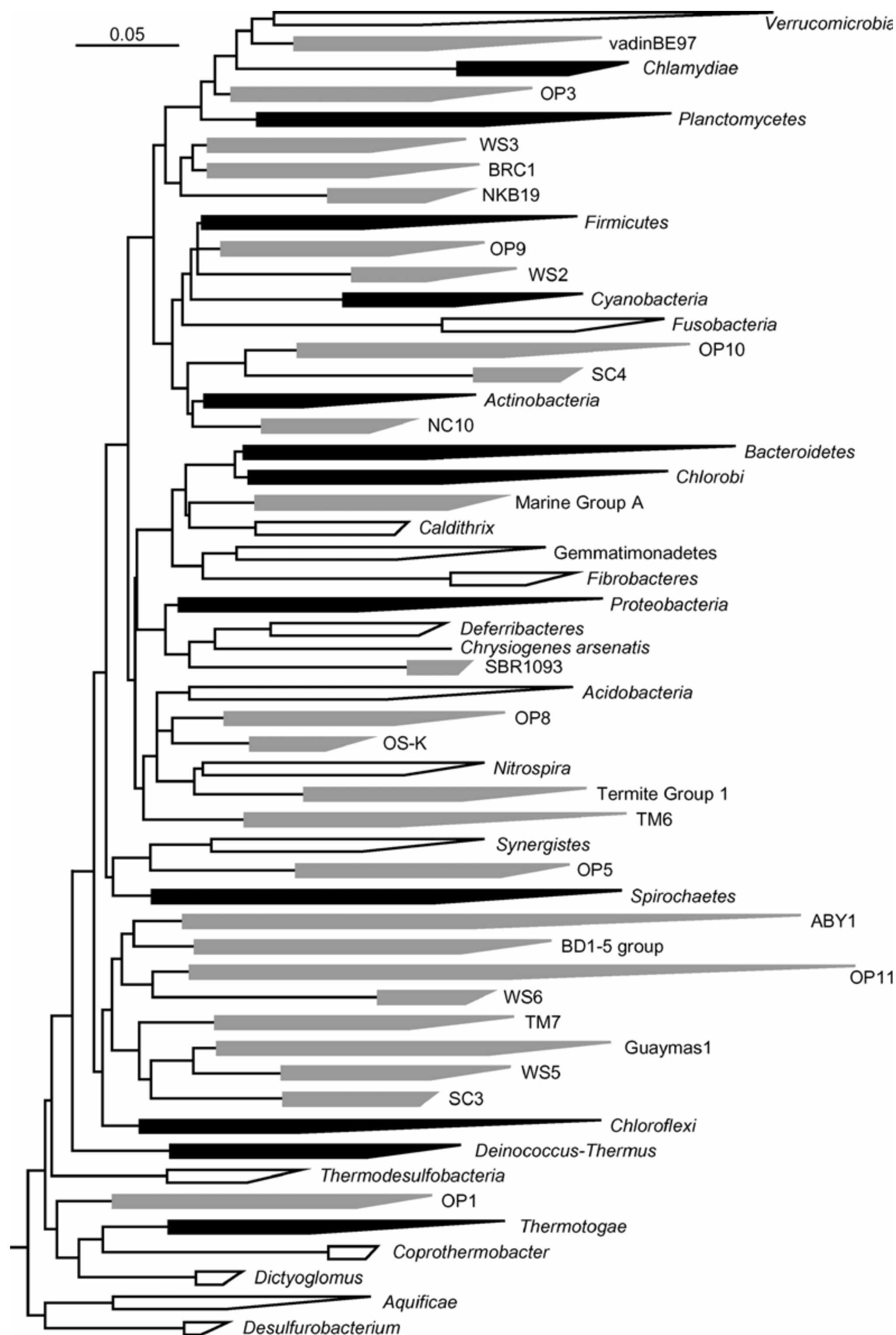

Figura 1. Árvore filogenética dos principais filos do domínio Bacteria baseada em sequências do gene de rRNA 16S. As barras em preto e em branco representam os filos bacterianos com representantes cultiváveis, e em cinza representa os filos “candidatos” que não contêm qualquer representante cultivado conhecido. Fonte: Rappé & Giovannoni, 2003

I. 4) Abordagens metagenômicas

A técnica que explora o conjunto dos genomas da microbiota total de uma amostra é denominada metagenoma, sendo que tal técnica acessa muito mais informações da diversidade microbiana que os procedimentos baseados em cultivos.

A partir da década de 1980, um grande número de métodos moleculares vem sendo desenvolvido para explorar a diversidade microbiana, cultivável ou não, em um determinado ambiente (Ward et al., 1990; Amann et al., 1995; Rodriguez-Valera, 2002). A maioria desses métodos é baseada na amplificação por meio da PCR e nas análises subseqüentes de genes de rRNA pelo seqüenciamento, biblioteca de clones e métodos fingerprints (RFLP, DGGE, e outros). Métodos baseados em cultivo são úteis para entender o potencial fisiológico de organismos isolados, mas não necessariamente fornece informação sobre a composição de comunidades microbianas. Os genomas bacterianos seqüenciados sem necessidade de cultivo têm feito da metagenômica uma abordagem comum para a avaliação de comunidade microbiana (Handelsman, 2004; DeLong, 2005).

A idéia de clonagem de DNA diretamente de amostras ambiental foi primeiro proposto por Pace et al., (1985) e, em 1991, foi reportada a primeira clonagem usando fago como vetor (Schmidt et al., 1991). O próximo passo foi a construção de uma biblioteca metagenômica com DNA derivado de uma mistura de organismos enriquecida de grama seca no laboratório (Healy et al., 1995). Os clones que expressaram atividade celulolítica foram selecionados dessa biblioteca (Healy et al., 1995). Em 1996, DeLong e colaboradores construíram uma biblioteca de procarioto de ambiente marinhos (Stein et al., 1996). Já em 1997, Kuske e colaboradores, comparando populações microbianas em solos áridos, no sudoeste dos Estados Unidos, construíram bibliotecas metagenômicas e seqüenciaram parcialmente o gene de rRNA 16S, nas quais verificaram uma considerável diversidade nas comunidades bacterianas, por meio de agrupamentos filogenéticos.

Após o trabalho sobre a bactéria *Haemophilus influenzae* – primeiro organismo a ter seu genoma completamente seqüenciado em 1995 –, o sequenciamento de amostras ambientais foi extensivamente discutido (Stein et al., 1996), e o termo metagenoma difundiu-se na literatura científica a partir de 1998 (Handelsman et al., 1998). Portanto, estudos independentes de cultivo e isolamento, baseados em amplificação e seqüenciamento de fragmentos do gene rRNA 16S, demonstraram que apenas uma pequena fração dos microrganismos (<10% em solos e <1% em ambientes aquáticos) é usualmente recuperada em estudos baseados em cultivo e isolamento (Staley & Konopka, 1995).

Atualmente, a construção de bibliotecas metagenômicas tem sido amplamente utilizada. Essas bibliotecas são bancos de clones bacterianos que carregam fragmentos originários dos genomas obtidos de todos os organismos, dependentes e independentes de cultivo, presentes no ambiente (Handelsman *et al.,* 2002, Lorenz & Schleper, 2002; Handelsman, 2004). Essa abordagem constitui uma poderosa ferramenta para estudar os genomas de uma microbiota que faz parte de um determinado ecossistema (Rondon *et al.,* 2000; Rodríguez-Valera, 2004; Sharma *et al.,* 2005). Portanto, é possível explorar e acessar, em diferentes ambientes, informações genéticas que podem ser obtidas independentes de cultivo, por meio do gene de rRNA 16S e expressão de novos metabólitos com interesse biotecnológico (Cottrell *et al.,* 2005; Riesenfeld *et al.,* 2004; Cowan *et al.,* 2005).

Na metagenômica, o DNA é extraído diretamente do ambiente e clonado em vetores, como cosmídios (Lopez-Garcia et al., 2004), fosmídios (Treusch et al., 2004) ou *bacterial artificial chromosome/*"cromossomo artificial bacteriano" – BAC – (Rondon et al., 2000). A vantagem dos vetores BAC é de manterem grandes insertos de DNA (maiores que 300 kb) estáveis em *Escherichia coli*, mesmo com número baixo de cópias do vetor BAC (Rondon et al., 2000). Além disso, a estratégia de clonagem em BAC possibilita clonar uma via metabólica inteira, permitindo a captura, a expressão e a detecção de produtos naturais a partir de uma biblioteca construída de DNA ambiental (Rondon et al., 1999). Essas bibliotecas vêm sendo amplamente utilizadas para revelar atividades metabólicas, como: antibacteriana, lipase, amilase, nuclease e hemolítica (Liles, 2003; Rondon et al., 2000; Schloss & Handelsman 2003), com potencial para descobrir novas enzimas (Lorenz & Schleper, 2002). Essa técnica apresenta grande interesse econômico e biotecnológico. Teoricamente, a biblioteca metagenômica contém clones que representam todo material genético de um único habitat, embora seja dependente da eficiência dos métodos de extração de DNA e clonagem (Eyers et al., 2004; Handelsman, 2004).

A informação obtida da biblioteca metagenômica pode ser usada para determinar a diversidade e a atividade da comunidade, a presença de microrganismos específicos ou a via de biossíntese de metabólitos secundários/produtos natural, como também a presença de genes individuais (Schloss & Handelsman 2003; Eyers et al., 2004; Handelsman, 2004). Muitos ambientes têm sido alvos para a construção de bibliotecas metagenômicas incluindo dentre outros: solo, fezes, habitats aquáticos. A área hospitalar tem sido usada para se conhecer o potencial genético de microrganismos envolvidos com saúde pública, como resistência a antibióticos e infecções nosocomiais (Riesenfeld et al., 2004). As bibliotecas metagenômicas funcionais têm identificado novos antibióticos (Rondon et al., 2000; Gillespie

et al., 2002; Brady et al., 2001, 2002), como também genes de resistência a antibióticos (Diaz-Torres et al., 2003), lipases (Henne et al., 2000), quitinases (Cottrell et al., 1999), proteína de membrana (Majernik et al., 2001), e genes que codificam enzimas do metabolismo (Henne et al., 1999).

Novos aspectos do metabolismo de microrganismos não cultiváveis foram descobertos usando abordagem metagenômica, como exemplo: estudos que revelam diferentes tipos de bactérias fototróficas marinhas (Beja *et al.,* 2000, 2001, 2005; Sabehi *et al.,* 2003); estudos que examinaram metabolismo de heterotróficos, incluindo a identificação de genes de quitinases de bactéria não cultivada de ambiente marinho (Cottrell *et al.*, 1999). No solo, micróbios com diferentes tipos de metabolismos envolvidos com álcool oxi-redutase (Knietsch *et al.*, 2003), como também lipases, amilases e nucleases (Rondon *et al.*, 2000).

Somente em 2004, os primeiros estudos de metagenomas em grande escala foram publicados (Tyson et al., 2004; Venter et al., 2004). O primeiro estudo foi o metagenoma de biofilme sob condições extremamente ácidas ($pH < 1$) em uma drenagem de mina ácida (Tyson et al., 2004). Essa análise permitiu importantes observações da estrutura de comunidade microbiana, pois de uma simples comunidade de bactérias e arquéias encontradas em um sistema de drenagem de mina ácida foi possível obter genomas em vários níveis de complexidade (Tyson et al., 2004).

Logo depois, surgiu o estudo de comunidades bacterianas em amostras ambientais de água de superfície do mar de Sargasso, no oceano Atlântico, próximo às Bermudas sendo considerado um dos mais completos estudos de metagenômica em grande escala (Venter et al., 2004). Esse estudo gerou dois milhões de seqüências, com mais de 1,6 bilhões de pares de base nessas seqüências de DNA. Baseado no relacionamento de seqüências do gene de rRNA 16S, essa análise sugere a existência de, pelo menos, 1800 espécies bacterianas, incluindo 148 previamente classificadas como filotipos ainda não conhecidos (Venter et al., 2004). Nesse estudo, também foram identificados mais de 1,2 milhões de genes que codificam proteínas, sendo, pelo menos 782, dentre elas, identificadas como novos fotoreceptores semelhantes à rodopsina, confirmando sua importância nesse ambiente (Venter et al., 2004).

No inicio de 2005, foram anunciados mais dois projetos, de diferentes ambientes: amostras de barbatanas de baleias coletadas a mais de 500 m de profundidade em água de dois diferentes oceanos (Pacífico e Península Antártica), e o outro com amostras da superfície do solo em uma fazenda de Minnesota (Tringe et al., 2005).

Em 2006, outro grande projeto usando a metagenoma foi publicado com amostras da microbiota intestinal de humanos (Gill et al., 2006). Neste trabalho foram analisados

aproximadamente 78 milhões de pares de base de seqüências amplificadas do gene de rRNA 16S obtidas de DNAs de fezes de humanos. Observou-se a presença de grupos dominantes de bactérias e de Archaea metanogênica (Gill et al., 2006).

Porém, apesar do grande avanço, a elucidação da composição filogenética das comunidades de procariotos em cada amostra ainda permanece como um dos desafios científicos da metagenômica. Atualmente, a metagenômica tem sido aplicada para identificar genes com potencial biotecnológico como na biossíntese de produtos farmacêuticos e outros usos industriais (Rondon *et al.,* 2000; Lorenz & Schleper, 2002; Streit & Schmitz, 2004). Em suma, a metagenômica proporciona uma via para acessar e conhecer a diversidade da comunidade microbiana de um determinado ambiente, sem necessidade de cultivar os microrganismos presentes nesses ambientes.

I. 5) O gênero *Bacillus*

O gênero *Bacillus* é caracterizado por bactérias Gram positivas, em forma de bastonetes, aeróbicas ou anaeróbicas facultativas formadoras de esporos, sendo fenotipicamente e filogeneticamente um táxon muito diverso. Atualmente, inclui mais de 100 espécies distribuídas amplamente na natureza e também associadas com doenças humanas, como, por exemplo, *B. cereus* e *B. anthracis,* pertencentes ao grupo *B. cereus*.

Esse gênero inclui bactérias termófilas e psicrófilas, acidófilas e alcalífilas que utilizam uma ampla faixa de fontes de carbono para seu crescimento heterotrófico, com conteúdo G+C entre 32-69% (Nazina et al., 2001). A maioria das espécies de *Bacillus* produz cápsula, e são móveis através dos flagelos peritríquios. Uma característica desse gênero é a capacidade de produzir endosporos – característica que os coloca entre os esporulados – esses endosporos são termorresistentes (toleram temperaturas de 55ºC a 70ºC) e também resistem à dissecação, à radiação, aos ácidos e a desinfetantes químicos. Esse gênero compreende espécies fenotipicamente heterogêneas exibindo uma ampla exigência nutricional, condições de crescimento, diversidade metabólica e composição de DNA (Claus & Berkeley, 1986; Joung & Cote, 2002). O metabolismo dessas bactérias pode ser respiratório, fermentativo, ou ambos, sendo a maioria produtora de catalase, além de oxidar uma ampla faixa de compostos orgânicos.

Membros desse gênero são amplamente usados em indústrias têxtil, farmacêutica, de cosmético e também na biorremediação. Várias espécies são produtoras de antibióticos, incluindo *B. brevis, B. cereus, B. licheniformis, B. polymyxa,* e *B. subtilis*. Os antibióticos

produzidos por essas espécies são bacitracina, polimixina, tirocidina, gramicidina e circulina. É interessante observar que a maioria desses antibióticos é constituída por peptídeos de baixo peso molecular, os quais possuem diferentes atividades biológicas, incluindo atividades antimicrobianas, antiviral e antitumoral. *B. subtilis, B. licheniformis* e *B. pumilus* produzem compostos biossurfactantes que reduzem a tensão superficial e dessa maneira são excelentes detergentes. Algumas espécies são colonizadoras da flora intestinal humanacausando diarréia, intoxicação gastrintestinal, e outras espécies são ecologicamente importantes como patógenos de insetos (Darby, 2005, Lee et al., 2007).

Abordagens fenotípicas, como propriedades bioquímicas, morfológicas e fisiológicas (Smith et al., 1952), formação de esporos (Gordon et al., 1973), composição de ácidos graxos (Kaneda, 1977), perfil enzimático (Baptist et al., 1978), dados fenotípicos de análise numérica (Priest et al., 1981), teste API (Berkeley et al., 1984; Logan & Berkeley 1984), e composição da parede celular (Stackebrandt et al., 1987), são extensivamente usadas para diferenciar e classificar as espécies de *Bacillus*. Baseados em estudos da heterogeneidade filogenética das seqüências do gene de rRNA 16S do gênero *Bacillus*, Roèssler et al., (1991) foram capazes de dividir as espécies desse gênero em quatro grupos, enquanto que Ash et al., (1991), estudando uma coleção de 51 espécies desse gênero, dividiram-no em cinco grupos distintos. A caracterização fenotípica e genotípica levou à descrição de vários novos gêneros derivados de *Bacillus*: *Alicyclobacillus* (Wisotzkey et al., 1992), *Paenibacillus* (Ash et al., 1993), *Aneurinibacillus* e *Brevibacillus* (Shida et al., 1996), Virgibacillus (Heyndrickx et al., 1998), *Gracilibacillus* e *Salibacillus* (Wainù et al., 1999), *Filobacillus* (Schlesner et al., 2001), *Geobacillus* (Nazina et al., 2001), *Ureibacillus* (Fortina et al., 2001), *Jeotgalibacillus* e *Marinibacillus* (Yoon et al., 2001).

Segundo Ash et al., (1991), no grupo I, algumas espécies são tão estritamente relacionadas que não podem ser facilmente distinguíveis como, por exemplo, grupo *B. cereus* incluindo *B. antracis*, *B. cereus, B.thuringiensis* e *B. mycoides* (Henderson et al., 1992). O Grupo II inclui *B. subtilis*, *B. pumilus*, *B. atrophaeus*, *B. licheniformis, B. amyloliquefaciens* e 23 outras espécies; o grupo III inclui *Bacillus* formadores de endosporos esféricos (*B. sphaericus*), junto com alguns táxons asporogênicos (os gêneros *Caryophanon, Xiguobacterium, Kurthia,* e *Planococcus*); o grupo IV, com dez representantes, compreende *B. polymyxa* e *B. macerans*, os quais têm sido re-classificados como sendo do gênero *Paenibacillus* (Ash et al., 1993); e o grupo V, com espécies classificadas em dois novos gêneros, *Aneurinibacillus* e *Brevibacillus* (Shida et al., 1996).

As relações filogenéticas das espécies do grupo *B. cereus* ainda continuam obscuras

(Cherif et al., 2003), apesar da diversidade genética desse grupo ter sido amplamente estudada por vários métodos. Os métodos mais utilizados para diferenciar esse grupo são: *multilocus enzyme electrophoresis*/"eletroforese de enzimas multilocus"(MEE), *pulse-field gel electrophoresis*/"gel de eletroforese de campo pulsado" (PFGE), *multilocus sequence typing* /"tipagem de seqüência de multilocus" (MLST; Helgason et al., 2004), dentre outros. Nesse grupo, seis espécies são descritas: *B. anthracis, B. cereus, B. mycoides, B. thuringiensis*, *B. weihenstephanensis* e *B. pseudomycoides* (Lechner et al., 1998; Nakamura, 1998). Espécies de *B. cereus* são bactérias patogênicas e *B. weihenstephanensis* cresce em temperaturas de 4ºC, dificultando a conservação de alimentos armazenados em baixas temperaturas (Mayr et al., 1999). Dentro desse grupo, *B. thuringiensis* é a espécie mais bem estudada devido ao seu uso no controle biológico (Schnepf et al., 1998), enquanto que *B. mycoides* é usada em processos de melhoramento de plantas (Petersen et al., 1995).

Baseado em estudos de hibridização DNA-DNA de todo o genoma e MEE, constatou-se que *B. anthracis, B. cereus* e *B. thuringiensis* pertencem a um único grupo e que as seqüências do gene de rRNA 16S dessas espécies compartilham um nível de similaridade >99% (Helgason et al., 2000). Por outro lado, a análise das regiões espaçadoras intergênicas (ITS) entre 16S-23S rDNA pôde diferenciar *B. thuringiensis* de *B. cereus*. Recentemente, estudos usando análises de *amplified fragment length polymorphism*/"polimorfismos de comprimento de fragmento amplificado"(AFLP) e MLST (Priest et al., 2004), colocaram *B. anthracis* como espécie geneticamente monomórfica quando comparada com as espécies *B. cereus* e *B. thuringiensis* (Daffonchio et al., 2000; Cherif et al., 2003; Priest et al., 2004). Os esporos de todas as espécies de *Bacillus* ocorrem naturalmente, sendo que os esporos de *B. cereus, B. thuringiensis* e *B. mycoides* são ubíquos, enquanto os de *B. anthracis* são limitados ao solo que tem o pH e conteúdo de cálcio adequado (Smith et al., 2000).

Espécies do grupo *B. subtilis* (*B. subtilis, B. pumilus, B. amyloliquefaciens* e *B. licheniformis*) são estritamente relacionadas e, portanto, não são facilmente distinguíveis uma da outra. Os esporos produzidos por esse grupo são elipsóides e, em geral, mesofilicos ou neutrofilicos. Essas espécies são usadas na indústria de produção de enzimas, antibióticos, solventes, componentes probióticos, substâncias antagonistas, surfactantes e degradantes de xenobióticos (Wu et al., 2006). Os testes fenotípicos usados para diferenciar as espécies desse grupo (Claus & Berkeley, 1986) indicam que somente algumas dessas espécies puderam ser diferenciadas por meio desses métodos, como por exemplo: *B. pumilus* é amilase negativo e *B. licheniformis* é propionato positivo, sendo que ambos crescem em temperatura superior a 55 ºC e são anaeróbios facultativos. Dados baseados na seqüência de rDNA 16S indicam que

B. subtilis compartilha 99,3% de similaridade no nível de gene de rRNA 16S com *B. atrophaeus* e 98,3% de similaridade com *B. licheniformis* e *B. amyloliquefaciens* (Ash et al., 1991).

Os métodos de tipagem molecular tiveram grandes avanços e revolucionaram o sistema de identificação e da taxonomia bacteriana. Porém, esses métodos se tornaram mais populares e amplamente acessíveis devido à sua reprodutibilidade, simplicidade e alto poder discriminatório. As estratégias mais comuns para analizar o relacionamento genético entre espécies do gênero *Bacillus* são baseadas em *fingerprinting* genômico, como rep-PCR, ITS e tDNA. Rep-PCR *fingerprinting* é baseado na amplificação de seqüências de DNA repetivivas, altamente conservadas, as quais estão presentes em múltiplas cópias ao longo do genoma da maioria das bactérias (Versalovic et al., 1991, 1994). Alternativamente, tDNA-PCR e ITS-PCR têm sido extensivamente utilizadas para diferenciação de linhagens bacterianas e ao nível de espécies (Clementino et al., 2001). tDNA-PCR é um método de identificação molecular rápido e tem sido amplamente aplicado para diferenciação de espécies de *Acinetobacter*, *Lactobacillus*, *Listeria* e *Bacillus* (Welsh & McClelland, 1991), enquanto que ITS-PCR explora os polimorfismos dentro da região espaçadora intergênica de 16S-23S rRNA e pode ser usado para caracterizar linhagens bacterianas sendo, conseqüentemente, importante como ferramenta para análises filogenéticas.

II) OBJETIVOS

Objetivo Geral

Analisar a comunidade bacteriana dependente e independente de cultivo em rejeitos de uma indústria de aço.

Objetivos Específicos

a) Cultivar, isolar, caracterizar filogeneticamente, analisar a diversidade genética e fenotípica de bactérias oriundas de diferentes rejeitos, recém produzidos (pré-tratamento de gusa – HMPT–, processo de refinamento de metal com lance – MRPL–, escória de aciaria – PTSS– e estação de tratamento de efluente – ETS) e de uma comunidade de *Bacillus* isolados do rejeito lama de alto forno – BFS –, recém-produzido.

b) Analisar a diversidade da comunidade bacteriana independente de cultivo (metagenoma) dos rejeitos recém produzidos e dos rejeitos depositados, há quatro anos, em ambiente adjacente a essa indústria.

III) CAPÍTULOS

CAPÍTULO I

Molecular characterization of early colonizer bacteria from wastes in a steel plant

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Molecular characterization of early colonizer bacteria from wastes in a steel plant

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Abstract

Aims: Forty-nine bacteria isolated from four newly-produced waste samples of a steel industry, which had a high content of CaO, MgO, Cr and P_2O_5, were characterized molecularly and phenotypically by susceptibility testing against heavy metals.

Methods and Results: Phylogenetic analysis using 16S rRNA gene sequences revealed that the isolates belonged to nine genera, *Pseudomonas*, *Micrococcus*, *Acinetobacter*, *Bacillus*, *Dietzia*, *Kocuria*, *Diaphorobacter*, *Staphylococcus* and *Brevibacillus*. Besides, some isolates could be affiliated to species: *M. luteus*, *A. junii*, *A. schindleri*, *B. cereus, K. marina, D. nitroreducens* and *S. warneri.* The bacteria that were characterized are taxonomically diverse, and *Pseudomonas* and *Micrococcus* predominated. Fingerprinting BOX-PCR revealed high genomic heterogeneity among the isolates. Among the heavy metal compounds, Zn, Ni, Pb and Cu were least toxic to the bacterial isolates, whereas Ag inhibited all isolates at 0.001 mM.

Conclusions: Heterotrophic bacteria, affiliated with several phylogentic groups, were able to colonize different wastes of a steel industry.

Significance and Impact of the Study: The present study extends our knowledge of the early colonizers bacteria populating siderurgic environments. Some of these bacteria could have potential for recycling siderurgic waste for steel production.

Keywords: BOX fingerprinting; heavy metal; 16S rRNA gene; steelmaking waste; colonizer.

Introduction

Wastes generated by mining and by mineral-ore processing, approximately 1.8 billion tons annually, constitute a potential source of contamination for the environment. These wastes include gases, dusts, solutions, sludge, and mineral materials, such as mine waste, along with ore processing and leaching residues (Ledin and Pedersen 1996).

Brazil has considerable mineral reserves and is a major exporter of iron ore. Eighty percent of Brazil's ore processing is focused on the production of iron ore (Barreto 2001). It is known that the world's steel industries produced more than 1.2 billion tons of steel in 2006, generating about 700 kg of waste per ton of steel produced. These wastes contain high concentrations of Zn, P, K, and S, which make recycling difficult (Li and Rutherford 1996). Some wastes are treated with chemical processes and are used for other purposes, but most are stored or discarded into the environment (Araújo 1997). This discarded material causes considerable environmental impact.

It is well established that microorganisms play a crucial role in decomposition, food chains, and biogeochemical cycling. They have significant role in the extraction and recovery of metals and are also involved in mineral weathering and biogeochemical cycling of nutrients (Gadd 2004). The use of bacterial communities to minimize the impacts caused by anthropogenic activities in natural habitats is a well-established biotechnological strategy and is used to recover metals from minerals containing Cu, Au, and U (Brierley 1978; Torma 1983; Rawlings 2002; Rawlings *et al*. 2003). Considering, that the microbial communities living in steelmaking waste have been rarely examined (Li *et al*. 2006), we investigated early colonizers bacteria from these wastes, using plate-culture methods. We used partial 16S rRNA gene sequencing, genomic fingerprinting, and heavy metals testing to assess cultivable heterotrophic bacteria found in the newly- produced steel wastes.

