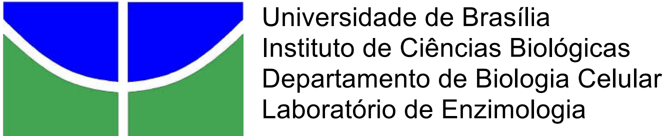

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Descrição do resistoma de solo de Cerrado *stricto sensu* e o potencial de dioxigenases metagenômicas na resistência antimicrobiana e em processos de biorremediação

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LISTA DE ABREVIAÇÕES

%	Porcentagem
aa	Aminoácido
ABC	*ATP-binding cassette*
ARG	Gene de resistência a antibióticos
BLASTp	*Protein BLAST*
CDO	Catecol dioxigenase
DNA	Ácido desoxirribonucleico
DO	Dioxigenase
FDA	*Food and Drug Administration*
GDO	Gentisato dioxigenase
HDO	Hidroxiquinol dioxigenase
IPTG	Isopropil β-D-tiogalactopiranosídeo
Kda	Quilodalton
L	Litro
M	Molar
MATE	*Multidrug and Toxic compound Extrusion*
MFS	*Major Facilitator Superfamily*
mg	Miligrama
mL	Mililitro
mM	Milimolar
MTT	Brometo de metiltiazolildifeniltetrazólio
NCBI	*National Center fot Biotechnology Information*
NGS	Sequenciamento de nova geração
nm	Nanômetro
^{o}C	Graus Celsius
OD	Densidade ótica
ORF	Fase aberta de leitura
pb	Pares de base
PCDO	Protocatecuato dioxigenase
PCR	Reação em cadeia da polimerase
RCD	*Ring-cleaving dioxygenase*
RND	*Resistance Nodulation Division*
rpm	Rotações por minuto
SDS-PAGE	Eletroforese em gel de poliacrilamida desnaturant
SMR	*Small Multidrug Resistance*
μL	Microlitro
μM	Micromolar

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RESUMO

A resistência antimicrobiana tem sido uma grande ameaça à saúde humana, dada a disseminação de genes de resistência a antibióticos (ARGs) em patógenos e micro-organismos clinicamente importantes, promovendo o aparecimento de cepas multirresistentes de difícil tratamento, complicado também pela queda na quantidade de novos antibióticos lançados no mercado. Assim, pesquisas por determinantes genéticos da resistência a antibióticos em isolados clínicos têm sido intensificadas desde os primeiros relatos de resistência antimicrobiana no começo do século XX. Neste contexto, a diversidade ambiental dos ARGs vem sendo subestimada, apesar do conhecimento de que a origem destes genes são os ambientes naturais, em particular o solo. Os micro-organismos presentes nesses ambientes são, em sua maioria, ainda não cultiváveis e consequentemente desconhecidos, o que os torna importantes fontes de novos genes e biomoléculas de interesse biotecnológico. Entretanto, analisar sua totalidade depende de técnicas independentes de cultivo. A metagenômica apresenta-se como uma ferramenta importante, já que acessa os micro-organismos de uma dada amostra ambiental pela extração do seu DNA total, permitindo identificar genes de interesse por abordagem de sequência ou funcional. Na pesquisa por novos ARGs, a metagenômica funcional é de grande valia para identificar novos mecanismos de resistência antimicrobiana e inferir a real diversidade de ARGs no meio ambiente, além de investigar os papéis ecológicos destes genes fora do ambiente clínico. Em trabalho anterior, observou-se a presença de três genes para putativas dioxigenases em dois clones resistentes isolados de biblioteca metagenômica de pequenos insertos (com insertos de 3-8 Kb) construída com DNA de amostras de solo de Cerrado. Nesta Tese, apresentam-se dois capítulos: o primeiro aborda a hipótese de que as dioxigenases putativas estão associadas ao fenótipo resistente observado. Dessa forma, três subclones foram construídos e denominados AMX3(2), AMX3(3) e CRB2(1), para posteriores análises fenotípicas e estruturais, onde CRB2(1) mostrou-se capaz de causar resistência à carbenicilina em *Escherichia coli* – adicionalmente, também mostrou-se capaz de resistir à ação do fenol. Em análises estruturais e enzimáticas, inferiu-se que o inserto é uma extradiol dioxigenase com domínio bicupina, sendo uma provável gentisato 1,2-dioxigenase (GDO). Estas enzimas são importantes no metabolismo de compostos aromáticos, o que as torna potenciais em processos de biorremediação. No segundo capítulo está descrita a análise das sequências de seis clones resistentes isolados de biblioteca metagenômica de grandes insertos (com insertos de mediana de 35 Kb), também construída com amostras de DNA de micro-organismos de solo de Cerrado. A anotação gênica somou um conjunto de dados de aproximadamente 0,27 Mb, num total de 172 prováveis genes. Tais ORFs foram agrupadas em oito diferentes classes, de acordo com funções selecionadas. Dentre elas, 1,2% eram referentes a enzimas previamente relacionadas à resistência antimicrobiana e 5,8% a transportadores, que também são importantes protagonistas na resistência a xenobióticos. Apesar da grande diversidade de funções observadas, a maior parte das ORFs foi anotada como hipotéticas, sendo fontes para descrição de possíveis novos ARGs, assim como de genes de interesse biotecnológico. Essas observações indicam que a diversidade de ARGs em ambientes naturais difere daquela classicamente encontrada em isolados clínicos, o que evidencia a importância em acessar micro-organismos ambientais na tentativa de revelar novos mecanismos de resistência a antibióticos, que vem a enriquecer o conhecimento sobre seus papéis ecológicos e auxiliar pesquisas das indústrias biotecnológicas, como na prospecção de antimicrobianos e em processos de biorremediação.

Palavras-chave: dioxigenase, metagenoma, resistência antimicrobiana, solo

ABSTRACT

Antimicrobial resistance poses a grave threat to human health given the dissemination of antibiotic resistance genes (ARGs) in pathogens and in clinically important microorganisms, promoting the rise of multiresistant strains of difficult treatment, also aggravated by the decreasing quantity of new antimicrobials launched onto the market. Hence, since the first reports of antimicrobial resistance in the beginning of the 20th century, the research for the genetic determinants of antimicrobial resistance have been intensified. In this context, the environmental diversity of ARGs was underestimated, despite the knowledge that such genes are environmental-born, especially on the soil. The majority of environmental microorganisms is not yet cultivable and remains unknown, being an important source for novel genes and biomolecules of biotechnological interest. However, analyzing them requires culture independent techniques. Metagenomics rise as a powerful tool, since it accesses the microorganisms from an environmental sample by the extraction of their total DNA, allowing for the identification of genes of interest by sequence and functional approaches. In the search for new ARGs, functional metagenomics is a valuable method to identify novel mechanisms for antibiotic resistance and infer the actual diversity of environmental ARGs, while exploring their ecological roles outside the clinical setting. In a former work, three putative dioxygenase genes were observed in two resistant clones isolated from a small-insert metagenomic library (insert size 3-8 Kb) constructed with DNA samples from Cerrado soil. In this Thesis, two chapters are provided: the former addresses the hypothesis in which putative dioxygenases are involved in the resistant phenotype observed. In this manner, three subclones were constructed and named AMX3(2), AMX3(3) and CRB2(1), for further phenotypical and structural analysis, where CRB2(1) proved resistant to carbenicillin in *Escherichia coli* – additionally, it was also able to resist the action of phenol. In the structural and enzymatic assays, the insert was presupposed as an extradiol dioxygenase with a bicupin domain, most probably a gentisate 1,2-dioxygenase (GDO). These are important enzymes on aromatic metabolism and potential resources in the bioremediation industry. The latter chapter describes the sequence analysis of six resistant clones isolated from a large-insert metagenomic library (insert size around 35 Kb), also constructed with DNA samples from Cerrado soil. Gene annotation comprised a 0.27 Mb dataset, summing 172 probable genes. Such ORFs were grouped in eight different classes, according to preselected functions. Within these, 1.2% was related to enzymatic mechanisms of resistance and 5.8% were classified as transporters, which also play important roles on xenobiotic resistance. Although a great diversity of functions were observed, a greater number of ORFs was classified as hypothetical, being sources for the description of novel ARGs as well as of other genes of biotechnological relevance. These observations imply that the diversity of environmental ARGs differ from that classically observed in clinical isolates, which shows the importance in accessing environmental microorganisms in the attempt to reveal new mechanisms to antibiotic resistance, which may enrich the knowledge on their ecological roles and aid biotechnological researches, such as in antimicrobial bioprospection and in bioremediation processes.

Key-words: dioxygenase, metagenome, antimicrobial resistance, soil.

INTRODUÇÃO

Antibióticos e resistência antimicrobiana – aspectos clínicos e ambientais

Doenças infecciosas são um grave problema em saúde pública sendo, até a atualidade, protagonistas nas taxas de mortalidade. No começo do século XX, eram também responsáveis pela baixa expectativa de vida e sobrevida da população.

Seu tratamento, até então, era realizado de forma experimental, até sofrer grande avanço na primeira década do século XX, a partir da descoberta de moléculas de origem microbiana com ação antibiótica, que teve seu marco histórico com o isolamento da penicilina por Alexander Fleming em 1929 (Fleming, 1929).

Assim, a chamada "era de ouro" dos antibióticos teve início nas décadas de 1930-40, dada pela introdução da sulfonamida (1938), penicilina (1942) e estreptomicina (1944) em saúde humana. A utilização dessas e de outras moléculas antimicrobianas revolucionou o tratamento de doenças infecciosas, algumas até então consideradas intratáveis e com alto grau de mortalidade (Davies, 2007).

À época, a resistência antimicrobiana, apesar de conhecida, não era considerada um revés grave, até o destaque de alguns casos de infecções refratárias às alternativas existentes, como uma epidemia de gastroenterite no Japão, causada pela dispersão de cepas multirresistentes de *Shigella spp* e a observação da rápida disseminação de *Staphylococcus aureus* resistentes a penicilinas, tetraciclinas e macrolídeos no Reino Unido, ambos nos anos 1950 (Davies, 2007; Hawkey, 2008).

A partir de então, estimulou-se a procura por novas moléculas antibióticas de origem natural e aqueles antibióticos semi-sintéticos de espectro mais amplo, construídos a partir de compostos naturais conhecidos. Paralelamente, foram intensificadas as buscas pelos determinantes da resistência antimicrobiana, que contou primeiramente com a identificação de uma penicilinase em 1940 (Abraham e Chain, 1940), capaz de destruir o antibiótico penicilina.

Daí em diante, o uso crescente e indiscriminado desses medicamentos, tanto em saúde humana e animal quanto na agricultura e pecuária, tem tornado obsoletos antibióticos antes eficazes, contribuindo também na rápida disseminação de micro-organismos multirresistentes. Com esta prática, a tendência é o esgotamento das alternativas terapêuticas existentes atualmente. Assim,

persiste o alerta para o uso racional destes compostos, além da necessidade de pesquisas e investimentos na busca por novos compostos antibióticos com diferentes mecanismos de ação.

Aliado ao uso racional está a necessidade de compostos antibióticos mais eficazes. Contudo, a incorporação de novos agentes antibióticos na clínica tem decrescido ao longo dos anos, em comparação a outras classes de quimioterápicos, como as drogas anti-neoplásicas. Por exemplo, nos últimos cinco anos, a agência norte-americana *Food and Drug Administration* (FDA) aprovou 37 novas drogas anti-câncer e apenas oito novas formulações antibióticas (disponível em www.fda.gov), conforme demonstra a Figura 1.

Figura 1. Novas drogas anti-neoplásicas e antibióticas aprovadas pela órgão de vigilância sanitária norte-americano (FDA) desde 2011. Os números acima das colunas representam novas formulações lançadas no mercado nos Estados Unidos para o tratamento de doenças infecciosas e diferentes tipos de câncer.

Ainda, nenhuma destas moléculas são membros de novas classes de antimicrobianos e sim derivados semi-sintéticos de estruturas pré-existentes. Dessa forma, nenhuma inovação ocorreu no desenvolvimento de antibióticos desde 2009, quando o medicamento *telavancin* (Vibativ®; Theravance Biopharma, Inc.) foi lançado no mercado. Esta formulação foi o primeiro antibiótico lipoglicopeptídeo aprovado pela agência norte-americana de vigilância sanitária – FDA – para tratamento de infecções complicadas de pele e pneumonia associada à ventilação mecânica (disponível em www.fda.gov).

Os mecanismos de resistência antimicrobiana vão desde a proteção ou substituição do sítio ativo, ao influxo reduzido dos antibióticos nas células (por exemplo, pela expressão de bombas de

efluxo) até a desintoxicação pela inativação do antibiótico por enzimas específicas (Wright, 2007; Bennett, 2008).

Deve-se salientar, entretanto, que os mecanismos abordados abaixo são os mais comuns na resistência antimicrobiana, que são os descritos nas cepas cultiváveis (e muitas vezes patogênicas). Não significa, entretanto, que a diversidade ambiental dos elementos de resistência não seja diferente e mais abrangente que este padrão.

Um dos principais mecanismos de resistência antimicrobiana está relacionado à degradação dos compostos por atividade enzimática. São diversos os já descritos em todas as classes antibióticas. Essas enzimas podem ter atividade de hidrólise, de transferência de grupos químicos, de redox e outros. O segundo grupo é o mais abundante em relação à presença de enzimas que provocam resistência antimicrobiana (Wright, 2005).

Exemplos clínicos bem descritos são a resistência antimicrobiana provocada por esterases na resistência aos antibióticos macrolídeos e por epoxidases aos antibióticos do grupo das fosfomicinas (Wright, 2005). Para os β-lactâmicos, as principais e mais descritas são as betalactamases.

Tais enzimas são o principal mecanismo de resistência aos antibióticos β-lactâmicos. As betalactamases são enzimas específicas responsáveis pela inativação hidrolítica do anel β-lactâmico dos antibióticos penicilínicos e cefalosporínicos (Davies, 1994; Walsh, 2000; Wright, 2005; Allen *et al.*, 2009).

A enzima é secretada pelas cepas resistentes no periplasma e destroem os antibióticos antes de se ligarem às proteínas de ligação à penicilina (PBPs); tratam-se de enzimas extremamente específicas e eficientes: uma molécula de betalactamase é capaz de hidrolizar 1.000 moléculas de penicilina por segundo (Walsh, 2000).

Ainda, o espectro de ação destas enzimas é modificado pela intensa alteração nos genes destas proteínas, criando afinidade a outros antibióticos β-lactâmicos. Tais mutações criam novos genes e promove a expressão de betalactamases de largo espectro (Davies, 1994).

Na tentativa de combater a infecção por bactérias resistentes produtoras destas enzimas, os inibidores de betalactamases foram introduzidos no tratamento farmacológico, em combinação com os antibióticos β-lactâmicos. Estas moléculas têm estrutura análoga à dos antibióticos β-

lactâmicos e são substrato para a ação das betalactamases, agindo, em sua maioria, na inibição irreversível da enzima (Davies, 1994).

O clavulanato, uma molécula natural, não possui atividade antibiótica significativa, porém mostrou-se muito eficiente como substrato para a ação das betalactamases. Em combinação com a amoxicilina, expandiu o espectro de ação deste antibiótico, promovendo maior utilização clínica do mesmo (Davies, 1994; Walsh, 2000).

Outra grande classe de proteínas importantes em resistência antimicrobiana são as bombas de efluxo. Essas estruturas são encontradas em quase todas as espécies bacterianas, codificadas tanto em cromossomos quanto em plasmídeos – dessa forma, medeiam tanto a resistência intrínseca quanto a adquirida. Ainda, agem tanto em multirresistência como também induzem patogenicidade e respondem ao estresse bacteriano (Sun *et al.*, 2014; Li *et al.*, 2015).

Em geral, são altamente eficientes em extrusar xenobióticos e possuem largo espectro de ação, além de agirem sinergicamente com outros mecanismos de resistência. Essas estruturas estão classificadas em cinco famílias: *Resistance Nodulation Division* (RND), *Major Facilitator Superfamily* (MFS), *ATP-binding Cassette transporters* (ABC), *Small Multidrug Resistance protein* (SMR) e *Multidrug and Toxic compound Extrusion family* (MATE). Destas, somente RND é exclusiva na multirresistência em gram-negativas, enquanto as outras famílias são encontradas tanto em gram-negativas quanto em positivas (Sun *et al.*, 2014; Li *et al.*, 2015).

Destaca-se membros das famílias RND e MFS como importantes atores na resistência a antibióticos em patógenos gram-negativos e gram-positivos, respectivamente (Li *et al.*, 2015).

A disseminação de patógenos multirresistentes, aliada à diminuição das opções farmacológicas capazes de controlar esta epidemia, é um grande problema em saúde pública. Dessa forma, é urgente a necessidade de moléculas antibióticas mais eficazes.

Ao encontro desta realidade, a pesquisa e identificação de novos mecanismos de resistência antimicrobiana podem fornecer novos alvos farmacológicos e estimular a síntese de novas drogas antibióticas para combater micro-organismos resistentes.

Como exemplo, pode-se citar um estudo recente que descreve uma estratégia para identificar novos antimicrobianos pela exploração de mecanismos de resistência. Truman e colaboradores exploraram o mecanismo de resistência a glicopeptídeos em *Streptomyces coelicolor* para desenvolver um novo protocolo de bioprospecção de moléculas antibióticas. Este protocolo

permitiu a identificação do cluster gênico para a biossíntese de ristocetina, até então desconhecido, e que, adicionalmente, codificam outras atividades enzimáticas (Truman *et al.*, 2014).

Mais tarde, em 2015, um novo antimicrobiano foi identificado em uma triagem independente de cultivo clássico, através de câmaras de difusão *in situ*, onde os micro-organismos são capazes de crescer utilizando os substratos de seu habitat natural. Assim, os autores isolaram a teixobactina, um antibiótico potente contra isolados Gram-positivos de importância clínica. Enfatiza, ainda, a ausência de mecanismos de resistência para este antibiótico em cepas de *Staphylococcus aureus* e *Mycobacterium tuberculosis*, embora também considerem que a resistência possa ser adquirida por transferência lateral de genes (Ling *et al.*, 2015).

Contudo, embora os genes de resistência a antibióticos (ARGs) em patógenos e em outras bactérias clinicamente importantes têm sido extensamente estudados, a sua diversidade ambiental continua pouco conhecida e portanto subestimada. Tanto antibióticos quanto ARGs estão associados a micro-organismos ambientais – assim, o meio ambiente, particularmente o solo, é um reservatório importante de tais genes e biocompostos. Entretanto, pouco se sabe sobre os papéis que antibióticos e genes de resistência exercem fora do ambiente clínico.

Em micro-organismos produtores de antibióticos, os clusters gênicos normalmente também codificam ARGs. Assim, infere-se que a principal função destes genes é o de conferir um fenótipo de autorresistência ao hospedeiro. No entanto, eles também podem estar envolvidos na regulação de vias biossintéticas. Em não-produtores, o fenótipo resistente pode estar associado a mutações em genes alvo que alteram vias metabólicas, resultando em diferentes níveis de resistência. A revisão publicada por Allen e colaboradores descreve exemplos de tais mutações em genes que codificam proteínas ribossomais e da DNA girase (Allen *et al.*, 2010).

Outras funções associadas a antibióticos também foram descritas. Em concentrações sub-inibitórias – e não tóxicas – essas moléculas agem na comunicação entre bactérias, que pode, por exemplo, entre outras funções, induzir a virulência. Adicionalmente, antibióticos podem ser uma fonte de energia para algumas bactérias, o que ajuda a explicar a grande diversidade de genes de resistência nos ambientes naturais (Martinez, 2008).

Nesse sentido, é urgente acessar micro-organismos de origem ambiental e explorar seu arsenal genético na tentativa de identificar novos genes e mecanismos de resistência a antibióticos e

explorar suas funções fora do ambiente clínico-hospitalar e, ainda, suas possíveis aplicações biotecnológicas.

Metagenômica

Contudo, a diversidade dos micro-organismos no meio ambiente representa um universo vasto e ainda pouco explorado. O conhecimento destes organismos é limitado devido à impossibilidade de cultivo da maioria dos micro-organismos em uma dada amostra ambiental. É estimado, por exemplo, que menos de 0,1% dos micro-organismos de solo são capazes de crescer pelos métodos tradicionais de cultivo adotados até então (Handelsman *et al.*, 1998).

Desse modo, métodos independentes de cultivo têm sido desenvolvidos para ultrapassar esta barreira e possibilitar o acesso universal aos micro-organismos presentes nas amostras ambientais. A metagenômica é uma destas técnicas e foi cunhada em 1998 por Jo Handelsman e colaboradores (Handelsman *et al.*, 1998). Esta técnica descreve o estudo da diversidade microbiana e seu leque funcional em uma dada amostra ambiental pelo acesso do seu DNA total (Handelsman *et al.*, 1998; Handelsman, 2004). Esta abordagem, juntamente com o desenvolvimento e avanço de tecnologias de sequenciamento de larga escala, revolucionou o campo da microbiologia ambiental.

Em linhas gerais, para acessar a diversidade taxonômica e funcional de micro-organismos originários dos ambientes naturais, a técnica da metagenômica utiliza duas abordagens: baseada em sequência e funcional.

A primeira envolve o sequenciamento direto do DNA total extraído de uma amostra ambiental para análises subsequentes. Conforme ilustrado na Figura 2, aplicações que utilizam esta abordagem incluem a identificação de variantes genéticas, descrição da diversidade e abundância funcional e taxonômica e a montagem de genomas microbianos, entre outros. Como o custo do sequenciamento de larga escala tem caído com a introdução das tecnologias de sequenciamento de nova geração, a abordagem por sequência se tornou mais vantajosa que a funcional. Ainda, a metagenômica por sequência é menos complexa e evita alguns vieses, já que os passos de clonagem e expressão não são necessários.

Figura 2. Abordagens metagenômicas para acessar a diversidade taxonômica e funcional dos micro-organismos de uma dada amostra ambiental. Em linhas gerais, o DNA total é extraído, fragmentado e então direcionado para análise baseada por sequência ou a funcional.

Todavia, a metagenômica funcional ainda é uma técnica robusta para identificar novos biocatalisadores de interesse provenientes de diferentes amostras ambientais. Exemplos recentes incluem enzimas com atividade amilolítica, celulolítica e lipolítica, com diversas aplicações industriais (Xu *et al.*, 2014; Alnoch *et al.*, 2015; Kanokratana *et al.*, 2015; Kim *et al.*, 2015; Pereira *et al.*, 2015; Prive *et al.*, 2015; Su *et al.*, 2015; Vester *et al.*, 2015), e também aquelas com potencial na utilização em processos de biorremediação (Lee *et al.*, 2008; Nagayama *et al.*, 2015). Esta abordagem é também uma ferramenta interessante na bioprospecção de antimicrobianos (O'mahony *et al.*, 2015; Takeshige *et al.*, 2015).

No contexto da pesquisa de ARGs, a abordagem funcional é utilizada para selecionar clones resistentes das bibliotecas metagenômicas e, então, explorar seu contexto genético. Assim, já que mais de 80% de todos os antibióticos utilizados na clínica são provenientes de micro-organismos de solo, este ambiente torna-se um importante reservatório tanto elementos de resistência antimicrobiana como para genes de biossíntese de moléculas antibióticas (D'costa *et al.*, 2007; Torres-Cortes *et al.*, 2011) e torna-se, desta maneira, o ambiente de escolha para a identificação de novos ARGs.

Consequentemente, explorar o chamado "resistoma" dos ambientes naturais, classificado como a coleção de elementos de resistência antimicrobiana de uma dada amostra, vem a ajudar a revelar a real diversidade e funções ecológicas dos ARGs, identificar novos mecanismos de resistência, assim como novas variantes de genes pré-existentes e levantar as seguintes questões: a

resistência antimicrobiana é a função primária destes genes? Qual a função ambiental dos ARGs? Há algum potencial biotecnológico nos ARGs ambientais?

Considerando a identificação de dioxigenases putativas em clones metagenômicos isolados previamente em triagem de resistência com nove antibióticos β-lactâmicos, que são parte das análises apresentadas nos Capítulo 1 desta Tese, faz-se necessária a contextualização quanto às características básicas e funcionais destas enzimas.

Dioxigenases

Assim, as dioxigenases são enzimas amplamente distribuídas tanto em eucariotos quanto em procariotos, sendo importantes no catabolismo de compostos aromáticos. Especificamente em micro-organismos, são essenciais na produção de energia e na reciclagem do ciclo do carbono. Em micro-organismos de solo são especialmente abundantes e estão intimamente relacionadas à degradação de xenobióticos, razão pela qual têm sido estudadas como alternativas de degradação de poluentes aromáticos em processos de biorremediação. (Broderick, 1999; Vaillancourt *et al.*, 2006; Urszula Guzik, 2013).

Diferentes vias catabólicas são responsáveis pela degradação de compostos aromáticos pela via aeróbia, geralmente via quatro principais intermediários: catecol, protocatecuato, gentisato e hidroquinona/hidroxiquinol, quando provenientes de compostos monocíclicos (Vaillancourt *et al.*, 2006; Urszula Guzik, 2013).

Estes intermediários são gerados por reações anteriores de ativação, que incorporam substituintes oxigenados ao anel, em geral pela incorporação de substituintes hidroxila. Assim, a degradação se dá primeiramente com a ação de monooxigenases ou dioxigenases hidroxilantes, que ativam o anel pela inserção de grupos hidroxila, permitindo a ação subsequente das dioxigenases responsáveis por clivar a estrutura, terminando assim o ciclo de quebra do anel aromático de intermediários catecólicos (Urszula Guzik, 2013).