Materials and methods

Steel plant wastes sampling and analysis

The Steel Plant of Acesita-Cia Aços Especiais Itabira is located in Timóteo (Minas Gerais state, Brazil). Samples were collected in four locals, where wastes were generated in an integrated steel mill after being submitted to different treatments and were named: hot metal pre-treatment (HMPT), metal refining process with lance (MRPL), pre-treated steelmaking slag (PTSS), a granulated slag; effluents-treatment station (ETS), the mud waste obtained

from the effluents station, in that order. The newly-produced wastes were collected in triplicate using sterilized bottles. These wastes were submitted to chemical, Mössbauer and X-Ray analyses.

Bacterial isolates

Wastes samples (1 g) were shaken in 9 mL of phosphate buffer for 24h at 37°C. Ten-fold serial dilutions of the suspension were prepared to a dilution up to 10^{-3}, and 0.1 ml of each dilution and the not diluted suspension were plated directly in triplicate on 10%-strength tryptic soy agar (TSA; Difco) and minimum medium M9 (44 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 1M $MgSO_4$, 1M $CaCl_2$ and 2 g glucose per liter), supplemented with cycloheximide (250 µg ml^{-1}) to inhibit fungal growth. Plates were incubated at 37°C, up to 7 days. The colonies were purified by restreaking prior to subsequent molecular and phenotypic analyses. All isolated bacteria were stored in tryptic soy broth plus glycerol 15% at -70 ºC. The morphology of isolated bacteria was examined microscopically based on Gram staining.

Heavy metals testing

The agar dilution method (nutrient agar, Difco) was used to determine the minimum inhibitory concentrations (MICs) for the heavy metals purchased from Merck: cadmium chloride, cobalt chloride, copper sulphate, lead nitrate, mercury bichloride, nickel chloride, zinc sulphate and silver nitrate.

DNA extraction and PCR amplification

Genomic DNA was prepared as previously described by Dramsi *et al*. (1995). For 16S rRNA gene sequences and BOX-PCR the reactions were performed with the primers 8F (5'-AGAGTTTGATYMTGGCTCAG-3'), and with 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Lane, 1991), and BOX-A1R (5'-CATACGGCAAGGCGACGCT-3') as described by Versalovic *et al*. (1994), respectively. Amplification reaction mixtures contained 0.2 mM of each dNTP, 0.5 μM of each primer, and 1 U of Taq polymerase (Phoneutria) and were carried out in a total volume of 20 μl. The program used was as described by Don *et al*. (1991).

Clustering analysis of fingerprinting PCR data

For the cluster analysis the data were converted into a binary matrix where the digit 1 represents the presence of DNA bands or the presence of a phenotypic character, and the digit

0 its absence. The similarity matrix was generated by Euclidean distances and the unweighted pair group mean averages (UPGMA) algorithm. Analysis of fingerprinting PCR data was performed using the software PAST (Paleontological Statistics Software Package) (Hammer *et al*. 2001).

16S rRNA gene sequencing and phylogenetic analysis

The sequences of 16S rDNA PCR products were automatically analyzed by using standard protocol with DYEnamic ET dye terminator kit (Amersham Biosciences) and the MegaBACE 1000 capillary sequencer (Amersham Biosciences). All the isolates were identified by phylogenetic analysis of their partial 16S rRNA gene sequences using GenBank BLASTN and RDP Classifier search tools. To accomplish this, the 16S rRNA gene sequences were basecalled, checked for quality, aligned and analyzed using Phred v.0.020425 (Ewing and Green 1998), Phrap v.0.990319 (Green 1994) and Consed 12.0 (Gordon *et al*. 1998). Phylogenetic relationships were inferred by MEGA 3.1 (Kumar *et al*. 2004) using the neighbor-joining method (Kimura 1980; Saitou and Nei 1987) and the Kimura 2-P model of sequence evolution.

The Unifrac metric method (Lozupone *et al*. 2006) was used to compare bacterial communities from the different wastes. First, a phylogenetic tree was constructed for the 16S rRNA gene sequences using the neighbor-joining method as implemented in MEGA 3. Second, a test was carried out to detect differences between isolates from distinct wastes, using the UniFrac statistics software that performed a principal components analysis.

Results

Characterization of steel plant wastes

The chemical analyses are given in Table 1. The phosphorus content found in these wastes was up to 2.11% (w/w).

Identification and phylogenetic analysis of isolates

Among 55 isolates recovered on 10% strength TSA culture medium, only 49 were able to grow after a second subculture; these were studied further. We were unable to directly cultivate any of the isolates on minimum medium M9. Differences in the total number of isolates recovered were observed among the four wastes: ETS gave 2.7×10^5 CFU/g, HMPT 6×10^2 CFU/g, MRPL 4×10^2 CFU/g and PTSS 2×10^2 CFU/g. Preliminary characterization

of isolates was performed on the basis of morphology and Gram staining; 51% of them were Gram-negative rods, 31% Gram-positive cocci, and 18% Gram-positive rods.

The 16S rDNA sequences used for phylogenetic analysis were approximately 628 nucleotides long and spanned the V2 and V5 variable regions, corresponding to *Escherichia coli* K12 16S rDNA. The phylogenetic tree formed using the neighbor-joining method showed monophyletic relationships among the isolates (Fig. 1). The 49 isolates fell into nine distinct phylogenetic clades, affiliated to three different phyla: *Proteobacteria* (*Pseudomonas* n = 18*, A. schindleri* n = 2, *A. junii n=4* and *D. nitroreducens* n = 1), *Actinobacteria* (*M.* luteus n = 11, *Dietzia* n = 4 and *K. marina* n = 2) and *Firmicutes* (*S. warneri* n = 1, *B. cereus* n=2*, Bacillus* n= 3 and *Brevibacillus* n = 1). *Proteobacteria* were more common in the ETS, whereas Gram-positive bacteria (*Actinobacteria* and *Firmicutes*) were more common in the HMPT and MRPL wastes.

HMPT gave the highest phylogenetic heterogeneity, with five genera, two of them, *Diaphorobacter* and *Staphylococcus* were exclusively found in this waste. Three out of the four genera (*Pseudomonas*, *Dietzia* and *Kocuria*) found in ETS were exclusive to this waste, whereas *Brevibacillus* was found only in PTSS. MRPL was the only waste that had no exclusive genus.

Pseudomonas isolates were recovered exclusively from the ETS waste. The highest degree of average similarity (99.9%) was observed among 17 *Pseudomonas* isolates sequences: *P. lindanilytica* (GenBank accession no. DQ916277), *P. pseudoalcaligenes* (GenBank accession no. DQ837704) and *P. mendocina* ATCC 25411 (GenBank accession no. AF094734); a slightly lower similarity (99%) was found for one ETS isolate: 2ETS and *P. alcaligenes* ATCC 12815 (GenBank accession no. AF390747).

The average degree of similarity between sequences of *Acinetobacter* isolates was 98.8%. Isolates 8HMPT and 12HMPT were most similar (99.7%) to *A. schindleri* (GenBank accession no. AJ275041), and *Acinetobacter* isolates (4PTSS, 1PTSS, I7HMPT, and 3MRPL) had 16S rDNA sequences identical to those of *A. junii* (GenBank accession no. AY87213).

Although *Micrococcus* isolates formed two branches within its clade, they had a high average similarity (99.8%) to the *M. luteus* sequences (GenBank accession no. AF542073 and DQ65943). The sequences of the *Dietzia* isolates had average similarities of 99.9 and 98.9% to *D. cinnamea* and *D. maris*, respectively.

The *Bacillus* isolates assigned to clade 6 were divided into two distinct subclades. Group A diverged from group B by 2.6%. However, the isolates within each of the groups were indistinguishable based on their 16S rDNA sequences.

We used the cluster environment analysis available in the UniFrac software package to compare the bacterial communities and specific features of the wastes. This analysis revealed that the bacterial community from ETS was most dissimilar when compared with the others, whereas the bacterial communities from HMPT and MRPL were closest (Fig. 2). The robustness of this result was confirmed by jackknife analysis ($P < 0.001$).

BOX-PCR genomic fingerprinting analysis

BOX fingerprinting generated profiles of 4 to 14 bands, ranging in size from approximately 150 bp to 4 kb, with distinct sets of patterns for each genus. Based on BOX-PCR fingerprints, the *Pseudomonas* isolates revealed three different banding profiles, which contained 10, seven and one member each; all isolates shared the same approximately 0.4 kb band (Fig. 3A). *Acinetobacter* isolates exhibited significant heterogeneity and five out of six isolates presented a common band of approximately 1.9 kb (Fig. 3B). Among the genera, *Micrococcus* BOX-PCR produced the most complex amplified banding patterns, which reflected a high degree of heterogeneity. Six *Micrococcus* isolates displayed identical genomic fingerprints (Fig. 3C, lanes 5-10). The *Kocuria* isolates (Fig. 3C, lanes 13-14) presented different profiles in this analysis, and they had a high similarity index in the 16S rDNA sequence (99.7%). BOX yielded three different banding profiles for the *Bacillus* isolates (Fig. 3D). Surprisingly, isolate 1MRPL did not produce bands with this technique. The *Dietzia* isolates presented the same profile in this analysis (Fig. 3E).

Results derived from BOX-PCR genomic fingerprinting of the 49 bacterial isolates were combined for cluster analysis. Five clusters were identified using a 50% cutoff value (Fig. 3F). Cluster A grouped the *Dietzia* isolates plus *Bacillus* isolate 9HMPT. Clusters B and D consisted exclusively of the *Pseudomonas* and *Acinetobacter* isolates, respectively. Cluster E grouped the *Micrococcus* isolates plus the *Pseudomonas* and *Brevibacillus* isolates.

Minimum inhibitory concentration of the heavy metal

MICs for eight heavy metals are given in Table 2. There was great variation in susceptibility to the metals among the bacterial genera: 90% of the *Pseudomonas* isolates presented MICs for Pb, Zn, Ni and Cd two times higher than those found for the *Acinetobacter* isolates. The *Bacillus* isolates presented high MICs for Cu, Pb, Zn, Co, Ni and Cd, with 90% of the isolates being inhibited by concentrations ranging from 0.5 to 2 mM. The *Micrococcus* isolates presented the highest MIC (4mM) for Zn. The *Dietzia* and *Pseudomonas* isolates exhibited the highest MICs for Pb and Hg, respectively, whereas the Hg was the most toxic

for *Diaphorobacter*. A dendrogram based on the MIC profiles revealed that almost all isolates exhibited a distinct profile for a combination of the used heavy metals. However, some isolates presented identical patterns, although obtained from different wastes (Fig. 4). The most of the isolates from ETS (17 out of 26) assembled in two clusters exclusive (clusters 1 and 5), whereas the isolates from wastes remaining scattered throughout the dendrogram (clusters 2, 3 and 4).

Discussion

This was one of the first attempts to characterize the nature of heterotrophic cultivable bacteria from steel plant wastes. The 49 isolates characterized by 16S rRNA gene sequencing undoubtedly represent only a fraction of the total bacterial community recoverable by our experimental design; several isolates were unable to grow in a second subculture, indicating clearly the difficulties to keep these bacteria a long term. Considering, that this type of waste has a low load of nutrients we choose minimum medium for retrieving the isolates; however, no isolates were directly obtained from this minimum medium. However, some isolates recovered on 10%-strength TSA medium were then able to grow on minimum medium.

As expected, 16S rRNA gene sequence analyses proved to be an efficient tool to identify bacterial isolates to the genus level (Drancourt *et al*. 2000; Pontes *et al*. 2007). Based on this molecular tool, nine bacterial genera found in the wastes were similar to those described in hostile environments (Nazina *et al*. 2002; Greenblatt *et al*. 2004; Pathom-aree *et al*. 2006; Foti *et al*. 2007; Khardenavis *et al*. 2007; von der Weid *et al*. 2007). Moreover, the bacterial community from ETS was more dissimilar when compared with the others, as illustrated by Unifrac and MIC profiles dendrogram analyses (Figs. 2 and 4). This result is in agreement with the sequence that the ore is processed in steel plant, indicating that wastes with similar chemical characteristics contained similar bacterial community.

Some of our isolates were affiliated to species *D. nitroreducens* and *K. marina*, recently described as new species (Khan and Hiraishi 2002; Kim *et al*. 2004). Approximately one half of the bacteria belong to γ-*Proteobacteria* and a significant proportion of the isolates belong to *Actinobacteria* (Fig. 1). Although *Pseudomonas* spp. are considered ubiquitous in the environment because they have simple nutritional requirements (Palleroni 1993), only two out of 18 *Pseudomonas* isolates grew on minimum medium, and all isolates were exclusively from ETS. In contrast, *Acinetobacter* and *Micrococcus* isolates were found in three of the four wastes. Therefore, they appear to be suited to these environments. The genus

Micrococcus seems to be well suited for long-term survival in extreme environments (Greenblatt et al. 2004). This may explain its isolation from steel waste samples. It is known that representatives of the genus *Acinetobacter* are ubiquitous and play an important role in environmental scenarios (Joly-Guillou 2005). The capability to accumulate phosphorus, one of the most abundant elements found in these wastes, was first discovered in members of this genus, suggesting their potential for enhanced biological phosphorus removal process (Seviour et al. 2003). *Bacillus* and *Dietzia* are distributed widely throughout nature and also present clinical relevance (Wilson *et al*. 1993; Toledo *et al*. 2006; Yassim *et al*. 2006). In addition, the genus *Bacillus* includes species of industrial, biotechnological and environmental interest.

The use in conjunction of two molecular techniques (16S rRNA gene and genomic fingerprinting analyses) can reveal hidden diversity that 16S rRNA gene sequencing is not able to detect (Healy *et al*. 2005). In fact, genomic fingerprinting analyses revealed significantly-higher genetic heterogeneity than 16S rDNA sequence analysis, as for example *Micrococcus* isolates that presented identical 16S rRNA gene sequences were quite diverse based on BOX-PCR analyses.

Bacterial metal tolerance has been shown to increase with increasing industrial contamination (Nies 1999). Among the heavy metals that we tested, Zn, Ni, Pb and Cu were less toxic, whereas Ag, Hg, Cd and Co were highly toxic for *Pseudomonas*, *Micrococcus*, *Acinetobacter* and *Bacillus* isolates, with the following order of toxicity: Zn > Cu > Ni > Pb > Co > Cd > Hg > Ag.

Despite of the nutritional environment be quite poor, we found several heterotrophic bacteria. The knowledge of the bacterial communities these wastes could be further studied for a better understanding of the microbial ecology of these environments.

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References

Araújo, L.A. (1997) Manual de Siderurgia, Volume I, Ed. ABM, S. Paulo, Brasil.

Barreto, M.L. (2001) Mineração e desenvolvimento sustentável: desafios para o Brasil. Rio de Janeiro: CETEM/MCT, 215p.

Brierley, J.A. (1978) Thermophilic iron-oxidizing bacteria found in copper leaching dumps. *Appl Environ Microbiol* **36,** 523–525.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* **19,** 4008.

Dramsi, S., Biswas, I. and Maguim, E. (1995) Entry of *Listeria monocytogenes* into hepatocytes requery express of InLB, a surface protein of the internal multigenic family. *Mol Microbiol* **16,** 251–261.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J. and Raoult, D. (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol* **38,** 3623–3630.

Ewing, B. and Green, P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* **8,** 186–194.

Foti, M.J., Sorokin, D.Y., Zacharova, E.E., Pimenov, N.V., Kuenen, J.G. and Muyz, G. (2007) Bacterial diversity and activity along a salinity gradient in soda lakes of the Kulunda Steppe (Altai, Russia) *Extremophiles* DOI 10.1007/s00792-007-0117-7

Gadd, G.M. (2004) Microbial influence on metal mobility and application for bioremediation. *Geoderma* **112,** 109–119.

Gordon, D., Abajian C. and Green P. (1998) Consed: a graphical tool for sequence finishing. *Genome Res* **8,** 195–202.

Greenblatt, C.L., Baum, J., Klein, B.Y., Nachshon, S., Koltunov, V. and Cano, R.J. (2004) *Micrococcus luteus* – Survival in Amber. *Microb Ecology* **48,** 120–127.

Green, P. (1994) PHRAP documentation. http://www.phrap.org

Hammer, Ø., Harper, D.A.T. and Ryan, P.D. (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* **4,** 1–9.

Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A., Nieto, R., Woods, C., Versalovic, J. and Lupski, J.R. (2005) Microbial DNA typing by automated repetitive-sequence-based PCR. *J Clin Microbiol* **43,** 199–207.

Joly-Guillou, M.L. (2005) Clinical impact and pathogenicity of *Acinetobacter. Clin Microbiol Infect* **11,** 868–873.

Khardenavis, A.A., Kapley, A. and Purohit, H.J. (2007) Simultaneous nitrification and

denitrification by diverse *Diaphorobacter* sp. *Appl Microbiol Biotechnol* **77,** 403–409.

Kim, S.B., Nedashkovskaya, O.I., Mikhailov, V.V., Han, S.K., Kim, K.O., Rhee, M.S. and Bae, K.S. (2004) *Kocuria marina* sp. nov., a novel actinobacterium isolated from marine sediment. *Int J Syst Evol Microbiol* **54,** 617–1620.

Khan, S.T. and Hiraishi, A. (2002) *Diaphorobacter nitroreducens* gen nov, sp nov, a poly (3-hydroxybutyrate)-degrading denitrifying bacterium isolated from activated sludge. *J Gen Appl Microbiol* **48,** 299–308.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16,** 111–120.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5,** 150–163.

Lane, D.J. (1991). 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons,
New York, N.Y., USA.

Ledin, M. and Pedersen, K. (1996) The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes. *Earth Sci Rev* **41,** 67–108.

Li, L.Y. and Rutherford, G.K. (1996) Effect of bauxite properties on the settling of red mud. *Inter J Min Proces* **48,** 169–182.

Li, S., Chen, Z., Qui, L., Wu, J. and Lai, Z. (2006) Isolation and identification of *Bacillus cereus* strain Jp-A and its capability in phenol degradation. *Ying Yong Sheng Tai Xue Bao* **17,** 920–4.

Lozupone, C., Hamady, M. and Knight, R. (2006) UniFrac – An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* **7,** 371–384.

Nazina, T.N., Grigor'yan, A.A., Xue, Y.F., Sokolova, D.Sh., Novikova, E.V., Tourova, T.P., Poltaraus, A.B., Belyaev, S.S. and Ivanov, M.V. (2002) Phylogenetic diversity of aerobic saprotrophic bacteria isolated from the daqing oil field. *Microbiology* **71,** 91–97.

Nies, D.H. (1999) Microbial heavy metal resistance. Appl Microbiol Biotechnol 51: 730–750.

Palleroni, N.J. (1993) *Pseudomonas* classification. A new case history in the taxonomy of gram-negative bacteria. *Anton Leeuw Int J G* **64,** 231–251.

Pathom-aree, W., Stach, J.E.M., Ward, A.C., Horikoshi, K., Bull, A.T. and Goodfellow, M. (2006) Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles* **10,** 181–189.

Pontes, D.S., Lima-Bittencourt, C.I., Chartone-Souza, E. and Nascimento, A.M.A. (2007) Molecular approaches: advantages and artifacts in assessing bacterial diversity. *J Indust Microbiol Biotechnol* **34,** 463-473.

Rawlings, D.E. (2002) Heavy metal mining using microbes. Annu Rev Microbiol 56: 65–91.

Rawlings, D.E., David, D. and Plessis, C. (2003) Biomineralization of metal-containing ores and concentrates. *Trends Biotechnol* **21,** 38–44.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4,** 406-425.

Seviour, R., Mino, T. and Onuki, M. (2003) The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol Rev* **27,** 99–127.

Toledo, F.L., Calvo, C., Rodelas, B. and González-López, J. (2006) Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Syst Appl Microbiol* **29,** 244–252.

Torma, A.E. (1983) Biotechnology applied to mining of metals. *Biotechnol Adv* **1,** 73-80.

Versalovic, J., Schneider, M., De Brujin, F.J. and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using the repetitive sequence based polymerase chain reaction. *Meth Mol Cell Biol* **5,** 25–40.

Von der Weid, I., Marques, J.M., Cunha, C.D., Lippi, K.R., Santos, S.C.C., Rosado, A.S., Lins, U. and Seldin, L. (2007). Identification and biodegradation potential of a novel strain of *Dietzia cinnamea* isolated from a petroleum-contaminated tropical soil. *Syst Appl Microbiol* **30,** 331–339.

Wilson, S.C. and Jones, K.C. (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environ Pollut* **81,** 229–249.

Yassim, A.F., Hupfer, H. and Schaal, P. (2006). *Dietzia cinnamea* sp. nov., a novel species isolated from a perianal swab of a patient with a bone marrow transplant. *Int J Syst Bacteriol* **56,** 641–645.

Table 1 Chemical features of the four waste types

Wastes	Elements/ Oxides (%)														
	C	SiO_2	CaO	MgO	Al_2O_3	MnO	ZnO	Cr	NiO	S	P_2O_5	FeT*	Fe^0	FeO	Fe_2O_3
HMPT	1.3	20.73	9.05	1.88	0.16	0.70	0.022	0.14	0.021	0.61	0.11	52.11	1.33	7.99	63.75
MRPL	1.07	1.39	13.55	1.60	0.054	2.96	0.032	9.7	2.35	0.012	0.02	38.00	2.50	7.90	42.01
PTSS	ND	11.47	47.94	8.72	3.92	2.20	ND	0.83	ND	0.23	2.11	17.17	ND	ND	ND
ETS	0.41	0.81	21.4	2.28	0.05	0.14	0.004	3.1	0.44	10.30	0.119	18.00	0.67	ND	24.78

FeT= $\sum$ Fei

ND, not determined.

Table 2 Minimum inhibitory concentration of heavy metals of bacterial isolates from steel plant wastes

Genera (n)[a]	MIC (mM)							
	Cu	Pb	Zn	Co	Ni	Cd	Hg	Ag
Pseudomonas (18)	(1, 2) [b]	(1, 1)	(0.25, 2)	(0.1, 0.25)	(2, 2)	(0.1, 0.5)	(0.03, 0.03)	(≤ 0.001, ≤ 0.001)
Acinetobacter (6)	(2, 2)	(0.5, 0.5)	(0.5, 1)	(0.25, 0.25)	(1, 1)	(0.1, 0.25)	(0.015, 0.03)	(≤ 0.001, ≤ 0.001)
Bacillus (5)	(2, 2)	(1, 1)	(1, 2)	(0.5, 0.5)	(2, 2)	(0.1, 2)	(0.015, 0.015)	(≤ 0.001, ≤ 0.001)
Micrococcus (11)	(2, 2)	(1, 1)	(2, 4)	(0.25, 0.25)	(2, 2)	(0.05, 0.03)	(0.015, 0.03)	(≤ 0.001, ≤ 0.001)
Dietzia (4)	2	2	0.25	0.05	1	0.015	0.03	≤ 0.001
	2	2	0.25	0.05	2	0.03	0.03	≤ 0.001
	2	2	0.25	0.05	2	0.03	0.03	≤ 0.001
	2	2	0.25	0.05	1	0.03	0.015	≤ 0.001
Kocuria (2)	0.5	1	2	0.25	1	0.015	0.015	≤ 0.001
	1	0.5	0.05	0.1	2	0.05	0.015	≤ 0.001
Diaphorobacter (1)	1	0.5	1	0.25	2	2	≤ 0.001	≤ 0.001
Staphylococcus (1)	2	1	2	0.25	2	0.05	0.015	≤ 0.001
Brevibacillus (1)	2	1	2	0.25	2	0.25	0.015	≤ 0.001

[a] Genera with sample size n ≤4 are given the MIC of the isolates.

[b] Minimum inhibitory concentration for 50% and 90% of isolates (MIC_{50} and MIC_{90}).

Cu, copper sulphate; Pb, lead nitrate; Zn, zinc sulphate; Co, cobalt chloride; Ni, nickel chloride; Cd, cadmium chloride; Hg, mercury bichloride; Ag, silver nitrate.

Figure legends

Figure 1. Neighbor-joining tree based on analysis of partial 16S rDNA sequences of bacterial isolates and related species. One-thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Spirochaeta fricana* was chosen to provide the outgroup sequence. The nucleotide sequences generated were deposited in the Genbank database with accession numbers EU151500 to EU151548.

Figure 2. UPGMA cluster of bacterial communities from steel plant wastes.

Figure 3. BOX-PCR patterns of the bacterial isolates by electrophoresis in a 1.5% agarose gel. Lane M, molecular size marker (1 Kb Plus-Invitrogen). (A) Isolates of *Pseudomonas*, lanes 1 to 18 (1ETS, 2ETS, 3ETS, 4ETS, 5ETS, 6ETS, 8ETS, 9ETS, 10ETS, 11ETS, 12ETS, 13ETS, 14ETS, 15ETS, 16ETS, 18ETS, 19ETS and 20ETS, respectively). (B) Isolates of *Acinetobacter,* lanes 1 to 6 (1PTSS, 4PTSS, 7HMPT, 3MRPL, 8HMPT and 12HMTS, respectively). (C) Isolates of *Micrococcus* lanes 1 to 11 (1HMPT, 2HMPT, 3HMPT, 4HMPT, 2MRPL, 21ETS, 22ETS, 23ETS, 24ETS, 25ETS and 26ETS, respectively) and *Kocuria* lanes 12 and 13 (27ETS and 28ETS, respectively). (D) Isolates of *Bacillus,* lanes 1 to 5 (9HMPT, 1MRPL, 4MRPL, 5MRPL and 6MRPL, respectively) and (E) Isolates of *Dietzia*, lanes 1 to 4 (29ETS, 30ETS, 31ETS and 32ETS, respectively). (F) Dendrogram constructed by UPGMA with the bacterial isolates.

Figure 4. Dendrogram constructed by UPGMA with the bacterial isolates according to heavy metal susceptibility profiles.

Figure 1

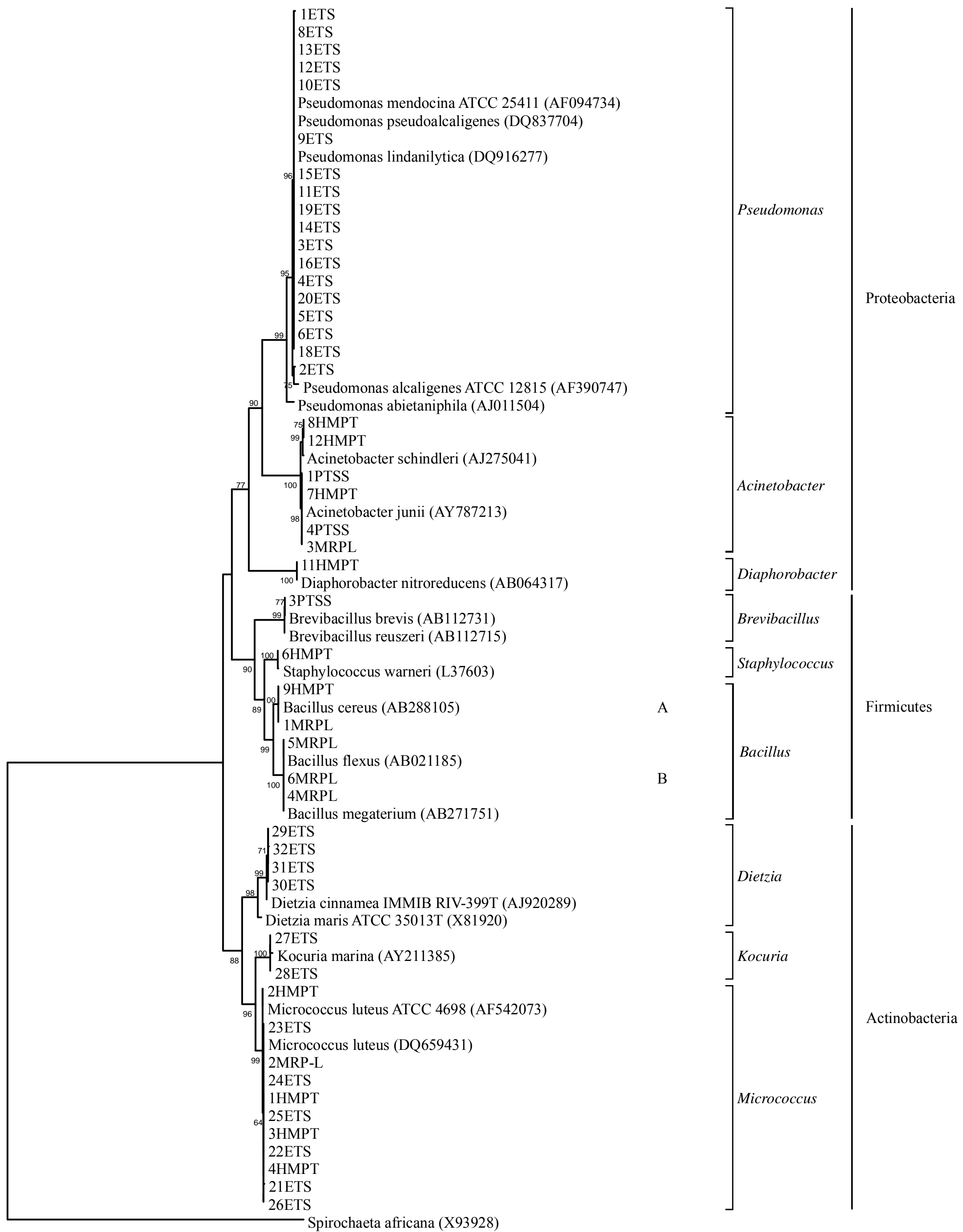


Figure 2

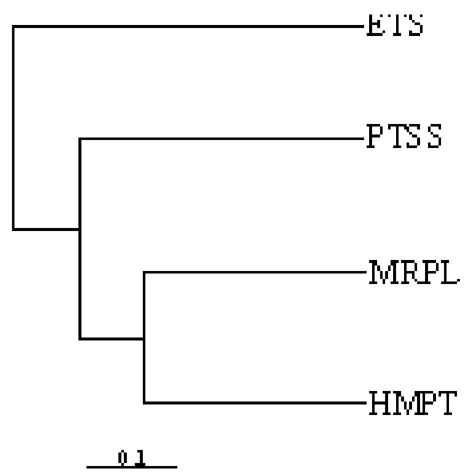


Figure 3

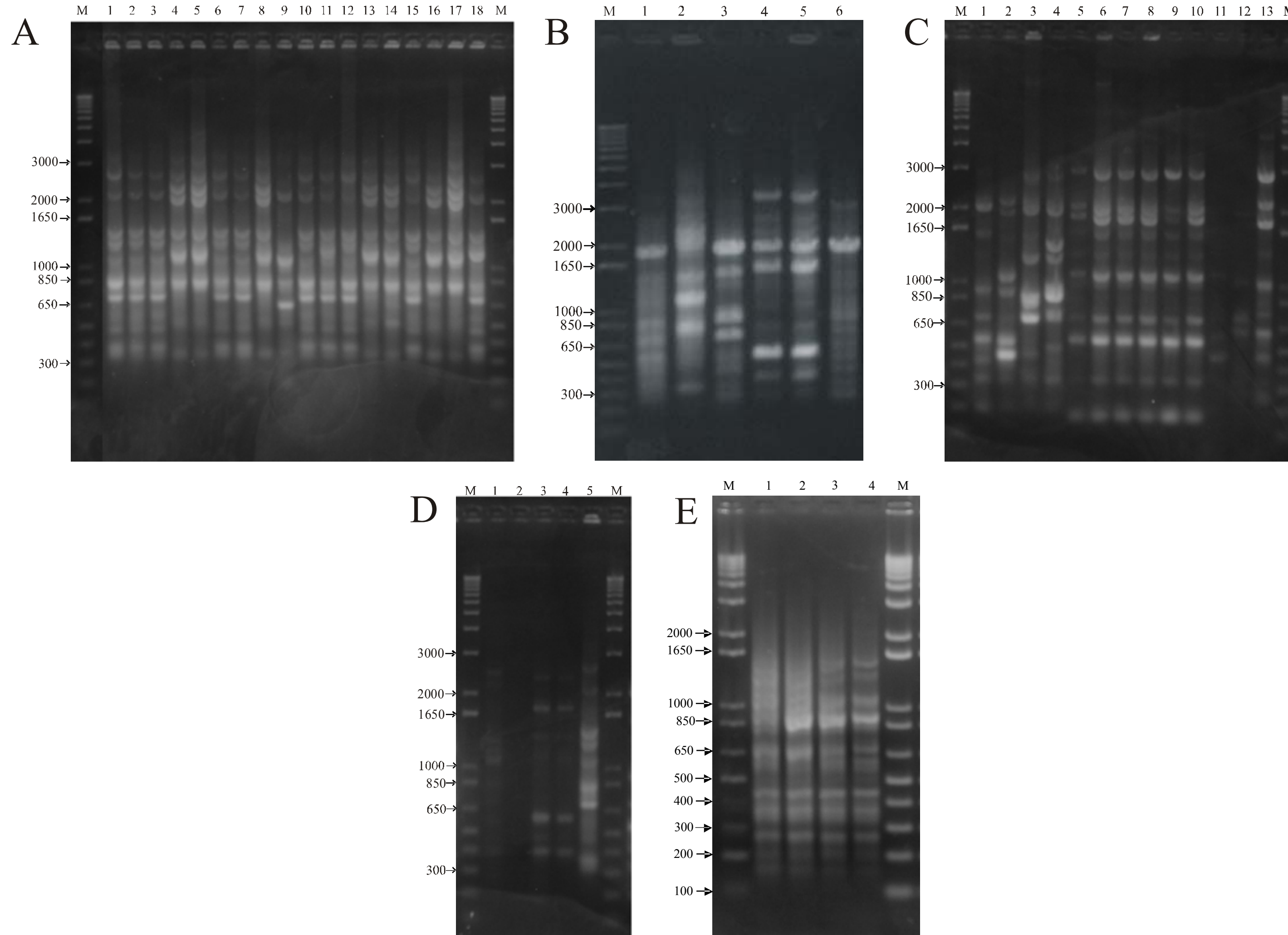


Figure 3F

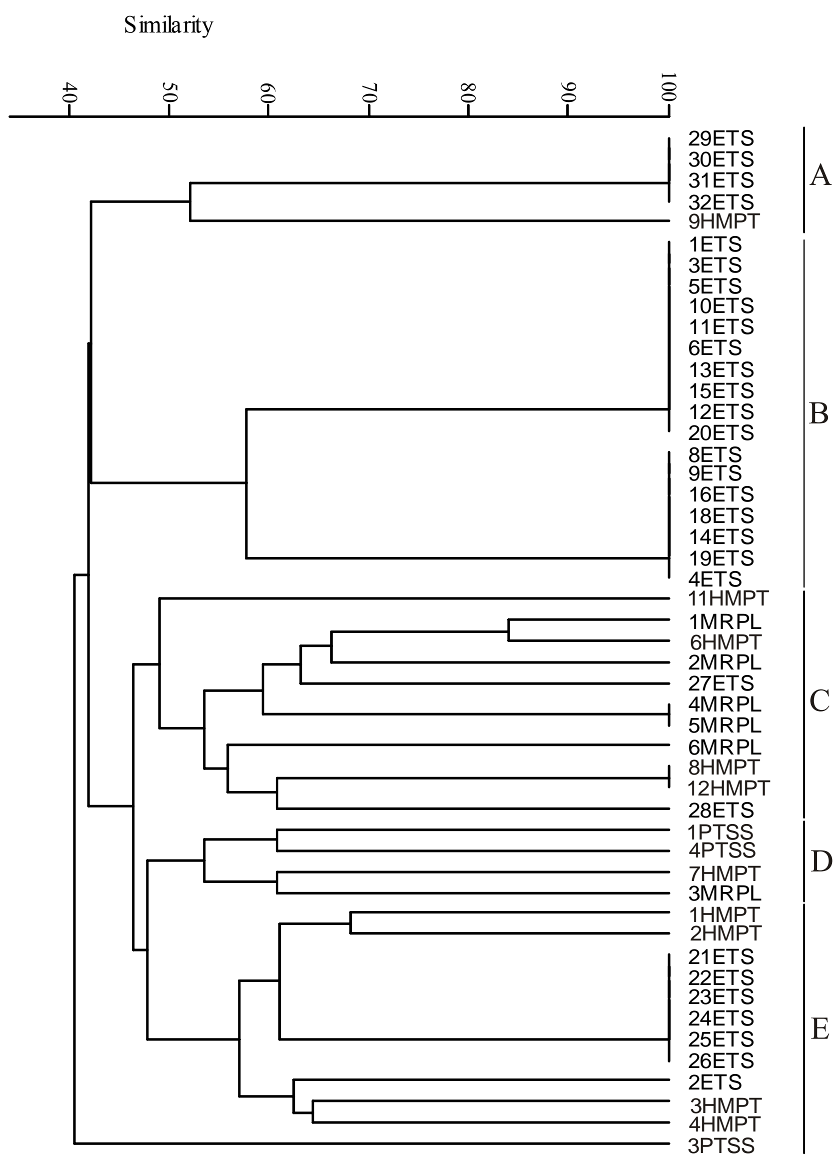
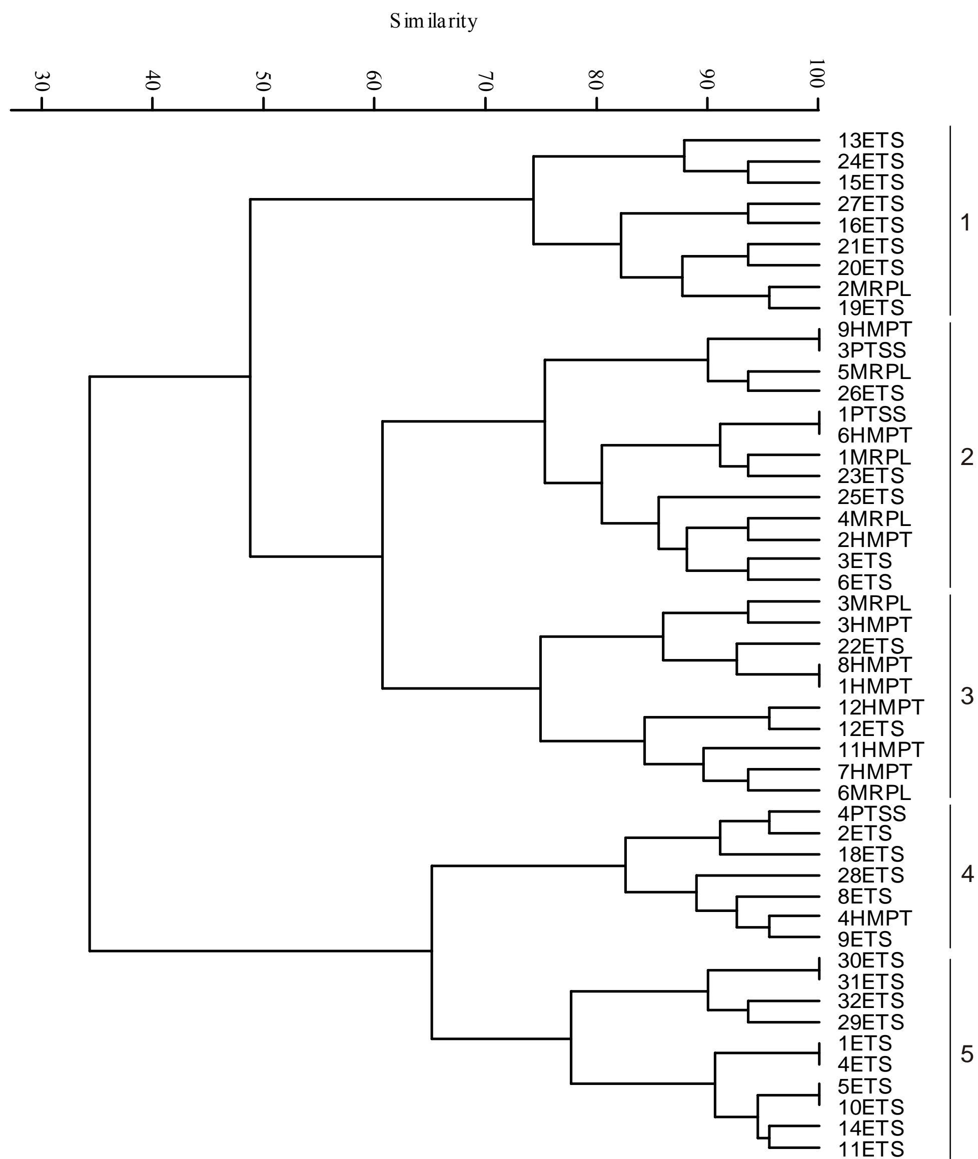


Figure 4



CAPÍTULO II

Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant wastes

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Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant waste

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Abstract

Background

Molecular studies of *Bacillus* diversity in various environments have been reported. However, investigations concerning *Bacillus* that live in steel plant environments have been rarely examined. Genotypic and phenotypic diversity and phylogenetic relationships among 40 bacterial isolates recovered from steel plant waste were investigated by classical and molecular methods.

Results

16S rDNA partial sequencing assigned all the isolates to the *Bacillus* genus, with close genetic relatedness to the *B. subtilis* and *B. cereus* groups and to the species *B. pumilus* and *B. sphaericus*. tDNA-intergenic spacer length polymorphisms (tDNA-PCR) and 16S-23S intergenic transcribed spacer region (ITS-PCR) failed to identify the isolates to the species level. Genomic diversity was investigated by molecular typing with rep (repetitive sequence)-based PCR using primer sets ERIC2 (enterobacterial repetitive intergenic consensus), $(GTG)_5$, and BOXAIR. Genotypic fingerprinting of the isolates reflected high intra- and interspecies diversity. ERIC-PCR fingerprinting clustered the isolates in the same way as the 16S rRNA gene phylogenetic tree; this technique has potential as a simple and useful tool for examining relationships among unknown *Bacillus* spp. Physiological, biochemical and heavy metal susceptibility profiles also indicated considerable phenotypic diversity. Among the heavy metal compounds tested, Zn, Pb and Cu were least toxic to the bacterial isolates, whereas Ag inhibited all isolates at 0.001 mM.

Conclusions

Since isolates with identical 16S rRNA gene sequences had different genomic fingerprints and also differed considerably in their physiological capabilities, the high diversity that we found is likely to be of ecological relevance.

Background

The genus *Bacillus* encompasses Gram-positive rod-shaped, endospore-forming aerobic or facultatively anaerobic bacteria; it is a phenotypically and phylogenetically diverse taxon [1]. Bacillus species are distributed widely throughout nature. Some are associated with human diseases, such as *B. cereus* and *B. anthracis*, which belong to the *Bacillus cereus* group [2]. Ask et al. [3] found that *Bacillus* species form five main phylogenetic groups. Two of these groups are the *Bacillus cereus* group (*B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides and B. weihenstephanensis*) and the *Bacillus subtilis* group (*B. subtilis, B. pumilus, B. atrophaeus, B. licheniformis and B. amyloliquefaciens*) [3].

Since the genus *Bacillus* includes species of industrial, biotechnological and environmental interest, as well as clinically important species, various studies have been made of the genetic diversity of *Bacillus* species. However, it is still difficult to identify and characterize new isolates, mainly because some species share morphological and biochemical characteristics [3, 4, 5], which makes it hard to separate them. In addition, species isolated from the environment have considerable physiological, DNA G + C content and nutritional requirement diversity [1, 3, 5-7]. Molecular approaches are increasingly being used for rapid species identification [8, 9]. Various techniques, including tDNA-PCR and ITS-PCR analysis, have been used for rapid identification and differentiation of bacterial species [10-12]. Repetitive element sequence-based PCR (rep-PCR) genomic fingerprinting has also been used for discriminating between a wide range of bacterial genera and species. This method can be used to generate more accurate information, since it is capable of screening several parts of the bacterial genome [13, 14].

Many studies have been made of *Bacillus* isolates originating from hospital, food and environmental samples [15], though little attention has been given to isolates recovered from

steelmaking waste. Little is known about the ecology and diversity of *Bacillus* spp. in this environment. We used biochemical (API 50 CHB/E) and molecular approaches (16S rDNA, ARDRA, tDNA, ITS, ERIC, BOX and $(GTG)_5$ to characterize, and differentiate 40 bacterial isolates obtained from steelmaking waste. We also evaluated their susceptibility to heavy metals and examined the phylogenetic relationships among them.

Results

Genetic characterization

Phylogenetic analysis

The 16S rDNA sequences used for phylogenetic analysis were 666 nucleotides long and spanned V2 to V5 variable regions, corresponding to *Escherichia coli* K12 16S rDNA. The 16S rDNA sequences of the reference strains that we used in our study were obtained from GenBank, except for *Bacillus amyloliquefaciens* ATCC 23842. The phylogenetic tree built based on these sequences revealed close relationships among the isolates and of the isolates with other members of the genus *Bacillus* (Fig. 1).

Based on 16S rDNA sequence analysis, 13 isolates clustered with *B. pumilus* strains (ATCC 7061 and 27142), sharing 99.8% similarity. Nine isolates had a sequence similarity of 99.9% with *B. amyloliquefaciens* ATCC 23842 strain. Slightly lower similarities (99.7% and 99.5%) were found for these nine isolates in comparisons with *B. subtilis* ATCC 6633 and *B. licheniformis* ATCC 14580, respectively. Seventeen isolates clustered with the *B. cereus* group (*B. cereus, B. mycoides* and *B. anthracis*) and shared 99.9% sequence similarity with this group. Fifteen of these 17 isolates had identical 16S rRNA gene sequences. The sequence of isolate 36BFS had 97.1% similarity with *B. sphaericus* ATCC 14577.

ARDRA analysis

The complete 16S rRNA gene amplicon was digested separately with three endonucleases that generated eight *Alu* I and four *Hha* I and *Mbo* I profiles. The similarity dendrogram for ARDRA is shown in Figure 2. Cluster 1 assembled 21 isolates plus the reference *B. pumilus*, *B. subtilis B. amyloliquefaciens* and *B. licheniformis* strains, with approximately 70% similarity. Eighteen of the 21 isolates had 100% similarity with the reference *B. subtilis* strain. Cluster 2 contained one isolate and no reference strain. Cluster 3 had one isolate and the *B. sphaericus* reference strain. Cluster 4 comprised 17 isolates plus the reference *B. cereus* strain.

Genomic fingerprinting

The resolution powers of ITS-PCR and tDNA-PCR were evaluated with the reference strains of *Bacillus* species identified from the 16S rRNA gene sequencing analyses (Fig. 1). The ITS and tDNA-PCR fingerprinting gave 10 and 11 distinct patterns, respectively (Figs. 3A and B). The *Bacillus* isolates, grouped based on 16S rRNA gene sequence phylogeny, had identical ITS and tDNA-PCR patterns, except for the tDNA-PCR of four isolates of the *B. cereus* group. These patterns differed from those of all the reference strains.

The fingerprint patterns of the 40 isolates generated by ERIC, $(GTG)_5$ and BOX-PCR were complex; they produced a large number of polymorphic bands of variable intensity (Figs. 4A, 5A and B). Thirty-six bands were identified by BOX-PCR (approximately 200 to 4000 bp), 34 bands by ERIC-PCR (200 to 4000 bp) and 49 bands by $(GTG)_5$-PCR (200 to 4000 bp). All reference strains had unique profiles in all genomic fingerprinting analyses; they did not match any of the patterns obtained for the isolates. Among the three sets of primers, $(GTG)_5$-PCR fingerprinting gave the broadest band pattern. BOX-PCR amplifications were negative for two isolates (8BFS and 31BFS), based on three independent

PCRs. Negative results were also observed for one isolate (33BFS) with ERIC-PCR fingerprinting. The dendrogram based on ERIC-PCR fingerprinting analysis (Fig. 4B) produced a clustering of isolates similar to that of the 16S rDNA phylogenetic tree. Only the 36BFS isolate grouped within cluster 1 with ERIC-PCR, far from its 16S rDNA tree cluster position (cluster 3, Fig. 1). The dendrogram generated from multi-rep-PCR fingerprinting (Fig. 6) revealed considerable genetic heterogeneity among the isolates and reference strains.

Morphological, biochemical and physiological characterization

Data from API 50CH/B and additional physiological tests are summarized in Table 2. All 40 isolates were found to be motile, Gram-positive, spore-forming rods. Among these isolates, 15 grew at 55ºC and 12 at 8ºC, while 32 and 13 isolates were able to grow in 7.5% and 15% NaCl, respectively. Only two of the isolates used citrate as a carbon source and three produced hydrogen sulphide. The substrates that were most often used were esculin and D-ribose. The substrates least frequently used were inulin and potassium gluconate. Ability to hydrolyze starch was tested with the 22 isolates that clustered within the *B. subtilis* group in an attempt to discriminate *B. pumilus* species. Among these 22 isolates, five were starch hydrolysis negative, placing them closer to *B. pumilus.*

These isolates were classified into specific genera by determining their API 50CH/B biochemical profiles. With a confidence level above 90%, the API 50 CH/B system assigned most of them (n=28) to the genus *Bacillus*. The remaining 12 isolates were identified to the species level by this system: *B. pumilus* (n=6), *B. cereus* (n=1), *B. coagulans* (n=1), *Brevibacillus laterosporus* (n=1) and *Aneurinibacillus aneurinilyticus* (n=3). Based on identification with the API data, the 40 isolates were highly similar to three genera of the Bacillaceae and Paenibacillaceae families. This system failed to identify all of the reference

strains to the species level.

In the heavy metal assays, the highest minimum inhibitory concentrations (MIC) were found for Cu and Pb (4mM, Table 3). The MIC levels were lower for Zn, Ni, Co and Hg, with 90% of the isolates being inhibited by concentrations ranging from 0.025 to 2 mM. All isolates were inhibited by Ag at the lowest concentration tested. A dendrogram based on the MIC profiles revealed distinct profiles of combinations of heavy metals for almost all isolates. The reference strains formed one exclusive cluster (Fig. 7). No significant relationship was found between isolates clustering based on this characteristic versus their phylogenetic position.

Discussion

It is well known that 16S rDNA sequences are good indicators of phylogenetic relationships of bacteria at intra- and intergeneric levels. In our study the 16S rRNA gene sequence analyses yielded very good identification of the isolates at the *Bacillus* genus level. As expected, analysis of 16S rRNA gene sequences alone was not sufficient to identify *Bacillus* species, as has also been reported in other studies [16, 17]. Identification of the bacteria using API CHB50 generally identified isolates to genus, and in some cases, species; however, identifications obtained by API versus 16S rDNA sequencing differed. Nevertheless, biochemical and physiological test data was useful to identify characteristics that are usually considered typical for the genus *Bacillus*, allowing phenotypic characterization of the isolates.

Since identifications based on 16S rRNA gene sequences are often incomplete, fingerprint methods were developed to characterize and distinguish *Bacillus* isolates [6, 10-12]. We used ARDRA and ITS-PCR, tDNA-PCR and rep-PCR genomic fingerprinting to examine phylogenetic relatedness among the isolates. Overall, the isolates phylogenetically

closest to *B. sphaericus*, *B, pumilus* and *B. subtilis* and *B. cereus* groups were clearly different from the reference strains, except in the ARDRA analysis.

ITS and tDNA-PCR are frequently used to identify bacterial species and to analyze their phylogenetic relationships [10]. Although, we attained sufficient resolution to differentiate between reference strains, it was not possible associate the *Bacillus* isolates with the reference strains based on this technique.

Considering that the genotype is less affected by environmental effects than the phenotype, the rep-PCR method was used to elucidate intraspecies diversity among the different *Bacillus* isolates. We found that isolates with the same partial 16S rDNA sequence often had quite dissimilar rep-PCR patterns. The primer specific for $(GTG)_5$ was less efficient than the BOX and ERIC primer sets for grouping the isolates. ERIC-PCR fingerprinting and 16S rDNA phylogenetic analyses gave similar clusters. ERIC-PCR fingerprinting clustered reference and environmental *B. cereus* strains in the same way as the 16S rDNA tree; consequently, the former appears to be a good approach for examining genetic relationships among unknown *Bacillus* isolates. When combined, all the rep-PCR methods were able to separate the environmental *Bacillus* species as a distinct group from the reference strains. There was less similarity among the reference strains than among the *Bacillus* isolates. This could reflect their different origins.

Although the API 50CH/B system failed to identify the isolates and reference strains to the species level, it was useful for their biochemical characterization and revealed significant variability among the isolates. Based on inability to hydrolyze starch, five out of 22 isolates that appeared grouped with the *B. subtilis* group were deduced to be affiliated with *B. pumilus*. The isolates that affiliated with *B. pumilus* by molecular analysis (16S rDNA) had greater physiological diversity.

Conclusions

Based on molecular characterization, most of the isolates were closely related to the species *B. pumilus*, *B. sphaericus*, and the *B. subtilis* and *B. cereus* groups. Despite the polyphasic approach, it was difficult to identify the environmental *Bacillus* isolates, implying that this genus is quite heterogeneous. We found high genetic and phenotypic heterogeneity in the *Bacillus* spp. isolates, despite their origin from a single steelmaking waste source.

Methods

Sampling and bacterial isolates

The blast furnace sludge (BFS) sample from a Steel Plant (Acesita-Cia Aços Especiais, Minas Gerais state, Brazil) was collected in triplicate using sterilized bottles. BFS is produced after the first step in the ore processing, when the raw material is subjected to high temperatures for ore separation. The chemical composition of BFS was determined by Mössbauer and X-ray analyses and contains: 38.72% FeT, 6.74% Si, 4.60% Zn, 2.29% Ca, 2.14% Mg, 1.81% Al, and traces of Cr, S, P and Mn (<1%).

Isolates were recovered from the BFS by blending 1 g wet weight of the sample with 9 ml of phosphate buffer and shaking at 37°C for 24h before serial dilution. The colonies grown on 1/10 strength tryptic soy agar (Difco) were purified by restreaking at the same medium at 37ºC to subsequent molecular and phenotypic analyses. Type strains of *B. subtilis* (ATCC 6633^T), *B. licheniformis* (ATCC 14580^T), *B. pumilus* (ATCC 7061^T), *B. amyloliquefaciens* (ATCC 23842), *B. cereus* (ATCC 11778) and *B. sphaericus* (ATCC 14577^T) were included as reference strains.

Characterization of the isolates

Morphological, physiological and biochemical characteristics

Morphological and physiological characterizations of the 40 isolates were based on the Gram reaction, shape, motility, endospore forming and growth at different temperatures and NaCl concentrations. Moreover, to confirm *B. subtilis* and *B. pumilus* molecular taxonomic identification, the ability of Bacillus isolates to hydrolyze starch was investigated. The isolates were also identified according to biochemical profiles with the commercial test kit API 50CH/B (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions, and conventional biochemical tests (citrate and indol utilization, and hydrogen sulphide production) were carried out as described [18]. Results were interpreted with the Analytical Profile Index (API) database of the Apiweb software (version 4.0; BioMérieux, Marcy l'Etoile, France).