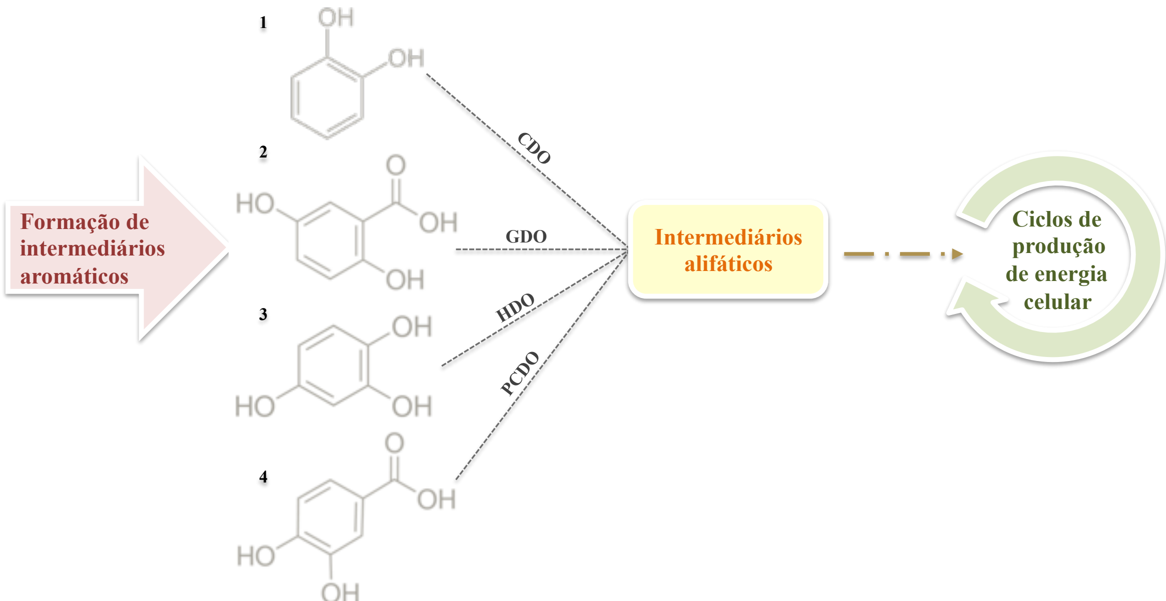


Figura 3. Parte do metabolismo de aromáticos por intra e extradiol dioxigenases. Os intermediários são gerados por reações anteriores de ativação do anel que são provenientes de ação enzimática, tanto de dioxigenases como de outras classes de enzimas. Os quatro intermediários da degradação aeróbia de compostos monocíclicos estão representados (1) catecol; (2) gentisato (ácido gentísico); (3) hidroquinona/hidroxiquinol e (4) protocatecuato (ácido protocatecuico). Estes são clivados, principalmente, por diferentes dioxigenases com distintas especificidades – catecol dioxigenase (CDO), gentisato dioxigenases (GDO), hidroxiquinol dioxigenases (HDO) e protocatecuato dioxigenases (PCDO). A primeira via pode ser direcionada via quebra intra ou extradiol, enquanto a via de GDO é extradiol e as vias de HDO e PCDO são intradiol. A ação destas enzimas forma intermediários alifáticos que, com o andamento da via metabólica, gera intermediários que entram nos ciclos de produção de energia celular (Vaillancourt *et al.*, 2006; Urszula Guzik, 2013).

O presente trabalho tem como foco o estudo de dioxigenases que clivam o anel benzênico – doravante descritas como RCD (*ring-cleaving dioxygenases)* – que, portanto, serão mais detalhadas a seguir.

Dentro deste grupo, existem duas classes de dioxigenases: intradiol e extradiol. Distinguem-se pela localização da clivagem, que nas intradiol ocorre entre os substituintes hidroxila (orto-clivagem) e, nas extradiol, adjacente a um destes substituintes (meta-clivagem). Ainda, diferenciam-se pelo cofator utilizado, já que intradiol dioxigenases utilizam Fe(III) não-heme enquanto extradiol utilizam, em geral, Fe(II) não-heme (Vaillancourt *et al.*, 2006; Fetzner, 2012; Urszula Guzik, 2013).

Nas duas classes há subclassificações, discriminadas por diferentes fatores como substratos, estruturas secundárias e terciárias e outros, conforme detalhado na Figura 4 abaixo.

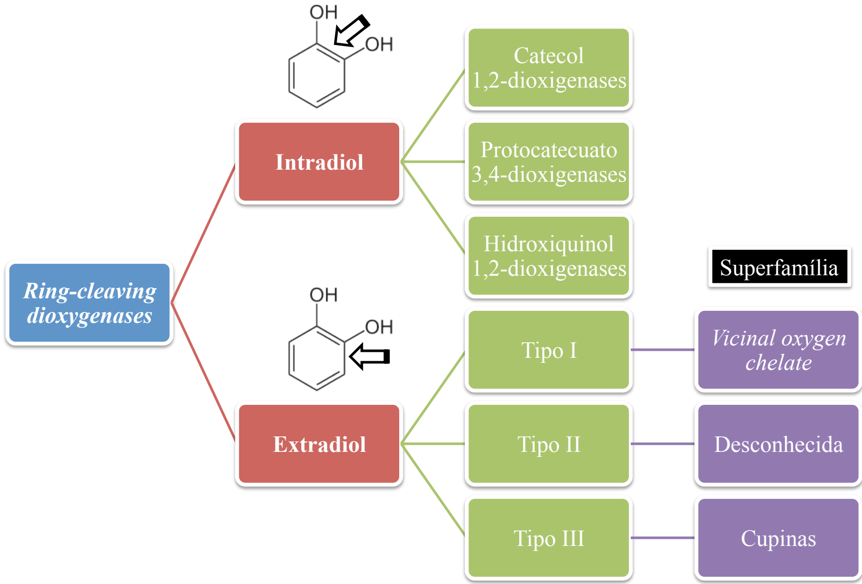


Figura 4. Classificação das dioxigenases RCD. O primeiro agrupamento divide as RCDs em intradiol e extradiol, de acordo com a localização da abertura do anel na posição orto e meta, respectivamente, conforme demonstram as setas na molécula de catecol. As intradiol dioxigenases são ainda subdivididas em duas classes estruturais, com características distintas: catecol 1,2-dioxigenases.hidroxiquinol 1,2-dioxigenases e protocatecuato 3,4-dioxigenases. As extradiol também estão divididas em três tipos, classificados como Tipo I, da superfamília *vicinal oxygen chelate*; Tipo II, de superfamília desconhecida; e Tipo III, da superfamília das cupinas (Vaillancourt *et al.*, 2006; Fetzner, 2012; Urszula Guzik, 2013).

Apesar de parecerem próximas, intra e extradiol dioxigenases têm linhagens distintas e não possuem similaridades de sequência ou estrutura, e algumas diferenças são bem evidentes. Extradiol dioxigenases, por exemplo, são enzimas mais abrangentes e clivam, inclusive, compostos não catecólicos, como o gentisato, e até aqueles não aromáticos. Por outro lado, intradiol dioxigenases restringem o leque de substratos àqueles aromáticos que possuem hidroxilas vicinais (Vaillancourt *et al.*, 2006).

A estrutura básica de compostos aromáticos é a presença do anel benzênico que, devido à sua configuração eletrônica ressonante, torna essas moléculas extremamente estáveis. Assim, compostos desta natureza são, por sua vez, altamente recalcitrantes e geralmente tóxicos. Muitos destes compostos são subprodutos de diversos processos industriais, sendo presentes também em defensivos agrícolas, derivados do petróleo, solventes, tintas, corantes e outros. Sendo assim, são vastamente despejados no meio ambiente, o que os tornam contaminantes com grande impacto ambiental, principalmente no solo e na água (Vaillancourt *et al.*, 2006).

Quando depositados nesses locais, são de difícil remoção e sua retirada é desafiadora; assim, compostos aromáticos tendem a acumular-se no meio ambiente. Apesar da existência de técnicas físico-químicas de remediação, elas são, muitas vezes, ineficientes.

Essas dificuldades estimularam a pesquisa por degradadores biológicos, ou seja, enzimas de origem microbiana que tenham, em sua natureza, capacidade de degradar anéis aromáticos, desestabilizando a estrutura, podendo tornar os subprodutos menos tóxicos ou facilitar sua remoção de ambientes contaminados. Este processo é chamado biorremediação.

Dessa forma, dioxigenases são enzimas potenciais para processos de biodegradação de aromáticos, devido à grande gama de substratos e especificidades possíveis (Broderick, 1999; Vaillancourt *et al.*, 2006; Urszula Guzik, 2013).

Nesta Tese, a abordagem dessas enzimas é dual, já que envolve a hipótese de que podem estar relacionadas à resistência antimicrobiana observada em testes anteriores, os quais identificaram clones metagenômicos resistentes a antibióticos β-lactâmicos que, na anotação gênica das ORFs nos insertos, observou-se a presença de dioxigenases putativas. Ainda, acessa a potencialidade de tais ORFs na quebra de compostos aromáticos, como o fenol, e sua possível utilização em processos de biorremediação de poluentes aromáticos em amostras ambientais contaminadas com este tipo de composto.

JUSTIFICATIVA

O conhecimento dos elementos de resistência antimicrobiana que envolvem micro-organismos patogênicos e aqueles implicados na saúde humana e animal é vasto, enquanto a diversidade de genes de resistência a antibióticos nos ambientes naturais permanece pouco estudada e, consequentemente, desconhecida.

Sabe-se, no entanto, que a origem de genes de resistência antimicrobiana é ambiental, sendo proeminentes nos solos. Dessa forma, torna-se urgente explorar estas amostras e descrever a coleção de genes de resistência antimicrobiana associados ao meio ambiente, o chamado "resistoma".

Para tanto, a técnica da metagenômica vem ao encontro desta necessidade, já que acessa os micro-organismos por uma abordagem independente de cultivo permitindo, assim, alcançar a totalidade do material genético de micro-organismos de uma dada amostra ambiental, auxiliando na identificação de novos genes e biocatalisadores de interesse.

Assim, enriquecer este conhecimento promove a identificação de novos mecanismos, ainda não relacionados ao fenótipo resistente dos micro-organismos, além de estimular o estudo das funções dos genes de resistência antimicrobiana, assim como de antibióticos, nos ambientes naturais.

Ainda, permite explorar o potencial biotecnológico de genes implicados em resistência baseados em suas funções primárias, como a utilização de certas enzimas, como as dioxigenases, em processos de biorremediação.

Capítulo 1

Dioxigenases metagenômicas putativas e sua implicação em resistência antimicrobiana e degradação de aromáticos

Os dados apresentados neste Capítulo foram publicados em versão modificada, intitulada **New dioxygenase from metagenomic library from Brazilian soil: insights into antibiotic resistance and bioremediation**, no periódico *Biotechnology Letters,* sob a seguinte referência: Biotechnol Lett. 2015 Sep;37(9):1809-17. doi: 10.1007/s10529-015-1861-x.

OBJETIVOS

Objetivo geral

Investigar a possível relação de três subclones construídos com insertos de dioxigenases putativas provenientes de biblioteca metagenômica de solo de Cerrado – denominados AMX3(2), AMX3(3) e CRB2(1) – na resistência antimicrobiana a β-lactâmicos, por meio de análises de sequência, fenotípicas e enzimáticas.

Objetivos específicos

1. Subclonar três ORFs relacionadas a enzimas dioxigenases em vetor de de expressão tipo pET, transformação em cepa de *Escherichia coli* de expressão, a fim de criar os subclones AMX3(2), AMX3(3) e CRB2(1);

2. Identificar domínios conservados, motivos de ligação ao cofator metálico e predição de estrutura secundária para os três subclones;

3. Submeter culturas de CRB2(1) a concentrações fixas de carbenicilina e fenol para testar a resistência do subclone aos componentes antibiótico e aromático;

4. Analisar a viabilidade celular de alíquotas de CRB2(1) em carbenicilina e fenol e compará-las àquelas do controle negativo, constituído pelo hospedeiro com o vetor intacto (pET24a);

5. Realizar ensaios de indução, solubilidade e purificação da proteína de CRB2(1) em condições nativas;

6. Realizar ensaios de caracterização enzimática da proteína de CRB2(1) com o substrato ácido gentísico.

MATERIAIS E MÉTODOS

Biblioteca metagenômica e triagem de resistência

Os clones utilizados nas análises a seguir são provenientes das triagens de resistência antimicrobiana de biblioteca metagenômica de pequenos insertos de solo de Cerrado *stricto sensu* (De Castro *et al.*, 2011), de tamanhos de inserto entre 3 a 8 Kb, realizadas previamente c om nove antibióticos β-lactâmicos, a citar: 16 μg amoxicilina mL^{-1}, 50 μg ampicilina mL^{-1}, 50 μg carbenicilina mL^{-1}, 16 μg cefamandol mL^{-1}, 20 μg cefoxitina mL^{-1}, 5 μg ceftazidima mL^{-1}, 50 μg cefalexina mL^{-1}, 50 μg penicilina G mL^{1} and 12.5 μg piperacilina mL^{1}. Este trabalho resultou no isolamento de 45 clones resistentes referentes aos ensaios realizados durante a dissertação de mestrado (Santos, 2011), onde quatro clones foram sequenciados e, na anotação gênica, identificados três ORFs de dioxigenases putativas.

Análises de bioinformática

As sequências de aminoácidos foram anotadas no programa Protein BLAST (Altschul *et al.*, 1990). Para identificação de resíduos conservados em CRB2(1), utilizou-se o programa Clustal Omega (Sievers *et al.*, 2011) para alinhamento múltiplo das sequências de aminoácidos do subclone com proteínas caracterizadas relacionadas, enquanto as estruturas secundárias foram preditas utilizando a plataforma PSIPRED – Protein Sequence Analysis Workbench (Jones, 1999). Finalmente, as árvores filogenéticas foram construídas no programa Mega 6.06 (Tamura *et al.*, 2013), pelo método de *Neighbor-joining* e *boostrap com 500* reamostragens.

Análises funcionais

Subclonagem de dioxigenases putativas

Na anotação de quatro clones sequenciados, provenientes da biblioteca de pequenos insertos, identificou-se três ORFs putativas, codificantes de possíveis enzimas dioxigenases. Estas ORFs, denominadas AMX3(2), AMX3(3) e CRB2(1) foram submetidas à otimização dos códons e subclonagem em vetor de expressão pET24a pela empresa GenOne (www.genone.com.br). Subsequentemente, os vetores de CRB2(1) e AMX3(3) foram transformados em cepas de *Escherichia coli* Turner DE(3) e BL21(DE3), respectivamente. Selecionou-se CRB2(1) para análises fenotípicas subsequentes.

Indução, expressão e purificação de dioxigenase metagenômica

As culturas foram crescidas até valor de OD_{600} entre 0,8 e 1,0, e a indução feita conforme discriminado abaixo. Às culturas induzidas, adicionou-se coquetel de inibidores de protease de acordo com as especificações do fabricante (Sigma-Aldrich®, EUA). A lise celular foi realizada por sonicação por até uma hora com pulsos de 30 segundos e a proteína de interesse purificada utilizando o *kit MagneHis Protein Purification System®* (Promega, EUA), sob condições nativas.

Para indução da expressão de CRB2(1), utilizou-se isopropil β-D-1-tiogalactopiranosídeo (IPTG) 1 mM ou lactose a 1% (para otimizar a solubilização da proteína no lisado bacteriano). A incubação de CRB2(1) foi realizada entre 20 e 28°C, com agitação de 130 rpm por, no mínimo, 16 horas. Às culturas, adicionou-se cisteína 1 mM e sulfato de amônio e ferro (II) 100 μM como fonte de íons Fe^{2+}.

A análise eletroforética foi realizada em SDS-PAGE em gel Tris-HCl 4-13% e a corrida em tampão desnaturante pH 8.3. As amostras foram centrifugadas, suspendidas em tampão de corrida desnaturante 1X e aquecidas por 5 minutos a 95°C.

Análise fenotípica e teste viabilidade celular

Para avaliar os fenótipos de resistência à carbenicilina e ao fenol, o subclone CRB2(1) foi cultivado na presença dos dois compostos, a 50 μg mL^{-1} e 0,1%, respectivamente, e a densidade

ótica foi monitorada por leituras periódicas. Para tanto, CRB2(1) e o controle negativo, representado pelo vetor pET24a intacto, foram incubados a 28°C e 130 rpm. Quando as culturas atingiram valores de OD_{600} próximos a 0,1, a expressão foi induzida adicionando IPTG na concentração final de 1 mM. Após 1 hora, os compostos antibiótico e aromático foram adicionados e a densidade celular monitorada de 1 a 96 horas. O ensaio foi realizado em quadriplicata.

A viabilidade celular foi avaliada adicionando 30 µL de uma solução 2 $mg.mL^{-1}$ de brometo de metiltiazolildifeniltetrazólio (MTT) a alíquotas de 200 µL das culturas de 24 horas e incubadas a 37°C por 1 hora.

Ensaio enzimático

A atividade de gentisato 1,2-dioxigenase foi avaliada por espectrofotometria pelo aumento da absorção a 330 nm, indicada pela formação de maleilpiruvato. A reação de 200 µL continha 50 µL da dioxigenase parcialmente purificada (~ 600 ng/µL), 130 µL de tampão fosfato salino (PBS) a 0,1 M, pH 7.7, acrescido de 100 µM de sulfato de amônio e ferro (II) e ácido 2,5-dihidroxibenzóico (ácido gentísico, 20 mM) como substrato. A composição das amostras do branco incluem todos os componentes acima, exceto a proteína purificada, que foi substituída pelo tampão de eluição.

As amostras foram previamente incubadas a 40°C por 1 hora e então a absorbância a 330 nm foi medida a cada 20 minutos por 4 horas. Este ensaio foi realizado em triplicata.

RESULTADOS

Análises de bioinformática – subclone CRB2(1)

O resultado da anotação gênica dos três insertos metagenômicos estão descritos na Tabela 1.

O clone CRB2(1) foi selecionado para testes subsequentes de acordo com os seguintes critérios: presença de domínios conservados, melhor desempenho nos ensaios de indução por IPTG e a presença de *hits* de dioxigenases na busca no programa BLASTp.

Embora a similaridade mais próxima na busca no Protein BLAST tenha sido uma proteína hipotética, o inserto de CRB2(1) possui domínio bicupina conservado, além da presença de exemplos de gentisato 1,2-dioxigenases (GDO) nos primeiros cem *hits*. Essas observações sugerem que CRB2(1) possa ser uma extradiol dioxigenase e, dessa forma, passível de ser caracterizada como uma nova dioxigenase metagenômica, como uma provável GDO.

Tabela 1. Anotação gênica do programa Protein BLAST para dioxigenases putativas de três subclones metagenômicos.

Subclone	Antibiótico de seleção	Best hit	e-value	Score	Similaridade (%)	Cobertura (%)	Domínios conservados	Tamanho	Massa molecular estimada (Kda)
AMX3(2)	Amoxicilina	Hypothetical protein (*Candidatus koribacter versatilis)*	2e-64	225	38	97	-	1077 pb 358 aa	39,8
AMX3(3)	Amoxicilina	Intradiol ring-cleavage dioxygenase (*Candidatus koribacter versatilis*)	2e-141	431	42	98	Peptidase_M14NE-CP-C_like superfamily	1674 pb 557 aa	61,9
CRB2(1)	Carbenicilina	Hypothetical protein (*Frankia sp.* Iso899)	2e-145	426	61	91	Cupin_2 superfamily	1068 pb 355 aa	39,5

Assim, as análises de bioinformática foram realizadas no sentido de confirmar a presença e localização dos domínios cupina indicados pela anotação gênica e identificar similaridades de sequência do subclone CRB2(1) com enzimas dioxigenases já caracterizadas, como características de estrutura secundária, a presença do motivo de ligação ao metal divalente e análise filogenética.

Como o domínio cupina é composto pela organização de folhas beta, realizou-se a predição de estrutura secundária de CRB2(1) no programa PSIPRED.

Via de regra, a presença de dois motivos conservados, dados pelos módulos $[G(X)_5HXH(X)_{3-4}E(X)_6G]$ e $[G(X)_{5-7}PXG(X)_2H(X)_3]$, denominados Motivo 1 e Motivo 2, respectivamente, constituem o domínio cupina (Dunwell *et al.*, 2001). Dessa forma, proteínas com este domínio duplicado são denominadas bicupinas.

Assim, de acordo com a ilustração da Figura 5, observa-se a presença dos motivos nos dois domínios, indicados pelos polígonos tracejados. As duas primeiras e duas últimas folhas beta de cada domínio contém os motivos 1 e 2, respectivamente, conforme é característico (Dunwell *et al.*, 2004; Vaillancourt *et al.*, 2006; Fetzner, 2012).

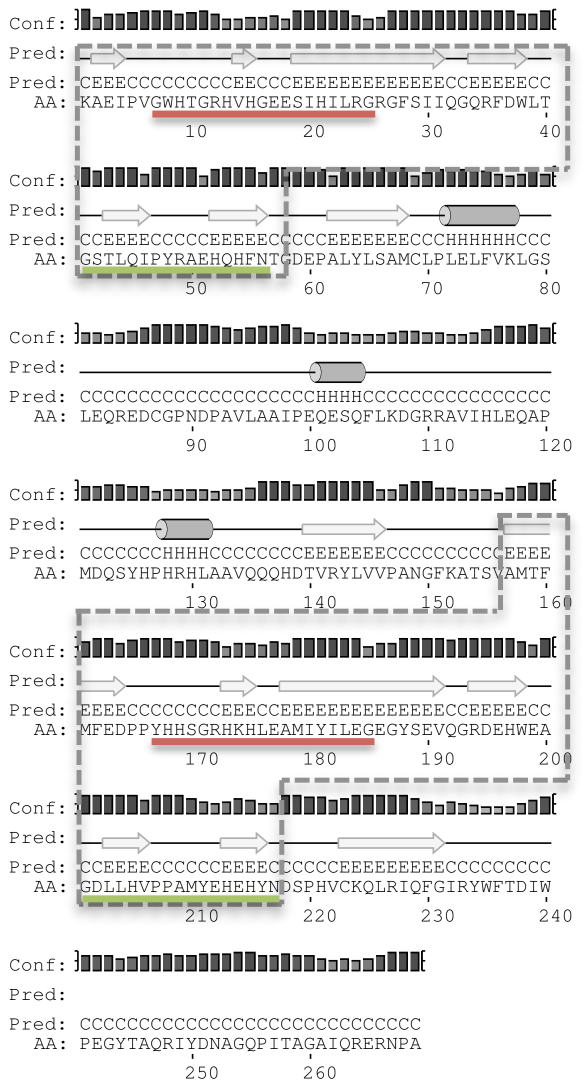


Figura 5. Predição de estrutura secundária da sequência de aminoácidos do subclone metagenômico CRB2(1) e localização dos domínios cupina. Cada domínio, indicado pelos polígonos tracejados, contém seis folhas beta, representadas pelas setas. A primeira e última folhas beta contém os motivos 1 e 2, respectivamente, representados pelas linhas vermelhas (M1) e verdes (M2). Esta estrutura secundária foi predita com o programa PSIPRED v3.3 (Jones, 1999) e mostra a presença de domínio bicupina na sequência de aminoácidos de CRB2(1), o que corrobora com a anotação gênica inicial.

Como a estrutura secundária identificou dois domínios cupina, realizou-se o alinhamento da sequência de aminoácidos de CRB2(1) com dioxigenases com domínio bicupina caracterizadas, incluindo gentisato 1,2-dioxigenases e 1-hidroxi-2-naftoato dioxigenase, que também é uma extradiol dioxigenase do Tipo III (Figura 6), para identificar os resíduos de ligação ao metal presente em todas as extradiol dioxigenases. Este domínio é denominado motivo 2-His-1-Glu, formado pela conservação de dois resíduos de histidina e um de glutamato (Vaillancourt *et al.*, 2006).

Em cupinas, há um resíduo adicional de histidina conservado que compõe o motivo de ligação ao metal. Assim, observa-se dois resíduos de histidina e um de glutamanto no Motivo 1 e outro de histidina no Motivo 2 (Dunwell *et al.*, 2004; Fetzner, 2012; Xu *et al.*, 2014).

Verifica-se, no alinhamento, a ausência do resíduo conservado de glutamato que, em gentisato 1,2-dioxigenases está geralmente substituído por um resíduo de alanina ou outro hidrofóbico ou polar (Fetzner, 2012). Em CRB2(1), este resíduo está substituído por uma serina, um aminoácido polar.

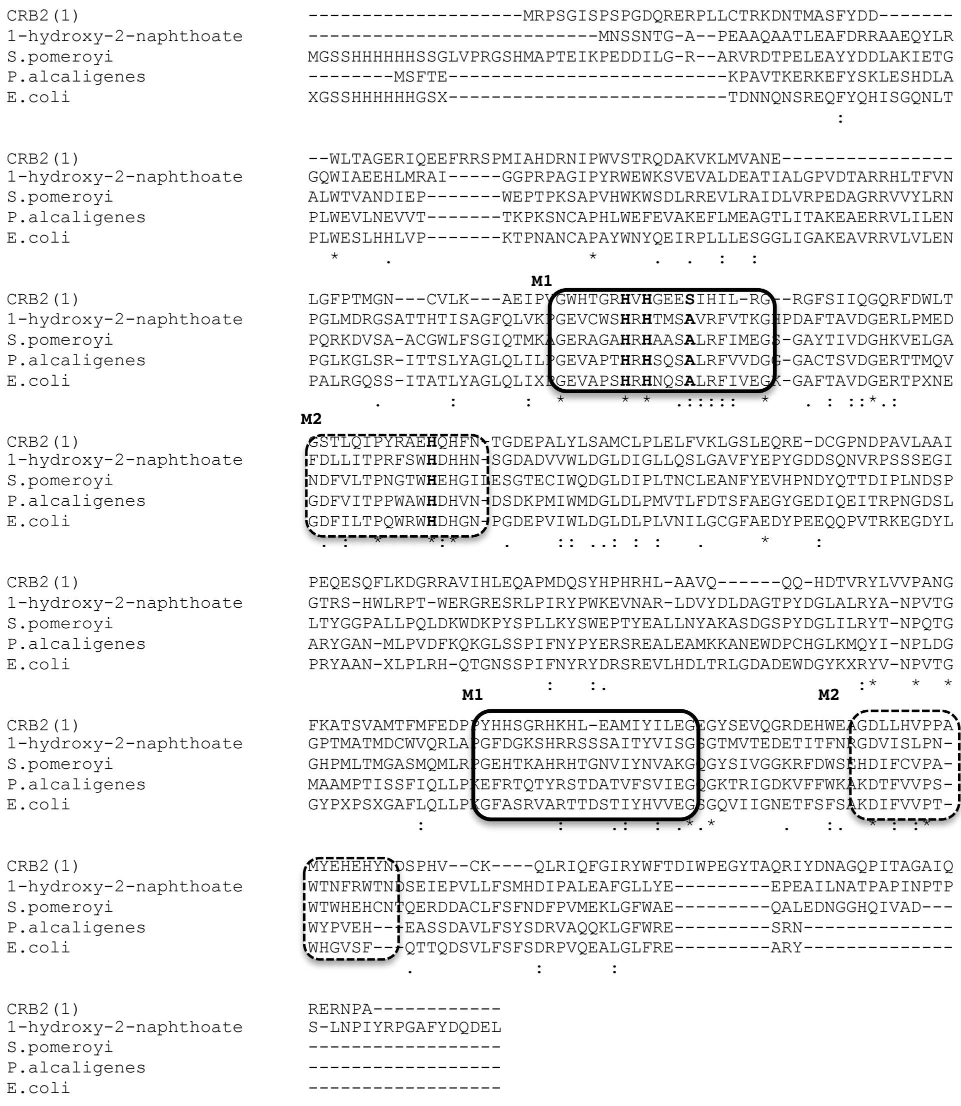


Figura 6. Alinhamento múltiplo das sequências de aminoácidos de CRB2(1) com enzimas gentisato 1,2-dioxigenases caracterizadas e uma 1-hidroxi-2-naftotato dioxigenase, todas com domínio bicupina. Os motivos cupina estão representados pelos retângulos, discriminados pelo motivo 1 (M1, representado pelos quadrados em negrito) e o motivo 2 (M2, representado pelos quadrados tracejados). As letras em negrito representam os resíduos conservados do domínio de ligação ao metal divalente dos domínios cupina (2-His-1-Glu no motivo 1 e 1-His no motivo 2). Nas gentisato 1,2-dioxigenases, o resíduo de glutamato é tipicamente substituído por um resíduo de alanina; entretanto, em CRB2(1) esse resíduo foi substituído por uma serina. As sequências foram obtidas no banco de dados do NCBI e incluem: 1-hydroxy-2-naphtoate de *Nocardioides sp.* K7 (BAA31235.2); gentisate 1,2-dioxygenase de *Silicibacter pomeroyi* (3BU7_A); gentisate 1,2-dioxygenase de *Escherichia coli* O57:H7 (2D40_A), e gentisate 1,2-dioxygenase de *Pseudomonas alcaligenes* (AAD49427.1).

Finalmente, a análise filogenética foi realizada a fim de corroborar as evidências de que CRB2(1) é uma extradiol dioxigenase, possivelmente um GDO. O cladograma distingue três grupos de proteínas: dioxigenases intradiol, extradiol e uma monooxigenase (Figura 7). A sequência de CRB2(1) agrupa ao clado de extradiol dioxigenases e está localizada junto com dioxigenases bicupinas conhecidas, incluindo GDOs bicupinas.

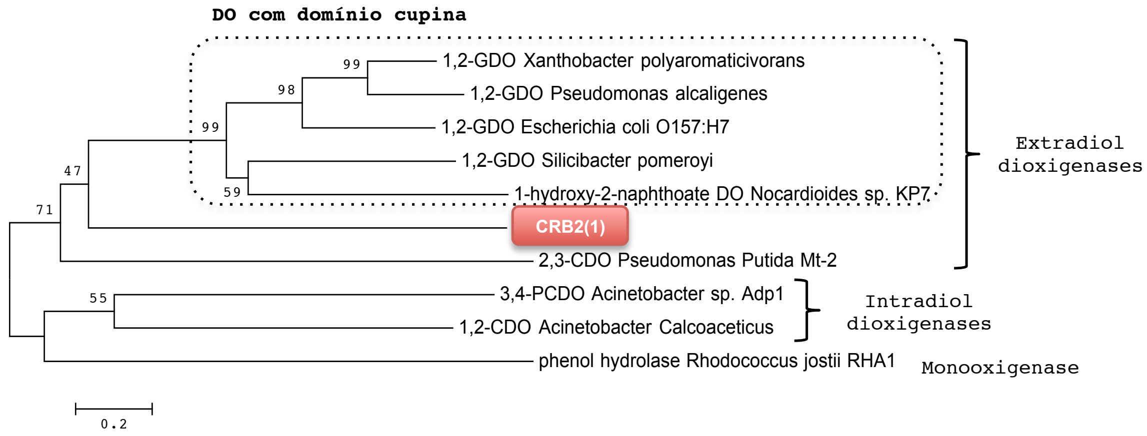


Figura 7. Análise filogenética da sequência de aminoácidos de CRB2(1). Três grupos distintos foram identificados e agrupados: extradiol dioxigenases, intradiol dioxigenases e uma monooxigenase, representada pela enzima fenol hidrolase. A sequência de CRB2(1), mostrada no quadro vermelho, agrupa com o clado das extradiol dioxigenases, próxima às dioxigenases com domínio cupina (DO). A árvore foi construída no programa Mega 6.06, utilizado o método Neighbor-joining e análise de *boostrap* com 500 reamostragens. As sequências foram obtidas no banco de dados do NCBI e incluem: gentisato 1,2-dioxigenase (1,2-GDO) de *Xanthobacter polyaromaticivorans* (BAC98955.1); gentisato 1,2-dioxigenase (1,2-GDO) de *Pseudomonas alcaligenes* (AAD49427.1); gentisato 1,2-dioxigenase (1,2-GDO) de *Escherichia coli* O157:H7 (2D40_A); gentisato 1,2-dioxigenase (1,2-GDO) de *Silicibacter pomeroyi* (3BU7); 1-hidroxi-naftoato-dioxigenase de *Nocardioides sp.* KP7 (BAA31235.2); catecol 2,3-dioxigenase (2,3-CDO) de *Pseudomonas putida* Mt-2 (1MPY_A); protocatecuato 3,4-dioxigenase (3,4-PCDO) de *Acinetobacter sp.* Adp1 (1EO2_A); catecol 1,2-dioxigenase (1,2-CDO) de *Acinetobacter calcoaceticus* (1DLM_B), e fenol hidrolase from *Rhodococcus jostii* RHA1 (YP_703833.1).

Análises funcionais – subclone CRB2(1)

Os insertos subclonados em vetor de expressão pET24a foram transformados em cepas de *Escherichia coli* Turner(DE3), para CRB2(1) e BL21(DE3) para AMX3(3). Os insertos têm tamanhos estimados de 1674 Kb e 1068 Kb para AMX3(3) e CRB2(1), respectivamente.

O subclone CRB2(1) foi selecionado para análises subsequentes. Dessa forma, sua indução foi realizada com lactose em concentração final de 1% para possibilitar a captura da proteína na fase solúvel do lisado bacteriano. O gel de poliacrilamida mostra as culturas induzidas do controle negativo, correspondente ao clone com vetor intacto, e as frações no tempo zero, após indução,

sobrenadante e proteína purificada. A análise do gel de SDS-PAGE sugere que a CRB2(1) é uma proteína com massa molecular estimada de 39,5 Kda, conforme predição das análises de sequência (Figura 8).

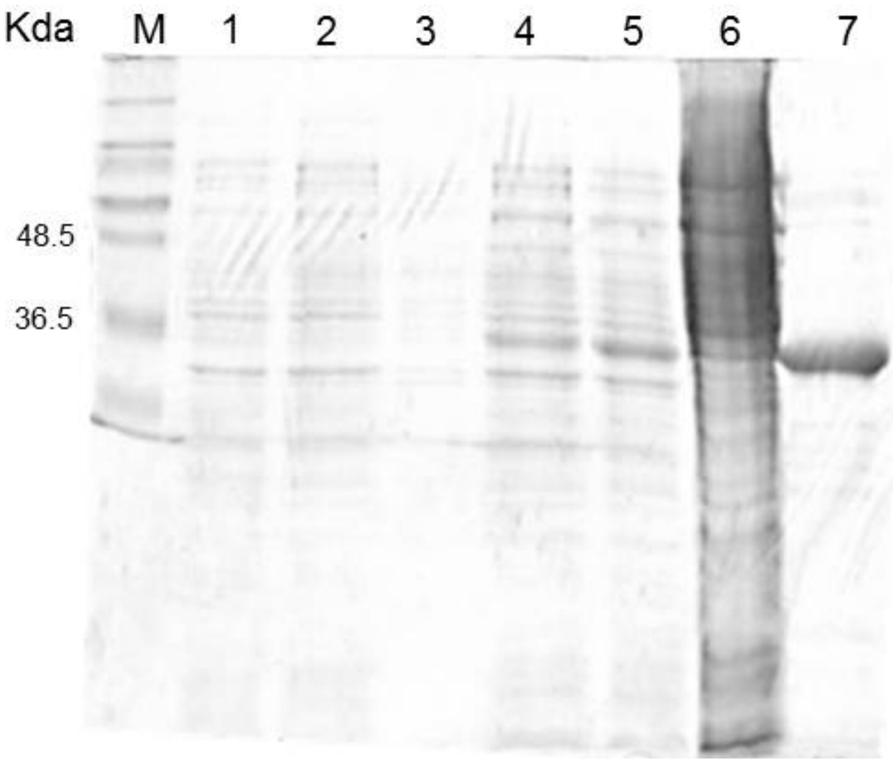
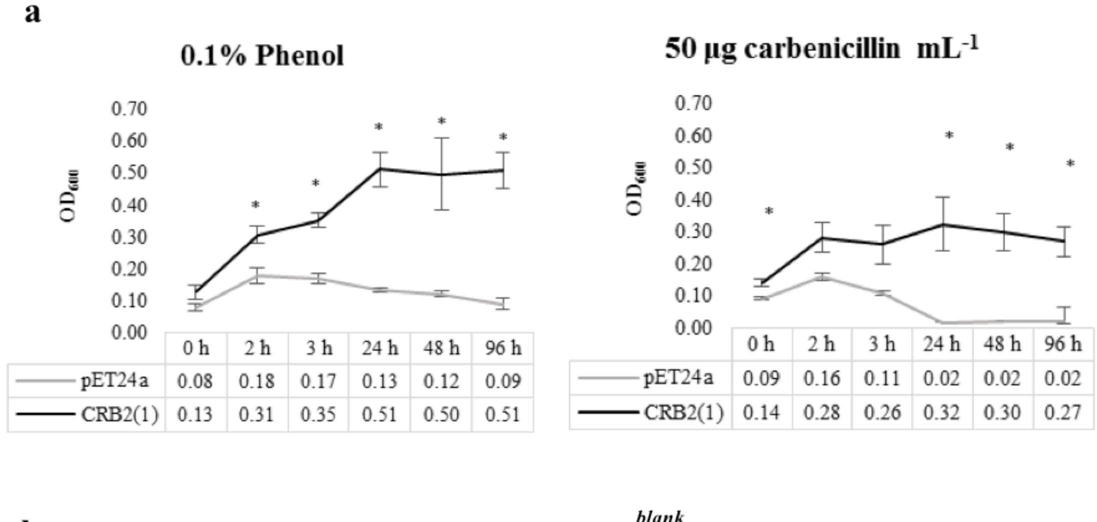


Figura 8. Corrida eletroforética em gel de poliacrilamida das frações induzidas, solúveis do controle negativo e subclone CRB2(1) e proteína purificada. M. Marcador; 1. Clone pET24a intacto tempo 0h; 2. Clone pET24a intacto induzido; 3. Clone pET24a intacto sobrenadante; 4. CRB2(1) tempo 0h; 5. CRB2(1) induzido; 6. CRB2(1) sobrenadante; 7. CRB2(1) proteína purificada. Verifica-se que a massa molecular estimada da proteína corresponde ao valor teórico previsto de 39,5 Kda.

Como as análises de bioinformática sugerem fortemente que o inserto de CRB2(1) é realmente uma gentisato 1,2-dioxigenase, a análise fenotípica incluiu ensaios de resistência do subclone à carbenicilina (antibiótico de seleção), para confirmar o fenótipo resistente inicial, e ao fenol, uma molécula aromática simples com efeitos citotóxicos.

A Figura 9a demonstra as diferenças comparativas do crescimento celular de CRB2(1) e pET24a intacto na presença de carbenicilina e fenol. Os resultados do teste de viabilidade celular estão demonstrados na Figura 9b, avaliada nas culturas de 24 h. Mudanças na coloração das amostras mostram a ação de células viáveis no reagente MTT e indicam a viabilidade celular, em detrimento dos controles negativos, onde não há mudança na coloração do meio. Esses resultados

demonstram o efeito dual de CRB2(1), que conferem resistência aos compostos antibiótico e aromático.



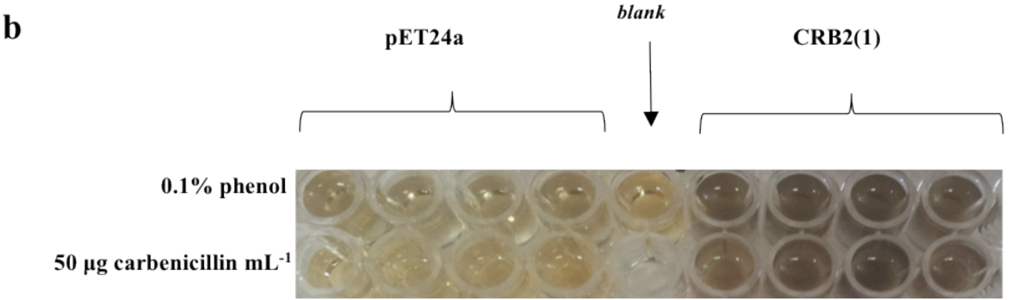


Figura 9. **a** Densidade celular em tempos pré-determinados para os clones com pET24a intacto e CRB2(1) em 50 µg carbenicilina ml^{-1} e 0,1% de fenol. No tempo 0 h, a expressão da proteína foi induzida adicionando IPTG e, uma hora depois, adicionou-se carbenicilina ou fenol às culturas. Diferenças claras foram observadas na turbidez e densidade ótica das culturas a partir do tempo 24 h. Os resultados estão expressos pela média das quadriplicatas. *$p<0,01$. **b** Ensaio de viabilidade celular. Alíquotas das culturas no tempo 24 h contendo pET24a intacto (*à esquerda*) e CRB2(1) (*à direita*) após incubação com fenol ou carbenicilina. Cada conjunto de quatro poços representa as replicatas. A redução do reagente MTT (coloração escura) indica a presença de células viáveis, o que é consistente com os resultados espectrofotométricos que mostram o aumento da densidade ótica das culturas de CRB2(1) e queda nas culturas de pET24a, quando expostas aos dois compostos.

Já que CRB2(1) foi identificada como uma possível gentisato 1,2-dioxigenase, utilizou-se ácido gentísico como substrato. O aumento da densidade ótica a 330 nm nas amostras contendo a proteína recombinante indicam o acúmulo de maleilpiruvato, formado pela quebra extradiol do anel aromático na molécula do ácido gentísico, conforme demonstra a Figura 10.

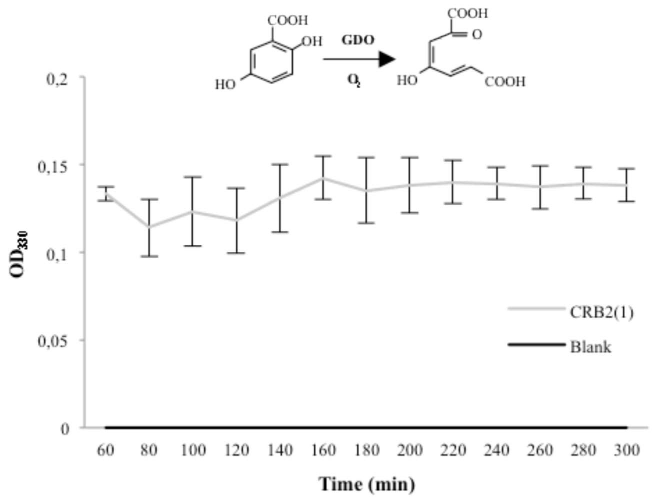


Figura 10. Ensaio enzimático cinético da proteína purificada de CRB2(1) incubada com ácido gentísico como substrato. A formação de maleilpiruvato pela quebra 2,3 de ácido gentísico na presença de oxigênio foi avaliada espectrofotometricamente. As leituras foram realizadas após 1h de incubação. As amostras do branco não continham a proteína. Os valores demonstrados são as médias aritméticas das amostras em triplicata, além da faixa de desvio padrão.

Análises de bioinformática – subclones AMX3(2) e AMX3(3)

As sequências de aminoácidos de AMX3(2) e AMX3(3) também foram anotadas e analisadas quanto à presença de resíduos conservados, estruturas secundárias e filogenia.

Assim, enquanto AMX3(3) possui maior similaridade com uma intradiol dioxigenase e, ainda, possui domínio conservado, o resultado de AMX3(2) é uma proteína hipotética, sem domínios conservados, conforme demonstra a Tabela 1.

Partindo do princípio que AMX3(3) é uma possível intradiol dioxigenase, conforme sugere a anotação pelo BLASTp, procedeu-se à investigação da conservação de motivos de ligação ao metal e de estruturas secundárias na sequência que possam ser compatíveis com as descritas para as famílias estruturais conhecidas de intradiol dioxigenases: catecol 1,2-dioxigenases/hidroxiquinol-1,2 dioxigenases e protocatecuato 3,4-dioxigenases (Urszula Guzik, 2013).

Dessa forma, a Figura 11 mostra o alinhamento de AMX3(3) com sequências caracterizadas de membros de cada classe citada acima. Observa-se a presença dos resíduos de ligação ao metal, altamente conservados em todas as intradiol dioxigenases já caracterizadas: duas histidinas e duas tirosinas (Urszula Guzik, 2013), conforme ilustra a figura abaixo.

```
AMX3(3)       ----MRMSNCGAIQRLILTITACCSM---------------TLAGTAQNTDTAPTSARTG
3,4 PCDO      -------------------------------MSQI---------------------IW--
1,2 CDO       MT--VKISHTADIQAFFNQVAGLDHAEGKPRFKQIILRVLQDTARLIEDLEITEDEFW--
1,2 HQDO      MSTPVSAEQQAREQDLVERVLRSFDATADPRLKQVMQALTRHLHAFLREVRLTEAEWE--

AMX3(3)       YRISGLVVSKTDGHPLDRARVLLRDSKSRQEPQSTVTSQDGKFVFENLKPGKYSLQGVKR
3,4 PCDO      ---GAYAQRNTEDHPPAY------------APGYKTSVLR---------SPKNALIS-IA
1,2 CDO       ---HAVDYLNRLGGRNEA--------------GLLAAGLGIEHFLDLLQDAKDAEAGLGG
1,2 HQDO      ---TGIGFLTDAGHVTNER---------RQEFILLSDVLGA-----SMQTIAMNNEAHGD
                  .    .                                              .

AMX3(3)       GFVTAAYDQHDQYSTAIVTGAGLD-TENL-------ILKLSPQAIISGRVLDEAGEPLRH
3,4 PCDO      ETLS------EVTAPHFSADKFGPKDNDLILNYAKDGLPIGERVIVHGYVRDQFGRPVKN
1,2 CDO       GTPR------TIEGPLYVAGAPLAQG----EVRMDDGTDPGVVMFLQGQVFDANGKPLAG
1,2 HQDO      ATEA------TVFGPFFVEGSPRIES----GGDIA-GGAAGEPCWVEGTVTDTDGNPVPD
                           .                          .    : * *  *.*:

AMX3(3)       ALLTLYRSSHLQG------------VDEILGASNAQTDDLGTYEFPSLQPGTYFLSVH--
3,4 PCDO      ALVEVWQANASGRYRHPNDQYIGAMDPNFGGCGRMLTDDNGYYVFRTIKPGPYPWRNR--
1,2 CDO       ATVDLWHANTQGTYSYFD-----STQSEFNLRRRIITDAEGRYRARSIVPSGYGCDPQGP
1,2 HQDO      ARIEVWEADDDGFYDVQY-----D-DDRTAARAHLLSGPDGGYAFWAITPTPYPIPHDGP
              * : ::.:.                  .     .  :   * *   :: *  *

AMX3(3)       ---------AQPWYAVHPHRQGDRTNSDGSDSEQANVERSLDVAYPVMYYENATETDAAT
3,4 PCDO      ---------INEWRPAHIHFSLIA------------------------------------
1,2 CDO       TQECLDLLGRHGQRPAHVHFFISA------------------------------------
1,2 HQDO      VGRMLAATGRSPMRASHLHFMVTA------------------------------------
                              * *

AMX3(3)       PIQIRGGEHLQIDMNLNPVPALRVIFHAPANQSHGFTVPQVEQPVFDGSTYIQTTPQFIA
3,4 PCDO      ---------------------------------DGWAQRLISQFYFEGDTLIDSCPILKT
1,2 CDO       ---------------------------------FGH-RHLTTQINFAGDKYLWDDFAYAT
1,2 HQDO      ---------------------------------PGR-RTLVTHIFVEGDELLDRDSVFGV
                                                *       :  . *.  :       .

AMX3(3)       PG-VVE-LT--------------G--IPAGRYNIHVAGPNAMTLLSGIELSKDTELDSSP
3,4 PCDO      IPSEQQ---RRALIALEDKSNFIEADSRCYRFDITLRGRRATYF----------END---
1,2 CDO       RDGLIGELRFVEDAAAARDRGVQGERFAELSFDFRLQGAQSPDA----------EAR---
1,2 HQDO      KDSLVKSFERQPAGAPTPGGREIDGPWSRVRFDIVLAPA---------------------
                                             ::: :

AMX3(3)       AEPLATVRVSVAAPGEGVIPRGLSVGLIHGRAFVGGFHEVNAKGESEIPQVPAGNYDVRV
3,4 PCDO      ----------------LT------------------------------------------
1,2 CDO       ----------------SHRPRALQEG----------------------------------
1,2 HQDO      ------------------------------------------------------------

AMX3(3)       IGGGKWYTIVGISADGAQVKGHSINVPAGASTTIALTVASGVEVQGVVKKAGKPFAEAMI
3,4 PCDO      ------------------------------------------------------------
1,2 CDO       ------------------------------------------------------------
1,2 HQDO      ------------------------------------------------------------

AMX3(3)       VLVPKDPEGNRDLFRRDQSDLDGTFSLRSVVPGSYTLVAIEDGWDLDWSRPEIIAAYRKR
3,4 PCDO      ------------------------------------------------------------
1,2 CDO       ------------------------------------------------------------
1,2 HQDO      ------------------------------------------------------------

AMX3(3)       GQPVQISSKTSGPVEVKEAIEVQPK
3,4 PCDO      -------------------------
1,2 CDO       -------------------------
1,2 HQDO      -------------------------
```

Figura 11. Alinhamento múltiplo da sequência de aminoácidos de AMX3(3) com enzimas intradiol dioxigenases caracterizadas de cada família conhecida. Verifica-se a conservação dos dois resíduos de histidina e tirosina, marcados em negrito, nas quatro sequências. As sequências foram baixadas do banco de dados do NCBI e incluem: 1,2 CDO - catecol 1,2-dioxigenase de *Pseudomonas arvilla* C-1 (2AZQ_A), 1,2 HQDO - hidroxiquinol 1,2-dioxigenase de *Nocardioides simplex* 3e (1TMX_A) e 3,4 PCDO - protocatecuato 3,4-dioxigenase subunidade beta de *Acinetobacter sp* Adp1 (1EO9_B).

Já a sequência de AMX3(2) foi alinhada separadamente de AMX3(3) para ilustrar a ausência de resíduos conservados quando comparadas a enzimas intradiol dioxigenases já caracterizadas. Assim, a Figura 12 ilustra o alinhamento múltiplo de AMX3(2) com as mesmas proteínas utilizadas no alinhamento anterior com a sequência de AMX3(3). Verifica-se a ausência parcial dos resíduos conservados clássicos para intradiol dioxigenases, ou seja, a conservação de dois resíduos de histidina e dois de tirosina.

Na referida ilustração estão destacados em negrito os resíduos de tirosina conservados. A localização do primeiro conjunto difere daquele encontrado no alinhamento com AMX3(3), que estão marcados em laranja. Nesta posição, é possível observar que o resíduo de AMX3(2), marcado em roxo, é uma fenilalanina, enquanto a tirosina está conservada nas outras sequências. O próximo conjunto de tirosinas conservado, marcado em negrito, é o mesmo observado no alinhamento de AMX3(3).

Para os resíduos de histidina, entretanto, não há conservação na sequência de AMX3(2). Aqueles encontrados conservados entre as três sequências caracterizadas de intradiol dioxigenases no alinhamento anterior (Figura 11) estão marcados em azul. Verifica-se neste caso, para AMX3(2), a mudança para resíduos de glutamato e metionina, marcados em vermelho (Figura 12).

```
AMX3(2)       MYGYPPVFYPGAADFASAETIQASPGQTVQDLVAARQPYYRVNIPVSSSDVTSGLNVTV
3,4 PCDO      ------------------------------------------------------------
1,2 CDO       --------------MT------------VKISHTADIQAFFNQVAG------------LD
1,2 HQDO      --------------MS----------TPVSAEQQAREQDLVERVLR------------SF

AMX3(2)       QSQAGPGYSLGYNPVAKRIEGFLPNGNYMVEATAFGPNVLSGMVNLKVDGTAAEAAPMTL
3,4 PCDO      -------MSQI-------------------------------------------------
1,2 CDO       HAEGKPRFKQIILRVLQDTAR---------------------------------------
1,2 HQDO      DATADPRLKQVMQALTRHLHA---------------------------------------
                      .

AMX3(2)       VPGGSIALDVKEHFTDTASRESMSWNNVKGTFTVRGPRAYLEARLEAADDFEPKNGTFLR
3,4 PCDO      -----------------------IWGAYAQRNTEDHPPAY--------------------
1,2 CDO       ------------LIEDLEITEDEFWHAVDYLNRLGGRNEA--------------------
1,2 HQDO      ------------FLREVRLTEAEWETGIGFLTDAGHVTNE--------------------

AMX3(2)       PPTGPNDESLVLENVLPGRYWLRLN-TSRGYVASASVGDVDVLRQPFVVTSGPSAPI---
3,4 PCDO      -------APGYKTSVLR---------SPKNALIS-IAETLSEVTAPHFSADKFGPKDNDL
1,2 CDO       ---------GLLAAGLGIEHFLDLLQDAKDAEAGLGGGTPRTIEGPLYVAGAPLAQG---
1,2 HQDO      ----RRQEFILLSDVLGA-----SMQTIAMNNEAHGDATEATVFGPFFVEGSPRIES---
                             *                 .        :  *

AMX3(2)       -EIEVRDD-----TAEIEGTVTG-IGPPSADLASPSSHPRAWV------YCIPL------
3,4 PCDO      ILNYAKDGLPIGERVIVHGYVRDQFGRPVKN-----ALVEVWQANASGRYRHPNDQYIGA
1,2 CDO       -EVRMDDGTDPGVVMFLQGQVFDANGKPLAG-----ATVDLWHANTQGTYSYFD-----S
1,2 HQDO      -GGDIA-GGAAGEPCWVEGTVTDTDGNPVPD-----ARIEVWEADDDGFYDVQY-----D
                              :.* *    * *        :    *       *

AMX3(2)       -PDSPGQFNQATVGGEGHFQDPMMAPGGYRILAFARQQLHLP------YRDAEAMKAFEA
3,4 PCDO      MDPNFGGCGRMLTDDNGYYVFRTIKPGPYPWRNR-----------INEWRPAHIHFSLIA
1,2 CDO       TQSEFNLRRRIITDAEGRYRARSIVPSGYGCDPQGPTQECLDLLGRHGQRPAHVHFFISA
1,2 HQDO      -DDRTAARAHLLSGPDGGYAFWAITPTPYPIPHDGPVGRMLAATGRSPMRASHLHFMVTA
                       :     :* :    : *  *                    * :.    . *

AMX3(2)       MGPVVHLSA-----GQKVSVEVPLISDSDAPEK---------------------------
3,4 PCDO      DGWAQRLISQFYFEGDTLIDSCPILKTIPSEQQ---RRALIALEDKSNFIEADSRCYRFD
1,2 CDO       FGH-RHLTTQINFAGDKYLWDDFAYATRDGLIGELRFVEDAAAARDRGVQGERFAELSFD
1,2 HQDO      PGR-RTLVTHIFVEGDELLDRDSVFGVKDSLVKSFERQPAGAPTPGGREIDGPWSRVRFD
               *    * :     *:             .

AMX3(2)       ------------------------
3,4 PCDO      ITLRGRRATYFENDLT--------
1,2 CDO       FRLQGAQSPDAEARSHRPRALQEG
1,2 HQDO      IVLAPA------------------
```

Figura 12. Alinhamento múltiplo da sequência de aminoácidos de AMX3(2) com enzimas intradiol dioxigenases caracterizadas. Verifica-se ausência de conservação dos resíduos de histidina, enquanto os de tirosina estão parcialmente conservados. Em negrito, é possível verificar a presença da conservação dos dois resíduos de tirosina, embora a localização de alguns difira da observada no alinhamento de AMX3(3). O primeiro conjunto de resíduos de tirosina, apesar de conservados em todas as proteínas, não estão no mesmo local do encontrado no alinhamento de AMX3(3) – estes, por sua vez, estão marcados em laranja. O resíduo em roxo mostra onde deveria estar a tirosina de AMX3(2), caso fosse conservada. Já os resíduos de histidina, conservados entre 1,2 CDO; 1,2 HQDO e 3,4 PCDO estão presentes e coloridos em azul – estes são os mesmos resíduos conservados no alinhamento de AMX3(3). Em vermelho, estão os resíduos de AMX3(2) onde deveriam estar os resíduos de histidina, caso houvesse conservação. As sequências foram baixadas do banco de dados do NCBI e incluem: catecol 1,2-dioxigenase de *Pseudomonas arvilla* C-1 (2AZQ_A), hidroxiquinol 1,2-dioxigenase de *Nocardioides simplex* 3e (1TMX_A) e protocatecuato 3,4-dioxigenase subunidade beta de *Acinetobacter sp* Adp1 (1EO9_B).