The minimal inhibitory concentration (MIC) was determined by a serial two-fold agar dilution method using nutrient agar (Difco) for the heavy metals: mercury chloride, silver nitrate, lead nitrate, cobalt chloride, copper sulphate, zinc sulphate and nickel chloride. All antimicrobials and heavy metals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Molecular identification

DNA extraction, PCR amplification, sequencing of 16S rDNA and phylogenetic analysis

Genomic DNA of isolates was prepared as described [19]. 16S rRNA genes were amplified using the primers 8F and 907R, and the amplification cycling conditions are given in Table 1. All sequencing reactions were performed using the Dynamic ET Dye (Amersham BioSciences) sequencing kit, and reactions were analyzed on a MegaBACE 1000 capillary sequencer (Amersham BioSciences). Sequences were compared with available databases using GenBank BLASTN and RDP Classifier search tools to determine approximate

phylogenetic affiliations. To accomplish this, the partial 16S rRNA gene sequences were basecalled, checked for quality, aligned and analyzed using Phred v.0.20425 [25], Phrap v.0.990319 [26] and Consed 12.0 [27] software. The phylogenetic relationships were inferred by MEGA 3.1 [28] using the Neighbor-Joining (N-J) method and the Kimura 2-P model of sequence evolution. The robustness of the phylogenetic tree topology was evaluated with 1,000 replicates of bootstrap analysis [29, 30]. The nucleotide sequences generated were deposited in the Genbank database with accession numbers EU689117 to EU689156.

ARDRA, tDNA-PCR, ITS-PCR and rep-PCR based DNA fingerprinting

The amplified 16S rDNA with the primers PA and U2 (Table 1) was digested separately with three restriction enzymes (*Alu* I, *Mbo* I and *Hna* I, New England Biolabs, Beverly, MA), which recognize sequences of four nucleotides, according to the supplier's recommendations. The restriction profiles were analyzed in an 8% polyacrylamide gel in Tris–Borate–EDTA (TBE) buffer.

Three variations of rep-PCR genomic fingerprinting were performed using the ERIC, BOX and $(GTG)_5$ primers. The primers and the amplification cycling conditions for the rep-PCR, tDNA and ITS-PCR are shown in Table 1. The reproducibility of the fingerprint profiles obtained was assessed in at least three separate experiments.

Cluster analysis

Cluster analysis of the pairwise similarity values was performed using the Euclidian similarity coefficient and unweighted pair-group method with arithmetic means (UPGMA) clustering technique. Analysis of data was performed using the software PAST [31].

Authors' contributions

DBF carried out laboratory work and wrote the draft of manuscript. MPR and PSC helped DBF with laboratory work. CIL-B was responsible for computational analysis together DBF. PSA was responsible for the *Bacillus sp.* samples from the steel plant wastes. ECS participated to the discussion of the results, and the manuscript draft. AMAN conceived and designed the study, coordinated the project and helped to write the final manuscript. All authors have read and approved the final manuscript.

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References

1. Claus D, Berkeley RCW: **Genus Bacillus Cohn 1872, 174AL**. In Bergey's Manual of Systematic Bacteriology, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe, J. G Holt. Baltimore: Williams & Wilkins (1986).
2. Lapidus A, Goltsman E, Auger S, Galleron N, Ségurens B, Dossat C, Land ML, Broussolle V, Brillard J, Guinebretiere MH, Sanchis V, Nguen-the C, Leredeus D, Richardson P, Wincker P, Weissenbach J, Ehrlich SD, Sorokin A: **Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity.** *Chem Biolog Interact* 2008, **171**:236–249.
3. Ash C, Farrow JAE, Dorsch M, Stackebrandt E, Collins MD: **Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA.** *Int J Syst Bacteriol* 1991, **41**:343–346.
4. Harrel LJ, Anderson GL, Wilson KH: **Genetic variability of *Bacillus anthracis* and related species.** *J Clin Microbiol* 1995, **33**:1947-1950.

5. Priest FG, Goodfellow M, Todd C: **A numerical classification of the genus *Bacillus*.** *J Gen Microbiol* 1988, **134**:1947–1982.
6. Dong X, Cote JC: **Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S–23S ITS nucleotide sequences.** *Int J Syst Evol Microbiol* 2003, **53**:695–704.
7. Gordon RE, Haynes WC, Pang CHN: **The genus Bacillus.** Agriculture Handbook no. 427. Washington, DC: US Government Printing Office (1973).
8. Goto K, Omura T, Hara Y, Sadaie Y: **Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*.** *J Gen Appl Microbiol* 2000, **46**:1–8.
9. Heyrman J, Rodríguez-Díaz M, Devos J, Felske A, Niall AL, De Vos P: ***Bacillus arenosi sp. nov.*, *Bacillus arvi sp. nov*. and *Bacillus humi sp. nov*., isolated from soil.** *Int J Syst Evol Microbiol* 2005, **55**:111–117.
10. Daffonchio D, Cherif A, Brusetti L, Rizzi A, Mora D, Boudabous A, Sara B: **Nature of polymorphisms in 16S–23S rRNA gene intergenic transcribed spacer fingerprinting of *Bacillus* and related genera.** *Appl Environ Microbiol* 2003, **69**:5128–5137.
11. Cherif A, Borin S, Rizzi A, Ouzari H, Boudabous A, Daffonchio D: *Bacillus anthracis* **Diverges from Related Clades of the *Bacillus cereus* Group in 16S-23S Ribosomal DNA Intergenic Transcribed Spacers Containing tRNA Genes.** *Appl Environ Microbiol* 2003, **69**:33–40.
12. Borin S, Daffonchio D, Sorlini S: **Single strand conformation polymorphism analysis of PCR-tDNA fingerprinting to address the identification of *Bacillus* species.** *FEMS Microbiol Lett* 1997, **157:**87–93.
13. Versalovic J, Koeuth T, Lupski JR: **Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes.** *Nucleic Acids Res* 1991, **19:** 6823–6831.
14. Versalovic J, Schneider M, De Bruijn F.J, Lupski, JR: **Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction**. *Meth Mol Cell Biol* 1994**, 5**: 25-40.
15. Turnbull PCB, Sirianni NM, LeBron CI, Samaan MN, Sutton FN, Reyes AE, Peruski LF: **MICs of Selected Antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest.** *J Clin Microbiol* 2003, **42**:3626–3634.

16. Lima-Bittencourt CI, Astolfi-Filho S, Chartone-Souza E, Santos FR, Nascimento AMA: **Analysis of *Chromobacterium* sp. natural isolates from different Brazilian ecosystems.** *BMC Microbiol*. 2007, **7**:1-9.

17. Pontes DS, Lima-Bittencourt CI, Chartone-Souza E, Nascimento, AMA: **Molecular approaches: advantages and artifacts in assessing bacterial diversity.** *J Ind Microbiol Biotechnol* 2007, 34:463–473

18. Smibert RM, Krieg NR: **Phenotypic characterization.** In: Gerhardt, P., Murray, R.G.E., Woods, W.A., Krieg, N.R. (Eds.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC 1994, pp. 607–654.

19. Dramsi S, Biswas I, Maguim E: **Entry of *Listeria monocytogenes* into hepatocytes Requery express of InLB, a surface protein of the internal multigenic family.** *Molec Microbiol* 1995, **16**:251-261.

20. Lane DJ: **16S/23S rRNA sequencing**, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. 1991. John Wiley and Sons, New York, N.Y., USA.

21. Kuske CR, Barns SM, Busch JD: **Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions.** *Appl Environ Microbiol* 1997, **63**: 3614-3621.

22. Lu JJ, Perng CL, Lee SY, Wan CC: **Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid.** *J Clin Microbiol* 2000, **38**: 2076-2080.

23. Welsh J, McClelland M: **Genomic fingerprints produced by PCR with consensus tRNA gene primers.** *Nucleic Acids Res* 1991, **19**:861-866.

24. Clementino MM, Filippis I, Nascimento CR, Branquinho R, Rocha CL, Martins OB: **PCR analysis of t-RNA intergenic spacer, 16S-23S internal transcribed spacer, and randomly amplified polymorphic DNA reveal inter- and intraspecific relationships of *Enterobacter cloacae* strains.** *J Clin Microbiol* 2001, **39**: 3865-3870.

25. Ewing B, Green P: **Base-calling of automated sequencer traces using phred. II. Error probabilities.** *Gen Res* 1998, **8:**186-194.

26. Green P: **PHRAP documentation.** 1994 http://www.phrap.org

27. Gordon D, Abajian C, Green P: **Consed: a graphical tool for sequence finishing.** *Genome Res* 1998, **8**:195-202.

28. Kimura M: **A simple method for estimating evolutionary rates of base**

substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980, **16**:111–120

29. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees.** Mol Biol Evol 1987, **4**:406-425

30. Kumar S, Tamura K, Nei M: **MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment.** *Brief Bioinform* 2004, **5**:150–163

31. Hammer Ø, Harper DAT, Ryan PD: **PAST: paleontological statistics software package for education and data analysis.** *Palaeontol Electronic* 2001, **4:**1-9.

Table 1. Primer sequences and amplification cycling conditions for the different PCR-based genomic DNA fingerprints and 16S rDNA.

Primer set	Primer	Nucleotide sequence 5'- 3'	References	Primer concentration (μM)	Thermal cycling conditions
16S rDNA	8F	AGAGTTTGATYMTGGCTCAG (271 to 290*)	[20]	0.5	Initial denaturation 94°C for 5 min, 94°C for 1 min, 57°C for 1 min and was then decreased by 1°C every two cycles until it was 49°C. Ten additional cycles were carried out at 49°C, 72°C for 3 min, final extension at 10 min at 72°C.
	907R	CCGTCAATTCMTTTRAGTTT (1356 to 1375*)	[20]		
	PA	TCCTGGCTCAGATTGAACGC (17 to 36*)	[21]		
	U2	ATCGGYTACCTTGTTACGACTTC (1513 to 1491*)	[22]		
tDNA	T5A	AGTCCGGTGCTCTAACCAACTGAG	[23]	0.5	Initial denaturation 94°C for 10 min, 30 cycles of 94°C for 30s, 50°C for 30s, 72°C for 1 min, final extension at 72°C for 10 min
	T3B	AGGTCGCGGGTTCGAATCC	[23]		
ITS	L1	CAAGGCATCCACCGT	[24]		
	G1	GAAGTCGTAACAAGG	[24]		
ERIC	ERIC2	AAGTAAGTGACTGGGGTGAGCG	[13]	0.5	Initial denaturation 94°C for 5 min, 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 3 min, final extension at 72°C for 10 min
BOX	BOXA1R	CATACGGCAAGGCGACGCT	[13]	0.4	
GTG	GTG_5	GTGGTGGTGGTGGTG	[13]	0.3	

*E. coli K12 16S rDNA gene

Table 2. Phenotypic characteristics of the isolates and reference strains used in this study.

Biochemical characteristic	Percentage of positive isolates									
	B. pumilus (n=5)*	*B. pumilus* ATCC 7061	*B. subtilis* group (n=17)	*B. subtilis* ATCC 6633	*B. amyloliquefaciens* ATCC 23842	*B. licheniformis* ATCC 14580	*B. cereus* group (n=17)	*B. cereus* ATCC 11778	*B. sphaericus* (n=1)	*B. sphaericus* ATCC 14577
Growth at 55 °C	60	-	47	-	-	+	24	-	-	-
Growth at 8 °C	20	-	12	-	+	+	53	-	-	-
Growth in 7.5% NaCl	100	+	71	+	+	+	76	+	-	-
Growth in 15% NaCl	80	-	24	-	+	+	29	-	-	-
Citrate	0	-	12	-	+	+	0	-	-	-
Hydrogen sulphide	0	-	18	-	-	-	0	-	-	-
Motility	100	+	100	+	+	+	100	+	+	+
Starch	0	-	100	+	+	+	71	+	+	-
Glycerol	100	+	41	+	-	+	35	+	+	-
L-arabinose	40	+	53	+	-	+	35	+	-	-
D-ribose	80	+	88	+	+	+	65	+	-	-
D-xylose	40	+	41	+	-	+	12	+	-	-
D-galactose	20	+	12	-	-	+	6	-	-	-
D-glucose	80	-	82	+	-	+	82	+	-	+
D-fructose	80	-	59	+	+	+	82	+	+	+
D-mannose	100	-	53	+	-	+	41	+	+	-
D-sorbose	20	-	0	-	-	-	6	-	-	-
L-rhamnose	20	-	0	-	-	+	6	-	-	-
Inositol	20	+	18	+	-	+	29	+	+	-
D-mannitol	80	+	53	+	+	+	47	+	+	-
D-sorbitol	40	+	24	+	+	+	35	+	+	-
Methyl-αD-Mannopyranoside	20	-	18	-	-	-	12	-	-	-
Methyl-αD-Glucopyranoside	20	+	29	+	-	+	29	+	-	-
N-Acetylglucosamine	80	+	41	-	-	+	82	-	+	+
Amygdalin	80	+	59	-	-	+	41	-	-	-
Arbutin	60	+	41	-	-	+	41	-	-	+
Esculin ferric citrate	100	+	100	+	-	+	100	+	+	+
Salicin	60	+	76	+	-	+	41	+	-	+
D-cellobiose	100	+	59	+	+	+	53	+	+	-
D-maltose	40	+	65	+	+	+	47	+	-	+
D-lactose	60	-	47	-	-	-	18	+	+	-
D-melibiose	20	-	6	+	-	+	12	-	-	-
D-saccharose	80	-	71	+	-	+	59	+	+	-
D-trehalose	80	-	59	+	-	+	71	+	+	+
Inulin	0	+	6	+	-	-	0	-	-	-
D-raffinose	20	+	18	+	-	+	0	+	-	-
Amidon	0	+	24	+	-	+	41	+	-	-
Glycogen	0	+	12	+	-	+	41	+	+	-
Gentiobiose	40	+	0	-	-	-	12	+	-	-
D-turanose	0	-	18	+	-	+	0	-	-	-
L-fucose	20	-	0	-	-	-	6	-	-	-
Potassium gluconate	20	-	0	-	-	-	0	-	-	-

* number isolates; + positive; - negative.

Table 3. Minimum inhibitory concentration of heavy metals of *Bacillus* isolates from steel plant wastes

Metal	MIC (mM)		
	Range	MIC_{50}	MIC_{90}
Cu	0.001-4	0.5	4
Pb	0.001-4	2	4
Zn	0.001-4	1	2
Ni	0.001-2	0.25	1
Co	0.001-1	0.25	0.25
Hg	0.001-1	≤ 0.001	0.025
Ag	0.001-1	≤ 0.001	≤ 0.001

Cu, copper sulphate; Pb, lead nitrate; Zn, zinc sulphate; Ni, nickel chloride; Co, cobalt chloride; Hg, mercury bichloride; Ag, silver nitrate.

Figure legends

Figure 1. Phylogenetic tree of members of the genus *Bacillus*, based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method, and genetic distances were computed by using Kimura's model. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. *Alicyclobacillus acidocaldarius* (AB089859) was used as outgroup.

Figure 2. UPGMA cluster analysis based on ARDRA patterns showing the relationship between the isolates and type strains.

Figure 3. (A) ITS- and (B) tDNA-PCR fingerprinting of isolates and reference strains. Lane M, molecular size marker (1 Kb Plus-Invitrogen). Lanes 1 to 40 (1BFS, 2BFS, 3BFS, 4BFS, 5BFS, 6BFS, 7BFS, 8BFS, 14BFS, 15BFS, 16BFS, 37BFS, 38BFS, 9BFS, 10BFS, 11BFS, 12BFS, 13BFS, 40BFS, 17BFS, 18BFS, 35BFS, 20BFS, 21BFS, 22BFS, 23BFS, 24BFS, 29BFS, 30BFS, 31BFS, 32BFS, 26BFS, 27BFS, 28BFS, 34BFS, 19BFS, 25BFS, 39BFS, 33BFS and 36BFS, respectively); lanes 41 to 46 ATCC strains (*B. pumilus* 7061, *B. cereus* 11778, *B. subtilis* 6633, *B. sphaericus* 14577, *B. licheniformis* 14580 and *B. amyloliquefaciens* 23842).

Figure 4. (A) ERIC-PCR fingerprinting patterns of the isolates and reference strains. Lane M, molecular size marker (1 Kb plus-Invitrogen). Lanes 1 to 40 (1BFS, 2BFS, 3BFS, 4BFS, 5BFS, 6BFS, 7BFS, 8BFS, 9BFS, 10BFS, 11BFS, 12BFS, 13BFS,14BFS, 15BFS, 16BFS, 17BFS, 18BFS, 19BFS, 20BFS, 21BFS, 22BFS, 23BFS, 24BFS, 25BFS, 26BFS, 27BFS, 28BFS, 29BFS, 30BFS, 31BFS, 32BFS, 37BFS, 38BFS, 39BFS, 40BFS, 33BFS, 34BFS, 35BFS, and 36BFS, respectively); lanes 41 to 46 ATCC strains (*B. pumilus* 7061, *B. cereus* 27877, *B. sphaericus* 14577, *B. licheniformis* 14580, *B. amyloliquefaciens* 23842 and *B. subtilis* 6633). (B) UPGMA cluster analysis of isolates and reference strains based on ERIC-PCR.

Figure 5. (A) $(GTG)_5$-PCR and (B) BOX-PCR fingerprinting patterns of the isolates and reference strains. Lane M, molecular size marker (1 Kb Plus-Invitrogen). Lanes 1 to 19 (1BFS, 2BFS, 3BFS, 4BFS, 5BFS, 6BFS, 7BFS, 8BFS, 9BFS, 10BFS, 11BFS, 12BFS, 13BFS,14BFS, 15BFS, 16BFS, 40 BFS, 17BFS, 18BFS); lane 20 and 21 (*B. pumilus* 7061 and *B. subtilis* 6633); lane 22 to 34, (19BFS, 20BFS, 21BFS, 22BFS, 23BFS, 24BFS, 25BFS, 26BFS, 27BFS, 28BFS, 29BFS, 30BFS, 31BFS); lane 35 and 36 (*B. cereus* 27877 and *B. sphaericus* 14577); lane 37 to 44 (32BFS, 33BFS, 34BFS, 35BFS, 36BFS, 37BFS, 38BFS

and 39BFS); lane 45 and 46 (*B. licheniformis* 14580 and *B. amyloliquefaciens* 23842, respectively).

Figure 6. UPGMA cluster analysis of isolates and reference strains based on multi rep-PCR global matrix of ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR. ERIC patterns. Numbers in parentheses identify the 16S rDNA sequence based phylogeny clusters obtained with the isolates.

Figure 7. Dendrogram constructed by UPGMA with the *Bacillus* isolates according to heavy metal suscepti susceptibility profiles.

Figure 1

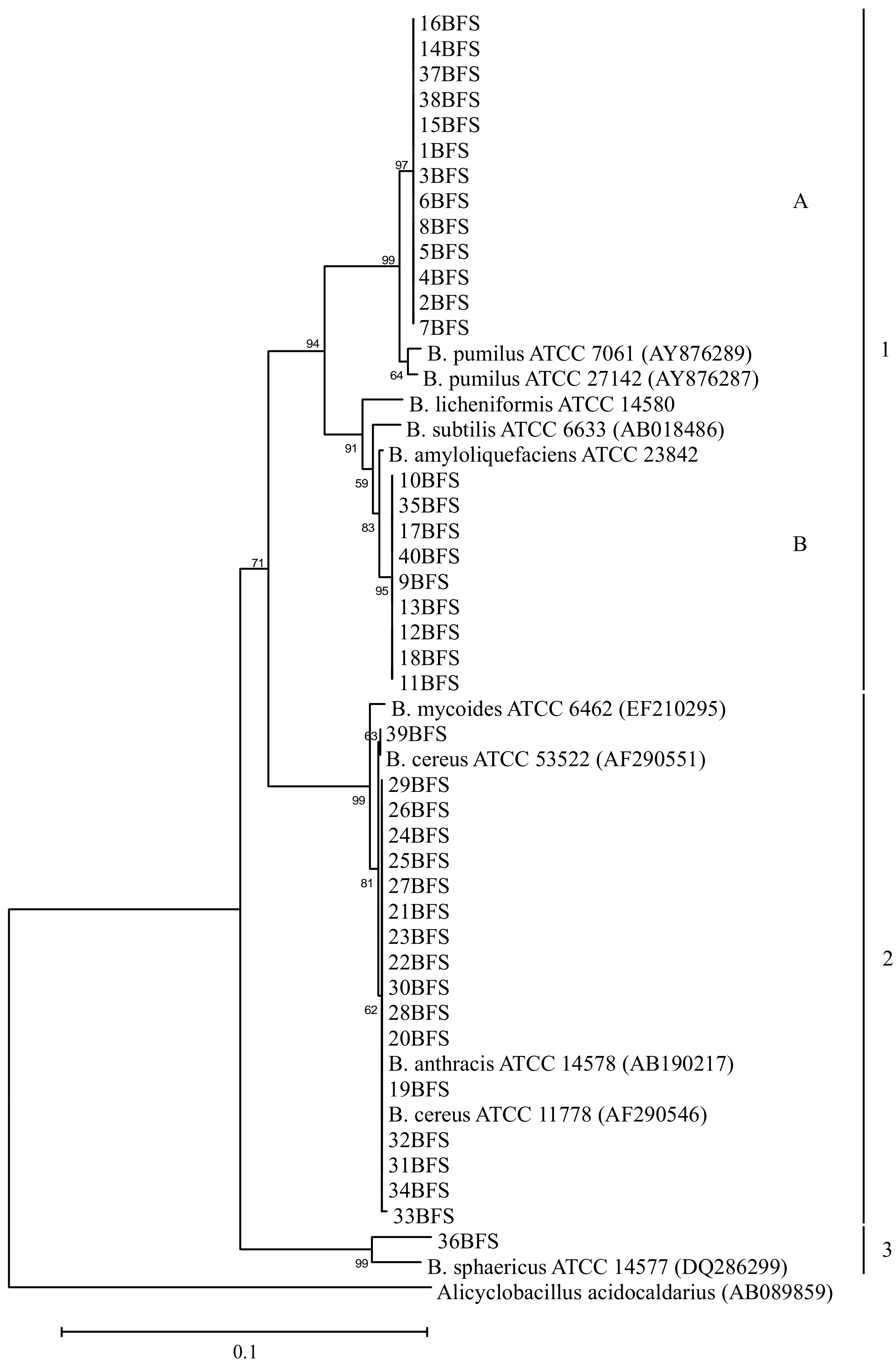


Figure 2

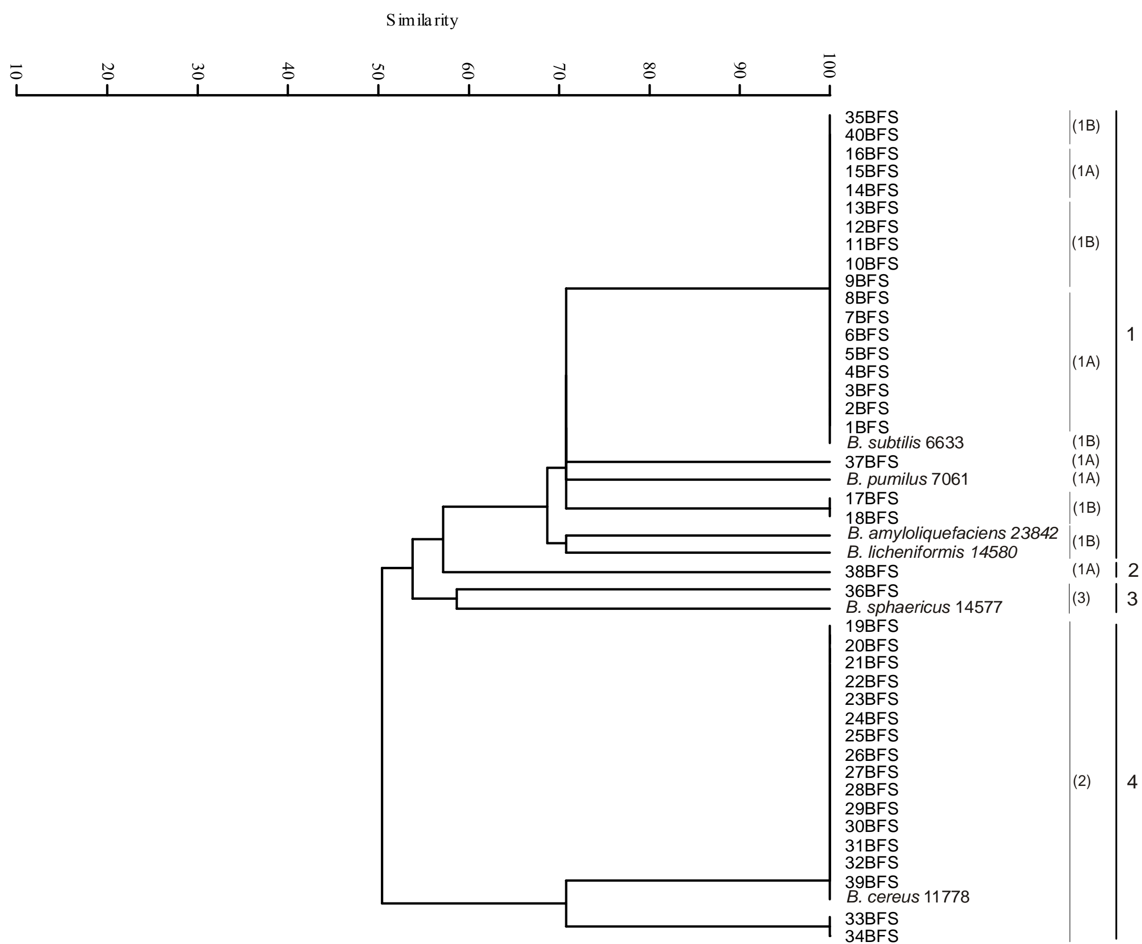


Figure 3

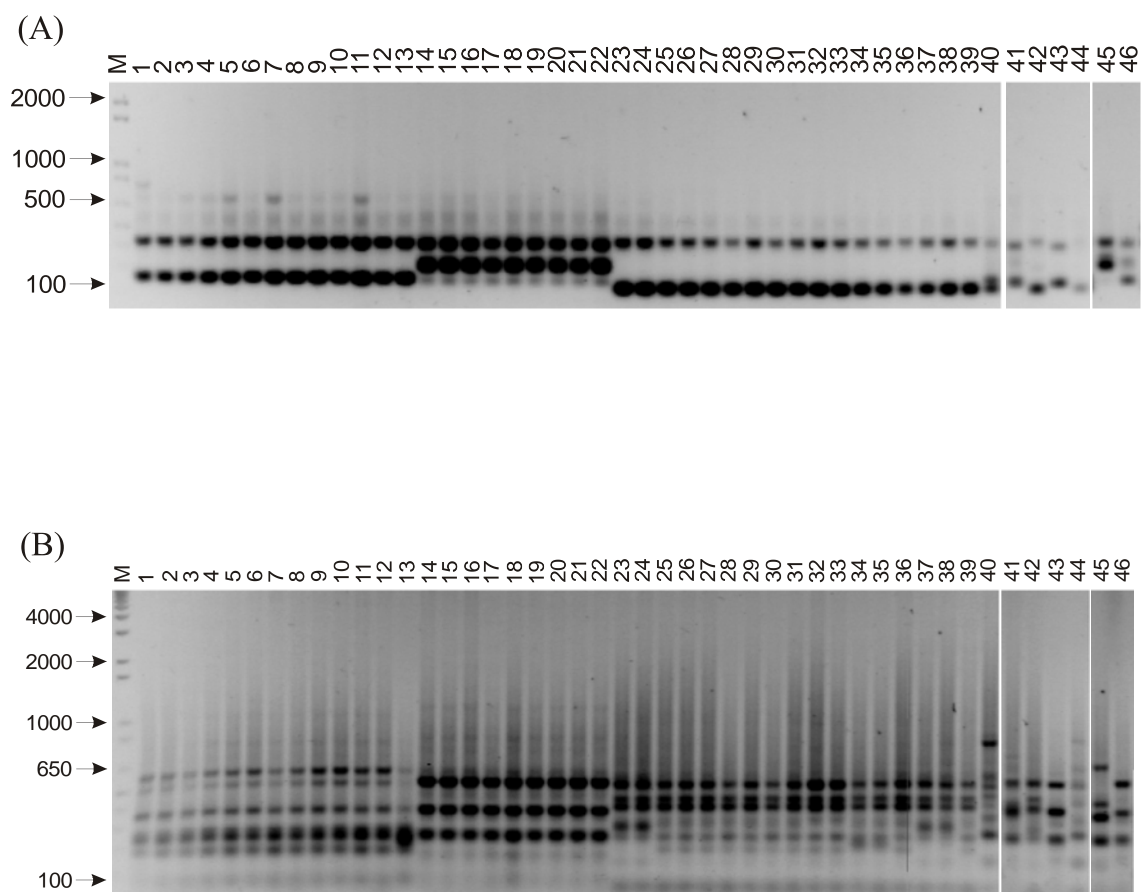
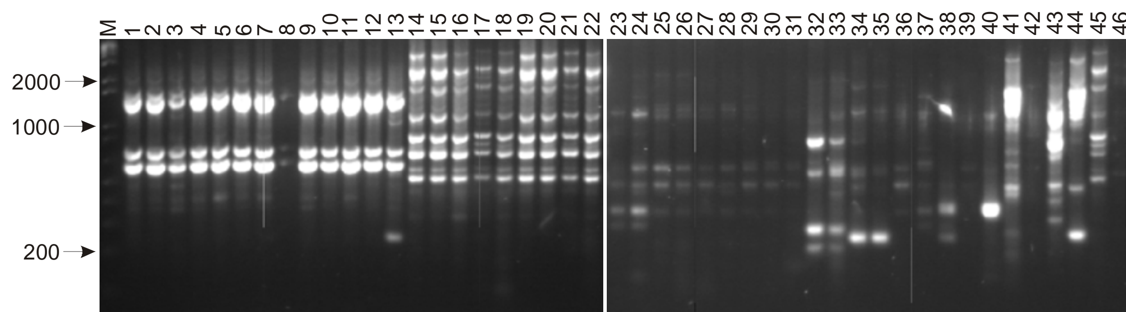


Figure 4.