As estruturas secundárias de AMX3(3) e AMX3(2) também foram preditas no programa PSIPRED e estão ilustradas na Figura 13. As duas proteínas metagenômicas contém uma grande quantidade de folhas beta – para AMX3(3) não há presença de alfa hélices, enquanto em AMX3(2) há apenas uma na porção C-terminal.

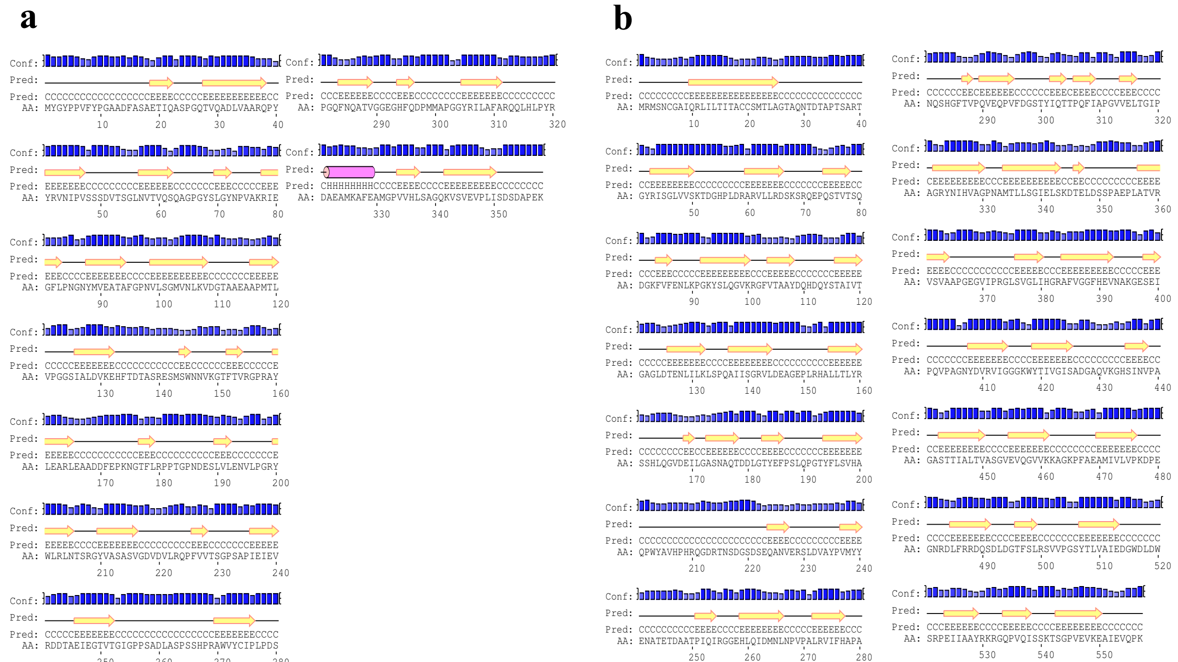


Figura 13. Predição de estrutura secundária de **a.** AMX3(2) e **b.** AMX3(3). Verifica-se a grande quantidade de folhas beta na estrutura das proteínas, sendo que AMX3(3) é totalmente constuída de folhas beta enquanto AMX3(2) possui apenas uma alfa hélice na porção C-terminal. As folhas beta estão representadas pelas setas e as alfa hélices pelos cilindros. Esta representação foi realizada no programa PSIPRED v3.3 (Tamura *et al.*, 2013).

Já a análise filogenética (Figura 14) foi realizada no sentido de evidenciar o agrupamento das duas proteínas em extra e intradiol dioxigenases ou em um possível *outgroup*, já que não há conservação de estrutura secundária característica nas duas e, ainda, ausência parcial de resíduos conservados de ligação ao metal para AMX3(2).

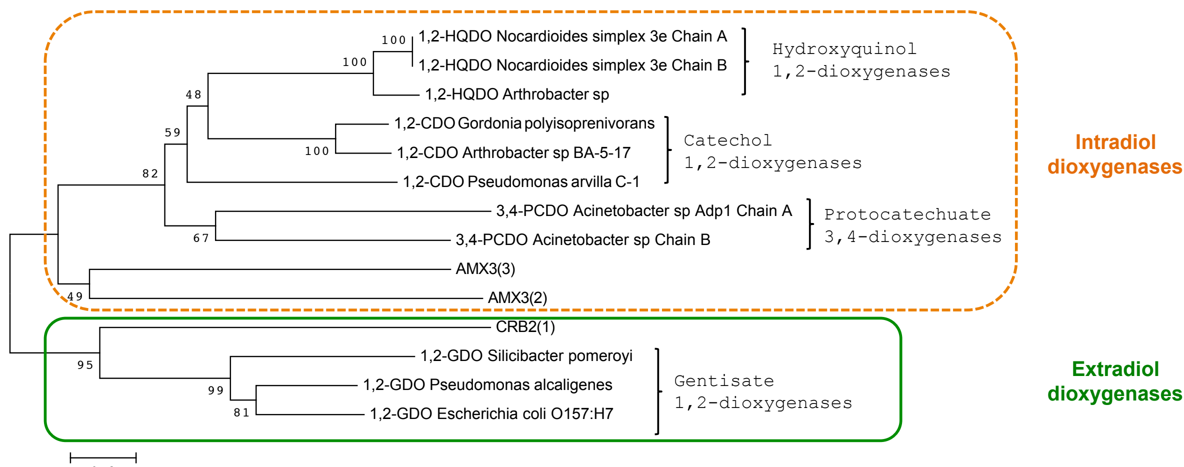


Figura 14. Análise filogenética de CRB2(1), AMX3(2) e AMX3(3) com enzimas dioxigenases, intra e extradiol caracterizadas. Observa-se a separação em dois grupos distintos, para intradiol (acima) e extradiol dioxigenases (abaixo). A sequência de CRB2(1) alinha novamente com o grupo de gentisato 1,2-dioxigenases, enquanto AMX3(3) e AMX3(2) agrupam com as intradiol dioxigenases, apesar de formarem um subgrupo distinto. As sequências foram baixadas do banco de dados do NCBI e incluem: hidroxiquinol 1,2-dioxigenase subunidade alfa de *Nocardioides simplex* 3e (1TMX_A); hidroxiquinol 1,2-dioxigenase subunidade beta de *Nocardioides simplex* 3e (1TMX_B); hidroxiquinol 1,2-dioxigenase de *Arthrobacter sp.* (BAA82713.1); catecol 1,2-dioxigenase subunidade alfa de *Pseudomonas arvilla C-1* (2AZQ_A); catecol 1,2-dioxigenase de *Gordonia polyisoprenivorans* (WP_014361202.1); catecol 1,2-dioxigenase de *Arthrobacter sp.* BA-5-17 (BAD11154.1); protocatecuato 3,4-dioxigenase subunidade alfa de *Acinetobacter sp.* Adp1(1EO9_A); protocatecuato 3,4-dioxigenase subunidade beta de *Acinetobacter sp.* Adp1(1EO9_B); gentisato 1,2-dioxigenase de *Silicibacter pomeroyi* (3BU7_A); gentisato 1,2-dioxigenase de *Pseudomonas alcaligenes* (AAD49427.1); gentisato 1,2-dioxigenase de *Escherichia coli* O157:H7 (2D40_A).

Verifica-se a separação das sequências em dois grupos distintos: extradiol e intradiol dioxigenases. A sequência de CRB2(1) agrupa em extradiol dioxigenases, conforme evidenciado anteriormente. Já AMX3(2) e AMX3(3) agrupam em intradiol dioxigenases, apesar de constituírem um subgrupo em separado.

DISCUSSÃO

A caracterização de sequências metagenômicas apresenta várias limitações. O mais proeminente é a baixa similaridade dos genes encontrados nos bancos de dados de sequência, onde muitos *hits* são proteínas hipotéticas ou putativas e, ainda, domínios conservados estão ausentes.

Entretanto, as triagens funcionais fornecem informações importantes sobre as sequências de um dado inserto ou gene metagenômico, já que os substratos utilizados nos ensaios iniciais são fortes indicativos para a classificação das ORFs identificadas.

No caso descrito nesta Tese, a resistência antimicrobiana por si só não foi capaz de sugerir qualquer ORF em particular como a responsável pelo fenótipo resistente observado, o que reforça o fato de que a diversidade ambiental de genes de resistência a antibioticos ainda é pouco conhecida.

Entretanto, a identificação de dioxigenases putativas nos insertos metagenômicos de dois clones resistentes sugerem o possível papel destas enzimas na resistência antimicrobiana. Dessa forma, os ensaios subsequentes às análises de bioinformática não só vieram a confirmar esta hipótese como também revelaram a potential habilidade desta enzima na quebra do anel aromático, dada pela observação da resistência do subclone CRB2(1) na presença de fenol.

Diversas gentisato 1,2-dioxigenases já foram caracterizadas, porém nenhuma, até o momento, é originária de ensaios metagenômicos. Neste trabalho, no entanto, descreve-se a caracterização inicial de uma gentisato 1,2-dioxigenase isolada de um clone metagenômico resistente a antibióticos β-lactâmicos, em especial à carbenicilina. Assim, as análises funcionais e de sequência indicam que o subclone CRB2(1) é uma provável GDO com domínio bicupina conservado.

É incerta a razão pela qual uma dioxigenase foi selecionada em uma triagem de resistência antimicrobiana ao invés de elementos de resistência a antibióticos bem caracterizados, como as betalactamases no caso de antibióticos β-lactâmicos. Esta observação é consistente com a ideia de que a diversidade de ARG, especialmente nos ambientes naturais, é bem maior que a esperada.

Na verdade, há fortes evidências de que ARGs não somente estão amplamente distribuídos no meio ambiente como também são anteriores à chamada “era antibiótica”, dada pela introdução de drogas antimicrobianas na saúde humana e animal a partir da década de 1930. Esta observação é demonstrada em um estudo metagenômico de amostras de solo congelado conservadas há milhares de anos, onde os autores identificaram genes de resistência a antibióticos (β-lactâmicos, tetraciclinas e glicopeptídeos), concluindo que a resistência antimicrobiana é um evento natural anterior à pressão

seletiva atual (D'costa *et al.*, 2011). Tal fato sugere que elementos de resistência clinicamente importantes são genes pré-existentes que foram selecionados pelo uso de antibióticos, com as cepas resistentes provavelmente adaptando-se às crescentes concentrações destes compostos.

Dessa maneira, a análise de ARGs provenientes de ambientes naturais pode identificar elementos de resistência antimicrobiana ainda não descritos e auxiliar na elucidação de novos mecanismos (enriquecendo também as bases de dados de sequências), possíveis papéis ecológicos, além de auxiliar na tentativa de prever como estes genes são transferidos para micro-organismos patogênicos, por exemplo, via elementos móveis tais como plasmídeos, transposons e integrons.

Consequentemente, mais esforços devem ser concentrados para identificar a origem dos ARGs, principalmente aqueles inseridos nos micro-organismos ainda não cultiváveis, que contêm uma ampla gama de genes e proteínas não caracterizados, muitos como potencial de causar resistência antimicrobiana.

Como exemplo, pode-se citar um estudo recente baseado em metagenômica funcional de amostras de solo que revelou o papel de um gene regulador de resposta na tolerância à carbenicilina em *Escherichia coli* (Allen et al., 2015), reforçando a necessidade de ampliar o conhecimento dos elementos de resistência antimicrobiana, especialmente aqueles originários dos ambientes naturais.

Contudo, a classificação de um determinado gene como um ARG é desafiadora, já que sua ação no fenótipo resistente pode não ser sua função primária, como mostra a ação de CRB2(1) em carbenicilina.

Os dados apresentados neste Capítulo estão descritos no artigo publicado em 2015 (Dos Santos *et al.*, 2015) e é, até onde se sabe, a primeira observação na qual uma enzima extradiol dioxigenase tem ação na resistência a antibióticos β-lactâmicos. Embora o mecanismo pelo qual a GDO metagenômica age no antibiótico ainda está a esclarecer, é provável que esta enzima atue na quebra do anel aromático da molécula de carbenicilina, inativando, assim, sua ação antimicrobiana. Esta ação pode revelar outro mecanismo de resistência à carbenicilina, que também pode ser transferível para outros antibióticos β-lactâmicos.

Caso esta hipótese seja confirmada, GDOs podem representar outra classe enzimática que age na quebra da molécula do antibiótico β-lactâmico, além das betalactamases, que têm grande impacto nas infecções de origem clínica.

A habilidade da GDO de CRB2(1) em quebrar anéis aromáticos, dada pelo ensaio cinético enzimático e sugerido pela resistência ao fenol, é um outra observação importante deste trabalho. Genes de resistência antimicrobiana são frequentemente encontrados associados a outros genes que conferem fenótipo similar, como aqueles de resistência a metais pesados e a amônias quaternárias. Assim, a degradação aromática pode atuar de maneira análoga, selecionando também outros genes e construindo cassetes de resistência a xenobióticos.

A resistência ao fenol do subclone metagenômico também sugere o potencial uso de dioxigenases, em especial esta GDO, em processos de biorremediação. Compostos aromáticos são poluentes ambientais importantes e a degradação enzimática destes compostos pode facilitar sua remoção de áreas contaminadas como este tipo de resíduo. Assim, acessar a diversidade funcional microbiana, especialmente daqueles micro-organismos ainda não cultiváveis e desconhecidos, ajuda a incrementar o leque de enzimas utilizadas neste tipo de processo biotecnológico.

Diante do exposto, é possível concluir que CRB2(1) apresenta características compatíveis com dioxigenases, em especial as extradiol dioxigenases do tipo III, da superfamília das cupinas. Assim, observa-se a presença de dois domínios cupina, o que caracterizou CRB2(1) como uma bicupina. Os motivos clássicos de ligação ao metal também estão presentes, dados pela conservação dos resíduos de histidina e glutamato – este último, em GDOs, pode estar mutado para resíduos polares ou hidrofóbicos (Fetzner, 2012).

Com tais observações, juntamente com a análise filogenética e enzimática, foi possível classificar CRB2(1) como uma extradiol dioxigenase e provável GDO. Dessa forma, esta é a primeira dioxigenase metagenômica parcialmente caracterizada e possivelmente a primeira GDO metagenômica.

O fato de ser isolada e confirmada a ação desta dioxigenase em resistência ao antibiótico carbenicilina demonstra o potencial, muitas vezes oculto, de proteinas com outras funções primárias caracterizadas em causar resistência antimicrobiana, sendo resultado de um possível “efeito colateral”. No caso de dioxigenases, há a possibilidade do mecanismo de resistência estar envolvido na capacidade destas enzimas em clivar anéis aromáticos e, dessa forma, desestabilizando a molécula de alguma forma.

Para os dois outros subclones, AMX3(2) e AMX3(3), realizou-se somente as análises de sequência, no sentido de verificar a presença de características conservadas relacionadas a enzimas dioxigenases e, assim, padronizar futuros testes fenotípicos.

Na anotação gênica inicial realizada no BLASTp (Tabela 1), AMX3(3) possui domínio conservado, em termos de superfamília, relacionado a enzimas intradiol dioxigenases. Já AMX3(2) não possui – entretanto, devido à presença de outros *hits* na anotação relacionados a estas enzimas, procedeu-se à análise desta ORF também.

Assim, os resultados das análises de alinhamento múltiplo, predição de estrutura secundária e filogenia mostram que AMX3(3) possui características conservadas para intradiol dioxigenases, demonstrada pela presença de resíduos conservados de ligação ao metal.

Entretanto, as outras análises não são tão precisas em indicar esta classificação.

A estrutura secundária das duas classes estruturais de intradiol dioxigenases – catecol 1,2-dioxigenases/hidroxiquinol 1,2-dioxigenases e protocatecuato 3,4-dioxigenases – possuem características particulares e altamente conservadas. Em 1,2-CDOs, por exemplo, o domínio N-terminal helicoidal é conservado em todas as enzimas já caracterizadas. Em 3,4-PCDO, a porção central é composta por uma série de pequenas alfa-hélices, circundadas por uma combinação de folhas beta que formam um beta-sanduíche (Urszula Guzik, 2013).

Ao analisar a Figura 13a, percebe-se que a estrutura secundária de AMX3(3) é composta somente de folhas beta, o que não é característico de nenhuma intradiol dioxigenase já descrita. Para comparação, a Figura 15 foi construída para ilustrar a predição de estrutura secundária de membros caracterizados de cada classe de intradiol dioxigenases.

Nesta ilustração, confirma-se a diferença estrutural entre catecol e hidroxiquinol 1,2-dioxigenases e protocatecuato 3,4-dioxigenases. Verifica-se também que a estrutura secundária dos dois subclones se assemelham mais àquela da protocatecuato 3,4-dioxigenase.

Já AMX3(2) apresenta mais diferenças que semelhanças em relação às características conservadas de intradiol dioxigenases. Além de não haver domínios conservados na estrutura, o alinhamento múltiplo não identifica os resíduos conservados de ligação ao metal presentes no alinhamento de AMX3(3). A estrutura secundária, por sua vez, assemelha-se à da enzima 3,4-PCDO.

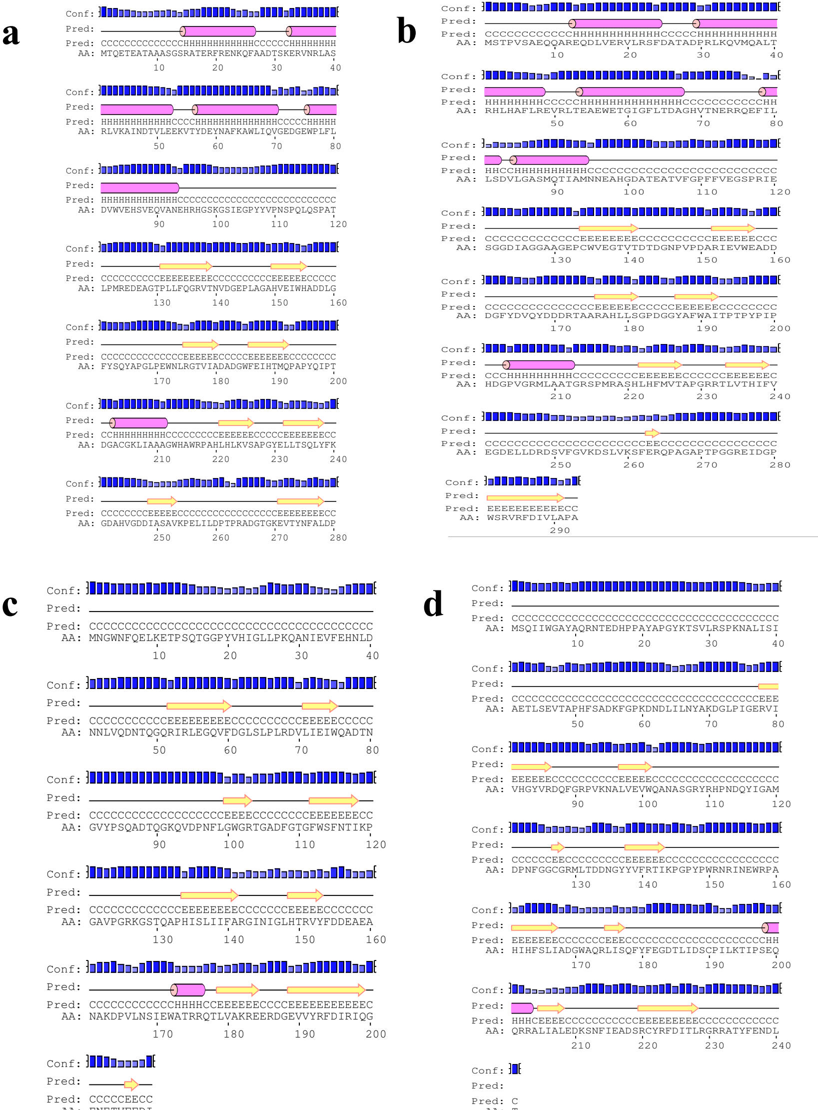


Figura 15. Predição de estrutura secundária de membros das classes estruturais conhecidas de intradiol dioxigenases. Verifica-se similaridade estrutural entre **(a)** catecol 1,2-dioxigenase e **(b)** hidroxiquinol 1,2-dioxigenase. Em **(c)** e **(d)** estão as estruturas das subunidades alfa e beta de uma protocatecuato 3,4-dioxigenase. Observa-se a semelhança estrutural entre as subunidades, enquanto diferem das duas primeiras enzimas. A predição foi realizadas no programa PSIPRED v 3.3 e as sequências baixadas do banco de dados do NCBI: **(a)** catecol 1,2-dioxigenase de *Arthrobacter sp.* BA-5-17 (BAD11154.1); **(b)** hidroxiquinol 1,2-dioxigenase de *Nocardioides simplex* 3e (1TMA_A); **(c)** protocatecuato 3,4-dioxigenase subunidade alfa de *Acinetobacter sp.* Adp1 (1EO9_A) e **(d)** protocatecuato 3,4-dioxigenase subunidade beta de *Acinetobacter sp.* Adp1 (1EO9_B).

A análise filogenética sugere a classificação, tanto de AMX3(3) quanto de AMX3(2), como intradiol dioxigenases, conforme ilustra a Figura 7. Contudo, esta análise foi realizada como auxiliar na interpretação dos outros resultados, já que é conhecido que a conservação de sequência primária entre estas enzimas é baixa, sendo a estrutura terciária mais conservada (Urszula Guzik, 2013).

A semelhança de AMX3(2) e AMX3(3) com 3,4-PCDO constitui um embate já que, caso realmente sejam compatíveis com uma das subunidades desta enzima, é possível que o subclone

não possua o fenótipo esperado, já que 3,4-PCDOs são heterodímeros, diferentemente dos outros membros, 1,2-CDO e 1,2-HDO, que são homodímeros (Urszula Guzik, 2013).

Ainda, há divergências quanto ao tamanho dos subclones quando comparados aos tamanhos médios dos membros das classes de intradiol dioxigenases. As subunidades de catecol 1,2-dioxigenases têm tamanho entre 29-38,6 Kda, enquanto as de hidroxiquinol 1,2-dioxigenases estão entre 29-42,5 Kda. Quanto a protocatecuato 3,4-dioxigenases, o tamanho médio total varia entre 97-700 Kda (Urszula Guzik, 2013). Assim, considerando os tamanhos estimados de AMX3(2) e AMX3(3), observa-se que AMX3(2) exclui-se da faixa de tamanho de PCDOs, enquanto AMX3(3) está incluso desta faixa, estimada em cerca de 48 a 350 Kda por subunidade.

Diante do exposto, verifica-se a necessidade de realizar os testes fenotípicos e enzimáticos para determinar tanto o fenótipo resistente, tratando-se da hipótese inicial, tanto aquele de metabolismo de aromáticos, pelos testes enzimáticos.

Ainda, sugere-se a predição de estrutura terciária de AMX3(2) e AMX3(3) para fins de comparação com estruturas caracterizadas de intradiol dioxigenases, já que é conhecida que a conservação de estrutura tridimensional é maior que estruturas secundárias ou similaridades de sequência primária.

Para todos os subclones, sugere-se ensaios enzimáticos subsequentes para caracterizar aspectos quantitativos, identificar inibidores e cofatores metálicos, além de observar a ação das enzimas, principalmente CRB2(1), em outros substratos aromáticos e moléculas antibióticas.

Como o mecanismo de resistência ainda não está claro, também faz-se necessário identificar se procede a hipótese de quebra do anel aromático nas moléculas de carbenicilina e fenol.

CONCLUSÕES E PERSPECTIVAS

A resistência antimicrobiana é, atualmente, um grande entrave no tratamento de doenças infecciosas, tanto em humanos quanto em animais. Como tal, os elementos de resistência antimicrobiana associados a micro-organismos de relevância clínica têm sido amplamente estudados. Contudo, a diversidade de genes de resistência a antibióticos nos ambientes naturais continua a ser subestimada, apesar do conhecimento de que a origem destes genes é ambiental.

Esta Tese de Doutorado teve por objetivo caracterizar ORFs isoladas de clones metagenômicos de solo de Cerrado previamente selecionados em triagens de resistência a antibióticos β-lactâmicos. Além disso, discute sobre a diversidade de genes de resistência em amostras ambientais pelo acesso a clones metagenômicos de biblioteca de grandes insertos.

Nas análises descritas, observou-se a capacidade de um gene, antes classificado como hipotético, em causar resistência à carbenicilina em *Escherichia coli*. Tal gene foi parcialmente caracterizado, tratando-se de uma extradiol dioxigenase, e uma provável gentisato 1,2-dioxigenase.

Até onde se sabe, esta enzima nunca foi relacionada à resistência antimicrobiana, o que evidencia a urgência em acessar micro-organismos ainda não cultiváveis de ambientes naturais, em especial o solo, na tentativa de identificar novos elementos de resistência antimicrobiana que tenham potencial em alcançar micro-organismos de importância clínica e que possam, futuramente, servir de matéria-prima para a síntese de novos compostos antibióticos.

A caracterização de novos genes de resistência também ajuda a enriquecer os bancos de dados de sequência.

Ainda, devido à natureza das dioxigenases, sugere-se acessar a potencialidade desta ORF no uso em processos de biorremediação de compostos aromáticos poluentes, já que observou-se não só a resistência à carbenicilina como ao também ao composto fenol.

Além disso, acessar estes genes levanta a discussão sobre o real papel de ARGs, e de antibióticos, nos ambientes naturais. As funções dos ARGs ambientais são equivalentes àquelas observadas no ambiente clínico, onde a concentração de antibióticos é alta e tóxica? Ou em habitats onde a pressão seletiva é menor os ARGs atuam em outras vias de regulação?

Finalmente, na análise dos clones de grandes insertos, verificou-se ampla diversidade de funções. No tocante à resistência antimicrobiana, poucas enzimas previamente relacionadas a este

fenótipo foram identificadas – ainda neste contexto, não foram observadas similaridades a betalactamases, que são o principal mecanismo de resistência a β-lactâmicos encontrado atualmente na clínica.

Em conclusão, a grande quantidade de genes hipotéticos reforça a ideia de que é urgente a caracterização de ARGs ambientais, para aprofundar o conhecimento dos seus mecanismos e espectro de ação e, principalmente, acessar o papel destes genes no contexto ambiental.

APÊNDICE II – Genes com possíveis aplicações biotecnológicas

Clone	Gene	Anotação BLASTp	Anotação Pfam	Descrição e possíveis aplicações
AMX5-fos	*orf15*	alpha/beta hydrolase	Abhydrolase_5 family	A aplicação biotecnológica de hidrolases é extensa. Participam como catalisadores nas indústrias têxteis, de papel, alimentícia além de serem potenciais em processos de biorremediação. Neste caso, a anotação em nível de superfamília é específica para esterases e lipases, que são também importantes na indústria de biocombustíveis
CRB2-fos	*orf25*	alpha/beta hydrolase	Abhydrolase_5 family	A aplicação biotecnológica de hidrolases é extensa. Participam como catalisadores nas indústrias têxteis, de papel, alimentícia além de serem potenciais em processos de biorremediação e na indústria de biocombustíveis
	orf8	anthranilate 1,2-dioxygenase small subunit	Ring hydroxylating beta subunit HAMP domain HD domain	Atuam na degradação de compostos aromáticos e são enzimas potencias em processos de remediação deste tipo de molécula, que são importantes poluentes ambientais, sendo também altamente recalcitrantes
PG2-fos	*orf37*	hydrolase	HIT domain	A aplicação biotecnológica de hidrolases é extensa. Participam como catalisadores nas indústrias têxteis, de papel, alimentícia além de serem potenciais em processos de biorremediação e na indústria de biocombustíveis

APÊNDICE III – Produção científica[1]

Ano	Tipo	Título	Periódico	Fator de impacto	Status	Referência
2014	Capítulo de livro	The Family *Cystobacteraceae*	The Prokaryotes	n/a	Publicado	(Dos Santos *et al.*, 2014)
2015	Artigo	New dioxygenase from metagenomic library from Brazilian soil: insights into antibiotic resistance and bioremediation	Biotechnology Letters	1.591	Publicado	(Dos Santos *et al.*, 2015)
2016	Artigo	Functional metagenomics as a tool for identification of new antibiotic resistance genes (ARGs) from natural environments	Microbial Ecology	2.973	Submetido, sob revisão	n/a

[1] Textos na íntegra disponíveis na seção “Anexos”

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Anexos

2 The Family *Cystobacteraceae*

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Abstract

Myxobacteria are a group of eubacteria classified in the order *Myxococcales*, further subdivided into two suborders and eight families. These unique microorganisms exhibit distinctive social behavior and morphogenetic characteristics, such as the ability to form myxospores and fruiting bodies. Cellular aggregation is an important feature that allows these microorganisms to adapt to almost every environment.

The genomic features of myxobacteria are also peculiar. Myxobacterial genomes are large, approximately 10 Mb, which may explain, in part, their unique morphophysiological behavior. Hence, myxobacteria are relevant targets for basic microbiological research, including the areas of ecology and morphogenesis. They are also interesting alternatives for the screening of new bioactive molecules, with production that may reach the levels of well-recognized bacterial producers such as *Actinomycetes* and *Bacillus* spp. Since the last decades of the twentieth century, a great effort has been made to isolate and characterize myxobacterial secondary metabolites. Antifungal and antibacterial agents are the most common compounds identified to date. However, other important metabolite activities, such as cytotoxicity to eukaryotic cell lines, suggest their potential applications in biotechnology and pharmaceutical industrial research.

Here, some important features of the genera and species of the *Cystobacteraceae* family, which was established in 1970, are described. The inclusion of new members and genera is still taking place as previously unknown myxobacteria are characterized. In addition, important ecological, genomic, phylogenetic, and morphophysiological questions are discussed and some of the genetic and physiological components that assure the ecological adaptability of these bacteria in highly variable habitats (both soil and water) are described. Finally, ongoing research and future perspectives are summarized.

Taxonomy

Cys.to.bac.ter.a' ce.ae M.L. masc. n. *Cystobacter* type genus of the family; -aceae ending to denote a family; M.L. fem. pl.n. *Cystobacteraceae* the *Cystobacter* family.

Type genus: *Cystobacter* (Schroeter 1886, p. 170).

Howard McCurdy established the *Cystobacteraceae* family in 1970 in a revised classification of cylindrical-shaped myxobacteria (McCurdy 1970). To better separate different cell morphologies, the *Cystobacteraceae* family was proposed to include tapered-ended myxobacteria, thus distinguishing this morphology from cylindrical cells of the *Polyangium* family. As such, the former *Cystobacter fuscus* (*Polyangium fuscum*) was re-established and became the type species of this new family. The type genus became *Cystobacter* (McCurdy 1970).

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Like all myxobacteria, the taxonomy is based primarily on the morphology of vegetative cells, swarms, fruiting bodies, and myxospores.

McCurdy (1970) describes the family as consisting of three genera based on sporangia characteristics: *Cystobacter* (sessile sporangia), *Podangium* (sporangia borne singly on an unbranched stalk), and *Stigmatella* (sporangia in clusters on branched or unbranched stalks). The type genus of the family, *Cystobacter*, was described by Schroeter in 1886 (Schroeter 1886) with two species: *Cystobacter fuscus* and *Cystobacter erectus*. However, in 1897, Thaxter (1897) described *Cystobacter fuscus* as a myxobacterium, later renamed *Polyangium fuscum*, because the shape and structure of its fruiting bodies are similar to those of *Polyangium vitellinum* Link 1809 (Reichenbach 2005c).

In 1970, McCurdy proposed the removal of species *Polyangium fuscum* from the *Polyangium* genus and redefinition of the family *Polyangiaceae* based on observations of the vegetative cells and myxospores. *Polyangiaceae* was changed to consist of myxobacteria with cylindrical cells, as described earlier.

One year later, McCurdy changed the name of the genus *Podangium* to *Melittangium*, based on an analysis performed with the type species of *Podangium*. In this study, he concluded that the type species of *Podangium* was a variant of *Stigmatella brunnea* and changed its name to *Stigmatella erecta*. McCurdy (1971a) proposed a new genus for the other species previously placed in the genus *Podangium*, in which *P. lichenicolum* would be considered the type species. As one of the species, *Podangium boletus*, was the type species of *Melittangium*, the name of the *Podangium* genus was changed to *Melittangium*.

The genus name *Archangium* was coined by Jahn (1924) The etymology (arch, primitive; angium, vessel) describes the primitive nature of *Archangium* fruiting bodies compared to the more elaborate structures found in other genera of fruiting myxobacteria (McDonald 1967). Jahn recognized five species. *Archangium gephyra* was a redesignation of another myxobacterial species, *Chondromyces serpens* Quehl (1906). This genus is considered problematic, because its definition rests on the morphology of its fruiting bodies, which are also produced by many other myxobacteria in place of the typical fruiting body (Reichenbach 2005c).

Until 1970, the genus *Stigmatella* consisted of a range of morphological types closely related to *Chondromyces*. The distinguishing features of its species were considered arbitrary (McCurdy 1971a) and not always consistent. In addition, there was no indication of their optimal growth conditions or whether they were grown and characterized in pure culture (Krzemieniewska and Krzemieniewski 1946). Thus, McCurdy proposed dividing *Chondromyces* into two genera: *Stigmatella* and *Chondromyces* (*Polyangiaceae*), with *Stigmatella* having tapered vegetative cells and encapsulated myxospores, and *Chondromyces* having cylindrical vegetative cells and similar myxospores (McCurdy 1971a).

Hyalangium was added to *Cystobacteraceae* in 2005 by Reichenbach (2005b). The members of this genus bear fruiting bodies with a distinct wall like *Cystobacter*. However, their vegetative cells, sporangioles, and swarm colonies differ morphologically from those of *Cystobacter*, which allowed the classification of *Hyalangium* as a new genus.

Anaeromyxobacter is described by Euzeby's list of bacterial names[1] as a genus within *Cystobacteraceae*. However, the genus belongs to the *Myxococcaceae* family according to the NCBI Taxonomy database and is a separate genus according to its describers (Sanford et al. 2002) and Shimkets et al. (2006). This genus does not appear in the last edition of *Prokaryotes*, because its characterization was not thorough enough for accurate placement (Shimkets et al. 2006). *Anaeromyxobacter* was the first anaerobic myxobacteria to be identified. Results of 16S sequence analysis revealed that it forms a deep branch in the *Myxococcales*, specifically related to the *Myxococcus* subgroup but outside any of the subgroup's three families (Sanford et al. 2002). Thus, its taxonomy is still controversial, and further studies are needed.

Short Descriptions of the Genera

The list of genera is presented according to Euzeby's list of bacterial names.

Anaeromyxobacter Sanford et al. 2002

An.aer.o.my.xo.bac.ter. Gr. pref. an, not or without; Gr. n. aer, air; Gr. n. myxa, slime; N.L. masc. n. bacter, rod; N.L. masc. n. Anaeromyxobacter, slime rod (living without air).

This genus consists of myxobacterial species capable of facultative anaerobic growth using terminal electron acceptors such as nitrate, fumarate, and chlorophenolic compounds. Sulfur compounds are not reduced, and oxygen is used at low concentrations. Cells are narrow rods (4–8-μm long, 0.25-μm wide) that exhibit gliding motility. Terminal ends of cells have pilus structures and form blebs periodically. Refractile cysts are visible in older cultures (Sanford et al. 2002).

Type species: *Anaeromyxobacter dehalogenans* (Sanford et al. 2002).

Archangium Jahn 1924

Ar. chan'. gium. Gr. Fem. n. arch. beginning, origin, primitive; Gr. neut. *angion* vessel, container.

Vegetative cells are long, slender, needle-shaped rods with tapered ends. Fruiting bodies without sporangioles are contorted strings of myxospores in hardened slime forming cushion-shaped masses; they vary in shape and size and may separate into packets under pressure. The myxospores are optically refractile and vary from fat rods with rounded ends to almost spherical in shape. Swarm colonies exhibit

[1] http://www.bacterio.cict.fr/

branched radial veins in a tough slime sheet. This genus cannot degrade chitin (Reichenbach 2005c).

Type species: *Archangium gephyra* (Jahn 1924).

Cystobacter Schroeter 1886

Gr. n. kustis, bladder; N.L. masc. n. bacter, rod; N.L. masc. n. Cystobacter, bladder-forming rod.

Vegetative cells are slender, tapered, flexible rods. Sporangia are sessile, occurring singly or in groups; they are rounded, elongate, or coiled, and surrounded by a definite slime envelope or membrane, either free or embedded in a second slimy layer. Microcysts are rod-shaped, phase-dense or refractile, and rigid. Vegetative colonies do not etch or erode agar media. Congo red is adsorbed. This genus hydrolyzes glycogen but does not utilize carbohydrates (McCurdy 1970).

Hyalangium Reichenbach 2005

*Hy. al. an'. gi. um.*Gr. fem. n. *hyalos* glass; Gr. neut. *angion* vessel, container; M.L. neut. n. *Hyalangium* glassy vessel.

Vegetative cells are delicate, slender rods with tapered ends. Fruiting bodies consist of small spherical sporangioles that are often empty; they appear glassy and transparent, and are arranged in extended dense sheets or in chains. Myxospores are short rods or exhibit an irregular spherical shape and are optical refractile. Swarm colonies form a thin but tough slime sheet with fine veins and adsorb Congo red to produce a purple red color. They are of the proteolytic-bacteriolytic nutritional type.

Type species: *Hyalangium minutum* (Reichenbach 2005b).

Melittangium (Jahn 1924)

r. n. melitta, bee; Gr. neut. n. angion, vessel; N.L. neut. n. *Melittangium*, a vessel resembling a honeycomb.

Vegetative cells are tapered rods. Sporangia are borne singly on a stalk. Microcysts are rod-shaped, and phase-dense or refractile. Vegetative colonies do not etch or erode agar media. Congo red is adsorbed (McCurdy 1971b).

Type species: *Melittangium boletus* (Jahn 1924).

Stigmatella (Berkeley and Curtis 1874)

Vegetative cells are rods with tapered ends. Sporangia are borne singly or in clusters on stalked fruiting bodies; the stalks often occur in groups arising from a common hypothallus. Myxospores are short, rigid, phase-dense, or refractile rods, surrounded by a slime capsule. Vegetative colonies do not etch, erode, or penetrate agar media. Congo red is adsorbed. Members of the genus are aerobic and most hydrolyze urea (McCurdy 1971a).

Type species: *Stigmatella aurantiaca* (Berkeley and Curtis 1874).

Phenetic Basis of the Taxonomy

Morphological characteristics of fruiting bodies, myxospores, and vegetative cells are used for the taxonomic classification of this family. Although aberrant fruiting bodies are common in cultures, members of *Cystobacteraceae* are characterized by fruiting bodies that consist of sporangioles with a distinct outer wall, often with stalks or naked masses of slime and myxospores (Reichenbach 2005c). Myxospores are short, fat rods. Vegetative cells are slender rods with tapered ends, either boat- or needle-shaped (Shimkets et al. 2006).

Genus taxonomy is determined by fruiting body and sporangiole morphology. Fruiting body morphology is used to differentiate *Archangium* from other genera. Members with fruiting bodies consisting of a naked mass of myxospores belong to the *Archangium* genus. Those with fruiting bodies composed of sporangioles with a distinct wall are differentiated by sporangia morphology: Members with sporangia borne on slime stalks belong to the *Melittangium* genus, whereas those with sessile sporangia belong to *Hyalangium* or *Cystobacter*. Finally, sporangiole morphology distinguishes *Hyalangium* and *Cystobacter*. *Cystobacter* has relatively large sporangioles, in more or less extended aggregates, often piled up, and sometimes surrounded by a translucent slime layer. However, sporangioles of *Hyalangium* are rather small, often glassy, translucent, and empty, and arranged in chains or sheets (Reichenbach 2005c).

The *Archangium* genus has a single species, *Archangium gephyra* (Jahn 1924). According to Reichenbach (2005c), several species have been described in the past; some are identical to *A. gephyra*, whereas others are clearly different organisms, and still others are not easily recognized, because they are poorly described. Part of the taxonomy problem is due to limited cultivation or comparative analysis with an insufficient number of strains.

As shown in Euzeby's list of bacterial names,[2] seven species comprise the *Cystobacter* genus: *C. fuscus* (type species), *C. armeniaca*, *C. badius*, *C. ferrugineus*, *C. minus*, *C. velatus*, *and C. violaceus*. However, *C. disciformis* and *C. gracilis* are also recognized by Reichenbach (2005c). Antibiotic sensitivity and enzymatic activities were used to differentiate species (McCurdy 1970), but species identification is currently based only on chitin degradation activity and morphological features of sporangioles and myxospores (Reichenbach 2005c).

Hyalangium minutum is the sole species of the genus *Hyalangium* (Reichenbach 2005c). Its glassy and translucent sporangioles are used for identification.

Fruiting body and sporangiole color and morphology are the phenotypic characteristics used to identify *Melittangium* species (McCurdy 1970; Reichenbach 2005, p. 117, 2005c). This genus comprises three species: (1) *M. boletus*, which produces yellow to brown spherical or flattened sporangia that resemble a mushroom pilus; (2) *M. lichenicola* which produces orange or red fruiting bodies that are single or arranged in an irregular

[2] Available at http://www.bacterio.cict.fr/c/cystobacter.html

mass; and (3) *M. alboraceum*, which produces a single irregular, pale orange, globe sporangium on a long, white, corkscrew-shaped sporangiophore (McCurdy 1970).

Three species are also recognized in the *Stigmatella* genus: *S. aurantiaca*, *S. erecta*, and *S. hybrida*. Sporangia of *S. aurantiaca* and *S. erecta* are similar, although fruiting bodies shows variable morphology in *S. aurantiaca*. However, vegetative colonies of *S. aurantiaca* and *S. erecta* exhibit different features. In *S. aurantiaca*, they are thin and flat with numerous radiating and concentric ridges, filamentous, and poorly defined. In *S. erecta*, vegetative colonies are at first thin and transparent, and later yellow or light flesh-colored with numerous radiating ridges and indefinite, thin edges.

Genomic Basis of the Taxonomy

The first 16S rRNA-based taxonomy of myxobacteria was reported by Shimkets and Woese, who used all cultivated genera described thus far (Shimkets and Woese 1992). The authors chose the molecular taxonomy approach in an attempt to solve some of the problems associated with phenotype-based taxonomy of myxobacteria.

At that time, fruiting body morphology was the primary basis of species classification, even though some genera form aberrant fruiting bodies or no fruiting bodies at all after continued cultivation (McCurdy 1971a, b; Reichenbach 2005c) Later, Spröer et al. (1999) used the same approach to determine the suitability of morphological criteria to affiliate myxobacteria strains to species. An analysis of 54 myxobacterial strains representing 21 morphological species demonstrated the phylogenetic coherence of myxobacteria, because strains classified on morphological grounds clustered next to their respective strains according to phylogenetic analysis.

Since the Shimkets and Woese analysis, several 16S rDNA molecular analyses of myxobacteria have been performed, including analyses of unculturable strains (Garcia et al. 2010, 2011; Jiang et al. 2007; Wu et al. 2005). Molecular taxonomy analysis performed by Garcia et al. (2010) showed the clustering of *Cystobacteraceae* species in a defined branch. However, *Anaeromyxobacter* strains were clustered in a separate branch within *Cystobacterinae*, constituting an anaerobic branch within Myxobacteria (branch XIII).

Two clusters of strains belonging to *Cystobacteraceae* were observed: the *Archangium-Cystobacter-Melittangium* cluster (branch XIV) and the *Hyalangium-Stigmatella* cluster (branch XV). In the first cluster (branch XIV), *Archangium* strains comprise a monophyletic group (bootstrap percentage = 99 %); however, *Cystobacter* strains comprise a polyphyletic cluster. Furthermore, many *Cystobacter* strains did not match to their corresponding type strains. Garcia et al. (2010) attributed these results to incorrect morphologic characterization. Fruiting body characteristics vary in different media and are often lost after several transfers in the same medium. *Melittangium* strains fall between polyphyletic branches of *Cystobacter*. The proposed neotype strain proposed by McCurdy, *M. lichenicola* (McCurdy 1971b), was evidently divergent from *M. alboraceum* and *M. boletus*. The current type strain of *M. lichenicola* was found in the *Corallococcus* cluster, suggesting a case of misclassification (Garcia et al. 2010).

According to Garcia et al. (2010), the *Hyalangium-Stigmatella* cluster (branch XV) doubtless represents a novel genus based on its 16S rDNA sequence, a unique branch in *Cystobacteraceae*, and unusual morphology. *Stigmatella* appears to be a homogenous group (bootstrap 100 %). All strains were matched with their corresponding types, indicating that the three species are distinct from each other. This cluster (branch XV) appears to represent a separate family, based on at least 4 % identity difference in 16S rDNA sequences and branching patterns with clusters XIV–XVI.

Similar to previous studies (Jiang et al. 2007; Sproer et al. 1999; Wu et al. 2005), the molecular taxonomy analysis of Garcia showed that myxobacteria are a phylogenetically coherent group. Most of the strains in the phylogenetic tree matched with the type or proposed neotype of *Cystobacteraceae* strains, except for members belonging to *Cystobacter* and *Melittangium*. The genus *Cystobacter* holds the most misclassified isolates, which may represent more than three genera.

To construct our 16S rRNA tree and compare it with the cited phylogenetic analysis, 17 sequences from *Cystobacteracea* family were used, including selected sequences from other suborders and families as well. ➋ *Table 2.1* shows the accession numbers used to construct the phylogenetic tree.

➋ *Figure 2.1* shows the clusterization of some species of the *Cystobacteraceae* family. The tree was constructed using Neighbor-joining algorithm and the type species are highlighted in bold letters. One of the species most recently included in this family, *Anaeromyxobacter dehalogenans*, is slightly divergent. However, the branching is consistent with the phylogenetic tree published in 2002 (Sanford et al. 2002). The deep branching of *H. minutum*, a new species characterized in 2005 (Reichenbach 2005b), can also be observed. *Stigmatella* and *Hyalangium* members are closely related to the *Archangium*, *Cystobacter*, and *Melittangium* members—this last branch is the most heterogeneous and includes *Cystobacter* and *Melittangium* species that were also misplaced in the phylogenetic tree performed by Garcia et al. (2010). It also shows the clusterization of species from *Cystobacter* and *Melittangium* genera, which is consistent with the *Cystobacter-Melittangium* branch previously described.

These discrepancies may be due to using partial 16S rRNA sequences, which can contribute to incorrect phylogenetic placement. Bootstrap indexes must be considered too, as some values are too low to be reliable for clusterization.

In general, morphology-based characterization alone is not conclusive for the classification of an isolate. Fruiting bodies, vegetative cells, and myxospore morphology are appropriate for genera identification but are frequently insufficient for species identification. Thus, it is highly recommended to combine phenotypic, chemo-physiological, and genetic characteristics for the classification of a strain.

Table 2.1
List of cataloged *Cystobacteraceae*[a] species

Genus	Species	Type strain	16S rRNA gene NCBI accession number	Genome sequencing
Cystobacter	*C. armeniaca*	CIP 109127; DSM 14710; JCM 12622	DQ768107	No
	C. badius	CIP 109126; DSM 14723; JCM 12623	DQ768108	No
	C. ferrugineus	DSM 14716; JCM 14717	AJ233901	No
	C. fuscus[b]	ATCC 25194; DSM 2262	DQ768109	Yes
	C. miniatus	CIP 109119; DSM 14712; JCM 12626	DQ768111	No
	C. minus	DSM 14751; JCM 12627	AJ233903	No
	C. velatus	CIP 109132; DSM 14718; JCM 12628	DQ768115	No
	C. violaceus	CIP 109131; DSM 14727; JCM 12629	DQ768114	No
Hyalangium	*H. minutum*[b]	CIP 109157; DSM 14724; JCM 12630	DQ768124	No
Melittangium	*M. alboraceum*	DSM 52894	AJ233907	No
	M. boletus[b]	DSM 14713; JCM 12633	AJ233908	No
	M. lichenicola	ATCC 25944	DQ768126	No
Stigmatella	*S. aurantiaca*[b]	ATCC 25190	DQ768127	Yes
	S. erecta	ATCC 25191; DSM 16858T	AJ970180	No
	S. hybrida	CIP 109130; DSM 14722; JCM 12640	DQ768129	No
Anaeromyxobacter	*A. dehalogenans*[b]	ATCC BAA-258; DSM 21875	AF382396	Yes
Archangium	*A. gephyra*[b]	ATCC 25201; DSM 2261	DQ768106	No
%				3/17 (17.64 %)

[a]According to the List of Prokaryotic names with Standing in Nomenclature (LPSN), available at: www.bacterio.cict.fr (accessed February 2013)
[b]Type species

Phenotypic Analysis

Cell and Colony Morphology

Members of the *Cystobacteraceae* family share many morphological and physiological features, the most important of which is the formation of fruiting bodies (and myxospores) under starvation conditions. Fruiting bodies are cell aggregations that enable the accumulation and dispersion of metabolites to ensure cell survival in stressful environments. This cooperative trait is one example of the exceptional social behavior of myxobacteria, which are also characterized by gliding motility and the production of a wide range of secondary metabolites.

As described in *Bergey's Manual of Determinative Bacteriology* (Reichenbach 2005c), vegetative cells are slender, flexible rods with tapering ends, often long and needle-shaped. The fruiting bodies can be naked masses of slime and myxospores that are of cartilaginous consistency, variable size, and irregular shape. However, in most cases, the fruiting bodies are made up of pale to deep brown sporangioles in various arrangements, with or without a slime stalk or pedicle. Myxospores are short, optically refractile rods, or spheroids. In the latter case, they are always slightly deformed and accompanied by short rods with rounded and tapered ends. Swarm colonies produce a firm slime layer with more or less conspicuous straight and often branched radial veins. The slime sheet, which may become tough, quickly adsorbs Congo red, giving the swarm a purple red color. All species utilize mono-, di-, and polysaccharides. Some species vigorously degrade chitin.

However, some differences can be seen between genera in the *Cystobacteraceae* family. A recent member, *Anaeromyxobacter dehalogenans*, was described in 2002 and designated to a new genus, *Anaeromyxobacter* (Sanford et al. 2002). It was the first myxobacteria identified as a facultative anaerobe; all other members are strict aerobes. The *Hyalangium* genus was proposed in 2005 (Reichenbach 2005b) and validated in 2007 (Euzeby 2007). Small delicate vegetative cells with glassy and transparent sporangioles characterize *Hyalangium minutum*, the only member of the genus so far.

Myxobacterial colonies exhibit a characteristic multicellular spreading behavior called swarming. Thus, their colonies are called swarms, and those of *Cystobacterineae* members are flat and thin-layered (Dawid 2000; Kaiser and Crosby 1983). Factors influencing the motility of myxobacteria include nutrient concentrations, temperature, and original cell density.