(A)



(B)

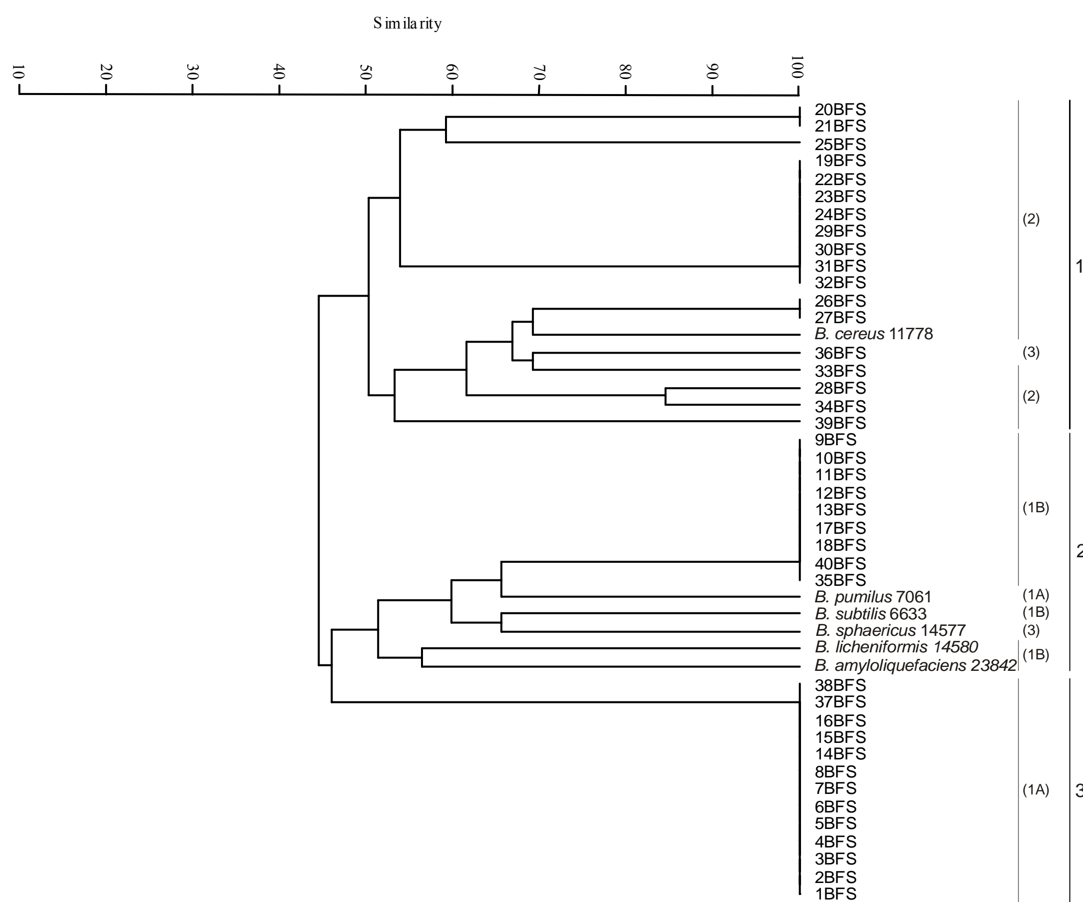


Figure 5.

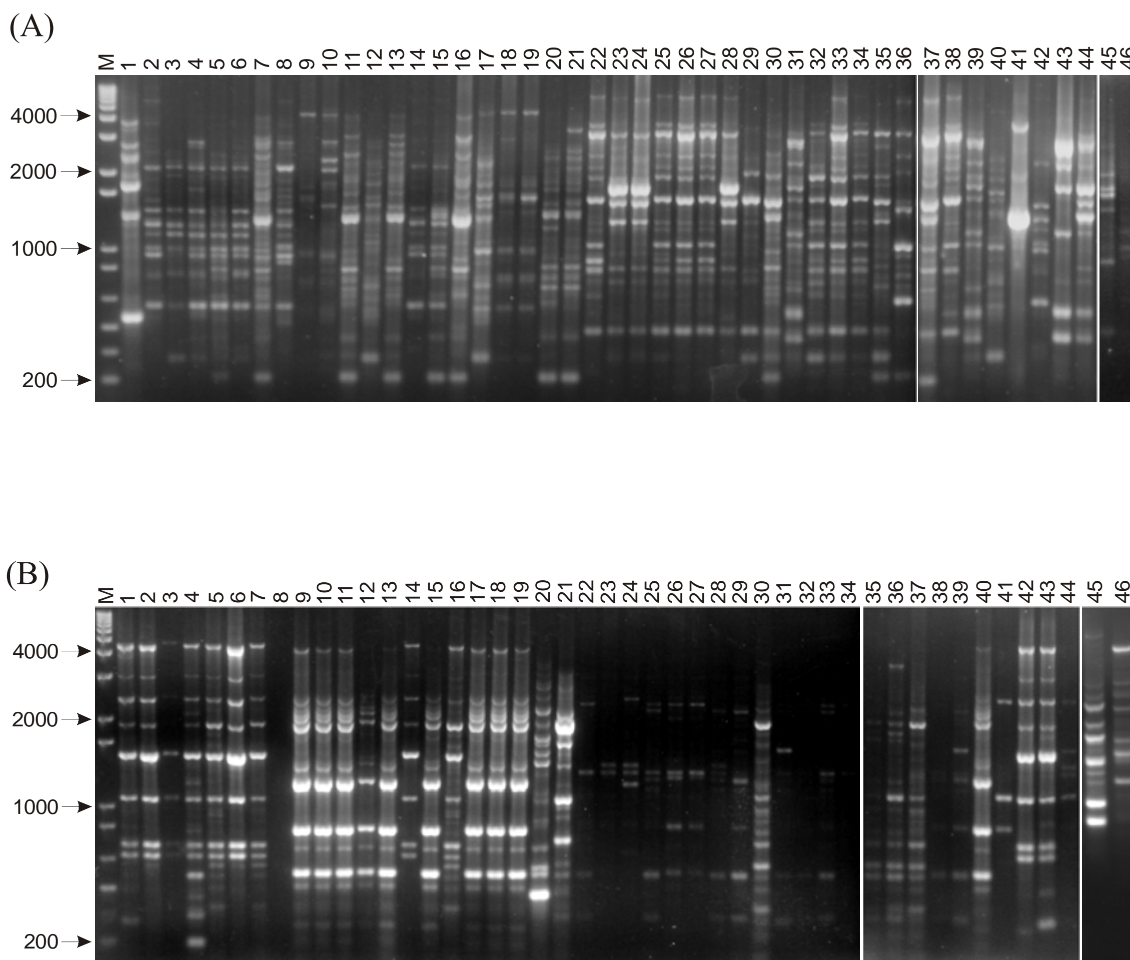


Figure 6.

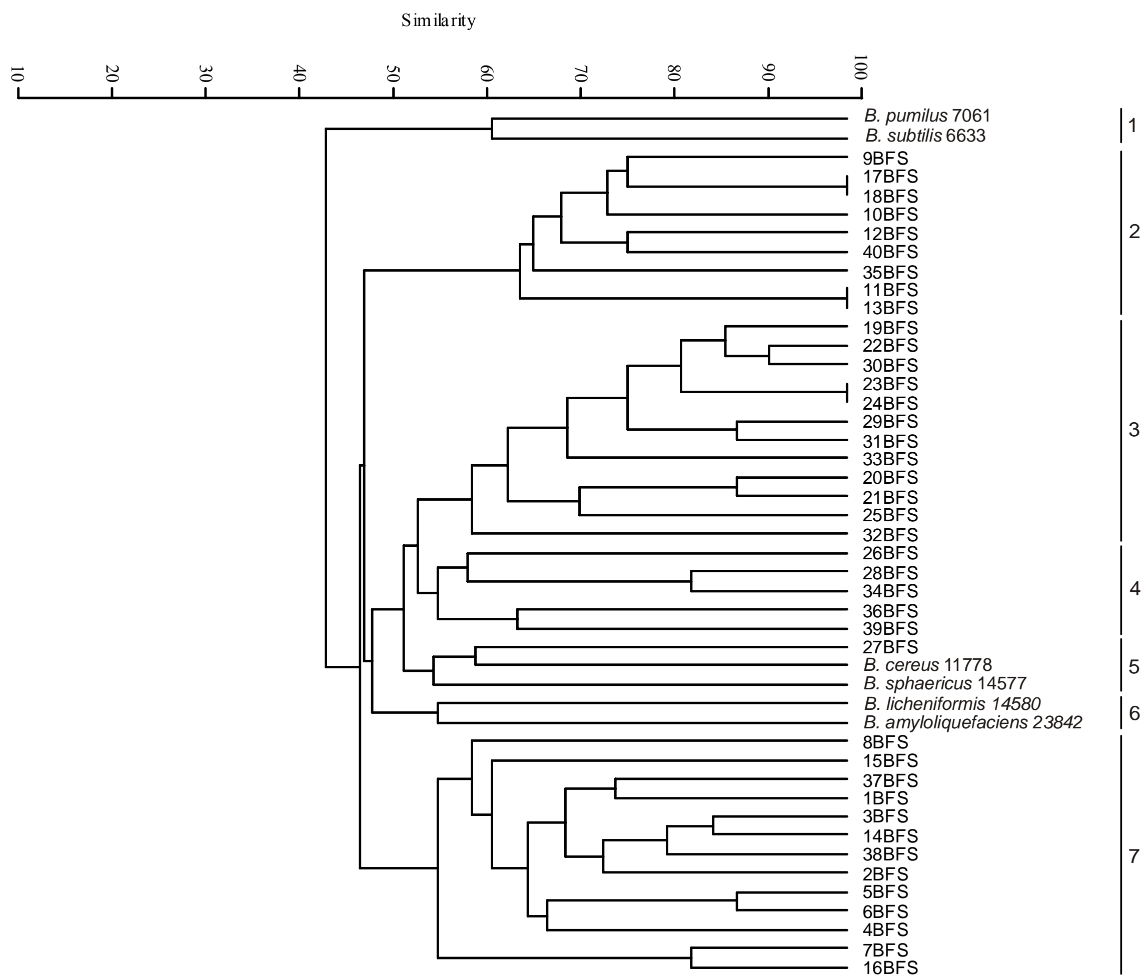
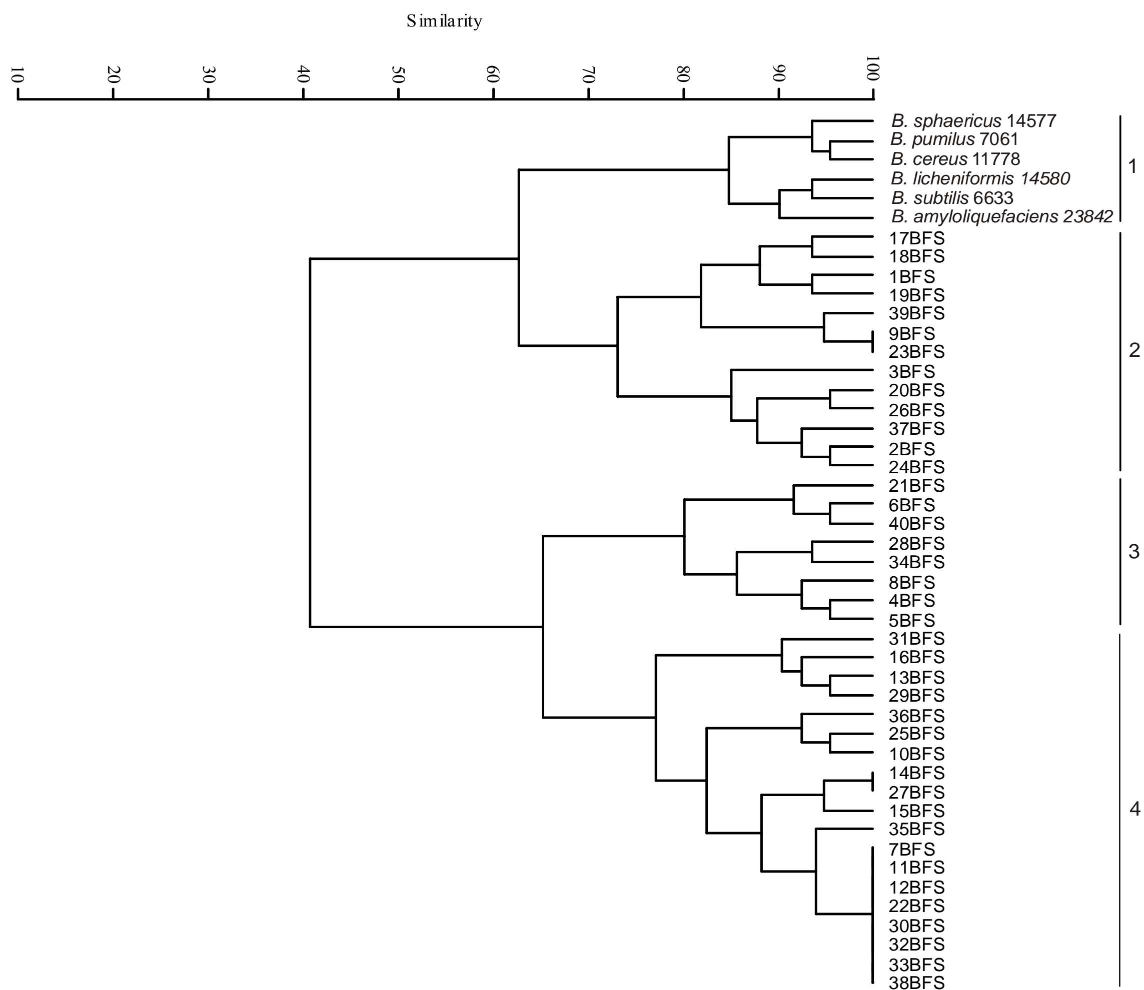


Figure 7



CAPÍTULO III

Molecular bacterial diversity and distribution in waste from a steel plant

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Molecular bacterial diversity and distribution in waste from a steel plant

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Abstract

We characterized the bacterial diversity of newly-produced waste (NPW) and waste deposited (WD) in a restricted land area, generated in the siderurgic industry, using the 16S rDNA clone library approach.

A total of 212 partial-length sequences were analyzed, revealing 123 distinct operational taxonomic units (OTUs) determined by the DOTUR program to 97% sequence similarity. Phylogenetic analysis of bacterial 16S rDNA sequences from the NPW and WD libraries demonstrated that *Gamma*-, *Beta*- and *Alphaproteobacteria*, *Actinobacteria*, *Planctomycetes*, *Firmicutes* and *Bacteroidetes* were represented in the two libraries. *Deltaproteobacteria*, *Acidobacteria*, *Chloroflexi*, *Deinococcus-thermus*, *Gemmatimonadetes* and candidate divisions OP10 and OD1 were present in the WD library and *Nitrospira* was only found in the NPW library. The abundance of sequences affiliated with *Gammaproteobacteria* was high in both libraries. Six OTUs previously unclassified may represent novel taxa. Based on diversity indices (Simpson, Shannon-Weaver, Chao1 and ACE), the SD library had a higher diversity. LIBSHUFF comparisons of the composition of the two libraries showed that they were significantly different. These results indicate that the bacterial communities in siderurgic waste are complex and require further investigation.

Key words: 16S rDNA, clone library, bacterial diversity, siderurgic waste

Introduction

It is well known that bacterial communities are essential in the function of all ecosystems playing a crucial role in the food chain and metal cycling in many environments. Current estimates indicate that more than 99% of the microorganisms present in many natural environments cannot be cultured with presently available technologies and therefore are not accessible for basic research or biotechnology (Rodriguez-Valera 2002; Streit and Schmitz 2004; Sharma et al. 2005). Regarding the great diversity presented by uncultured microorganisms, the culture limitations and difficulties of manipulation of microorganisms in laboratory environments, metagenomic techniques have been applied to environmental samples (Amann et al. 1995; Pace 1997; Eyers et al. 2004; Handelsman 2004). The construction of genomic libraries using the amplification of 16S rRNA gene and other functional genes in order to study the total biodiversity contributes to expanding the knowledge on bacterial phylogeny and ecology. In general, the use of 16S rRNA gene clone libraries to map the diversity of uncultured bacteria has provided much information about the taxa present in an environmental sample (Handelsman 2004; Cowan et al. 2005).

Mining is a basic activity that utilizes natural resources and is fundamental for economics and social development of many countries. The amount of waste generated by mining and mineral ore processing exceeds 1.8 billion tons annually. It is known that the steel industries generate about 700 kg of waste per ton of steel produced. These wastes include gases, dust solutions, sludge and mineral materials such as mine waste, ore processing and leach residues (Ledin and Pedersen 1996) containing high concentrations of Zn, P, K, S and Cr^{+6} that make their recycling difficult (Li and Rutherford 1996), and constituting a potential source of contamination of the environment (Ledin and Pedersen 1996). Some wastes are treated by chemical processes and are used for other purposes, but most are stored or discarded into the environment (Araújo 1997). This discarded material causes a considerable environmental impact.

Despite the major impact of the steel industry on the environment, the bacterial community in steel plant waste remains poorly understood. Thus, the objective of this study was to investigate the uncultured bacterial diversity present in newly-produced waste (NPW) and waste deposited (WD) for 4 years in a restricted land area. To this end, the bacterial communities were analyzed using a phylogenetic approach based on metagenomic clones of the 16S rDNA libraries. The composition of the communities was also compared using phylogenetic statistics methods to assess the similarity of the sequence libraries. This is the

first molecular characterization of uncultured members of the bacterial community associated with siderurgic waste.

Materials and methods

Study area

The steel plant of Acesita-Cia Aços Especiais Itabira (ArcelorMittal) is located in Timóteo (Minas Gerais State, Brazil). This industry produces grain oriented silicon steel, no oriented grain silicon steel, carbon steels and stainless steels (series 300 and 400). Another studied area was the deposit site in Timóteo (Minas Gerais State, Brazil), which receives more than 1 ton of waste daily from the same steel plant.

Environmental sampling and DNA extraction

In November 2006, NPW and WD samples were collected in triplicate using sterile bottles. The metagenomic DNA was extracted from NPW and WD samples using the PowerMax^{TM} Soil DNA Isolation Kit (MoBio Laboratories, Inc), according to the manufacturer's instructions.

PCR amplification and clone library construction

Polymerase chain reaction amplification of the 16S rRNA genes was performed using primers 8F (5'-AGAGTTTGATYMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Lane 1991). Amplification was carried out in a 20 µL reaction mixture containing 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 50 ng DNA template and 1 U Taq DNA polymerase (Phoneutria, Brazil) with reaction buffer supplied by the manufacturer. The program used was as described by Don et al. (1991). 16S rRNA gene amplicons were visualized on 1% (w/v) agarose gel run in Tris borate–EDTA buffer, stained with ethidium bromide and purified directly with the Qiaquick PCR purification kit (Qiagen, Valencia, Calif.). Purified amplicons were cloned into the TOPO TA cloning system according to the manufacturer's instructions (Invitrogen) and transformed into competent *Escherichia coli* MC1061 cells. At least 300 positive clones from each clone library (selected by blue and white screening) were transferred to 96-well plates and incubated overnight at 37ºC in Luria-

Bertani medium containing ampicillin (100 µg mL^{-1}) and 20% glycerol and stored at –80°C. Plasmids containing 16S rDNA inserts were extracted using a standard alkaline lysis method (Ausubel *et al*., 1990) and inserts were subsequently PCR-amplified with primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) specific for the vector.

16S rRNA gene sequencing and phylogenetic analysis

Sequencing was performed with the MegaBACE 1000 capillary sequencer (Amersham Biosciences) using standard protocols with a DYEnamic ET dye terminator kit (Amersham Biosciences). Sequences (600 pb) were compared against sequences held in the Ribosomal Database Project (RDP) using Compare Library and against sequences held in GenBank, using BLASTN to search for similar homologous sequences of 16S rRNA gene partial sequences in clone libraries. To accomplish this, the 16S rRNA gene sequences were base-called, checked for quality, aligned and analyzed using Phred v.0.020425 (Ewing and Green 1998), Phrap v.0.990319 (Green 1994) and Consed 12.0 (Gordon et al. 1998). To detect and omit chimeric DNAs, the CHECK-CHIMERA program of the RDP (Maidak et al. 2001) was used. Phylogenetic relationships were inferred by MEGA 3.1 (Kumar et al. 2004) using the neighbor-joining method (Kimura 1980; Saitou and Nei 1987) and the Kimura 2-P model of sequence evolution.

DOTUR software (Schloss & Handelsman, 2005) was used to assign sequences to operational taxonomic units (OTUs). Duplicate sequences were grouped into an OTU at distance values of 0.03 (≥97% sequence similarity) corresponding to the species level and only one sequence was used for further analysis (Stackebrandt and Goebel 1994). The nucleotide sequences generated were deposited in the Genbank database with accession numbers EU447446 to EU447657.

Estimation of bacterial diversity and clone library similarity

Rarefaction analysis was performed and diversity indices were calculated with the DOTUR program (Schloss and Handelsman 2005) to characterize the bacterial diversity of the waste samples. The diversity indices included the Chao1 richness estimates, the abundance-based

coverage estimator (ACE), the Simpson diversity index ($1/D$), the Shannon-Weaver diversity index and the number of OTU analysis. Rarefaction curves having 95% confidence intervals were constructed by comparing the number of clones in each 16S rRNA gene library. The coverage (C) of each clone library, a measure of captured diversity, was calculated according to the equation: $C = 1-(n/N)$, where n is the number of different OTU types from a clone library that were encountered only once and N is the total sequenced number of clones in the library (Good 1953).

The phylogenetic diversity in both libraries was compared using LIBSHUFF software (Singleton et al. 2001). This program uses Good's coverage formula (Good, 1953) to generate homologous and heterologous coverage curves (C_X and C_{XY}, respectively) for the two 16S rDNA libraries (X and Y) at a similarity level or evolutive distance (D). To determine whether the coverage curves $C_X(D)$ and $C_{XY}(D)$ were significantly different, the Crámer-von Mises (Pettitt, 1982) test was used to determine the distance between the two curves. The curves were then considered significantly different if $P < 0.05$.

Results

In total, 82 and 152 clones displaying 16S rRNA gene inserts were sequenced from DNA extracted from NPW and WD libraries, respectively. Sixteen NPW and six WD clones were identified as chimeric sequences and excluded from further analyses, resulting in 66 and 146 clones for the libraries, respectively. A total of 123 OTUs were identified, 22 and 101 from NPW and WD libraries, respectively. Comparative analysis of the retrieved sequences showed that all clones were grouped within the Bacteria domain.

Phylogenetic analysis

The composition of the bacterial communities and the frequency of occurrence of each of the identified phyla are shown in Figure 1. BLAST and RDP analyses associated 116 OTUs to 12 phyla and seven OTUs to unclassified groups; most of the sequences were closely related to sequences of uncultured bacterial clones (Tables 1 and 2).

The NPW library contained 22 OTUs, which were classified into six phyla: *Proteobacteria* and *Firmicutes* and the minority phyla *Actinobacteria*, *Planctomycetes*, *Bacteroidetes* and *Nitrospira* represented by only one clone (Table 1). The WD library presented a higher diversity and comprised 101 OTUs, which were grouped into 11 phyla:

Chloroflexi, *Gemmatimonadetes*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes* and *Deinococcus-thermus*; candidate divisions OP10 and OD1 were minority phyla represented by one to six clones, whereas the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* predominated (Table 2).

Proteobacteria dominated both libraries including 12 and 62 OTUs represented by 52 NSWP clones and 96 WD clones, respectively (Fig. 1 and Tables 1 and 2). *Gammaproteobacteria* were the most commonly sampled group followed by *Alpha-*, *Beta-*, and *Deltaproteobacteria* classes (Fig. 2). Seven OTUs (44 of the 66 clones in the NPW library) and 31 OTUs (53 of the 146 clones in the WD library) were affiliated to families *Enterobacteriaceae*, *Pseudomonadaceae*, *Moraxellaceae* and *Xanthomonadaceae* (Fig. 3a). The remaining proteobacterial clones (*Alpha*, *Beta*, and *Delta*) comprised between 2% and 21%. The *Betaproteobacteria* presented the highest values of 13% (NPW) and 18% (WD) with four OTUs (NPW) affiliated to the families *Comamonadaceae* and 13 OTUs (WD) associated with families *Hydrogenophilaceae*, *Incertae sedis 5*, *Rhodocyclaceae* and *Comamonadaceae*. *Alphaproteobacteria* were numerically dominant, including 14 OTUs (20 of the 146 total clones). Among them, five OTUs were phylogenetically associated with the families *Phyllobacteriaceae*, *Caulobacteraceae*, *Rhodospirillaceae* and *Acetobacteraceae*. Nine remaining OTUs could not be assigned to an existing family and were only classified to class level. Only one OTU (1 of the 66 total clones) was classified as *Alphaproteobacteria* in the NPW library. The class *Deltaproteobacteria* occurred exclusively in the WD clone library and was represented by only one OTU (3 of the 146 total clones). OTUs (WD60 and WD61), unclassified *Proteobacteria* (Table 2), were affiliated to *Deltaproteobacteria* (Fig. 3a).

Firmicutes was represented by 11 OTUs (17 of the 212 total clones), which shared a phylogenetic affiliation with the class *Bacilli* (Fig. 3b). Eleven OTUs represented by 15 clones were affiliated with *Actinobacteria*. Information concerning the additional phyla is given in the phylogenetic tree (Fig. 3b). In addition, one (WD99) of seven OTUs for which no clear phylogenetic affiliation could be inferred by BLAST and RDP analyses, was clearly associated with clade of *Gemmatimonadetes* by phylogenetic tree analysis (Fig. 3b). The six OTUs remaining had no new phylogenetic associations. Thus, these bacteria have the potential to be classified as a new phylum.

Species richness and diversity

Rarefaction curves were obtained by plotting the number of OTUs observed against the

number of clones sequenced (Fig. 4). A decrease in the rate of OTU detection was observed on the NPW curve indicating that the major part of the diversity in the library was detected. This result was further supported by calculating the coverage of the library, which was 75%. On the other hand, the WD curve did not reach a plateau indicating that the diversity in this library is high (Fig. 4). The analysis of the rarefaction curves is also in agreement with the diversity indices calculated with the Chao1 and ACE estimators (Table 3). Both Chao1 and ACE non-parametric richness estimators predicted that the OTU richness of the WD library was far greater than that observed for the NPW library. The values of the Shannon-Weaver index obtained for the NPW and WD libraries were 2.27 and 4.4, respectively (Table 3), showing a considerable difference between the two libraries and indicating that there is a high species diversity in the WD sample.

Comparison of the bacterial compositions between the clone libraries

In order to determine whether the structures of the two bacterial communities were the same or different, we applied the LIBSHUFF algorithm (Fig. 5). Pairwise comparisons of each NPW clone library to every other library revealed that the bacterial community composition differed significantly between the two libraries (NPW, P =0.008 and WD, P= 0.001 for each combination; Fig. 5). More information was obtained on the differences between clone libraries by examining the distribution of $(C_X - C_{XY})^2$ as a function of evolutionary distance (D). The coverage curves for representative pairs of clone libraries clearly showed major differences at low levels of genetic distance ($D < 0.2$; Fig. 5) and these differences were clear for WD (Fig. 5B). LIBSHUFF analysis also indicated that the WD library was characterized by deeper divergence than the NPW library.

Discussion

It is notoriously difficult to isolate and cultivate bacteria representative of bacterial communities present in natural environments, especially in extreme environments. Hence, we use the metagenomic library approach that can provide information on the phylogeny and distribution of uncultured bacteria. With 16S rRNA gene sequence phylogenetic analysis, we were able to characterize a number of bacteria inhabiting waste environments and a great diversity of bacteria was detected. To the authors' knowledge, this is the first metagenomic library of waste.

The constructed libraries were mainly composed of *Proteobacteria* (Fig. 1). The majority of the sequences retrieved were closely affiliated to *Beta-, Delta-* and *Gammaproteobacteria* classes (Fig. 2) that include very diverse phenotypes, lifestyles, and trophic capabilities (Kersters et al. 2003). Moreover, other sequences were associated with phylogenetic groups such as *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Gemmatimonadetes, Nitrospira*, *Firmicutes*, *Acidobacteria*, *Chloroflexi*, *Deinococcus-Thermus*, *Planctomycetes* and the candidate divisions OP10 and OD1. The great diversity of bacteria indicates that there is a wide spectrum of metabolic activities being carried out in these environments.

Although *Proteobacteria* were dominant in the bacterial communities, differences were observed in the relative distribution and abundance of the different proteobacterial classes, with predominance of *Gammaproteobacteria*. The *Proteobacteria* form the largest and most variable group of culturable bacteria, comprising the uncultured *Gamma-* and *Alphaproteobacteria* mainly found in habitats of acid metal leaching (Hugenholtz et al. 1998). Thus, our results are in agreement with the literature since high incidences were found of *Gamma-* (NPW, 85% and WD, 55%) and *Alphaproteobacteria* reaching 21% in the WD library. We also found sequences associated with unclassified proteobacteria, indicating the presence of novel bacterial species.

Actinobacteria and *Firmicutes* phyla are considered high and low GC-content Gram-positive, respectively. The relative numbers of clones affiliated to *Actinobacteria* are higher in the WD library than in the NPW library. They have been reported as minor components of bacterial communities in xenobiotic-contaminated natural environments and play important roles in the degradation of xenobiotic compounds (Greene et al. 2000). In contrast, the relative number of clones associated with *Firmicutes* was higher in the NPW library than in the WD library. Members of this phylum are highly diverse in phenotypic characteristics due to promiscuous plasmid exchange across species and genera (Dunbar et al. 2002; Ventura et al. 2007).

Sequences affiliated with *Nitrospira* were only observed in the NPW library. This phylum contains a limited number of cultivable bacteria with varied metabolic capacities, including lithotrophic, nitrification, sulfate reduction, and oxidation of Fe (II) (Garrity and Holt 2001). *Bacteriodetes* are ubiquitous in a variety of environments such as sediments; soil and sea water and are typical degraders, hydrolyzing complex organic molecules or fermenting under strict anaerobic conditions (Shah et al. 2005). The presence of the *Deinococcus-thermus* phylum is especially interesting since *Deinococcus* species have been

isolated from extreme environments such as radioactive residues and thermal springs (Makarova et al. 2001). Bacteria from the *Acidobacteria* phylum have been described based on 16S rDNA sequences in a wide range of environments (abundant in soil and sediments) and their physiology and ecology are little known (Hugenhotz et al. 1998; Barns et al. 1999; Quaiser et al. 2003; Janssen, 2006). Members of the *Chloroflexi* phylum are filamentous anoxygenic phototrophic bacteria that can be thermophilic, and they are frequently found in hotsprings (Hanada, 2003), but environmental studies have revealed several phylotypes of these green non-sulfur bacteria in anoxic zones of systems of stratified freshwater (Gich et al. 2001). Many *Planctomycetes* species are facultative aerobic chemoorganotrophs, which grow by fermentation or respiration, but others are strict anaerobic autotrophs that carry out the anaerobic oxidation of ammonia using nitrite (van Niftrik et al. 2004).

Several statistical approaches can be used to analyze bacterial diversity estimated from the number of species found in relatively small samples (Hughes et al. 2001). Clonal diversity was evaluated using Simpson's and Shannon's diversity indices (Table 1). Both indices indicated a high diversity level of the bacterial communities represented by the NPW and WD libraries. The Shannon index is determined by OTU richness while the Simpson index is highly influenced by the abundance of the most common OTU found in the sample (Hughes and Bohannan 2004). Regarding the species richness estimated by the ACE and Chao1 estimators, the values were higher for the WD bacterial community. The coverage values retrieved for these libraries (WD, 46% and NPW, 75%) can be considered to be low and indicate that both libraries were only partially sampled and reflect a higher diversity of the WD. The rarefaction curve (Fig. 4) suggests that sampling of more clones is needed in order to achieve a better coverage of the bacterial diversity present in the WD sample. However, the LIBSHUFF analysis (Fig. 5) shows that the number of clones sequenced in each library was sufficient to retrieve the most abundant deep phylogenetic groups. For values of evolutionary distance (*D*) higher than 0.2 (cut-off value for group sequences at the phylum level), the homologous coverage was approximately 84% for the WD library and more than 90% for the NPW library.

One of the drawbacks of analysis based on OTUs is the reduction in potentially valuable information relative to the phylogenetic relationships that are lost. That information is not lost in phylogenetic analysis using the LIBSHUFF software that compares the genetic diversity found in different communities (Singleton et al. 2001). As a consequence, the LIBSHUFF analysis is complementary to the richness estimators since it does not determine OTUs and thus allows the determination of differences in the taxonomic composition of each community

(Martin 2002). In this study, the LIBSHUFF analysis revealed significant differences between the two samples indicating a strong heterogeneity in the phylogenetic composition of the two libraries. This result indicates that a significant population composition shift occurred in the bacterial communities during the period of adaptation of the WD bacterial community.

Considering that understanding the taxonomic composition of environmental communities is an important indicator of their evolution and environmental adaptation, these results can be used as a starting point for more in-depth studies, with the potential to mediate industrial processes regarding ecological and economic aspects.

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References

Amann, R.I., Ludwig, W., and Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of microbial cells without cultivation. Microbiol. Rev. **59**(1): 143–169.

Araújo, L.A. 1997. Manual de Siderurgia, Volume I, Ed. ABM, S. Paulo, Brasil.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, H., and Strujil, K., Eds., 1990. Current Protocols in Molecular Biology, vol. 1. Greene Publishing Associates and Wiley–Interscience, New York, NY.

Barns, S.M., Takala, S.L., and Kuske, C.R. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. **65**(4): 1731–1737.

Cowan, D., Meyer, Q., Stafford, W., Muyanga, S., Cameron, R. and Wittwer, P. 2005. Metagenomic gene discovery: past, present and future. Trends Biotechnol. **23**(6): 321–329.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19**(14): 4008.

Dunbar, J., Barns, S.M., Ticknor, L.O., and Kuske, C.R. 2002. Empirical and theoretical

bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. **68**(6): 3035–3045.

Ewing, B., and Green, P. 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8**(3): 186–194.

Eyers, L., George, I., Stenuit, B., Agathos, S.N. and Fantroussi, S. 2004. Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. Appl. Microbiol. Biotechnol. **66**(2): 123–130.

Garrity, G.M., and Holt, J.G. 2001. The road map to the manual. In: Krieg, NR.; Holt, JG. (Ed.). Bergey's manual of systematic bacteriology. Baltimore: Williams & Wilkins, p. 119-154.

Gich, F., Garcia-Gil, J., and Overmann, J. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177**(1): 1–10.

Good, I.J. 1953. The population frequencies of species and the estimation of population parameters. Biometrika **40**(3-4): 237–262.

Gordon, D., Abajian, C., and Green, P. 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8**(3): 195–202.

Green, P. (1994) PHRAP documentation. http://www.phrap.org.

Greene, E.A., Kay, J.G., Jaber, K., Stehmeier, L.G., and Voordouw, G. 2000. Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. Appl. Environ. Microbiol. **66**(12): 5282–5289.

Hanada, S. 2003. Filamentous anoxygenic phototrophs in hot springs. Microbes Environm. **18**(: 51–61.

Handelsman, J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. **68**(4): 669–685.

Hugenholtz, P., Brett, M., and Pace, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180**(24): 4765–4774.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., and Bohannan, B.J.M. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. **67**(10): 4399–4406.

Hughes, J.B., and Bohannan, B.J.M. 2004. Application of ecological diversity statistics in microbial ecology. In Molecular Microbial Ecology Manual, vol. 2, 2nd edn, pp. 1321–1344. Edited by G. A. Kowalchuk, F. J. de Bruijn, I. M. Head, A.D.L Akkermans and J. D. van Elsas. Dordrecht: Kluwer Academic Publishers.

Janssen, P.H. 2006. Identitying the dominant soil bacterial taxa in libraries of 16S rRNA and

16S rRNA genes. Appl. Environ. Microbiol. **72**(3): 1719–1728.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., and Stackebrandt, E. 2003. Introduction to the *Proteobacteria*. *In* M. Dworkin et al. (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.12, 28 March 2003. [Online.] Springer-Verlag, New York, N.Y. http://link.springer-ny.com/service/books/10125.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**: 111–120.

Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform **5**(2):150–163.

Lane, D.J. 1991. 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics (Stackebrandt E & Goodfellow M, eds), pp. 115–175. Wiley, Chichester.

Ledin, M., and Pedersen, K. 1996. The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes. Earth Sci. Rev. **41**(1-2): 67–108.

Li, LY, and Rutherford, G.K. 1996. Effect of bauxite properties on the settling of red mud. Inter. J. Min. Proces. **48**(3-4): 169–182.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, Jr. C.T., Saxman, P.R. and Farris, R.J. 2001. The RDP-II (Ribosomal database project). Nucleic Acids Res **29**(1): 173–174.

Makarova, K.S., Aravind, L., Wolf, Y.I., Tatusov, R.L., Minton, K.W., Koonin, E.V. and Daly, M.J. 2001. Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. Microbiol. Mol. Biol. Rev. **65**(1): 44–79.

Martin, A.P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol **68**(8): 3673–3682.

Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. Science **276**(5313): 734-739.

Pettitt, A.N. 1982. Cramer-von Mises statistic, in: S. Kotz, N.L. Johnson (Eds.), Encyclopedia of Statistical Sciences, Wiley-Interscience, New York, N.Y, pp. 220-221.

Quaiser, A., Ochsenreiter, T., Lanz, C., Schuster, S.C., Treusch, A., Eck, J. and Schleper, C. 2003. *Acidobacteria* form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. Mol. Microbiol. **50**(2): 563–575.

Rodríguez-Valera, F. 2002. Approaches to prokaryotic biodiversity: a population genetics perspective. Environ. Microbiol. **4**(11): 628-633

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**(4): 406–425.

Sharma, R., Ranjan, R., Kishor, R.K. and Grover, A. 2005. 'Unculturable' bacterial diversity: an untapped resource. Current Science **89**(1): 72–77.

Shah, H.N., Gharbia, S.E. and Olsen, I. Bacteroides, Prevotella, and Porphyromonas 2005. In *Topley & Wilson's Microbiology and Microbial Infections, Volume 80*. 10th edition. Edited by: Borrelio SP, Murray PR and Funke G. London, Hodder Arnold:1913-1944.

Schloss, P.D., and Handelsman, J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71**(3): 1501–1506.

Singleton, D.R., Furlong, M.A., Rathbun, S.L. and Whitman, W.B. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples, Appl. Environ. Microbiol. **67**(9): 437–4376.

Stackebrandt, E., and Goebel, B.M. 1994. Taxonomic note: a place for DNA: DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. System Bacteriol. **44**(4): 846–849.

Streit, W.R., and Schmitz, R.A. 2004. Metagenomics – the key to the uncultured microbes. Curr. Opin. Microbiol. **7**(5): 492–498.

van Niftrik, L.A., Fuerst, J.A., Sinninghe Damste, J.S., Kuenen, J.G., Jetten, M.S., and Strous, M. 2004. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. FEMS Microbiol. Lett. **233**(1): 7–13.

Ventura, M., Canchaya, A., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F., and van Sinderen, D. 2007. Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. Microbiol. Mol. Biol. Rev. **71**(3): 495–548.

Table 1. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from NPW.

Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identify (%)	Habitat of closest relative
Gammaproteobacteria	NPW1	25	uncultured gamma proteobacterium S15D-MN30	AJ583184	99	Ground waters of the deep
	NPW2	12	uncultured bacterium EV818BHEB5102702SAS56	DQ256355	91	subsurface water
	NPW3	2	uncultured bacterium clone AA4 8	EF379149	99	sediment of an old industrial laguna
	NPW4	2	Pseudomonas sp. 2zhy	AM411620	99	deep sea bacterium
	NPW5	1	uncultured bacterium L3-17C7	AY048885	98	bacterial rhizosphere
	NPW6	1	uncultured bacterium 47mm12	AY796034	95	Gold mine borehole water
	NPW7	1	uncultured gamma proteobacterium CLi132	AF529326	94	PCE-contaminated site
Betaproteobacteria	NPW8	4	unc. bacterium 654953	DQ404881	99	contaminated subsurface sediments
	NPW9	1	uncultured beta proteobacterium XME18	EF061949	100	Mangrove sediment
	NPW10	1	uncultured beta proteobacterium 233	AB252904	83	Iron-oxidation biofilm at Shibayama lagoon
	NPW11	1	uncultured bacterium EV818BHEB5102702SAS62	DQ256349	99	subsurface water
Alphaproteobacteria	NPW12	1	uncultured alpha proteobacterium A12 WMSP2	DQ450755	94	Water-saturated environment
Firmicutes	NPW13	5	uncultured Paenibacillus sp. ACf23	AM489498	94	Soil Mycoremediation
	NPW14	1	uncultured bacterium DA036	AJ000981	62	Grassland soils
	NPW15	1	uncultured bacterium EV818CFSSAHH200	DQ337010	76	subsurface water
	NPW16	1	unc. Firmicutes S15B-MN30	AJ583201	98	radioactive waste disposal site
Bacteroidetes	NPW17	1	uncultured bacterium A25B8	DQ447181	84	gas fed lab-scale reactor
Actinobacteria	NPW18	1	Streptomyces sp. IH32-1	EF157833	92	Deep-sea sediments
Planctomycetes	NPW19	1	unc. Bacterium 028F09-P-BN-P5	BX294807	99	Evry municipal wastewater treatment
Nitrospira	NPW20	1	uncultured Nitrospirae bacterium 9	AB252940	95	Iron-oxidation biofilm at Shibayama lagoon
unknown	NPW21	1	uncultured bacterium B1NR70D4	AY957889	96	chlorine or monochloramine residual
	NPW22	1	uncultured candidate division WS6 bacterium 062-DZ87	DQ329652	36	hypersaline microbial mat

Table 2. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from WD.

Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identify (%)	Habitat of closest relative
Gammaproteobacteria	WD1	9	uncultured bacterium SEW-C-K11	EF659152	99	raw sewage
	WD2	8	uncultured bacterium CJRC109	DQ202182	99	fluidized bed reactor
	WD3	3	uncultured nterobacteriaceae bacterium	DQ234100	98	danshui river estuary
	WD4	3	unc.Citrobacter sp. FRC71	EU268599	98	uranium contaminated subsurface sediments
	WD5	2	uncultured bacterium 91-80	EF157085	98	natural asphalts
	WD6	2	uncultured Luteimonas sp. XJ100	EF648150	99	aerobic activated sludge
	WD7	2	uncultured Enterobacteriaceae bacterium 7150D1B15	EF562128	99	molybdenum Mine
	WD8	1	uncultured bacterium P13-80	EU287173	89	arctic surface sediment
	WD9	1	uncultured gamma proteobacterium EB1041	AY395360	86	pasture soil
	WD10	1	uncultured bacterium LLR3	EU247480	94	soil polluted with 2,4-dichlorophenol
	WD11	1	uncultured beta proteobacterium MKC23	EF173354	98	hydrocarbon-contaminated soil
	WD12	1	uncultured bacterium Kas172B	EF203204	98	sediment
	WD13	1	uncultured bacterium 35-8	DQ833482	94	sediment of Guanting Reservoir
	WD14	1	uncultured bacterium ORCA-3N112	DQ823176	92	oregon Caves National Monument
	WD15	1	uncultured bacterium LLD18	EU247460	91	soil polluted with 2,4-dichlorophenol
	WD16	1	uncultured gamma proteobacterium BJS81-135	AB239034	96	deep-sea cold seep sediments
	WD17	1	uncultured bacterium MIZ12	AB179503	96	sedimentary rock
	WD18	1	uncultured bacterium R	DQ655709	98	freshwater zone of shallow coastal lagoon
	WD19	1	uncultured gamma proteobacterium 4P36	AJ871058	94	soil
	WD20	1	uncultured bacterium 217	DQ158103	95	phenol-degrading soil
	WD21	1	uncultured bacterium H6	DQ328618	94	acid mine drainage site
	WD22	1	uncultured bacterium G3DCM-185	EU037365	97	chromium contaminated soil
	WD23	1	uncultured bacterium G2DCM-250	EU037302	95	chromium contaminated soil
	WD24	1	uncultured gamma proteobacterium PA-11	EF491590	91	deep-sea sediments
	WD25	1	uncultured bacterium 0442	DQ346902	94	TNT-containing bioreactor
	WD26	1	uncultured bacterium D17	EF590063	93	nitrobenzene-contaminated river sediment
	WD27	1	uncultured bacterium 24-ORF04	DQ376561	95	aerobic sequencing batch reactor (SBR)
	WD28	1	uncultured soil bacterium SalCon28	EF101805	99	bioreactor microbial community
	WD29	1	uncultured bacterium SEW-C-M09	EF659162	93	raw sewage influent
	WD30	1	uncultured bacterium YCC57	EF205483	100	geothermal regions
	WD31	1	uncultured bacterium ORS10C-c09	EF392913	97	ohio River sediments

Table 2 - Continued

Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identify (%)	Habitat of closest relative
Betaproteobacteria	WD32	5	uncultured soil bacterium MJK30	EF540387	97	oil-shale chemical industry
	WD33	2	uncultured soil bacterium 21B-MJK	EF540375	98	oil-shale chemical industry
	WD34	1	uncultured soil bacterium UC11	DQ297980	95	hydrocarbon contaminated soil
	WD35	1	uncultured bacterium 5B-14	DQ906815	95	subsurface soil
	WD36	1	uncultured bacterium JH-WHS153	EF492931	98	iron-manganese nodule
	WD37	1	uncultured bacterium NAK2-b1	DQ867044	98	natural gas field
	WD38	1	uncultured soil bacterium PAH-Feed-31	DQ123771	97	PAH-contaminated soil
	WD39	1	uncultured beta proteobacterium OS-C12	EF612409	99	lead-zinc mine tailings
	WD40	1	uncultured bacterium FCPS464	EF516381	89	grassland soil
	WD41	1	uncultured soil bacterium 3-18MK	EF540414	98	oil-shale chemical industry
	WD42	1	uncultured bacterium FCPO505	EF516568	81	grassland soil
	WD43	1	uncultured bacterium JH-GY28	DQ537533	89	soil
	WD44	1	uncultured beta proteobacterium KCM-B-81	AJ581593	94	soil of non-ferrous metal smelting
Alphaproteobacteria	WD45	4	uncultured alpha proteobacterium Gitt-KF-194	AJ532683	97	uranium mill tailings
	WD46	3	uncultured bacterium 1700-3	AY425763	99	volcanic deposit from 1700
	WD47	2	uncultured alpha proteobacterium AKYH1214	AY921890	91	farm soil adjacent to a silage storage bunker
	WD48	1	uncultured sludge bacterium A39	AF234724	96	industrial sewage treatment plant
	WD49	1	uncultured bacterium FCPS581	EF516586	96	grassland soil
	WD50	1	uncultured bacterium MojaveB69	EF071541	96	desert
	WD51	1	uncultured alpha proteobacterium A12-WMSP2	DQ450755	99	water-saturated environment
	WD52	1	uncultured bacterium clone ORSFAB-c03	EF393195	94	ohio River sediments
	WD53	1	uncultured bacterium HB55	EF648061	98	aerobic activated sludge
	WD54	1	uncultured soil bacterium PK-XIII	EF540444	99	oil-shale chemical industry
	WD55	1	uncultured bacterium DSSD53	AY328751	81	drinking water distribution system
	WD56	1	uncultured soil bacterium 711-1	AY326602	96	soil
	WD57	1	uncultured green ferromanganous micronodule MND8	AF292999	92	sediments
	WD58	1	uncultured bacterium 101-114	EF157242	75	natural asphalts
Deltaproteobacteria	WD59	3	uncultured bacterium ES3-33	DQ444157	97	River sediment
Proteobacteria , unclassified	WD60	1	uncultured bacterium LaP15L84	EF667837	89	river sediment
	WD61	1	uncultured soil bacterium PAH-Bio-57	DQ123722	91	PAH-contaminated soil
Actinobacteria	WD62	3	uncultured actinobacterium M2-D03	EU051976	99	savanna soil
	WD63	3	uncultured bacterium YSK16S-13	EF612976	80	acid mine drainage
	WD64	1	uncultured actinobacterium SIMO-1661	AY711027	96	sediment
	WD65	1	uncultured actinobacterium CLi20	AF529327	98	PCE-contaminated site
	WD66	1	uncultured bacterium WET-E15	EF658849	98	urban freshwater creek

Table 2 – Continued

Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identify (%)	Habitat of closest relative
	WD67	1	uncultured bacterium SK47	AY753393	90	metal surfaces
	WD68	1	uncultured actinobacterium XMAC202	DQ990349	92	mangrove sediment
	WD69	1	uncultured actinobacterium C3B-G10	EU052207	96	savanna soil
	WD70	1	uncultured Actinobacteridae bacterium ACF30	AJ555204	90	soil
	WD71	1	uncultured actinobacterium SBRA95	AF387310	96	EBPR sludge
Firmicutes	WD72	2	Bacillus sp. WL-3	AY601723	98	mannanase-producing strain
	WD73	2	Bacillus sp. SAFN-018	AY167812	99	spacecraft facilities
	WD74	1	uncultured bacterium FFCH2290	EU134681	85	soil
	WD75	1	Paenibacillus lautus JCM 9073	AB073188	98	soil
	WD76	1	uncultured bacterium 8-sw-su5-3	DQ981820	94	river water
	WD77	1	Paenibacillus sp. CCBAU ZL506	DQ512475	98	soil
	WD78	1	uncultured Paenibacillus sp. ACf23	AM489498	94	soil
Chloroflexi	WD79	2	uncultured Chloroflexi bacterium UH-10	AB265904	98	anoxic rice field soil
	WD80	2	uncultured bacterium pLW-96	DQ067035	98	sediment of Lake Washington
	WD81	1	uncultured soil bacterium C043	AF507690	83	pinyon-juniper forest soil
	WD82	1	uncultured bacterium DTB5	EF205543	67	geothermal spring mat
Gemmatimonadetes	WD83	2	uncultured Gemmatimonadetes bacterium AKYH1080	AY921753	96	farm soil adjacent to a silage storage bunker
	WD84	1	uncultured Gemmatimonadetes bacterium AKYH1514	AY921705	90	farm soil adjacent to a silage storage bunker
	WD85	1	uncultured Gemmatimonadetes bacterium FI-2F-H04	EF220455	94	unvegetated soil environments
	WD86	1	uncultured Gemmatimonadetes bacterium AKYG1759	AY921941	89	farm soil adjacent to a silage storage bunker
Planctomycetes	WD87	1	uncultured planctomycete GASP-WB2W2-E05	EF074221	97	pasture
	WD88	1	undentified bacterium Sai4P3-62	AJ518738	94	sediment
	WD89	1	uncultured planctomycete glen99-16	AY150880	97	surface coal mining
Bacteroidetes	WD90	1	uncultured CFB group bacterium S15D-MN4	AJ583192	94	radioactive waste disposal site
	WD91	1	uncultured bacterium CJRA42	DQ202147	77	reactor contaminated with nitrate and uranium
	WD92	1	uncultured Bacteroidetes bacterium GASP-KB1S1-G04	EU297512	95	burned native tallgrass priarie
Deinococcus-thermus	WD93	1	uncultured bacterium G2DMC-110	DQ899883	97	chromium contaminated wastes
Acidobacteria	WD94	1	uncultured Acidobacteria GASP-WA2W1-B07	EF072812	98	pasture site
Candidate divison OP10	WD95	2	uncultured candidate division OP10 HAVOmat40	EF032775	65	lava cave microbial mat
Candidate divison OD1	WD96	1	uncultured candidate division OD1 MVP-35	DQ676307	36	suboxic freshwater-pond bacterioplankton
unknown	WD97	1	uncultured bacterium RA13C7	AF407406	52	reactor system treating monochlorobenzene
	WD98	1	uncultured bacterium FFCH4570	EU133990	76	soil grass
	WD99	1	uncultured soil bacterium 3	AY493975	53	soil
	WD100	1	uncultured bacterium FFCH18280	EU134278	70	soil grass
	WD101	1	uncultured bacterium TSBW02	AB186889	74	polychlorinated dioxin dechlorinating

Table 3. Sequence diversity and library coverage estimations.