On nutrient-rich media, colonies may show a round, compact morphology, like those of common bacteria. However, on commonly used media with relatively low nutrient concentrations, colonies appear as a sheet of cells and excreted slime, revealing patterns of veins, rings, or even depressions in the agar. This is commonly observed in the swarms of *Cystobacter*, *Archangium*, *Melittangium*, and *Stigmatella*. Swarms of

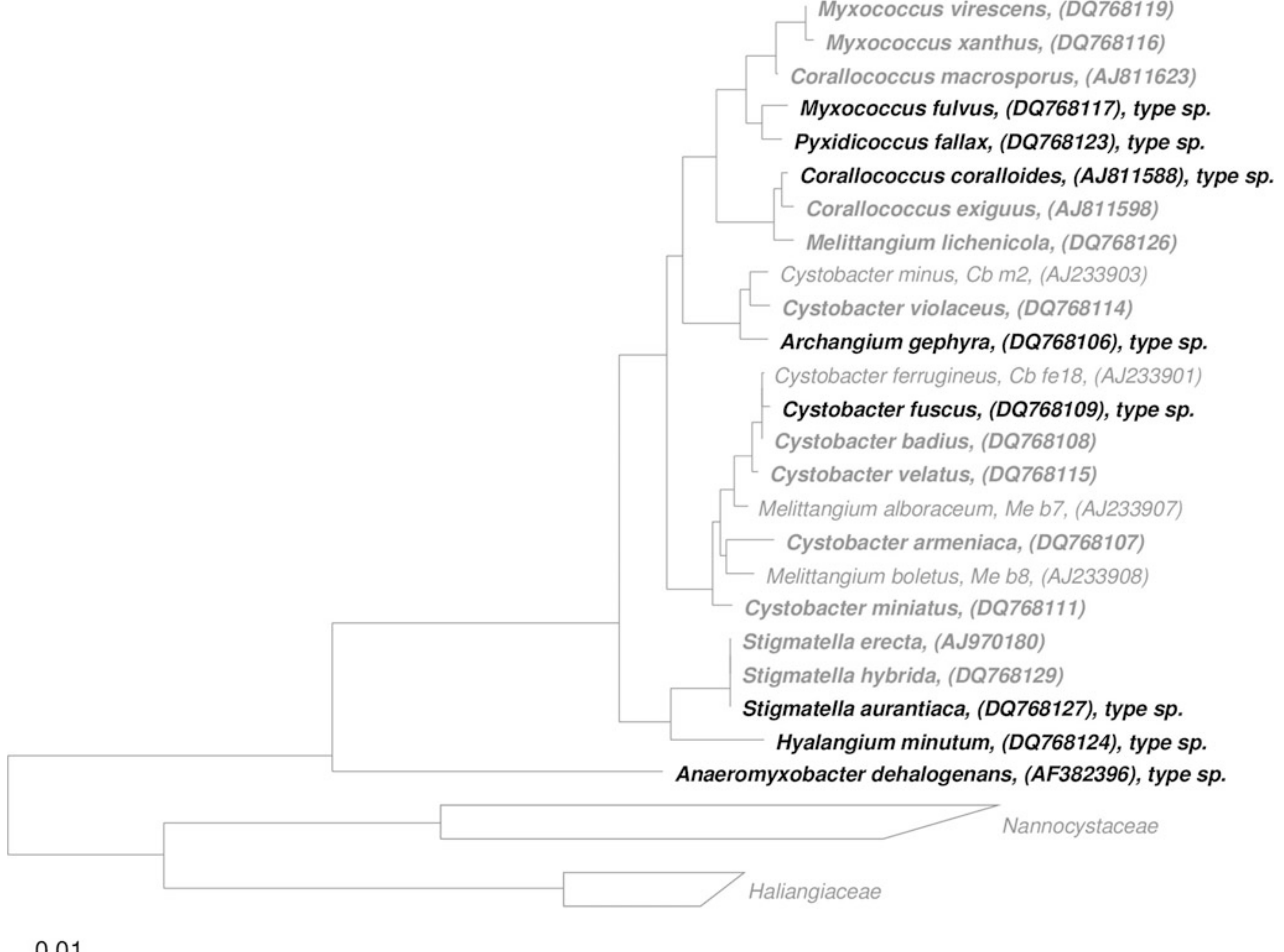

Fig. 2.1

Phylogenetic reconstruction of the family *Cystobacteraceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Archangium, *Cystobacter*, and *Melittangium* can become very tough and strongly adherent to the medium (Shimkets et al. 2006). On some media, colonies are pigmented, probably due to the presence of melanoid pigments. Depending on the specific medium, colonies of some *Cystobacter*, *Archangium*, and *Stigmatella* species may appear purple, red, or even black.

According to Dawid (2000), fruiting bodies are diverse, ranging from globular to unusual shapes, with hardened slimes like those of *Archangium*. In most species, spores are enclosed in sporangioles, which may be embedded in the substrate (as in *Cystobacter*) or associated with slime stems (as in *Melittangium* and *Stigmatella*). The fruiting body ranges from 10 to 1,000 μm, and its color may be white, brown, bright yellow, orange, red, brown, or lilac (Shimkets et al. 2006).

In *Stigmatella aurantiaca*, fruiting body development is enhanced by light, whereas in other genera, factors such as nutrient concentration, pH, cations, or temperature influence this process (Dworkin 1996). Fruiting bodies of *Cystobacteraceae* are briefly described below (adapted from Shimkets et al. 2006). Morphological changes that occur during the life cycle of myxobacteria are illustrated in the *Fig. 2.2*.

As previously mentioned, fruiting bodies have not been described in *Anaeromyxobacter*. Those of *Archangium* are naked masses of slime and myxospores, or hard and cartilaginous. In *Melittangium* and *Stigmatella erecta*, the fruiting body consists of a single sporangiole on top of a stalk, whereas in *Cystobacter*, it consists of a group of sporangioles on or in the substrate. In *Hyalangium*, the fruiting body consists of a group of small spherical sporangioles often empty, glassy, and

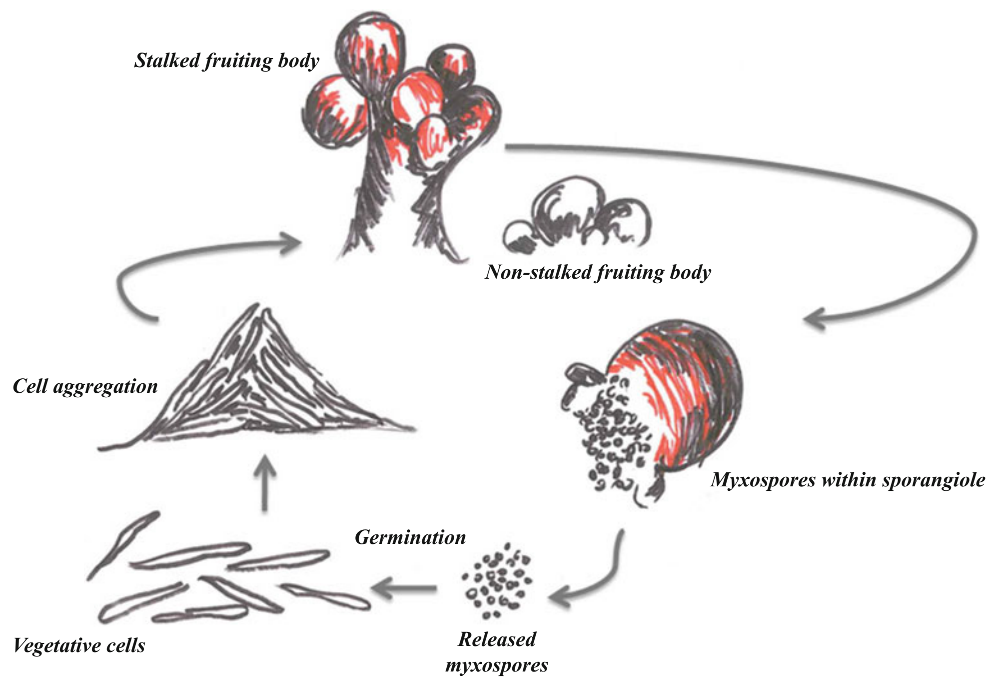

Fig. 2.2
Morphological changes of myxobacteria during their characteristic life cycle. Under stress conditions, vegetative cells aggregate to form multicellular structures known as fruiting bodies. The figure shows two morphologies: stalked (as seen in *Stigmatella aurantiaca*) and non-stalked fruiting bodies. Within these structures, cells continue to change to form myxospores, which can endure stressful circumstances (e.g., low nutrient levels) for long periods. When conditions improve, the myxospores germinate into vegetative cells

transparent, arranged in extended, dense sheets or in chains. In *Stigmatella aurantiaca*, it appears as a cluster of sporangioles on top of an unbranched stalk.

Thus, *Cystobacteraceae* family members exhibit Gram-negative, tapered-ended, needle-shaped vegetative cells with gliding motility, convertible to refractile or phase-dense myxospores. Fruiting bodies, when present, are of variable sizes and shapes, often brown to red-colored, and can be structured or not on pedicles or stalks (McCurdy 1970; Reichenbach 2005a). *Table 2.2* summarizes the information described above.

Isolation, Enrichment, and Maintenance Procedures

Members of *Cystobacteraceae* are commonly found in soils, especially near the base of stems or between roots, but are also found on decaying plant material, tree bark, and the dung of herbivorous animals. Attempts to isolate these organisms from those habitats often fail, in particular because of the slime matrix produced by the cells, which makes cell dispersion difficult (Shimkets et al. 2006). When nutrients are scarce, cells aggregate into fruiting bodies, within which some differentiate into spores. When nutrients are sufficient, cells grow actively on surfaces as a swarming colony. Among the *Cystobacteraceae* genera, only *Anaeromyxobacter*, a microaerophilic organism, does not produce fruiting bodies (Huntley et al. 2011). *Stigmatella aurantiaca* digests chitin, and its growth is enhanced by glucose (Reichenbach and Dworkin 1969). *Archangium gephyra* can be isolated from cold environments such as the Swedish arctic tundra, Alaska, Iceland, and soils of northern Canada. On the other hand, some species such as *Stigmatella aurantiaca*, *Cystobacter*, *Melittangium*, and *Archangium* are found in hot biotopes like desert soils. *Archangium* and *Cystobacter* have also been found in coastal samples from North and South America (Dawid 2000).

Oligotrophic media are better for characterizing an organism as a myxobacterium (some protocols are described in Supplement 1). On these types of media, colonies develop as delicate swarmings. On rich media, colonies appear as compact structures, not easily recognizable as myxobacteria (Shimkets et al. 2006).

It is possible to isolate members of *Cystobacteraceae* directly from their natural habitats by soaking the substrate (dung, leaves, or bark) in water containing up to 0.08 mg/ml cycloheximide for a few hours in Petri dishes lined with filter paper. This system should be wrapped in a sterile plastic bag or placed in an incubator to reduce the chance of contamination. The cultures should be moistened periodically and maintained at room temperature or higher (approximately 30 °C) to prevent fungal growth. Cultures should be inspected daily for the presence of fruiting bodies. Alternatively, pigmented colonies may be detected, especially on the surface of the filter paper (Shimkets et al. 2006). When using soils as natural samples, the general procedure is the same, except for the initial step, in which soils are incubated in the presence of sterile dung pellets.

Table 2.2
Morphophysiological features of *Cystobacteraceae* members according to type species

Genus	*Anaeromyxobacter*	*Archangium*	*Cystobacter*	*Hyalangium*	*Melittangium*	*Stigmatella*
Vegetative cell morphology	4–8 × 0.25 μm narrow rods; pilus at terminal ends	0.8 × 6–15 μm needle-shaped rods with tapered ends	0.6–0.8 × 3–20 μm needle-shaped rods	0.6–0.7 × 3–6 μm delicate rods with tapered ends	0.7–4.5 × 10.5 μm rods with tapered round ends	0.6–1.0 by 4–10 μm rods with tapered ends
Pigmentation	Red colonies in fumarate-grown cells	Reddish to violet	Diffuse dark brown to red	Brownish fruiting bodies	White to yellowish sporangiophores	Yellowish pigmentation on agar; reddish sporangia
Spores and multicellular morphology	Refractile microcysts (older cultures); fruiting bodies are not characteristic	No sporangioles, fruiting bodies' size varies, cushion-shaped myxospores	Sessile, oval sporangia in gelatinous matrix	Potato-like sporangioles, often translucent or glassy	Mushroom-like sporangia, brown-colored	Stalked fruiting bodies, ≤400 μm; unbranched; pedicles may be present
Growth	Facultative anaerobe	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Type species	*Anaeromyxobacter dehalogenans*	*Archangium gephyra*	*Cystobacter fuscus*	*Hyalangium minutum*	*Melittangium boletus*	*Stigmatella aurantiaca*
Type strain	ATCC BAA-258; DSM 21875	ATCC 25201; DSM 2261	ATCC 25194; DSM 2262	CIP 109157; DSM 14724; JCM 12630	DSM 14713; JCM 12633	ATCC 25190
pH	7.0 optimum	ND	6.8–8.2	ND	4.0–8.5	7.0–7.2
Temperature	30 °C optimum		18–40 °C, 30 °C optimum	ND	20–31 °C	18–37 °C, 30 °C optimum
Catalase	ND	ND	Positive	ND	Positive	Positive
Oxidase	ND	ND	Negative	ND	Negative	Negative
Nitrate reduction	Yes	ND	No	ND	No	No
G+C content	ND	68 mol%	68 mol%	ND	ND	68 mol%
Metabolism	2-chlorophenol best for halorespiration; does not reduce sulfur compounds	Cellulolytic, proteolytic; does not degrade chitin	Non-cellulolytic; hydrolyzes starch, nucleic acids	Does not degrade chitin	Non-cellulolytic; hydrolyzes starch, nucleic acids	Hydrolyzes starch, nucleic acids

- **Isolation from soils with bait.** Soil samples should be placed in a Petri dish, moistened with distilled water, and mixed with sterile dung pellets. Vegetative cells will colonize the dung pellets, resulting in the development of fruiting bodies. This method is useful for the isolation of *Cystobacter*, *Stigmatella*, *Archangium*, and *Melittangium* species.
- **Isolation from swarms**. This method is based on the bacteriolytic properties of *Cystobacteraceae*. Small samples, preferentially soil or vegetal debris, should be inoculated onto a plate of water agar (WAT) previously inoculated with an organism preyed upon by *Cystobacteraceae* (e.g., *Escherichia coli*) as parallel, cross, or circular streaks. The streaks should be made in the center (cross or circular) or ends of the plate (parallel). Moistening the sample facilitates inoculation, and the sample should not be scattered over the medium. To minimize fungal contamination, use WCX agar, which contains cycloheximide (25–100 μg/ml) (Brockman and Boyd 1963) (protocol described in Supplement 1).

According to a recent paper by Garcia et al. (2009), the isolation and cultivation of members of the suborder *Cystobacterineae*, to which the *Cystobacteraceae* family belongs, is likely to be more successful than that of other myxobacteria. Commonly used culture media include CY and VY/2 agar (Shimkets et al. 2006), mineral salts agar, and anaerobic medium. A buffered yeast agar (modified version of VY/2 agar) is a useful transfer medium, because it supports spore germination and promotes the spreading of the swarm colony and fruiting body formation. This is also a suitable medium to preserve the cultures (Garcia et al. 2009) (protocol described in Supplement 1).

Because the cultures must sometimes be incubated for long periods (e.g., 1–4 weeks), it may be wise to prepare thick agar

plates and incubate the organisms in humid chambers or incubators that have a source of humidity. Generally, the best culture conditions are:

- pH: 6.5–8.5
- Temperature: 30–36 °C, although some species of *Cystobacter* and *Archangium* tolerate higher temperatures, nearly 40 °C
- Oxygen: with the exception of the microaerophile *Anaeromyxobacter*, which is a microaerophile, all *Cystobacteraceae* are strict aerobes

Anaeromyxobacter dehalogenans is a motile facultative anaerobic/microaerophilic Gram-negative rod that uses acetate as an electron donor and chlorinated phenols as electron acceptors. Its colonies produce a red pigment, and to date, no fruiting bodies have been described (Sanford et al. 2002). Two of the media commonly used to grow this organism are anaerobic medium and mineral salts medium (protocol described in ➋ Supplement 2) (Loffler et al. 1996; Sanford and Tiedje 1996/1997). This organism is metabolically versatile and considered an important bioremediation agent. *Anaeromyxobacter dehalogenans* can reduce a variety of toxic compounds, such as soluble U(VI) to sparingly soluble, immobile U(IV) (Thomas et al. 2010) and Se(IV) to Se(0) (He and Yao 2011).

Ecology

Main Habitats

Previous studies have reviewed the ecological distribution of myxobacteria based upon enrichment and classical isolation (Dawid 2000; Parish 1984; Reichenbach 1993, 1999). Noted for the formation of multicellular fruiting bodies containing myxospores that resist desiccation and freezing, as well as temperatures up to 60 °C when suspended in liquids (Holt et al. 1994), myxobacteria are found in both moderate and extreme environments.

In terms of global distribution, Dawid (2000) reported the isolation of myxobacteria from numerous countries across all continents. Regarding climate, myxomycetes have been isolated from Antarctic soil, arctic tundra, steppes, deserts, bogs, a range of altitudes, warm and humid climates, warm and dry climates, tropical climates, and temperate climates (Shimkets 1990; Shimkets et al. 2006). The greatest abundance and diversity of myxobacteria are associated with warm semiarid zones such as the southwest United States, northern India, and Egypt (Reichenbach 1999).

A recent metagenomic survey from Cerrado soil in Middle-western Brazil showed that 16S rDNA sequences from *Myxococcales* order comprised 1.3 % of all rDNA sequences analyzed; however, no sequences from *Cystobacteraceae* were detected. Nevertheless, the metagenome sequence analysis identified *Cystobacteraceae*, *Kofleriaceae*, *Myxococcaceae*, *Nannocystaceae*, and *Polyangiaceae* families. With respect to function, these sequences appear to be closely related to RNA metabolism, as determined by a comparison with sequences in the SEED database (Castro de, Bustamante and Kruger, Unpublished results).

Most myxobacteria are aerobic mesophiles (Reichenbach 1999); however, psychrotrophs (Zhukova 1963) and psychrophiles have also been described (Dawid et al. 1988). Regarding pH, the most common range for growth in culture is 6.8–7.8, although isolation from acid soils (pH 2.5) (Dawid 1979) and alkaline lakes (pH 9.5) in east Africa (Reichenbach 1999) has been reported.

Observed predominantly in the topmost layers of aerated soils, myxobacteria are principally observed in neutral or slightly alkaline soils (McCurdy 1969), compost (Singh 1947), herbivore dung (especially that of rabbits, hares, deer, moose, sheep, and goats), rotting wood or tree bark (Shimkets et al. 2006), and decomposing lichens and insects (Reichenbach 1999). Specific species can adapt to different substrates according to climatic and edaphic characteristics (Shimkets et al. 2006). The attraction of myxobacteria to such habitats may reflect micropredation (Shimkets 1990), with dead and living microorganisms in these typically rich microbial communities serving as secondary substrates for degradation by host-secreted enzymes (Shimkets et al. 2006). Myxobacteria also include cellulose decomposers; thus, attraction to such habitats may reflect the abundance of degradable plant residues. The presence of myxobacteria on dung collected from snow suggests their ability to pass through the animal digestive system (Rückert 1975). This was confirmed under laboratory conditions by pipette feeding of myxobacterial fruiting bodies to white mice (Kühlwein 1950). The involvement of myxomycetes in soil microbial communities is limited, but their involvement in microbial predation and secondary metabolite antibiotic production is common (Reichenbach et al. 1988).

Myxobacteria also appear to settle in specific habitats through the movement of soil and dust particles by air currents, as suggested by reports of air contamination (Wu et al. 1968) and their presence on plant leaf surfaces (Ruckert 1981). Freshwater habitats can also support myxobacteria (Hook 1977). Although their presence may simply be a result of run-off from soil, cultivation of strains in aquatic conditions has been demonstrated (Shimkets et al. 2006). The isolation of halotolerant myxobacteria from marine environments has also been described, but this is less common (Dawid 2000; Iizuka et al. 1998; Li et al. 2002).

It should be noted that culture-dependent identification methods for myxobacteria may be limited in their precision and sensitivity as a result of unsuitable growth media, slow growth of cells into colonies, and microbial contamination. In contrast, culture-independent methods (e.g., characterization of 16S rDNA genes) are promising alternatives, with potentially greater resolution. For example, the diversity of halotolerant myxobacteria in oceanic sediments has been shown to be considerable based upon this approach, with 69 highly diverse marine myxobacteria-related 16S rRNA gene sequences characterized from Japanese oceanic sediments (Wu et al. 2005).

Given their unique features and highly adaptable phenotypes (e.g., sporulation and gliding motility), myxobacteria are able to survive in nearly any environment, although the main habitat of this ubiquitous group of microorganisms appears to be soil. Their ability to grow on decaying plant matter, decomposed substrates, and living and dead microorganisms (Reichenbach 1999) depends on an extensive enzymatic supply, consisting of different types of molecules with a broad range in activities, from proteolytic to signaling.

The distribution of myxobacteria among marine habits remains unclear. A survey published in 2010 described a 16S rRNA myxobacteria-enriched library constructed with marine samples (Jiang et al. 2010). The results of phylogenetic analysis showed a distinct separation between marine and terrestrial myxobacteria, which is consistent with the previous hypothesis of environmental selection and adaptation of these microorganisms. However, some questions regarding marine myxobacteria remain. Are their nutritional requirements similar to those of soil myxobacteria? How do the two types react to saline concentration? Is saline concentration essential for morphogenetic changes? Are marine myxobacteria able to form fruiting bodies and myxospores, and do they use the same mechanism as soil myxobacteria? Isolation methods must be improved to compare morphological and physiological features of soil and marine myxobacteria and to correlate those features with phylogenetic characteristics.

With a specific focus on the family *Cystobacteraceae* (encompassing the genera *Anaeromyxobacter*, *Archangium*, *Cystobacter*, *Hyalangium*, *Melittangium*, and *Stigmatella*), a summary of common substrate sources for these myxobacteria is provided.

1. ***Anaeromyxobacter***
   Members of the *Anaeromyxobacter* genus are distributed throughout the environment (Sanford et al. 2002), but undisturbed and contaminated soils and sediments appear to be their principal habitat. Studies have reported isolates obtained from anaerobic enrichment cultures of soil samples in Michigan and rainforest soils of Cameroon (Sanford et al. 2002) and the presence of this genus in acidic subsurface sediments (North et al. 2004). *Anaeromyxobacter* spp. appear to use a variety of respiratory electron acceptors, including soluble hexavalent uranium U(VI), nitrate, ferric iron, and manganese oxide (He and Sanford 2003; Sanford et al. 2002; Wu et al. 2006). Given the capability of *A. dehalogenans* for metabolic reduction of soluble U(VI) to sparingly soluble, immobile U(IV) (Marshall et al. 2009; Sanford et al. 2007; Wu et al. 2006), which is important for containment at uranium-contaminated locations, 16 s rRNA gene sequence analyses were conducted to examine its abundance in uranium-contaminated soils at the U.S. Department of Energy Field-scale Subsurface Research Challenge (IFC) site near Oak Ridge, Tennessee (Cardenas et al. 2008; North et al. 2004; Petrie et al. 2003). Numerous *Anaeromyxobacter* spp. from the IFC site samples were characterized via sequencing of 16S rRNA gene clone libraries and quantitative PCR (Thomas et al. 2009).
2. ***Archangium***
   Members of the genus *Archangium* are commonly isolated from soil and herbivore dung (Holt et al. 1994; Shimkets et al. 2006). The presence of *A. gephyra* has been reported in grassland soils in Southern Chile (Ruckert 1978), rock, bark, rotting wood, dung (Dawid 1979), and plant leaf samples (Ruckert 1981). Isolation agar with 0.5 % NaCl stimulates the development of *A. gephyra* (Ruckert 1978), indicating some degree of salt tolerance, which most myxobacteria lack. *Archangium serpens* has also been detected in soil and herbivore dung (Shimkets et al. 2006). Culture-independent characterization by 16S rDNA gene analysis has also identified sequences phylogenetically close to *A. gephyra* in Chinese soils (Wu et al. 2005).
3. ***Cystobacter***
   Results of culture-based identification show that *C. fuscus*, *C. ferrugineus*, *C. gracilis*, *C. velatus*, *and C. violaceus* are common in soil and herbivore dung. *Cystobacter disciformis* is common in moderately acidic soils and alkaline peat bogs, and its presence was originally reported in muskrat and deer dung (Reichenbach 2005a). Phylogenetic analysis of 16S rDNA genes from genomic DNA extracted from soil samples from Jinan, China, has identified sequences homologous to *C. ferrugineus*, *C. fuscus*, *C. violaceus*, and *C. minus* (Wu et al. 2005).
4. ***Hyalangium***
   Hyalangium minutum is relatively common in typical myxobacterial habitats. The translucent fruiting bodies can be overlooked or suffer degradation; therefore, its abundance can be underestimated when based solely upon culture-based identification approaches. The type strain NOCB-2 originated from a soil sample obtained from the mountains of Izu and Manazuru peninsula, Japan, and the type strain Hy m4 originated from soil samples containing rotting wood from Iowa, USA (Reichenbach 2005b).
5. ***Melittangium***
   Melittangium boletus also occurs in habitats typically associated with myxobacteria, such as soil containing wood and rotting bark. The type strain Me b8 was isolated from such soil material in Uttar Pradesh, India (Reichenbach 2005a). Culture-independent 16S rDNA gene-based identification of *M. alboraceum* and *M. boletus* in soil samples in China has been reported (Wu et al. 2005).
6. ***Stigmatella***
   Bark, rotting wood, and herbivore dung are also typical substrates for members of the genus *Stigmatella*. The species *S. aurantiaca* has been isolated from bark and rotting wood; its occurrence is common in North America (McCurdy 1969; Nellis and Garner 1964; Reichenbach and Dworkin 1969) but rare in Europe (Krzemieniewska and Krzemieniewski 1946). The more frequent isolation from substrates in North America may reflect the more humid summers (Shimkets et al. 2006). Characterization of 16S rRNA gene sequences has identified sequences homologous to *S. aurantiaca* from soil samples in China (Wu et al. 2005).

The presence of *Stigmatella erecta* has been reported in herbivore dung (Shimkets et al. 2006) and soil (Wu et al. 2005).

Molecular Analysis

Genome Structure

Anaeromyxobacter, *Cystobacter*, *Melittangium*, and *Stigmatella* are the *Cystobacteraceae* genera that have at least one fully sequenced genome. Two strains of *Anaeromyxobacter dehalogenans* have been sequenced: 2CP-1 (GenBank NC_011891.1) and 2CP-C (GenBank NC_007760.1). Analysis of these genomes show that *A. dehalogenans* possesses one circular chromosome (approximately 5.00 Mb, 74.7 % GC) with approximately 4,500 genes and 4,400 protein-coding sequences. The two strains have two copies each of 16S, 5S, and 23S rRNA. Each copy of 16S rRNA is identical within the genome of each strain, and there is 99 % similarity between strains. The distribution of genes into clusters of orthologous groups (COG) functional categories showed that the highest number of genes are categorized as hypothetical (1,490, 34.3 %), followed by signal transduction (339, 7.8 %); energy production and conversion (296, 6.8 %); cell wall, membrane, and envelope biogenesis (257, 5.9 %); amino acid transport and metabolism (231, 5.3 %); transcription (214, 4.9 %); translation and ribosome structure (198, 4.6 %); posttranslational modification, protein turnover, and chaperones (187, 4.3 %); inorganic ion transport and metabolism (178, 4.1 %); replication, recombination, and DNA repair (169, 3.9 %); carbohydrate transport and metabolism (143, 3.3 %); lipid transport and metabolism (127, 2.9 %); coenzyme transport and metabolism (120, 2.8 %); secondary metabolite biosynthesis, transport, and catabolism (93, 2.1 %); nucleotide transport and metabolism (70, 1.6 %); defense mechanisms (70, 1.6 %); intracellular traffic, secretion, and vesicular transport (63, 1.4 %); cell motility (59, 1.4 %); cell cycle control, cell division, and chromosome partitioning (38, 0.9 %); chromatin structure and dynamics (2, <0.1 %); and RNA processing and modification (2, <0.1 %) (Huntley et al. 2011). Extrachromosomal elements were not related.

Assembly and annotation of *Cystobacter fuscus* (strain DSM2262) are in progress (GenBank ANAH00000000.1). Its 12.44-Mb genome (68.6 % GC) contains 10,372 genes and 10,372 protein-coding sequences. *Melittangium boletus* (strain DSM1473) sequences were submitted by the Max Planck Institute for Terrestrial Microbiology on February 15, 2013 (BioProject PRJNA189498). Data regarding genome size, GC content, genes, and protein-coding sequences are not yet available.