Measurement	NPW	WD
OTUs	22	101
Chao1 estimator	62	364
ACE estimator	82	454
Shannon index diversity	2.27	4.4
Simpson index diversity	0.18	0.01
Good's estimator of coverage (%)	75	46

Figure captions

Figure 1. Phylogenetic distribution of 16S rDNA sequences generated from (A) NPW and (B) WD samples.

Figure 2. Distribution of clones (%) in the different classes of *Proteobacteria* among the total *Proteobacteria* found in the NPW and WD clone libraries.

Figure 3. Neighbor-joining tree, obtained using an alignment of 600 nucleotides of the 16S rRNA gene sequences, showing the phylogenetic relationships between the environmental clones derived from NPW and WD libraries and related organisms. Numbers of clones in each OTU are given in parentheses. Bootstrap values above 50 are shown. The scale bar indicates 10% estimated sequence. Separate phylogenetic trees are shown for (a) *Proteobacteria* and (b) additional phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Nitrospira*, *Chloroflexi*, *Gemmatimonadetes, Chloroflexi*, *Deinococcus-thermus*, and candidate divisions OP10 and OD1. *Halobacterium salinarum* was used as outgroup.

Figure 4. Rarefaction curves generated for 16S rRNA genes in NPW and WD libraries. Clones were grouped into OTUs at a level of sequence similarity of ≥97%.

Figure 5. Homologous (♦) and heterologous (□) coverage curves for 16S rRNA gene sequences libraries from NPW and WD. Solid lines indicate the value of $(C_X - C_{XY})^2$ for the original samples and broken lines show the $P < 0.05$ value of $(C_X - C_{XY})^2$ for the randomized samples. (A) NPW as homologous coverage and WD as heterologous coverage, and (B) WD as homologous coverage and NPW as heterologous coverage. Taxonomic distances (*D*) were calculated using the Jukes-Cantor correction.

Figure 1

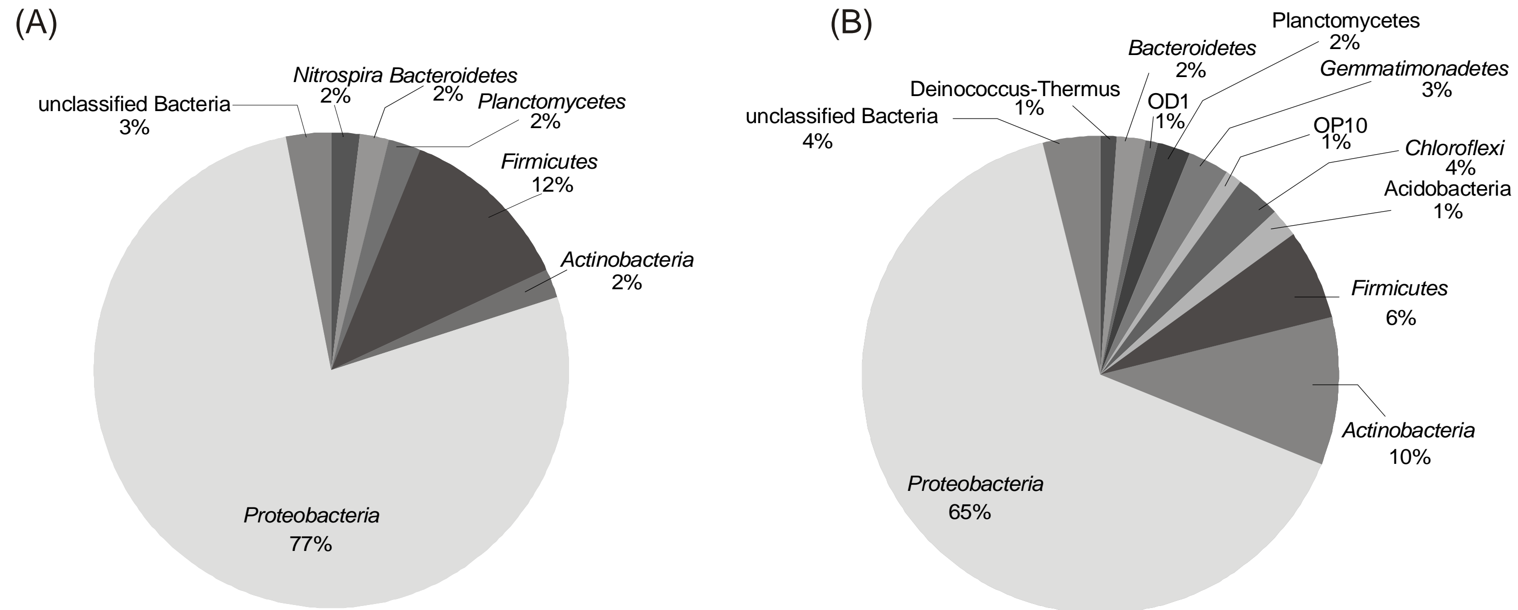


Figure 2

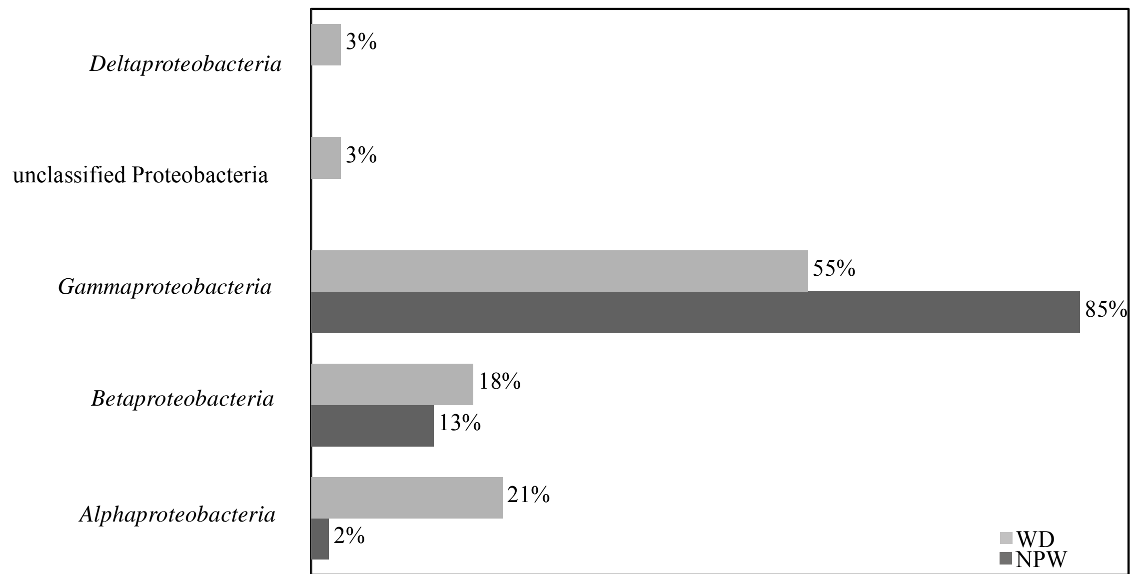


Figure 3.

(a)

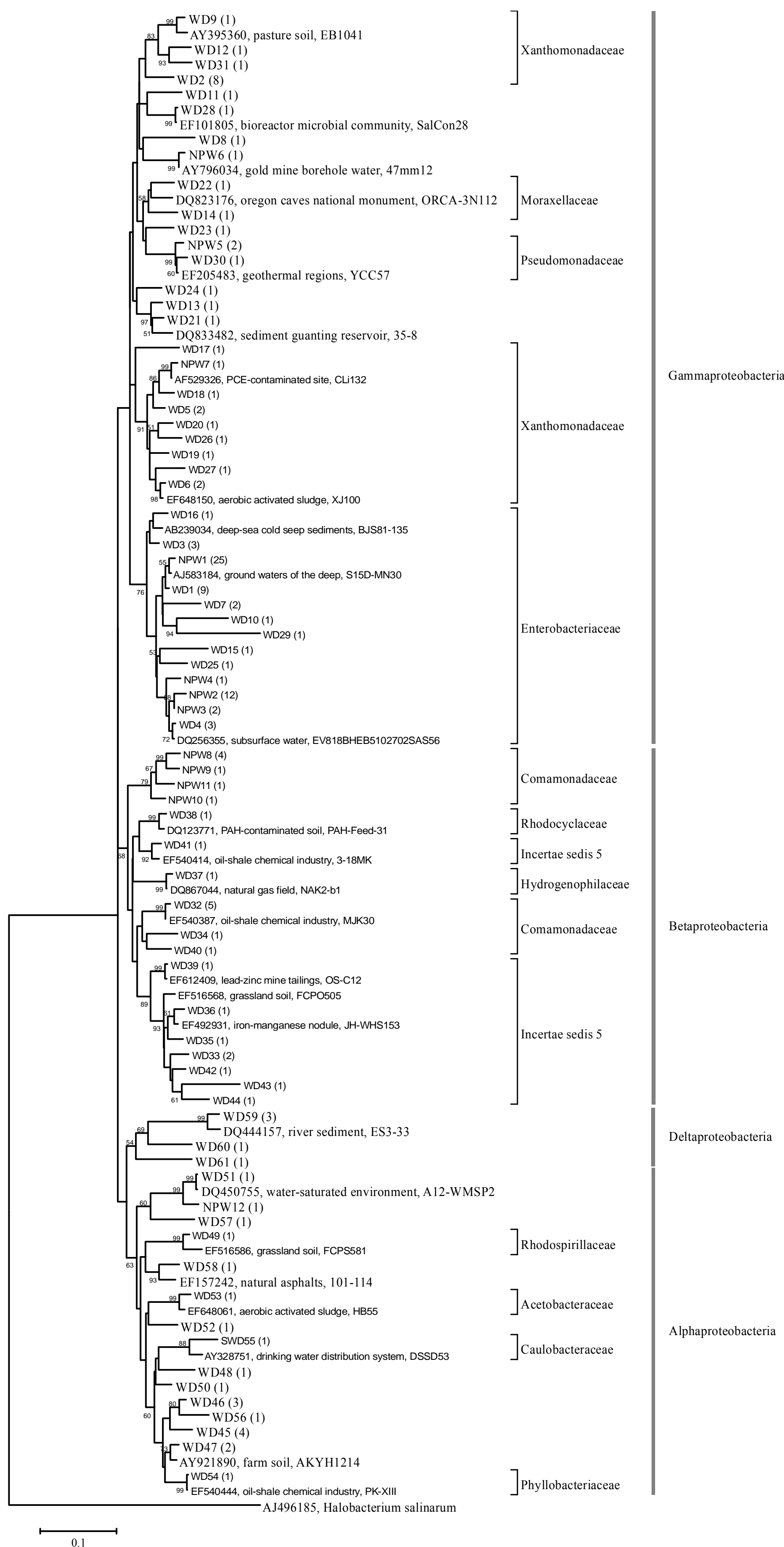


Figure 3

(b)

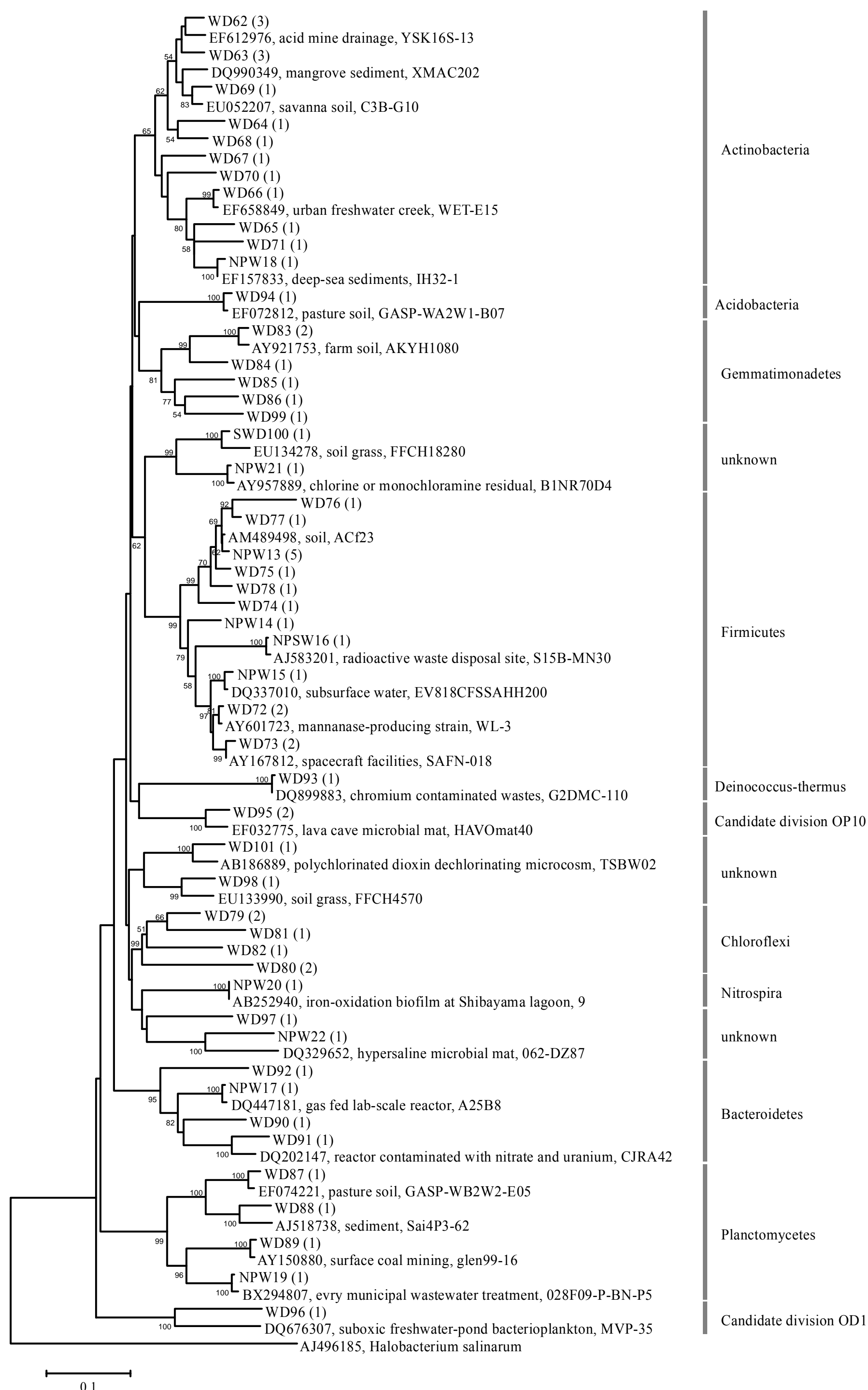


Figure 4.

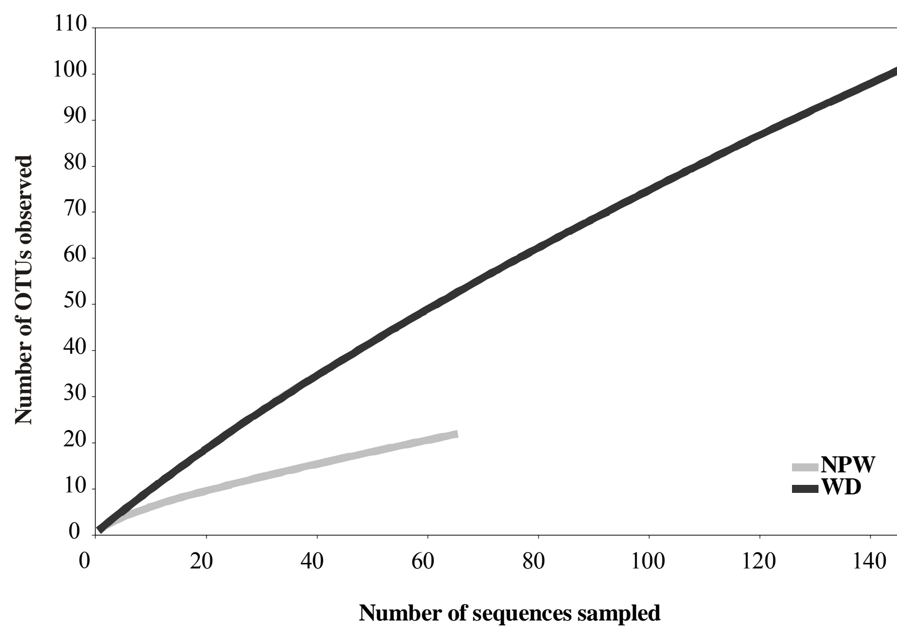
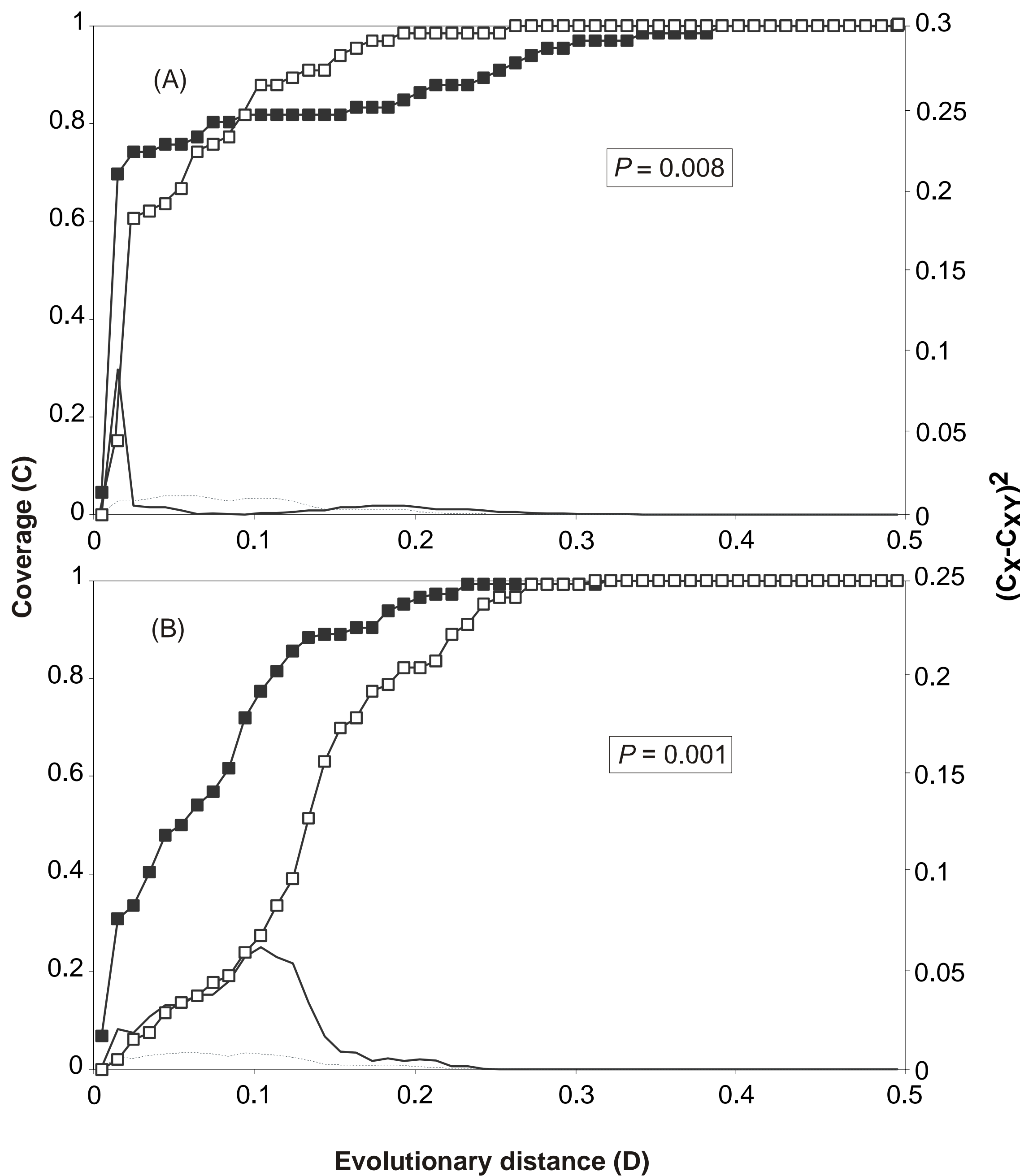


Figure 5



IV) DISCUSSÃO GERAL

Neste estudo foram caracterizadas e identificadas bactérias recuperadas de cinco tipos de rejeitos de uma indústria de aço. Esses rejeitos são produzidos durante os processos de redução e refino do aço e depois são transportados e depositados em área adjacente à indústria, onde permanecem por tempo indeterminado. As indústrias siderúrgicas brasileiras produzem entre 30 e 34 milhões de toneladas de aço anuais, gerando, infelizmente, 700 Kg de resíduos por tonelada de aço produzida. O grande problema é a presença de altas concentrações de Zn, Pb, P, K e S e outros elementos que dificultam a reciclagem desses rejeitos e constituem fonte potencial de contaminação ambiental. Portanto, a recuperação desses rejeitos, antieconômicos e poluidores do ambiente, deve ser considerada como prioridade e parte dos processos de mineração e siderúrgico. Considerando a escassez de estudos sobre o conhecimento das comunidades bacterianas cultiváveis e não cultiváveis presentes nesses rejeitos, este trabalho propôs investigá-las por meio de técnicas clássicas e moleculares.

Cinqüenta e cinco isolados bacterianos, considerados como primeiros colonizadores nesse ambiente, foram recuperados dos rejeitos HMPT, MRPL, PTSS e ETS, sendo apenas 49 deles posteriormente estudados devido à dificuldade de sobrevivência após subcultivos. Esses isolados foram identificados por meio da análise de seqüências parciais do gene de rRNA 16S e classificados, na sua maioria, em nível de gênero. Esta análise permitiu afiliar esses isolados em nove gêneros: *Pseudomonas*, *Micrococcus, Acinetobacter, Bacillus*, *Dietzia*, *Kocuria, Diaphorobacter*, *Staphylococcus e Brevibacillus.* Dentre os isolados, os gêneros *Pseudomonas* e *Micrococcus* foram os mais freqüentemente identificados. No rejeito ETS encontrou-se o maior número de isolados, sendo que o gênero *Pseudomonas* foi exclusivo desse rejeito, apesar dessas bactérias serem consideradas ubíquas na natureza. Ao contrário, os gêneros *Micrococcus* e *Acinetobacter* foram encontrados em três dos quatros rejeitos estudados. O rejeito HMPT apresentou a maior diversidade sendo representada por cinco gêneros: *Diaphorobacter*, *Staphylococcus*, *Brevibacillus, Kocuria* e *Dietzia.* Os dois primeiros encontrados somente neste rejeito e afiliados às espécies *Diaphorobacter nitroreducens* e *Staphylococcus warneri*. E os isolados pertencentes ao gênero *Kocuria* afiliados à espécie *Kocuria marina*. O gênero *Bacillus* foi predominante no rejeito MRPL. Deve-se, ainda, destacar que *Diaphorobacter nitroreducens* é uma nova bactéria, pertencente à família *Comamonadaceae* (*betaproteobacteria*), descrita por Khan e Hiraishi (2002) e *Kocuria marina* é uma nova espécie de actinobactéria (Kim et al., 2004).

Análise do dendrograma gerado por BOX-PCR mostrou uma alta diversidade genômica entre os isolados, incluindo aqueles isolados que apresentaram sequências idênticas do gene de rRNA 16S. Dentre os metais pesados, Ag, Hg, Cd e Co foram altamente tóxicos para os isolados, enquanto Zn, Ni, Pb e Cu mostraram menor toxicidade.

Bacillus isolados do rejeito BFS

No rejeito BFS foram recuperadas 63 isolados bacterianos os quais foram identificados como sendo do gênero *Bacillus* (n=59), *Pseudomonas* (n=2), *Staphylolococcus* (n=1) e *Aerococcus* (n=1), por meio da análise de seqüências parciais do gene de rRNA 16S. Devido a predominância do gênero *Bacillus* no rejeito BFS e sua importância biotecnológica (indústria farmacêutica e biorremediação) 40 desses isolados foram investigados numa abordagem polifásica, levando à caracterização em níveis molecular e fisiológico. Esses isolados foram analisados por meio das seguintes técnicas moleculares: sequenciamento parcial do gene de rRNA 16S e *fingerprinting* genômico – ARDRA, ITS, tDNA, rep-PCR (ERIC, BOX e GTG) –, testes bioquímicos e fisiológicos, incluindo susceptibilidade a metais pesados. Baseado nas seqüências parciais do gene de rRNA 16S e no ensaio de hidrólise de amido, esses isolados se afiliaram ao gênero *Bacillus* sendo intimamente relacionados aos grupos *B. subtilis* (*B. subtilis*, *B. licheniformis* e *B. amyloliquefaciens*), e *B. cereus* (*B. cereus*, *B. mycoides, B. antracis,*) e às espécies *B. pumilus* e *B. sphaericus.* A alta similaridade da seqüência do gene de rRNA16S, neste caso, dificulta sua identificação em nível de espécie. Entretanto, a análise filogenética da seqüência deste gene vem sendo amplamente utilizada em pesquisas taxonômicas devido ao fácil acesso aos bancos de dados, como GenBank e Ribosomal Database Project (RDPII – *online*).

Numa tentativa de melhorar a resolução da afiliação desses isolados avaliou-se a força discriminatória do tDNA e ITS, freqüentemente usados para a identificação de espécies de *Bacillus*. A análise eletroforética dos amplicons de ITS e tDNA-PCR mostrou ser espécie-específica para as linhagens referência e os isolados, agrupados de acordo com a árvore filogenética do rDNA 16S, apresentaram perfis únicos mas distintos das linhagens referência, não permitindo, portanto, uma afiliação mais precisa dos isolados. A fim de avaliar a diversidade genética intra-interespecifica o *fingerprinting* genômico foi analisado usando rep-PCR. A análise dos perfis gerados por BOX, GTG, e ERIC-PCR foi complexa e revelou uma grande heterogeneidade genética entre os isolados do gênero *Bacillus,* confirmando que este método pode ser usado para caracterização de populações do gênero *Bacillus*. Diversos estudos para determinar as relações entre isolados do gênero *Bacillus* têm empregado análises

como ERIC, BOX e $(GTG)_5$ – regiões altamente conservadas e repetidas. O uso desses oligonucleotídeos específicos é vantajoso porque, em uma única reação, obtém-se um número elevado de bandas, permitindo a detecção de polimorfismo suficientemente elevado para a análise genética das bactérias.

Estes isolados apresentaram diferentes perfis fisiológicos, incluindo susceptibilidade aos metais pesados. Embora o sistema API não tenha contribuído para identificar os isolados até o nível de espécie, dificuldade já relatada com o uso desse sistema, ele evidenciou grande diversidade fenotípica dentro dessa população. Como as bactérias pertencentes ao gênero *Bacillus* são geralmente usadas em biotecnologia e biorremediação é importante ampliar o seu conhecimento sobre a estrutura e o comportamento em ambientes hostis.