The genome analysis of *Stigmatella aurantiaca* strain DW4/3-1 revealed one circular chromosome (approximately 10.26 Mb; 67.5 % GC) with 8,500 genes and 8,500 protein-coding sequences, three 16S rRNA gene copies; two 5S rRNA gene copies; and one 23S rRNA gene (GenBank CP002271.1; AAMD00000000). The sequences of the three 16S rRNA and two 5S rRNA gene copies of strain DW4/3-1 are identical. No extrachromosomal elements were related. COG analysis categorized the highest number of genes as hypothetical (4,014; 41.1 %), followed by signal transduction (674, 8.1 %); transcription (352, 4.2 %); cell wall, membrane, and envelope biogenesis (329, 3.9 %); replication, recombination, and DNA repair (326, 3.9 %); amino acid transport and metabolism (324, 3.9 %); carbohydrate transport and metabolism (306, 3.7 %); posttranslational modification, protein turnover, and chaperones (272, 3.3 %); secondary metabolite biosynthesis, transport, and catabolism (261, 3.3 %); energy production and conversion (257, 3.1 %); inorganic ion transport and metabolism (254, 3.0 %); translation and ribosome structure (228, 2.7 %); lipid transport metabolism (188, 2.3 %); coenzyme transport and metabolism (168, 2.0 %); nucleotide transport and metabolism (102, 1.2 %); intracellular traffic, secretion, and vesicular transport (93, 1.1 %); defense mechanisms (89, 1.1 %); cell motility (58, 0.7 %); cell cycle control, cell division, chromosome partitioning (49, 0.6 %); RNA processing and modification (4, <0.1 %); chromatin structure and dynamics (3, <0.1 %); and cytoskeleton (1, <0.1 %) (Huntley et al. 2011).

Metabolism and Biotechnological Relevances

Overall and Secondary Metabolism

Soil is the most common habitat for members of the *Cystobacteraceae* family, but they are also frequently found in the dung of herbivorous animals and on decaying plant material, tree bark, and the surface of plant leaves. These bacteria are also adapted to extreme environments, including extremely acidic soil and conditions that are extremely hot and dry, or extremely cold and nutrient-poor (Dawid 2000). The family consists primarily of aerobic organisms. The exception is the genus *Anaeromyxobacter*, which consists of anaerobic or microaerophilic organisms (Wenzel and Muller 2009b). The metabolism of aerobic members is similar to that of other aerobic eubacteria; however, little is known regarding metabolic pathways of extremophile members.

The soil- and plant-associated species secrete exoenzymes that catalyze the hydrolysis of biological macromolecules and entire microorganisms (e.g., bacteria and yeasts) as sources of carbon, nitrogen, and energy. The most important enzymes secreted by this bacterial family under natural conditions are hydrolases such as proteases, nucleases, lipases, and glucanases. Chitinases, amylases, and xylanases have also been described. Regarding cellulose degradation and utilization, some myxobacteria have been categorized as cellulose degraders (Group I, represented by the suborder Sorangineae) or those unable to use cellulose (Group II), which represents most myxobacterial species, including the *Cystobacteraceae* family (Dawid 2000).

Whole genome sequencing projects have contributed to the description and discovery of metabolic pathways in microorganisms, including myxobacteria such as *S. aurantiaca* and *C. fuscus*. This approach enabled the identification of respiratory chain proteins, the enzyme NADH oxidase, cytochromes, and the complete tricarboxylic acid cycle of *Cystobacteraceae* aerobes, as well as the classic metabolic pathways of carbohydrate metabolism (glycolysis, gluconeogenesis, and pentose phosphate pathway), energy metabolism (oxidative phosphorylation), lipid metabolism, nucleotide metabolism, amino acid metabolism, glycan biosynthesis, and metabolism of cofactors and vitamins.

Most metabolism studies have focused on the remarkable secondary metabolism of these organisms (Gross 2007). *Cystobacteraceae* secondary metabolism is responsible for the production of a large number of bioactive molecules with antifungal, antibiotic, and antitumor activities (Wenzel and Muller 2005). Most secondary metabolites characterized to date are polyketides or nonribosomal peptides, which are synthesized by the two multifunctional enzyme systems polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS), respectively. A hybrid PKS-NRPS system synthesizes hybrid polyketide-nonribosomal peptide metabolites. The biosynthesis initiates through the coupling of simple precursors (i.e., acyl-coenzyme A thioesters for polyketide metabolites, and both proteinogenic and non-proteinogenic amino acids for nonribosomal peptide metabolites). Chain extension intermediates undergo various processing reactions during the assembly process and may be further modified after release from the multienzyme (Wenzel and Muller 2009b).

According to studies of *Bacillus* and actinomycete species, PKS and NRPS act as molecular assembly lines. A PKS consists of an acyl transferase for selection of the specific building block, a ketosynthase to catalyze carbon–carbon bond formation, and an acyl carrier protein, to which the chain is assembled. Other domains have been also described as a ketoreductase, a dehydratase, and an enoyl reductase. These enzymes together determine the final redox-state at the β-carbon of each chain extension unit, as well as C-, O-, and N-methyltransferases. The NRPS system involves adenylation, condensation (or heterocyclization) assembled in a peptidyl carrier protein, complemented by the processing enzymes epimerase, *N*- and *C*-methyltransferases, and an oxidase. The final products are released from the carrier proteins through the action of a thioesterase (Wenzel and Muller 2009b). During a cycle of chain extension, each module of the biosynthetic pathway is used only once before passing the nascent molecule to the downstream module; therefore, the number of modules and their order determine the structure of the secondary metabolite (➲ *Fig. 2.3*). In the model organisms mentioned above, these genes are organized as clusters exhibiting colinearity of the genetic organization and the enzymatic transformations. This common type of genomic organization enables the reconstruction of the biosynthetic pathway by determining the gene sequence within the clusters (Gross 2007).

In contrast, secondary metabolite biosynthesis in myxobacteria differs from this model. It often begins with precursors other than acetate or propionate, such as benzoate, isovalerate, isobutyrate, 2-methylbutyrate, dehydro-isobutyrate, 3-hydroxyvalerate, pipecolic acid, or polyunsaturated fatty acids. Whole genome sequencing of myxobacteria has revealed that gene order within a cluster does not represent the order of enzymatic transformations, making it difficult to predict the structures of the secondary metabolites. Thus, understanding the biosynthesis of these secondary metabolites remains a challenge, and more studies are needed to characterize this process (Li and Muller 2009; Wenzel and Muller 2009b).

Secondary metabolism in the well-characterized species *Stigmatella aurantiaca* includes three main biosynthesis and degradation pathways: terpenoid and polyketide biosynthesis, antibiotic biosynthesis, and xenobiotic degradation (➲ *Table 2.3*). This species is a potential candidate for bioremediation of environments contaminated by phenolic compounds. The *S. aurantiaca* strain Sg a 15 produces structurally different molecules that inhibit the mitochondrial respiratory chain (e.g., aurachins) (➲ *Fig. 2.4*). Aurachins are quinoline alkaloids that target mitochondrial complexes I and II and the cytochrome B6/f complex. These compounds exhibit antifungal, antibacterial, and antiplasmodial activities (Pistorius et al. 2011b).

The biosynthetic pathway by which the aurachins are produced has been studied using a combination of approaches including genome analysis, transposon mutagenesis, and in vivo inactivation. Aurachin synthesis starts with the precursor anthranilate, which is activated and transferred to the acyl carrier protein (ACP). Subsequent condensation of anthraniloyl-ACP with two malonyl units produces one of the major aurachin types, aurachin D (Li and Muller 2009; Pistorius et al. 2011a) (➲ *Fig. 2.5*).

At least three different loci on the *S. aurantiaca* chromosome have been identified as encoding proteins involved in the synthesis of the four aurachin types (locus I to III). Genes at locus I (*auaA*–*auaE*) are responsible for the synthesis of aurachin D. AuaA is a prenyltransferase with activity demonstrated in vitro. The other genes encode proteins with homology to ACP (AuaB), β-ketoacyl-ACP synthase II (AuaC and AuaD), and benzoate:coenzyme A ligase (AuaE). Thus, aurachin biosynthesis is a complex and intricate metabolic pathway that requires the action of a set of proteins encoded by different genetic loci (Pistorius et al. 2011a, b).

The species *A. dehalogenans* appears to be an unusual myxobacterium, because it grows under anaerobic or microaerophilic conditions, and its genome does not appear to contain typical secondary metabolite gene clusters (Thomas et al. 2008). This species has received considerable attention because of its ability to oxidize organic matter in both pristine and contaminated environments (e.g., uranium sediments). Another noteworthy feature of *A. dehalogenans* is the ability of to derive energy from reductive dechlorination of chlorophenols and its use of a broad range of electron acceptors including fumarate, nitrate, Fe (III), and U(VI) (Sanford et al. 2002).

Fig. 2.3
Modular organization of polyketide biosynthesis enzymes showing the six successive steps of extension leading to erythromycin production by *Saccharopolyspora erythraea*. Individual domains include ketoreductase (KR); dehydratase (DH); enoyl reductase (ER); ketosynthase (KS), which catalyzes carbon–carbon bond formation; acyl carrier protein (ACP), to which the chain is assembled; and thioesterase (TE), which releases the final products from the carrier proteins. Module 1, KS/KT/KR; Module 2, KS/AT/KR; Module 3, KS/AT; Module 4, KS/AT/DH/ER/KR; Module 5, KS/AT/KR; and Module 6, KS/AT/KR/ACP/TE

However, little is known regarding its overall metabolism and physiology. Chao (Chao et al. 2010) used a proteomic approach to characterize 50 metabolic pathways of *A. dehalogenans* during its growth using fumarate as primary electron acceptor. The study revealed a range of enzymes involved in carbohydrate and amino acid metabolism, including the fermentation of lysine to butyrate. However, many issues must be addressed for a complete understanding of its metabolism.

Applications

Myxobacteria produce an extensive arsenal of secondary metabolites, similar to well-known bacterial producers such as actinomycetes, pseudomonads, *Bacillus spp.*, yeasts, and filamentous fungi. The core structures of these secondary metabolites are promising tools for the pharmaceutical and biotechnological industry, because they appear to have unique cellular targets (Weissman and Muller 2009). Many possess antifungal and antibacterial properties, and others are herbicidal, cytostatic to eukaryotic cells, or antineoplastic.

Most of the bioactive compounds described thus far are produced by *Myxococcus xanthus*, *Sorangium cellulosum*, or *Chondromyces* species, with *Sorangium spp.* being the most prominent (Wenzel and Muller 2009a). However, these species are all members of myxobacteria families other than *Cystobacteraceae*. In this chapter, the discussion of bioproducts and their pharmaceutical and biotechnological applications will focus on *Cystobacteraceae* members, particularly *Stigmatella aurantiaca*. Two strains are of great importance: *S. aurantiaca* DW4/3-1 and *S. aurantiaca* Sg a15. Some of these metabolites will be briefly discussed. Most are electron transport inhibitors that target microbial respiration.

A group of researchers from the German Research Center for Biotechnology (GBF) in Braunschweig, Germany, published several papers in the 1980s describing the isolation and characterization of novel molecules, mainly antibiotics, derived from myxobacteria. Of these primary references, three are of great value for understanding *S. aurantiaca* myxalamids (Gerth et al. 1983), stigmatellin (Kunze et al. 1984), and aurachins (Kunze et al. 1987). Two other compounds from members of the *Cystobacteraceae* family should also be mentioned: cystothiazole (Ojika et al. 1998) (from *Cystobacter fuscus*) and melithiazols (Sasse et al. 1999) (from *Melittangium lichenicola* and others).

Although the 1980s were extremely important for myxobacterial antibiotic screening and identification, the molecular techniques available today are much more efficient. Genetic screening for biosynthetic modules (such as PKS and NRPS) and gene clusters are the central theme of several works on secondary metabolite identification in microorganisms. Many of these studies relied on mutagenesis and/or gene inactivation strategies. Recent studies have focused on identifying hybrid biosynthetic clusters of secondary metabolites from myxobacteria, mainly PKS/NRPS, which may contribute to more complex and rare molecular structures.

Molecular approaches offer an additional advantage over genetic screening. Myxobacteria are slow growing, making chemical and functional screenings more difficult. Furthermore, molecular techniques enable culture-independent screening, even though only 15 myxobacterial genomes have been completely sequenced according to the NCBI Microbial Genome Database[3].

[3] Accession in 01.21.13, available at: www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html

Table 2.3
Secondary metabolism pathways of *Stigmatella aurantiaca*

Metabolism of terpenoids and polyketides
Terpenoid backbone biosynthesis
Sesquiterpenoid and triterpenoid biosynthesis
Carotenoid biosynthesis
Limonene and pinene degradation
Geraniol degradation
Type I polyketide structures
Polyketide sugar unit biosynthesis
Biosynthesis of siderophore group nonribosomal peptides
Biosynthesis of other secondary metabolites
Penicillin and cephalosporin biosynthesis
Streptomycin biosynthesis
Novobiocin biosynthesis
Xenobiotic biodegradation and metabolism
Benzoate degradation
Aminobenzoate degradation
Fluorobenzoate degradation
Chloroalkane and chloroalkene degradation
Chlorocyclohexane and chlorobenzene degradationToluene degradation
Xylene degradation
Nitrotoluene degradation
Ethylbenzene degradation
Styrene degradation
Caprolactam degradation
Bisphenol degradation
Dioxin degradation
Naphthalene degradation
Polycyclic aromatic hydrocarbon degradation
Steroid degradation

Myxalamids

Although myxalamids antibiotics were first isolated from *Myxococcus xanthus* Mx x12, their production was also observed in *Stigmatella aurantiaca* Sg a15. This antibiotic is actually a mixture of four compounds (myxalamid A–D). The main compound, myxalamid B, has a spectrum of activity that includes molds, yeasts, and Gram-positive bacteria (Gerth et al. 1983). However, this range was considered narrow in comparison to myxothiazol, an antifungal compound isolated in 1980 (Gerth et al. 1980).

Stigmatellin

This metabolite was identified in an antibiotic screening of *Stigmatella aurantiaca* Sg a15 in 1984. It is active against yeast, filamentous fungi, and many Gram-positive bacteria. Because it possessed a chemical structure that differs completely from other known metabolites of myxobacteria, it was considered a new compound and named stigmatellin (Kunze et al. 1984).

Aurachins

Aurachin antibiotics are the third structurally different family of metabolites produced by *Stigmatella aurantiaca* Sg a15. Similar to myxalamids, aurachins are a mixture consisting of 13 compounds; the three major components are aurachin A–C. At the time of the first isolation, they were classified as new quinoline alkaloids and named aurachins (Kunze et al. 1987).

Myxochromide S

More recently, the screening of secondary metabolites has included genomic and molecular processes. One example is the screening for hybrid gene clusters of secondary metabolites in *Stigmatella aurantiaca* DW4/3-1. As described above, most of the myxobacterial machinery of secondary metabolites relies on large enzymatic modules, mainly PKS and NRPS. In 2005, three metabolites of *Stigmatella aurantiaca*, named myxochromide S_{1-3}, were identified. The myxochromide biosynthetic pathway is encoded by a hybrid PKS/NRPS cluster in a three-gene operon (Wenzel et al. 2005).

Aurafurons

These compounds were initially isolated by high performance liquid chromatography-diode array detector analysis of cell extracts from *Stigmatella* and *Archangium* strains (Kunze et al. 2005). They were characterized as new polyketides and named aurafuron A and B. Aurafuron A was isolated from *Archangium gephyra* Ar 10844, and aurafuron B from *Stigmatella aurantiaca* DW4/3-1. Their spectra of activity differ: Aurafuron A is active against some filamentous fungi, and aurafuron B is weakly active against some Gram-positive bacteria. However, both were shown to be cytotoxic to a mouse cell line. This cytotoxic activity has been of great interest since the isolation and characterization of epothilones, a new class of microtubule inhibitors first extracted from *Sorangium cellulosum* So ce90, a myxobacterium from the *Sorangiaceae* family (Gerth et al. 1996). The epothilone analog Ixabepilone (Ixempra™) was approved by the US Food and Drug Administration in 2007 for the treatment of advanced breast cancer.

Melithiazols and *Cystothiazoles*

Using a classic antibiotic screening approach, antifungal activity was observed in the cultures of two *Melittangium lichenicola*

Fig. 2.4
Chemical structures of the four major aurachins from *Stigmatella aurantiaca*. A) Aurachin D (C-type); B) Aurachin C (C-type); C) Aurachin B (A-type); and D) Aurachin A (A-type)

Fig. 2.5
General pathway of aurachin biosynthesis

strains (Me l26 and Me l46). The new compounds, named melithiazol A, B, and C, were also found in other myxobacteria. They are related to the previously characterized myxothiazol but are less toxic. However, antibacterial activity was not observed (Sasse et al. 1999). Cystothiazoles were identified as antibiotics in 1998 in a screen for bioactive compounds in myxobacteria. They were purified from a *Cystobacter fuscus* culture and characterized as new bithiazole-type antibiotics (Ojika et al. 1998).

Although most secondary metabolites of *Cystobacteraceae* members are antibiotics, some also show cytotoxic activity. However, further studies are needed to determine any potential

applications in cancer treatment. These molecules are extremely complex and diverse, motivating both the chemical manipulation of these molecules to construct semisynthetic analogs and the search for new secondary metabolites from these organisms. ➲ *Table 2.4* shows the structures and biosynthetic clusters of the main antibiotic compounds described above.

Concluding Remarks and Perspectives

Myxobacteria are a singular group of microorganisms that are characterized by their environmental adaptability, which is related to their multicellular behavior and complex social traits. The morphological changes that take place during their life cycle include cellular aggregation and fruiting body formation. The study of myxobacteria can contribute to the understanding of bacterial communication, social behavior, and adaptation to stressful conditions. In addition, these microorganisms have proved to be efficient producers of bioactive molecules.

Since the 1980s, many studies have described the characterization and classification of myxobacteria. In 2002, the first anaerobic myxobacterium was identified in this group, which was previously thought to consist of strict aerobes, suggesting an as yet undiscovered diversity in these bacteria. Two new members of the *Cystobacteraceae* family, *Anaeromyxobacter dehalogenans* and *Hyalangium minutum*, were recently described and characterized.

Cultivation and isolation of these slow-growing bacteria are challenging. Therefore, cultivation-independent methods are important tools to screen for metabolic pathway gene clusters that regulate morphogenetic changes during stressful conditions (e.g., starvation) and other physiological and adaptive events, such as secondary metabolite production. Determining the genetic mechanisms that regulate formation of the characteristic fruiting bodies and myxospores is of great importance to microbial ecology studies. The extremely large genomes of myxobacteria may account for some of their unique features; therefore, whole genome sequencing may be important to identify the genetic basis for such traits.

In addition to the screening and isolation of new active compounds, manipulating myxobacterial secondary metabolite core structures may produce semisynthetic molecules with a broader spectrum of activity, less toxicity, and higher efficiency. This is a promising alternative for antibiotic development, as commercially available antibiotics are becoming obsolete, primarily because of multidrug-resistant pathogens. This process could also contribute to anticancer drug development, as molecules with cytotoxic actions are being isolated and characterized. In particular, the secondary metabolites of *Stigmatella aurantiaca* should be noted for the diversity of their molecular structures, which increases the arsenal of active core structures that may be useful in the pharmaceutical and biotechnology industries.

Finally, research focusing on the lifestyle of these microorganisms could provide insights into the molecular pathways underlying the unique features of myxobacteria.

Supplement 1

WAT agar and WCX agar

$CaCl_2 \cdot 2H_2O$ 0.1 % (w/v)
Agar 1.5 % (w/v)
HEPES 20 mM

Prepare medium with distilled water, adjust pH to 7.2, and autoclave. After cooling, add cycloheximide (final concentration 25 mg/ml) from a filter-sterilized stock solution to produce the WCX agar. Because the pH is difficult to adjust in this unbuffered medium, adding 20 μM *N*-2-(hydroxy-ethyl)piperazine-*N*′-2-ethane-sulfonic acid (HEPES) is advisable.

The cultures should be incubated at 30 °C for 8–21 days, with daily inspection for swarms and/or fruiting bodies. *Cystobacter* species are fast spreaders and may reach the streaks within 1–2 days after inoculation. *Cystobacter* and *Archangium* swarms usually develop tough slime sheets, with branched and radiating veins (Shimkets et al. 2006).

VY/2 agar

Bakers' yeast (commercial yeast cake) 0.5 % (w/v)
$CaCl_2 \cdot 2H_2O$ 0.1 % (w/v)
Cyanocobalamin 0.5 mg/μl
Agar 1.5 % (w/v)

Prepare medium in distilled water, adjust pH to 7.2, and autoclave. The yeast may be stored as an autoclaved stock suspension for several weeks. To obtain a uniform suspension of yeast cells in the agar, add the yeast to the molten medium.

CY agar

Casitone (Difco) 0.3 % (w/v)
Yeast extract (Difco) 0.1 % (w/v)
$CaCl_2 \cdot 2H_2O$ 0.1 % (w/v)
Agar 1.5 % (w/v)

Prepare medium in distilled water, adjust the pH to 7.2, and autoclave.

Buffered Yeast Agar (Modified Version of VY/2 Agar)

Add the following to 1 l distilled water: 5-g baker's yeast, 1-g $CaCl_2 \cdot 2H_2O$, 5-mM HEPES, 10-g Bacto Agar. Adjust pH to 7.0 with KOH, and autoclave. After the medium cools, add 0.5-μg/ml vitamin B_{12} (filter-sterilized).

Table 2.4
Structure and general characteristics of some myxobacterial antibiotic compounds

Compound	Strain	Structure	Genetic cluster	Observation	References
Myxalamid B	*Stigmatella aurantiaca* Sg a15	$(H_3C)_2HC$; OH; O; NH; OH	PKS/NRPS	The substituted alkyl radical (pink structure) is the only one that differs among myxalamid compounds	Gerth et al. (1983), Silakowski et al. (2001)
Stigmatellin	*Stigmatella aurantiaca* Sg a15	OCH_3; O; CH_3O; O; OH; OCH_3; OCH_3	PKS	This compound has no natural derivatives so far	Gaitatzis et al. (2002), Kunze et al. (1984)
Aurachin A	*Stigmatella aurantiaca* Sg a15	HO; O; N^+; O^-	Non-PKS or NRPS	Structures of A and B are similar (pink). Aurachin C has slightly different rings (orange)	Kunze et al. (1987), Pistorius et al. (2011b)
Aurachin B		OH; N^+; O^-			
Aurachin C		O; N; OH			

Table 2.4 (continued)

Compound	Strain	Structure	Genetic cluster	Observation	References
Myxochromide S1	*Stigmatella aurantiaca* DW4/3-1		PKS/ NRPS	Compounds differ in the alkyl substitutions (shown in orange)	Wenzel et al. (2005)
Myxochromide S2					
Myxochromide S3					
Aurafuron B (2a)	*Stigmatella aurantiaca* DW4/3-1		PKS	Substitution and chiral differences are seen between the two aurafuron B molecules (identical structure in blue)	Frank et al. (2007), Kunze et al. (2005)
Aurafuron B (2b)					

Melithiazol A	*Melittangium lichenicola* Me l26 and Me l46		PKS/NRPS	The three structures have similar rings (shown in green). A and B differ in one double bond	Sasse et al. (1999), Weinig et al. (2003)
Melithiazol B					
Melithiazol C					
Cystothiazole A	*Cystobacter fuscus*		PKS/NRPS	Different radicals are seen in the core structure (shown in purple)	Feng et al. (2005), Ojika et al. (1998), Suzuki et al. (2003)
Cystothiazole B					

Abbreviations: NRPS nonribosomal peptide synthetase, *PKS* polyketide synthase

Supplement 2

Anaerobic Medium

Add the following (per liter): NaCl, 1.0 g; $MgCl_2$•$6H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g; $CaCl_2$•$2H_2O$, 0.015 g; resazurin, 1 mg; trace element solution A, 1 ml; Trace element solution B, 1 ml; Na_2S•$9H_2O$, 0.048 g; L-cysteine, 0.035 g; $NaHCO_3$, 2.52 g; vitamin solution, 10 ml.

Trace Element Solution A

Add the following (per liter): HCl (25 % [wt/wt] solution), 10 ml; $FeCl_2$•$4H_2O$, 1.5 g; $CoCl_2$•$6H_2O$, 0.19 g; $MnCl_2$•$4H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; Na_2MoO_4•$2H_2O$, 36 mg; $NiCl_2$•$6H_2O$, 24 mg; and $CuCl_2$•$2H_2O$, 2 mg.

Trace Element Solution B

Add the following (per liter): Na_2SeO_3, 6 mg; Na_2WO_4•$2H_2O$, 8 mg; NaOH, 0.5 g.

Vitamin Solution (Wolin et al. 1963)

After autoclaving, add the following to the medium (mg/l distilled water): biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; riboflavin, 5; thiamine, 5; nicotinic acid, 5; pantothenic acid, 5; vitamin B_{12} 0.1; p-aminobenzoic acid, 5; thioctic acid, 5. Add reductants to the medium after it has been boiled and cooled to room temperature. Flux the headspace with oxygen-free N_2: CO_2 (80:20) and adjust pH to 7.2–7.3 by varying the flow of CO_2 (Wolin et al. 1963).

Mineral Salts Medium (Sanford and Tiedje 1996/1997)

Add the following (per liter): $CaC1_2$•$2H_20$, 0.015 g; $MgC1_2$•$6H_20$, 0.02 g; $FeSO_4$•$7H_20$, 0.007 g; and Na_2SO_4, 0.005 g. Add 2-mM potassium phosphate buffer (pH 7.2–7.5).

Trace Metals Solution

Add the following to give the final concentration per liter: $MnCl_2$•$4H_20$, 5 mg; H_3BO_3, 0.5 mg; $ZnC1_2$, 0.5 mg; $COC1_2$•$6H_20$, 0.5 mg; $NiSO_4$•$6H_20$, 0.5 mg; $CuC1_2$•$2H_20$, 0.3 mg; $NaMoO_4$•$2H_20$, 0.1 mg. Add NH_4C1 (final concentration 8 mM). Add 10-mM $NaHCO_3$ to buffer the headspace containing N_2:CO_2 (95:5).

Vitamin Solution (Wolin et al. 1963)

After autoclaving the medium, add the following (mg/l distilled water): biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; riboflavin, 5; thiamine, 5; nicotinic acid, 5; pantothenic acid, 5; vitamin B_{12} 0.1; p-aminobenzoic acid, 5; thioctic acid, 5 (Wolin et al. 1963).

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ORIGINAL RESEARCH PAPER

New dioxygenase from metagenomic library from Brazilian soil: insights into antibiotic resistance and bioremediation

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Abstract
Objectives Putative new dioxygenases were identified in a metagenomic β-lactam-resistance screening and, given their key role on aromatic metabolism, we raise the hypothesis that these enzymes maybe concomitantly related to antibiotic resistance and aromatic degradation.
Results ORFs of three putative dioxygenases were isolated from resistant metagenomic clones. One of them, CRB2(1), was subcloned into pET24a expression vector and subjected to downstream phenotypic and bioinformatics analyses that demonstrated the "dual effect" of our metagenomic dioxygenase, on antibiotic and aromatic resistance. Furthermore, initial characterization assays strongly suggests that CRB2(1) open-reading frame is an extradiol-dioxygenase, most probably a bicupin domain gentisate 1,2-dioxygenase. This observation is, to our knowledge, the first description of a metagenomic dioxygenase and its action on β-lactam resistance.
Conclusion Unraveling the diversity of antibiotic resistance elements on the environment could not only identify new genes and mechanisms in which bacteria can resist to antibiotics, but also contribute to biotechnology processes, such as in bioremediation.

Keywords Antibiotic resistance · Aromatic metabolism · Dioxygenase · Metagenome · Soil

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Introduction

Resistance to antibiotics has been reported since the 1950s, shortly after their introduction for the treatment of infectious diseases (Davies 2007). The characterization of antibiotic resistance genes (ARGs) focuses primarily on clinical isolates; however, environmental habitats harbor an unappreciated and vast diversity of ARGs that are not yet well explored.

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Therefore, it is urgent to identify and characterize ARGs from natural environments to better understand their diversity outside the clinical setting. We asked whether it was possible to identify new ARGs unrelated to clinical antibiotic resistance, and what their functions might be in natural environments.

In contrast to pathogenic microorganisms, most of which can be readily isolated and identified, only an estimated 0.1–1 % of soil microorganisms can be cultivated using traditional methods. This represents a major drawback for detecting ARGs in soil microorganisms, leaving culture-independent methods such as metagenomics as potential tools for accessing these microorganisms (Handelsman 2004). Here, we explore ARG diversity in a metagenomic library constructed from soil samples obtained in the Cerrado (de Castro et al. 2011), a savannah-like biome in the midwestern region of Brazil. In a functional screening performed with β-lactam antibiotics, 62 clones were isolated, and four were selected for sequencing reactions. Downstream analysis showed a large diversity of open reading frames (ORFs) inside the metagenomic inserts and assigned putative functions. In particular, genes encoding dioxygenase enzymes were frequently identified in the metagenomic clones.

Ring-cleaving dioxygenases play important roles in aromatic metabolism, both in eukaryotes and prokaryotes. These enzymes catalyze the incorporation of both atoms of O_2 into the aromatic ring, thereby cleaving it. Dioxygenases can be classified as intradiol dioxygenases, which catalyze ortho cleavage, and extradiol dioxygenases, which catalyze meta cleavage. Both classes of enzymes have distinct features and specificities (Arora et al. 2009), with extradiol dioxygenases generally being a more versatile group, cleaving a broader number of substrates (Vaillancourt et al. 2006).

Aromatic compounds are major pollutants discharged into the environment from different sources, including agricultural and industrial processes. These molecules are extremely stable in the environment, and existing physico-chemical procedures are often inefficient in their removal. Bioremediation is an efficient, cost-effective alternative that harness microbial degradation pathways to remove these persistent environmental pollutants via enzymatic catabolism (Arora et al. 2009).

This work tests the hypothesis that dioxygenases play a role in the resistance to β-lactamic antibiotics, and explores the dual effect of these enzymes in the resistance phenotype and aromatic metabolism, their primary function.

Materials and methods

Metagenomic libraries

The metagenomic libraries used in this work were previously constructed with Cerrado *stricto* sensu soil samples, as described by de Castro et al. (2011).

Resistance screening and subcloning

The functional screening of the metagenomic libraries, performed in lysogeny broth (LB) agar plates supplemented with nine β-lactamic antibiotics (16 µg amoxicillin ml^{-1}, 50 µg ampicillin ml^{-1}, 50 µg carbenicillin ml^{-1}, 16 µg cefamandole ml^{-1}, 20 µg cefoxitin ml^{-1}, 5 µg ceftazidime ml^{-1}, 50 µg cephalexin ml^{-1}, 50 µg penicillin G ml^{-1} and 12.5 µg piperacillin ml^{-1}), identified 62 resistant clones. Putative dioxygenase ORFs were subcloned into a pET24a vector, and transformed in *Escherichia coli* Tuner (DE3) cells.

Sequence analysis

Amino acid sequences were annotated using Protein BLAST (Altschul et al. 1990). Multiple alignments of the CRB2(1) amino acid sequence and related proteins were carried out using Clustal Omega (Sievers et al. 2011). Secondary structure was predicted using PSIPRED Protein Sequence Analysis Workbench (Buchan et al. 2013; Jones 1999). A phylogenetic tree was constructed with Mega 6.06 program (Tamura et al. 2013) using the neighbor-joining method and bootstrap analysis.

Expression and purification of putative metagenomic dioxygenase

To characterize the subclone, CRB2(1), that performed best in vector-based gene expression assays, cultures of it were grown to an OD_{600}, of 0.6 and protein induction was carried out by overnight incubation with 1 % (w/v) lactose at 20 °C. The bacteria were lysed by sonication, and the protein of interest

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Table 1 Protein BLAST annotation for putative dioxygenase open reading frames in three metagenomic subclones

Subclone	Selection antibiotic	Best hit	e value	Score	Similarity (%)	Coverage (%)	Conserved domains	Size	Estimated molecular mass (kDa)
AMX3(2)	Amoxicillin	Hypothetical protein (*Candidatus koribacter versatilis*)	2e-64	225	38	97	–	1077 bp 358 aa	39.8
AMX3(3)	Amoxicillin	Intradiol ring-cleavage dioxygenase (*Candidatus koribacter versatilis*)	2e-141	431	42	98	Peptidase_M14NE-CP-C_like superfamily	1674 bp 557 aa	61.9
CRB2(1)	Carbenicillin	Hypothetical protein (*Frankia* sp. Iso899)	2e-145	426	61	91	Cupin_2 superfamily	1068 bp 355 aa	39.5

was purified using the MagneHis Protein Purification System (Promega, Madison, WI, USA) under native conditions. SDS-PAGE analysis was performed in 4–13 % Tris–HCl gel and ran in denaturing running buffer pH 8.3. Samples were centrifuged, suspended in denaturing loading buffer 1× and heated for 5 min at 95 °C.

Phenotypic analysis and cell viability test

In order to evaluate resistance phenotypes to a β-lactamic antibiotic and phenol, CRB2(1) was grown in the presence of both compounds. Growth was monitored from the OD_{600} value. Cells carrying the expression vector containing subclone CRB2(1) were cultured at 28 °C with shaking (130 rpm). When cultures reached $OD_{600} = 0.1$, protein expression was induced by adding IPTG. After 1 h, 50 µg carbenicillin ml^{-1} and 0.1 % phenol were added, and OD_{600} was monitored from 1 to 96 h. This phenotypic assay was performed in quadruplicate. Cell viability was evaluated by adding 30 µl 2 mg MTT ml^{-1} to 200 µl of the 24 h cultures and incubating at 37 °C for 1 h.

Enzymatic assay

Gentisate 1,2-dioxygenase (GDO) activity was evaluated spectrophotometrically following the increase in absorption at 330 nm indicating maleylpyruvate formation. The 200 µl reaction mixture contained 50 µl purified putative dioxygenase (∼35 µg), 130 µl phosphate buffered saline (0.1 M, pH 7.7) supplemented with 100 µM ferrous ammonium sulfate, and 2,5-dihydroxybenzoic acid (gentisic acid; 10 mM) as substrate. The samples were previously incubated at 40 °C for 1 h and then the absorbancy was measured every 20 min for 4 h. This assay was performed in triplicate. Blank samples consist of all the components but the purified protein, replaced by the elution buffer.

Results

Sequence analysis

Within the ORFs on the four selected metagenomics clones—AMX3, CFX12, CRB2 and PG17—we observed three putative dioxygenase genes. After subcloning processes, we constructed the respective subclones, named AMX3(2), AMX3(3) and CRB2(1) (Table 1). CRB2(1) subclone was selected for further analysis following some criteria—presence of conserved domains, better performance on IPTG induction assays and the presence of dioxygenase hits on the BLAST search.

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Although the closest match from the BLAST search was a hypothetical protein, the CRB2(1) insert has a conserved bicupin domain, and GDO proteins were present within the first hundred Blastp hits. These findings suggest that CRB2(1) is an extradiol dioxygenase and could be further characterized as a novel GDO.

To identify the location of the cupin domains in the CRB2(1) protein, we used PSIPRED to predict its secondary structure (Fig. 1). The diverse cupin superfamily, which includes catalytic and non-catalytic proteins, is characterized by a six-stranded β-barrel fold. Catalytic members include dioxygenase enzymes. Two conserved motifs [$G(X)_5HXH(X)_{3-4}E(X)_6G$ and $G(X)_{5-7}PXG(X)_2H(X)_3$] comprise a cupin fold (Dunwell et al. 2001). Proteins with two cupin domains are called bicupins.

Because the secondary structure prediction identified two cupin domains, we aligned the CRB2(1) amino acid sequence with known bicupin dioxygenases (Fig. 2). Although all GDOs described to date contain two cupin domains (Adams et al. 2006; Chen et al. 2008; Hirano et al. 2007), the metal-binding sites are generally active in only one domain (Chen et al. 2008; Dunwell et al. 2004). Conserved metal-binding residues are present in both motifs of the cupin domain: two histidines and one glutamate in motif 1, and a histidine in motif 2. However, in GDOs the glutamate in motif 1 is typically replaced by alanine or another polar or hydrophobic residue (Fetzner 2012). In CRB2(1), an alanine-to-serine mutation occurred. The metal-binding site was conserved and is possibly active only in the *N*-terminal cupin domain of CRB2(1). Of the bicupin dioxygenases shown in Fig. 2, only the GDO of *Silicibacter pomeroyi* contains active metal-binding sites in both domains (Chen et al. 2008).

The phylogenetic tree distinguishes three groups of proteins: extradiol and intradiol dioxygenases and a monooxygenase (Fig. 3). CRB2(1) clusters with the extradiol dioxygenases and is placed along known cupin dioxygenases, including bicupin GDOs.

Functional analysis

To determine the resistance phenotype of CRB2(1), cells bearing the subclone were incubated with carbenicillin, the antibiotic initially used to select the CRB2 metagenomic clone, and phenol, a simple

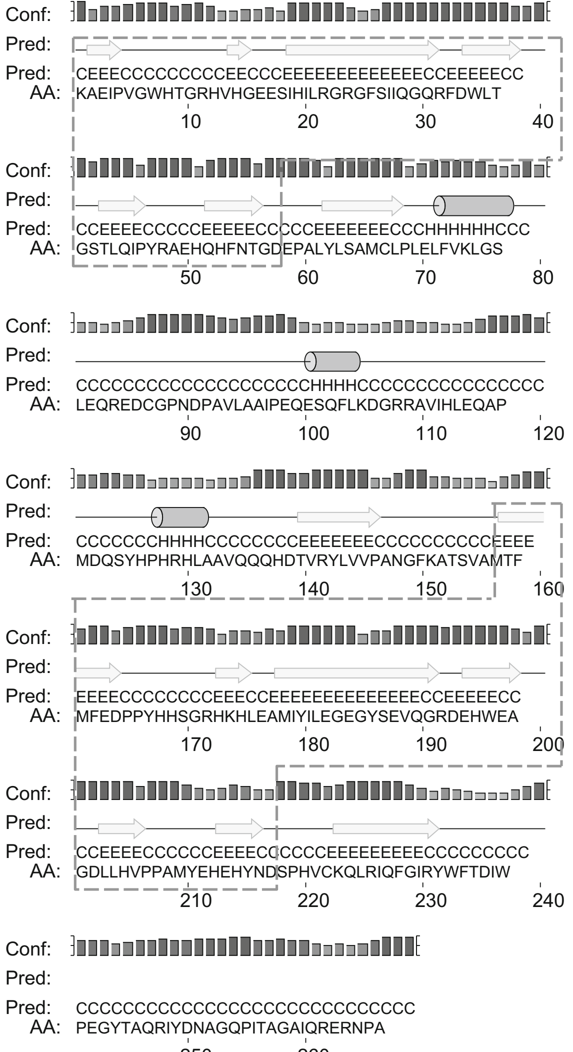

Fig. 1 Secondary structure prediction for the metagenomic clone CRB2(1) and characterization of cupin domains, represented by the *dashed polygons*. Each cupin domain contains six β-sheets, represented by *arrows*. The first and last two β-sheets contain motifs 1 and 2, respectively, for each cupin domain. This secondary structure was predicted with PSIPRED v3.3 and shows a bicupin domain-containing protein in the CRB2(1) amino acid sequence

hydroxylated aromatic molecule with cytotoxic effects.

Figure 4a demonstrates clear differences on cellular growth when CRB2(1) subclone and intact pET24a are compared. Then cell viability was evaluated after 24 h exposure to carbenicillin or phenol (Fig. 4b). Changes in the samples coloration shows the action of

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Fig. 2 Using the Clustal Omega multiple alignment tool, the CRB2(1) amino acid sequence was aligned with characterized gentisate 1,2-dioxygenases and a 1-hydroxy-2-naphtoate dioxygenase, all containing bicupin domains. Cupin motifs are represented by *rectangles*, divided by motif 1 (M1, represented by *bold squares*) and motif 2 (M2, represented by *dashed squares*). *Bold letters* represent conserved residues of the metal-binding motif of cupin domains (2-His-1Glu on motif 1 and 1-His on motif 2). In gentisate 1,2-dioxygenases the glutamate residue is typically replaced by an alanine; however, in CRB2(1) the glutamate residue was by a serine residue. Sequences downloaded from NCBI include 1-hydroxy-2-naphthoate from *Nocardioides sp.* K7 (BAA31235.2); gentisate 1,2-dioxygenase from *Silicibacter pomeroyi* (3BU7_A); gentisate 1,2-dioxygenase from *Escherichia coli* O57:H7 (2D40_A), and gentisate 1,2-dioxygenase from *Pseudomonas alcaligenes* (AAD49427.1)

living cells on MTT reagent and indicates cellular viability. These results demonstrate the dual effect of CRB2(1), conferring resistance to both an antibiotic and phenol.

Expression of the recombinant protein was induced with 1 % lactose at 20 °C for protein solubility, allowing for purification under native conditions. SDS-PAGE analysis of CRB2(1) suggests that it was a monomeric enzyme with an estimated molecular mass of 39.5 kDa, as predicted by sequence data (Fig. 5).

We then carried out an enzyme kinetics assay to evaluate the ability of the purified protein to cleave hydroxylated aromatic rings. Since we identified CRB2(1) as a potential GDO, gentisic acid was used as the substrate. Increased optical density at 330 nm in

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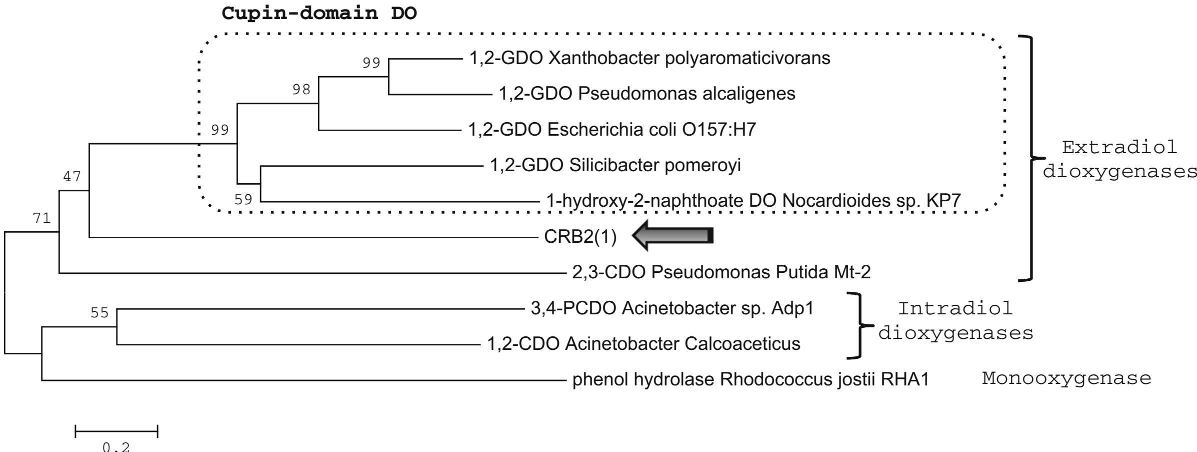

Fig. 3 Phylogenetic analysis of CRB2(1). Three distinct groups were identified and branched together: extradiol dioxygenases, intradiol dioxygenases, and a monooxygenase, represented by a phenol hydrolase. CRB2(1), indicated by the *arrow*, clusters in the extradiol dioxygenase group, along the subgroup of cupin domain dioxygenases (DO). The phylogenetic tree was constructed with the Mega 6.06 program, using the neighbor-joining method and bootstrap analysis (500 replicates). Sequences retrieved from NCBI include gentisate 1,2-dioxygenase (1,2-GDO) from *Xanthobacter polyaromaticivorans* (BAC98955.1); gentisate 1,2-dioxygenase (1,2-GDO) from *Pseudomonas alcaligenes* (AAD49427.1); gentisate 1,2-dioxygenase (1,2-GDO) from *Escherichia coli* O157:H7 (2D40_A); gentisate 1,2-dioxygenase (1,2-GDO) from *Silicibacter pomeroyi* (3BU7); 1-hydroxy-naphtoate-dioxygenase from *Nocardioides sp.* KP7 (BAA31235.2); catechol 2,3-dioxygenase (2,3-CDO) from *Pseudomonas putida* Mt-2 (1MPY_A); protocatechuate 3,4-dioxygenase (3,4-PCDO) from *Acinetobacter sp.* Adp1 (1EO2_A); catechol 1,2-dioxygenase (1,2-CDO) from *Acinetobacter calcoaceticus* (1DLM_B), and phenol hydrolase from *Rhodococcus jostii* RHA1 (YP_703833.1)

samples containing the recombinant protein indicated the accumulation of maleylpyruvate, formed by extradiol cleavage of gentisic acid (Fig. 6).

Discussion

Characterizing metagenomic DNA sequences presents some challenges. The most prominent is low sequence similarities with matches in existing databases; many are hypothetical or putative proteins, and conserved domains are generally absent. However, functional screening provides important clues about these sequences, with the substrate used in the initial assay being a strong indication for the classification of the ORF inside the insert. In this case, antibiotic resistance per se did not point to any particular ORF as the one responsible for the phenotype observed since the diversity of ARGs in the environment is not yet well understood. Our identification of putative dioxygenase genes in three metagenomic inserts suggested a possible role on the resistance phenotype. Subsequent tests not only confirm antibiotic resistance but also revealed the ability to cleave aromatic rings.

Several GDOs have been characterized, but none was identified in metagenomic sequences. Here we describe the initial characterization of a new dioxygenase isolated from an antibiotic-resistant metagenomic clone. Sequence and functional analyses indicate that CRB2(1) is likely a GDO with a conserved bicupin domain.

It is unclear why a dioxygenase was selected by a β-lactam-resistance screening of clones rather than well-characterized antibiotic resistance elements, such as β-lactamases. This finding supports the idea that ARG diversity, especially in the environment, is much greater than previously thought. In fact there is strong evidence that ARGs are not only widespread in the environment but predate the antibiotic era, as demonstrated by a metagenomic survey of ancient environmental DNA samples (D'Costa et al. 2011). This suggests that clinically important resistance elements were pre-existing genes that were selected by antibiotic use, with resistant strains likely adapting to increasing concentrations of these toxic compounds. Therefore, analysis of ARGs from natural environments may identify new resistance elements and help explain how these ARGs were transferred to

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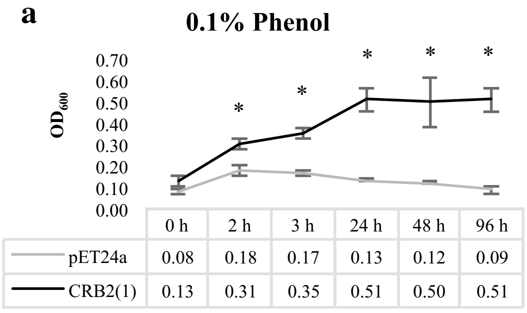

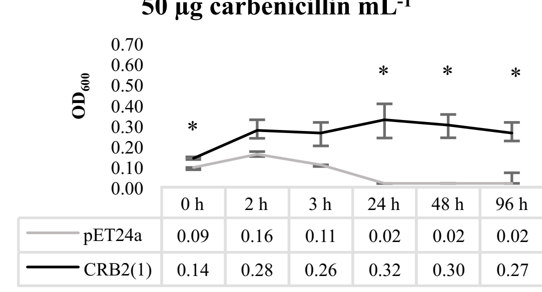

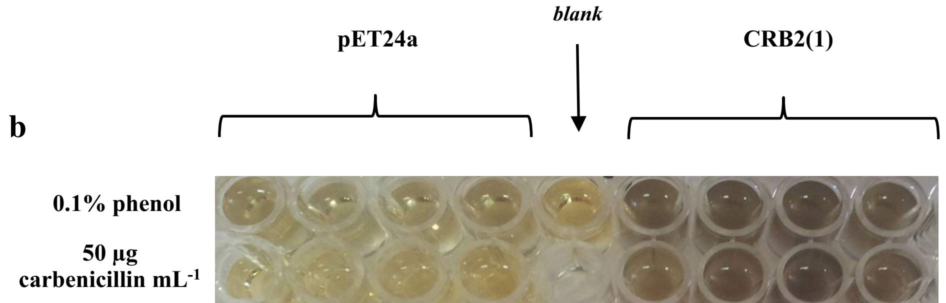

Fig. 4 **a** Cell density over time for cells containing pET24a vector containing CRB2(1) or empty vector on 50 µg carbenicillin ml^{-1} or 0.1 % phenol. At 0 h, protein expression was induced by adding IPTG, and 1 h later phenol or carbenicillin was added. Clear differences in turbidity and optical density values were observed by the 24 h time point. Results are expressed as mean values of quadruplicate samples. *$p < 0{,}01$. **b** Cell viability assay. Aliquots were obtained at the 24 h time point of cultures carrying the empty pET24a vector (*left*) and or vector containing the CRB2(1) subclone (*right*) after incubation with phenol or carbenicillin (each set of four wells represents assay replicates). MTT reduction (*dark color*) indicates viable cells, which is consistent with spectrophotometry results showing increased cell density for cells carrying the vector containing CRB2(1) and decreased cell density for cells carrying the empty vector after exposure to both compounds

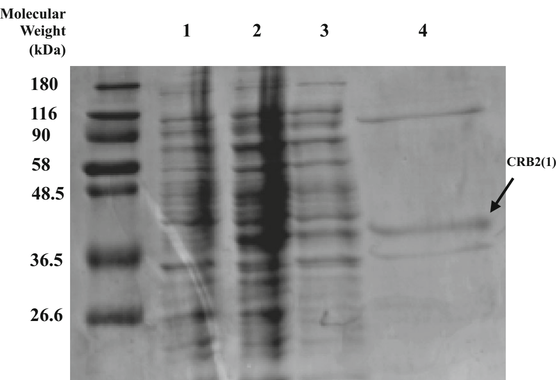

Fig. 5 SDS-PAGE analysis of the CRB2(1) subclone. *Lane 1*, cells carrying the empty pET24a (without CRB2(1) gene insert) after induction with 1 % lactose; *lane 2*, cells carrying pET24a vector with CRB2(1) after induction with 1 % lactose; *lane 3*, crude lysate supernatant from cells carrying vector with CRB2(1); *lane 4*, purified protein from CRB2(1) subclone. *Arrow* indicates the protein of interest. CRB2(1) insert band is absent in *lane 1*, showing the successful lactose induction and purification of CRB2(1) subclone

pathogenic microorganisms (e.g., via mobile elements such as transposons or integrons).

More attention should be turned to the origins of ARGs, especially those carried by non-cultivable microorganisms, which harbor a vast range of uncharacterized proteins, many with potential to cause antimicrobial resistance. For example, a recent functional metagenomic study on antimicrobial resistance in soil (Allen et al. 2015) revealed the role of a response-regulator gene on carbenicillin tolerance in *E. coli*, reinforcing the need to amplify our knowledge on antibiotic resistance elements, specially those from natural environments.

However, classification of a particular gene as an ARG can be difficult since its role in resistance may not be the primary function of the protein, as evidenced by our metagenomic clone.

This report is, to our knowledge, the first to show β-lactam-resistance activity of a dioxygenase. Although

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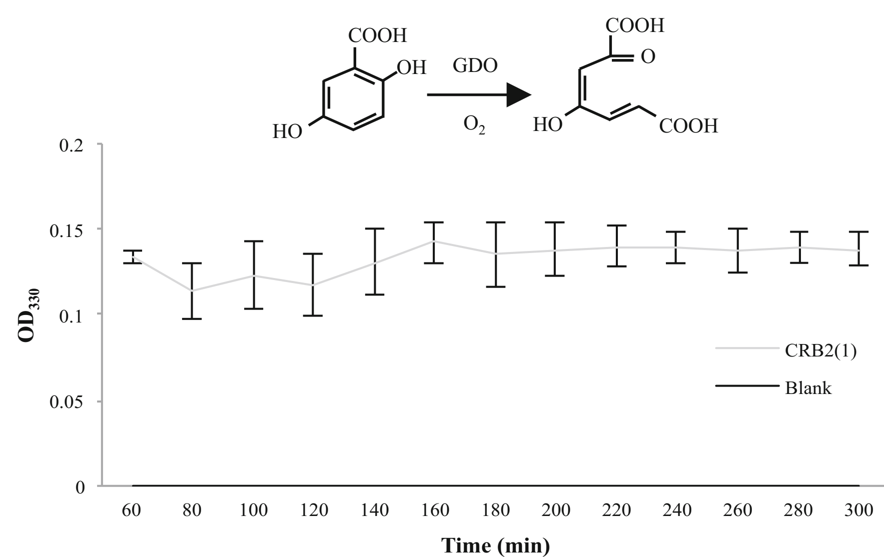

Fig. 6 Enzyme kinetics assay using purified CRB2(1) protein incubated with gentisic acid as substrate. Maleylpyruvate formation from the 2,3-cleavage of gentisic acid in the presence of oxygen was evaluated by spectrophotometry. The reads were performed after the 1 h incubation procedure. Blank samples lacked the purified protein. Results are expressed as mean ± SD of triplicate samples

the mechanism of antibiotic resistance is unclear, dioxygenases may act on the carbenicillin molecule by disrupting the aromatic ring, thereby inactivating its antibiotic activity. If so, dioxygenases represent another type of cleaving enzyme that confers β-lactam resistance, in addition to β-lactamases, which play an important role in clinical infections.

The ability of this protein to degrade aromatic rings, as evidenced by phenol resistance and the kinetic assay, is another important finding of this work. Antimicrobial resistance genes are frequently found in association with other genes that confer similar phenotypes, such as resistance to heavy metals or quaternary ammonium. Aromatic degradation may play an analogous role, selecting other genes to construct xenobiotic resistance gene cassettes. The phenol resistance of this metagenomic clone also suggests its possible use in bioremediation. Aromatic compounds are important environmental pollutants, and degradation by microbial enzymes can facilitate their removal from contaminated areas. Thus, accessing microbial diversity, especially that of non-cultivable and consequently unknown microorganisms, could increase the range of microbial enzymes used in this field.

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