Diversidade bacteriana independente de cultivo

A aplicação de técnicas moleculares no estudo de ecologia microbiana sofreu recentes avanços por meio da construção de bibliotecas metagenômicas, constituindo-se numa poderosa abordagem para explorar a diversidade microbiana no ambiente, fornecendo dados, inclusive, sobre os microrganismos ainda não cultiváveis. Por meio dessa técnica, foram analisadas a estrutura e composição da comunidade bacteriana presente em amostras de rejeitos de uma indústria de aço.

Segundo as análises filogenéticas das seqüências de rDNA 16S dos clones das bibiotecas NPW e WD foram identificados 10 filos bacterianos e duas candidatas a divisões. Na biblioteca NPW foram encontradas seis filos: *Proteobacteria, Firmicutes Nitrospira, Actinobacteria, Bacteriodetes e Chloroflexi* e na biblioteca WD pôde-se identificar nove filos: *Proteobacteria, Firmicutes Actinobacteria, Planctomycetes, Bacteriodetes, Acidobacteria, Deinococcus-thermus, Chloroflexi, Gemmatimonadetes*, e duas candidatas a divisões, OD1 e OP10. Observa-se, portanto, que todos os filos encontrados na biblioteca NPW, com exceção de *Nitrospira*, são encontrados também em WD. Em ambas bibliotecas pôde-se observar que um número reduzido de OTUs, cujas seqüências do gene de rRNA 16S não foram afiliadas com outras seqüências dos bancos de dados (RDP e BLASTn), podem representar candidatos a novas divisões. *Proteobacteria* foi o filo que predominou nas duas bibliotecas. Considerando os índices de diversidade de Shannon e de Simpson e estimadores não paramétricos (chao1 e ACE) observou-se que a biblioteca WD apresentou maior diversidade genética. Pela análise de cobertura genética, através do programa Libshuff, observou-se diferença significativa na composição das duas bibliotecas. A diversidade bacteriana

encontrada na biblioteca WD poderia ser esperada, uma vez que esses rejeitos encontram-se depositados há cerca de quatro anos, em ambiente adjacente à Siderúrgica, e submetidos a colonização e sucessivos crescimentos bacterianos.

V) CONCLUSÃO GERAL

Ao nosso conhecimento, esta foi a primeira caracterização da diversidade bacteriana em rejeitos de uma indústria de aço. Os resultados evidenciaram diversidade dentre as bactérias dependentes de cultivo e, por meio de testes bioquímicos e fisiológicos e métodos moleculares, foram identificadas bactérias de diversos gêneros, com predominância de *Bacillus, Pseudomonas* e *Micrococcus*. Outros gêneros identificados foram: *Diaphorobacter, Kocuria*, *Staphylococcus*, *Acinetobacter*, *Dietzia* e *Brevibacillus*. A partir das análises de *fingerprinting* BOX, ERIC e GTG detectou-se uma grande heterogeneidade dentre os isolados do gênero *Bacillus*, ressaltando-se que o *fingerprinting* ERIC é uma ferramenta útil para o agrupamento preliminar de *Bacillus* desconhecidos. As bibliotecas metagenômicas NPW e WD mostraram considerável diversidade da microbiota cultivável ou não cultivável desses rejeitos, salientando-se que a complexidade dessas comunidades bacterianas ainda não é explorada. Portanto, o conhecimento dessa diversidade é relevante e pode representar o ponto de partida para estudos ecológicos das comunidades desses rejeitos e abrir perspectivas futuras para aplicações biotecnológicas, como biolixiviação.

VI) REFERÊNCIAS

Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V. & Polz, M. F. (2004). Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J. Bacteriol.* 186 (9): 2629–2635.

Almeida, A.S. (1992). Controle e recuperação ambiental na mina de Cana Brava – Goiás. São Paulo, IPT. (Boletim IPT, 66).

Amann R. I, Ludwig W & Schleifer K.H (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59: 143–169.

Ash C, Farrow JAE, Dorsch M, Stackebrandt E, Collins MD (1991). Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol*, 41:343–346.

Ash, C., Priest, F.G. & Collins, M. D. (1993). Molecular identification of rRNA group 3 Bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie Van Leeuwenhoek* 64, 253-260.

Barreto, M. L. Mineração e desenvolvimento sustentável: desafios para o Brasil (2001). Rio de Janeiro: CETEM/MCT, 215p.

Baptist, J. N., Mandel, M. & Gherna, R. L. (1978). Comparative zone electrophoresis of enzymes in the genus Bacillus. *Int J Syst Bacteriol*,28, 229-244.

Berkeley, R.C.W., Logan, N.A., Shute, L.A. & Capey, A.G. (1984). Identification of Bacillus species. In Methods in Microbiology vol. 16 ed. Bergan, T. pp. 291-328. London: Academic Press.

Bitar, O. Y. (1997). Avaliação da recuperação de áreas degradadas para mineração Região Metropolitana de São Paulo-SP.

Brady S. F., Chao C. J. & Clardy J. (2002). New natural product families from an environmental DNA (eDNA) gene cluster. *J Am Chem Soc,* 124:9968-9969.

Brady S.F, Chao CJ, Handelsman J & Clardy J (2001). Cloning and heterologous expression of a natural product biosynthetic gene cluster from eDNA. Org Lett, 3:1981-1984.

Claus, D. & Berkeley, R.C.W. (1986) The genus Bacillus. In Bergey's Manual of Systematic Bacteriology Vol. 2 ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1105-1140. Baltimore: Williams & Wilkins.

Clementino, M. M., Filippis, I., Nascimento, C. R., Branquinho, R., Rocha, C. L., & Martins, O. B. (2001) PCR Analyses of tRNA Intergenic Spacer, 16S-23S Internal

Transcribed Spacer, and Randomly Amplified Polymorphic DNA Reveal Inter- and Intraspecific Relationships of Enterobacter cloacae Strains. *J Clin Microbiol,* November; 39(11): 3865–3870.

Cherif, A. Brusetti, L. Borin, S. Rizzi, A. Boudabous, A. Khyami-Horani, H. & Daffonchio, D. (2003). Genetic relationship in the 'Bacillus cereus group' by rep-PCR fingerprinting and sequencing of a Bacillus anthracis-specific rep-PCR fragment. *J Appl. Microbiol,* 94, 1108–1119.

Cottrell M.T, Waidner L.A, Yu L, Kirchman, D.L. (2005). Bacterial diversity of metagenômica and PCR libraries from the Delaware River. *Environ Microbiol,* 7: 1883-1895.

Cottrell M. T, Moore JA & Kirchman D. L (1999). Chitinases from uncultured marine microorganisms. *Appl Environ Microbiol,* 65:2553-2557.

Cowan, D., Meyer, Q., Stafford, W., Muyanga, S., Cameron, R. & Wittwer, P. (2005). Metagenomic gene discovery: past, present and future. *TRENDS in Biotechnol,* 23 321-329

Chun, J., Huq, A. & Colwell, R. R. (1999). Analysis of 16S-23S rRNA intergênica spacer regions of Vibrio cholerae and Vibrio mimicus. *Appl Environ Microbiol*. 65: 2202-2208.

Beja, O., Suzuki, M.T., Koonin, E.V., Aravind, L., Hadd, A., Nguyen, L.P., Villacorta, R., Amjadi, M., Garrigues, C., Jovanovich, S. B., Feldman, R. A. & DeLong, E. F. (2000) Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environ Microbiol* 2:516–529.

Beja, O., Spudich, E.N., Spudich, J.L., Leclerc, M. & DeLong, E.F. (2001) Proteorhodopsin phototrophy in the ocean. *Nature,* 411: 786–789.

Beja, O., Suzuki, M. T., Heidelberg, J. F., Nelson, W. C., Preston, C. M., Hamada, T., Eisen, J. A., Fraser, C. M. & DeLong, E. F. (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature,* 415: 630–633.

Dahllöf, I. (2002) Molecular community analysis of microbial diversity. *Curr. Opinion Biotechnol.*, 13: 213-217.

Daffonchio D, Cherif A, Brusetti L, Rizzi A, Mora D, Boudabous A, Sara B (2003). Nature of polymorphisms in 16S–23S rRNA gene intergenic transcribed spacer fingerprinting of *Bacillus* and related genera. *Appl Environ Microbiol,* 69:5128–5137.

Darby, C. (2005). Interactions with microbial pathogens. *WormBook*. 6:1-15

Davison, J. (1999) Genetic exchange between bacteria in the environment. *Plasmid*, 42: 73-91.

DeLong, E.F. (2002). Microbial population genomics and ecology. *Curr. Opinion*

Biotechnol., 5:520–524.

Delong E. F. (2005). Microbial community genomics in the ocean. *Nat Rev Microbiol* 3:459–469

Dias, E. G.C.S. (2001) Avaliação de impacto ambiental de projetos de mineração no Estado de São Paulo: a etapa de acompanhamento. Tese (Doutorado em Engenharia Mineral) Universidade de São Paulo.

Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N,Wilson M, Mullany P (2003). Novel tetracycline resistance determinant from the oral metagenome. *Antimicrob Agents Chemother,* 47:1430-1432.

Dobrindt, U., Agerer, F., Michaelis, K., Janka, A., Buchrieser, C., Samuelson M., Svanborg, C., Gottschalk, G., Karch, H. & Hacker, J. (2003). Analysis of Genome Plasticity in Pathogenic and Commensal Escherichia coli Isolates by Use of DNA Arrays. *J. Bacteriol,* 185:1831–1840.

Dobrindt, U & Hacker, J. (2001) Whole genome plasticity in pathogenic bacteria. *Curr. Opinion. Microbiol.*, 4: 550-557.

Dolzani, L., Tonin, E., Lagatolla, C. & Monti-Bragadin, C. (1994). Typing of *Staphylococcus aureus* by amplification of the 16S-23S intergênica spacer sequences. *FEMS Microbiol. Lett.* 119: 167-174.

Dunbar, J., Takala, S., Barns, S. M., Davis, J. A. & Kuske, C. R. (1999). Levels of Bacterial Community Diversity in Four Arid Soils Compared by Cultivation and 16S rRNA Gene Cloning. *Appl. Environ. Microbiol. 65*(4): 1662-1669.

Eyers L, George I, Stenuit B, Agathos S.N & Fantroussi S.E. (2004). Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. *Appl Microbiol Biotechnol,* 66: 123–130.

Fogel, G.B., Collins, C.R., Li, J. & Brunk, C.F. (1999). Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population. *Microb Ecol.*, 38:93–113.

Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001). Ureibacillus gen. nov., a new genus to accommodate Bacillus thermosphaericus (Andersson et al. 1995), emendation of Ureibacillus thermosphaericus and description of Ureibacillus terrenus sp. nov. *Int J Syst Evol Microbiol,* 51:447–455.

França, R. J., Figueiredo, R. F. & Filho, B. C. (2004). Remoção de Metais de Lodo de Esgoto por Biolixiviação. *Engenharia Civil,* 19: 31-40.

Gill, S. R., Pop, m., DeBoy, R. T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S.,

Gordon, J.I., Relman, D. A., Fraser-Liggett, C. M., Nelson, K. E. (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. *Science*, 312:1355-1359.

Gillespie DE, Brady SF, Bettermann AD, Cianciotto NP, Liles MR, Rondon MR, Clardy J, Goodman RM & Handelsman J (2002). Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. *Appl Environ Microbiol,* 68:4301-4306.

Gorab, E. (2001). Evolução dos genes nucleares de RNA ribossômico. In: *Biologia Molecular e Evolução* (Matioli, S. R. ed.). ed. Holos, Ribeirão Preto, pp. 64-69.

Gordon, R.E., Haynes, W.C. & Pang, C.H. (1973) The genus Bacillus. Agriculture Handbook no. 427. Washington, DC: US Government Printing Office.

Grahn, N.; Olofsson, M.; Ellnebo-Svedlund, K.; Monstein, H. J. & Jonasson, J. (2003) Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by pyrosequencing of cloned amplicons. *FEMS Microbiol. Lett.*, 219: 87-91.

Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J. & Goodman, R.M. (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.*, 5:245–249.

Handelsman, J., Liles, M., Mann, D., Riesenfeld, C. & Goodman, R.M. (2002) Cloning the metagenome: cultureindependent access to the diversity and functions of the uncultivated microbial world. *Funct Microbial Genomics* 33: 241-255.

Handelsman J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and Molecular Biol Rev,* 68:669-685.

Healy, F. G., Ray, R. M. Aldrich, H. C. Wilkie, A. C. I. L. O. & Shanmugam, K. T.. (1995). Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. *Appl. Microbiol. Biotechnol.* 43:667–674.

Helgason, E., kstad, O.A., Caugant, D. A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. & Kolst, A.B. (2000). *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* - One Species on the Basis of Genetic Evidence. *Appl Environ Microbiol,* 66: 2627-2630.

Helgason, E., Tourasse, N.J., Meisal, R., Caugant, D.A., Kolstø, A.B., (2004). Multilocus sequence typing scheme for bacteria of the Bacillus cereus group. *Appl Environ Microbiol*, 70:191–201.

Henderson, I., Dongzheng, Y. & Turnbull, P.C.B. (1995). Diferentiation of Bacillus anthracis and other Bacillus cereus group' bacteria using IS231-derived sequences. *FEMS Micro-biol. Lett*. 128, 113-118.

Henne A, Schmitz RA, Bomeke M, Gottschalk G. & Daniel R (2000). Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. *Appl Environ Microbiol*, 66:3113-3116.

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N.A. (1998) Virgibacillus: a new genus to accommodate Bacillus pantothenticus (Proom and Knight 1950). Emended description of Virgibacillus pantothenticus. *Int J Syst Bacteriol*, 48, 99-106.

Hugenholtz, P., Pitulle, C., Hershberger, K. L. & Pace, N. (1998). Novel Division Level Bacterial Diversity in a Yellowstone Hot Spring. *J. Bacteriol.* 180 (2): 366–376.

Joung, K.B. & Côté, J.C. (2002). Evaluation of ribosomal RNA gene restriction patterns for the classifcation of Bacillus species and related genera. *J. Appl. Microbiol*, 92, 97-108.

Khan S.T., Hiraishi A. (2002) *Diaphorobacter nitroreducens* gen nov, sp nov, a poly (3-hydroxybutyrate)-degrading denitrifying bacterium isolated from activated sludge. *J Gen Appl Microbiol,* 48:299–308

Kaneda, T. (1977) Fatty acids of the genus Bacillus: an example of branched-chain preference. *Bacteriol Rev,* 41: 391-418.

Kim, S.B., Nedashkovskaya O. I., Mikhailov V.V., Han S.K., Kim K.O., Rhee M.S., Bae K.S. (2004). *Kocuria marina* sp. nov., a novel actinobacterium isolated from marine sediment. *Int J Syst Evol Microbiol,* 54: 617–1620

Keller, M. & Zengler, K. (2004). Tapping into microbial diversity. *Nat. Rev. Microbiol.* 2: 141-150.

Kemp, P. F. & Aller, J. Y. (2004). Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. *FEMS Microbiol. Ecol.* 47: 161-177.

Klappenbach, J. A., Saxman, P. R., Cole, J. R. & Schmidt, T. M. (2001). Rrndb: the ribosomal RNA operon copy number database. *Nucleic Acid Research.* 29: 181-184.

Knietsch, A., Bowien, S., Whited, G., Gottschalk, G., & Danie, R. (2003). Identification and Characterization of Coenzyme B12-Dependent Glycerol Dehydratase- and Diol Dehydratase-Encoding Genes from Metagenomic DNA Libraries Derived from Enrichment Cultures. *Appl Environ Microbiol,* 69:3048–3060.

Kuske, C.R., Barns, S.M., and Busch, J.D. (1997). Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. *Appl Environ Microbiol,* 63: 3614–3621.

Lawrence, J. G. (1999). Gene transfer, speciation, and the evolution of bacterial genomes. *Curr. Opin. Microbiol.* 2: 519-523.

Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiessner-Gunkel, E.,

Stewart, G.S. & Scherer, S. (1998) Bacillus weihenstephanensis sp. nov. is a new psychrotolerant species of the Bacillus cereus group. *Int J Syst Bacteriol*, 48: 1373–1382.

Ledin M & Pedersen K (1996). The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes. *Earth Sci Rev* 41: 67–108.

Lee K, Costerton J.W, Ravel J, Auerbach R.K, Wagner D.M, Keim P, Leid J.G. (2007). Phenotypic and functional characterization of Bacillus anthracis biofilms. *Microbiol,* 153:1693-1701.

Liles, M.R., Manske, B.F., Bintrim, S.B., Handelsman, J., and Goodman, R.M. (2003) A census of rRNA genes and linked genomic sequences within a soil metagenomic library. *Appl Environ Microbiol,* 69: 2684–2691.

Lopez-Garcia P, Brochier C, Moreira D, Rodriguez-Valera F (2004) Comparative analysis of a genome fragment of an uncultivated mesopelagic crenarchaeote reveals multiple horizontal gene transfers. *Environ Microbiol,* 6:19–34.

Logan, N.A. & Berkeley, R.C.W. (1984) Identification of Bacillus strains using API system. *J General Microbiol,* 130, 1871-1882.

Lorenz, P. and Schleper, C. (2002). Metagenome- a challenging source of enzyme discovery. *J Mol Catalysis B: Enzymatic*. *19–20*: 13–19

Machado, I. F. (1989). Recursos minerais, política e sociedade. São Paulo: Edgard Brücher. O meio ambiente e a mineração. In: Economia mineral do Brasil. Coord. Barboza, F. L. M. E GURMENDI, A. C. Brasília: DNPM.1995.

Majernik A, Gottschalk G & Daniel R (2001). Screening of environmental DNA libraries for the presence of genes conferring Naþ(Liþ)/Hþ antiporter activity on Escherichia coli: characterization of the recovered genes and the corresponding gene products. *J Bacteriol,* 183:6645-6653.

Mayr, R., Eppert, I. & Scherer, S. (1999). Incidence and identification of psychrotrophic (C-tolerant) Bacillus spp. in German HTST pasteurized milk. *Milchwissenschaft* 54, 26–30.

McInerney, J.O., Wernecke, M., Mullarkey, M. & Powell, R. (2002) Bacteria and Archaea: Molecular techniques reveal astonishing novel diversity. *Biodiversity* 3:3-10.

Myers, N. (1996). Environmental services of biodiversity. *Proc. Natl. Acad. Sci. USA,* 93, 2764-2769.

Nakamura, L.K. (1998) Bacillus pseudomycoides sp. nov. *Int J Syst Bacteriol*, 48: 1031–1034.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, EV Grigoryan, AA Ivanova, AE

Lysenko, AM Petrunyaka, VV Osipov, GA Belyaev SS & Ivanov, MV (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of Geobacillus subterraneus gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius and Bacillus thermodenitrificans to Geobacillus as the new combinations G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans. *Int J Syst Evol Microbiol* 51, 433–446.

Olsen, G. J.; Lane, D. J.; Giovannoni, S. J. & Pace, R. N. (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Ann. Rev. Microbiol.*, 40: 337-365.

Osorio, C. R.; Collins, M. D.; Romalde, J. L. & Toranzo, A. E. (2005). Variation in 16S-23S rRNA Intergenic Spacer regions in *Photobacterium danselae:* a Mosaic-Like Struture. *Appl. Environ. Microbiology,* 71:636-645.

Oren, A. (2004). Prokaryote diversity and taxonomy: current status and future challengs. *Phil. Trans. R. Soc. Lond. B,* 359:623-638.

Pace, N. R. (1997). A Molecular view of microbial diversity and the biosphere. *Science. 276*: 734-740.

Pace, N. R., Stahl, D. A., Lane, D. J. & Olsen, G. J. (1985). Analyzing natural microbial populations by rRNA sequences. *ASM News* 51:4–12.

Petersen, D.J., Shishido, M., Brian Holl, F. & Chanway, C.P. (1995). Use of species- and strain-specific PCR primers for identification of conifer root-associated Bacillus spp. *FEMS Microbiol Let,* 133, 71–76.

Priest, F.G., Goodfellow, M. & Todd, C. (1981) The genus Bacillus: a numerical analysis. In The Aerobic Endospore-forming bacteria: classification and identification ed. Berkeley, R.C.W. and Goodfellow, M. pp. 91-103. New York: Academic Press.

Rappé, M. S. & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annu. Rev. Microbiol.* 57:369-394.

Riesenfeld, C.S., Schloss, P.D. & Handelsman1, J. (2004). METAGENOMICS: Genomic Analysis of Microbial Communities. *Annu. Rev. Genet.,* 38:525–52.

Robertson, C.E., Harris, J.K., Spear, J.R. & Pace, N.R. (2005). Phylogenetic diversity and ecology of environmental Archaea. *Curr Opinion Biotechnol,* 8:1-5.

Rodríguez-Valera, F. (2002). Approaches to prokaryotic biodiversity: a population genetics perspective. *Environm Microbiol* 231: 153-158.

Rodríguez-Valera, F. (2004). Environmental genomics, the big picture? *FEMS Microbiol*

Lett. 231: 153-158.

Rössler D, Ludwig W, Schleifer KH, Lin C, McGill TJ, Wisotzkey JD, Jurtshuk P Jr, Fox GE. (1991) Phylogenetic diversity in the genus Bacillus as seen by 16S rRNA sequencing studies. *Syst ApplMicrobiol*, 14: 266-269.

Rosière, C.A. (1992). Classificação genética de minérios de ferro - problemas e vícios - Proposta de uma classificação tipológica para indústria. In: Seminário de Redução de Minério de Ferro, 28, 1997. Vitória. *Anais* . São Paulo: ABM, 295-302.

Rosselló-Mora, R & Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol. Rev.*, 25: 39-67.

Rondon, M. R., Goodman, R. M. & Handelsman, J. (1999). The Earth's bounty: assessing and accessing soil microbial diversity. *TIBTECH*. *17*: 403-409.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., Minor, C. Tiong, C. L., Gilman, M., Osburne M. S., Clardy, Handelman, J. & Goodman R. M. (2000). Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl Environ Microbiol*. *66*:2541-2547.

RDPII – http://rdp.cme.msu.edu/index.jsp

Sabehi, G., Massana, R., Bielawski, J.P., Rosenberg, M., Delong, E.F. & Beja, O. (2003) Novel proteorhodopsin variants from the Mediterranean and Red Seas. *Environ Microbiol* 5: 842–849.

Schleifer, K. (2004). Microbial Diversity: Facts, Problems and Prospects. *System. Appl. Microbiol.,*27: 3-9.

Schlegel, H. G. & H. W. Jannasch. 1992. Prokaryotes and their habitats, p. 75–125. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, vol. I. Springer-Verlag, New York, N.Y.

Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Völker, H. & Thomm, M. (2001). Filobacillus milensis gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glutype peptidoglycan. *Int J Syst Evol Microbiol* 51, 425–431.

Schmidt, T. M., DeLong, E. F. & Pace, N. R. (1991). Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* 173:4371–4378.

Schloss PD & Handelsman J (2003) Biotechnological prospects from metagenomics. *Curr Opin Microbiol* 14:303–310.

Sharma, R., Ranjan, R., Kishor, R.K. & Grover, A. (2005). 'Unculturable' bacterial

diversity: An untapped resource. *Curr Science*, 89:72-77.

Shida, O., Takagi, H., Kadowaki, K. & Komagata, K. (1996) Proposal for two new genera, Brevibacillus gen. nov. and Aneurinibacillus gen. nov. *Int J Syst Bacteriol*, 46: 939-946.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. & Dean, D.H. (1998) Bacillus thuringiensis and its pesticidal crystal proteins. *Microbiol Molec Biol Rev,* 62:775–806.

Stackebrandt, E., Ludwig, W., Weizenegger, M. et al. (1987) Comparative 16S rRNA oligonucleotide analyses and murein types of round-spore-forming bacilli and non-spore-forming relatives. *J General Microbiol,* 133: 2523-2529.

Staley, J. (1998). Microbial Diversity and the biosphere.www.bdt.org.br/oea/sib/staley.

Staley, J. T. & Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol,* 39:321–346.

Stein, J. L., Marsh, T. L. Wu, K. Y. Shizuya, H. & DeLong, E. F. (1996). Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment front a planktonic marine archaeon. *J. Bacteriol.* 178:591–599.

Streit, W. R. & Schmitz, R. A. (2004). Metagenomics – the key to the uncultured microbes. *Curr. Opin. Microbiol. 7*:492–498

Smith, K.L., DeVos, V., Bryden, H., Price, L.M., Hugh-Jones, M.E. & Keim, P. (2000) Bacillus anthracis diversity in Kruger National Park. *J Clin Microbiol*, 38: 3780–3784.

Tringe, S.G, von Mering, C., Kobayashi, A., Salamov, A. A, Chen, K,, Chang, H. W., Podar, M., Short, J. M., Mathur, E. J, Detter, J. C., Bork, P., Hugenholtz, P., Rubin, E.M. (2005) Comparative metagenomics of microbial communities. *Science* 308, 554–557.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M. Solovyev, V. V., Rubin, E. M., Rokhsar, D. S. & Banfield, J. F. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428, 37–43.

Torsvik, V., Ovreas, L. & Thingstad, T. F. (2002). Prokaryotic Diversity – Magnitude, dynamics, and controlling factors. *Science*. 296: 1064-1066.

Treusch, A. H., Kletzin, A., Raddatz, G., Ochsenreiter, T., Quaiser, A., Meurer, G., Schuster, S. C. & Schleper, C. (2004) Characterization of large-insert DNA libraries from soil for environmental genomic studies of Archaea. *Environm Microbiol,* 6(9): 970-980.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu DY, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers Y.

H & Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74.

Versalovic, J.; Schneider, M.; De Bruijn, F. J.; Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth Mol Cel Biol,* 5: 25-40.

Wainù, M., Tindall, B.J., Schumann, P. & Ingvorsen, K. (1999) Gracilibacillus gen. nov., with description of Gracilibacillus halotolerans gen. nov., sp. nov., transfer of Bacillus dipsosauri to Gracilibacillus dipsauri comb. nov. and Bacillus salexigens to the genus Salibacillus gen. nov., as Salibacillus salexigens comb. nov. *Inter J Sys Bacteriol,* 49:821-831.

Ward, D.M, Weller, R. & Bateson, M. M (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 344:63–65.

Welsh J, McClelland M. (1991). Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucleic Acids Res,*19:861–866.

Whitman, W., B., Coleman, D. C. & Wiebe, W., J. (1998) Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U. S. A.,* 95: 6578-6583.

Wisotzkey, J.D., Jurtshuk, P. Jr, Fox, G.E., Deinhard, G. & Poralla, K. (1992) Comparative sequence analyses on the 16S rRNA (rDNA) of Bacillus acidocaldarius, Bacillus acidoterrestris, and Bacillus cycloheptanicus and proposal for creation of a new genus, Alicyclobacillus gen. nov. *Inter J Sys Bacteriol,* 42:263-269.

Woese, C. R. (1987) Bacterial Evolution. *Microbiol. Rev.*, 51: 221-271.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eucarya*. *Proceedings of the National Academy of Sciences of the USA* 87: 4576–4579.

Wu, X.Y., Walker, M., Vanselow, B., Chao, R. L & Chin, J (2006). Characterization of mesophilic bacilli in faeces of feedlot cattle. Journal of Applied Microbiology ISSN 1364-5072

Xu, J. (2006) Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Mol Ecol* 15, 1713–1731.

Yoon, J.H., Weiss, N., Lee, K.C., Lee, I.S., Kang, K. H. & Park, Y. H. (2001). Jeotgalibacillus alimentarius gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of Bacillus marinus Ru¨ger 1983 as Marinibacillus marinus gen. nov., comb. nov. *Int J Syst Evol Microbiol,* 51, 2087–2093.

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