ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR
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AMANDA SIMÃO DIAS

# CARACTERIZAÇÃO DE ISOLADOS BACTERIANOS MARINHOS EM RELAÇÃO À SUA SUSCETIBILIDADE A ANTIMICROBIANOS E AO SEU POTENCIAL DE AÇÃO ANTIMICROBIANA

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**SUMÁRIO**

## Resumo

Os oceanos sofrem contaminações diárias por diferentes tipos de compostos químicos, como fármacos antimicrobianos, devido a diversas atividades humanas, favorecendo o surgimento de microrganismos resistentes e a presença de genes de resistência a antimicrobianos (ARGs) nestes ambientes e alterando a composição de comunidades microbianas. Pesquisas nos ambientes marinhos voltadas à detecção da resistência antimicrobiana e à busca por novas moléculas com potencial antimicrobiano têm ganhado destaque. Neste estudo, investigou-se o perfil de resistência antimicrobiana de bactérias de sedimento marinho profundo, em condição planctônica e de biofilme; a detecção da presença de genes de resistência através do sequenciamento de genoma completo; a avaliação dos níveis de expressão relativa de genes de resistência detectados nestes isolados, por PCR quantitativa, em diferentes condições de cultivo semelhantes às do seu ambiente de origem e a habilidade destes microrganismos de inibir outras cepas bacterianas e/ou fúngicas. Nossos resultados indicaram a presença de dois *Paenibacillus* sp. resistentes a clindamicina (MET16 e MET17) e eritromicina (MET 17), além de seis *Pseudomonas* sp. resistentes a aztreonam e um (MD330.9) também a ceftazidima, em concentrações elevadas. Também foi observada uma alta tolerância a antimicrobianos destes isolados quando em condição de biofilme. Nos genomas dos isolados de *Pseudomonas* sp. foi detectado a presença dos genes *mexE* e *mexF*, pertencentes ao operon MexEF-OprN, responsável pela expressão de um mecanismo de bomba de efluxo. A modulação das condições de cultivo empregadas não mostrou variações significativas na expressão destes genes para a maioria dos isolados. Quanto a capacidade de inibição de microrganismos, cinco *Pseudomonas* sp. apresentaram a capacidade de inibir microrganismos, principalmente fungos unicelulares e filamentosos. A análise genômica indicou clusters gênicos voltados a produção do sideróforo Piochelina, que apresenta ação antifúngica descrita. Em função deste resultado, uma análise quantitativa da produção de sideróforos totais foi realizada, indicando que o isolado de Pseudomonas com maior ação antifúngica (MD330.9), foi o que mais produziu sideróforos e, inicialmente, o único que apresentou resistência a dois antibióticos. Nossos resultados fornecem dados inéditos a respeito do perfil de resistência a antimicrobianos em bactérias de ambiente marinho profundo, a presença e a expressão de genes de resistência em bactérias do gênero Pseudomonas de ambientes marinhos, bem como sobre o potencial de produção de moléculas antimicrobianas por esses isolados.



Palavras-chave: bactérias marinhas; resistência microbiana; tolerância ao biofilme; ação antimicrobiana; microbiologia ambiental.

**Abstract**

The oceans are contaminated daily by different types of chemical compounds, such as antimicrobial drugs, due to various human activities, favoring the emergence of resistant microorganisms and the presence of antimicrobial resistance genes (ARGs) in these environments and altering the composition of microbial communities. Research in marine environments aimed at detecting antimicrobial resistance and searching for new molecules with antimicrobial potential has gained prominence. In this study, we investigated the antimicrobial resistance profile of bacteria from deep marine sediments, in planktonic and biofilm conditions; the detection of the presence of resistance genes through whole genome sequencing; the evaluation of the relative expression levels of resistance genes detected in these isolates, by quantitative PCR, under different culture conditions similar to those of their environment of origin; and the ability of these microorganisms to inhibit other bacterial and/or fungal strains. Our results indicated the presence of two Paenibacillus sp. resistant to clindamycin (MET16 and MET17) and erythromycin (MET 17), in addition to six Pseudomonas sp. resistant to aztreonam and one (MD330.9) also to ceftazidime, at high concentrations. A high tolerance to antimicrobials was also observed in these isolates when in biofilm condition. The presence of the mexE and mexF genes, belonging to the MexEF-OprN operon, responsible for the expression of an efflux pump mechanism, was detected in the genomes of the Pseudomonas sp. isolates. The modulation of the culture conditions employed did not show significant variations in the expression of these genes for most of the isolates. Regarding the capacity to inhibit microorganisms, five Pseudomonas sp. showed the capacity to inhibit microorganisms, mainly unicellular and filamentous fungi. The genomic analysis indicated gene clusters focused on the production of the siderophore Piochelin, which has a described antifungal action. Based on this result, a quantitative analysis of total siderophore production was performed, indicating that the Pseudomonas isolate with the highest antifungal activity (MD330.9) was the one that produced the most siderophores and, initially, the only one that showed resistance to two antibiotics. Our results provide unprecedented data on the antimicrobial resistance profile of deep-sea bacteria, the presence and expression of resistance genes in Pseudomonas bacteria from marine environments, as well as the potential for production of antimicrobial molecules by these isolates.

Keywords: marine bacteria; microbial resistance; biofilm tolerance; antimicrobial action; environmental microbiology.

# 1. INTRODUÇÃO

## 1.1 Comunidades microbianas de ambientes marinhos

Comunidades microbianas são sistemas complexos dispersos por todos os tipos de ecossistemas naturais. Encontrados em solos, águas ou na microbiota de outros seres, estes organismos provêm recursos que são aproveitados pelo ambiente assim como pela sociedade (MAIER; PEPPER, 2009; RAMÍREZFLANDRES; GONZÁLES; ULLOA, 2019; NAYFACH *et al*., 2021). Os ecossistemas naturais geram cerca de 128 trilhões de dólares em serviços globais por ano. Neste contexto, os ecossistemas marinhos apresentam a segunda maior contribuição, com cerca de 49,7 trilhões ao ano, só perdendo para os ecossistemas terrestres (COSTANZA *et al*., 2014).

Ambientes marinhos abrigam uma grande parcela dos microrganismos do planeta, visto que mais de 70% da superfície terrestre é coberta por oceanos (NOAA, 2020). Eles estão presentes de formas muito diversificadas, seja como microrganismos planctônicos, associados a sedimentos, em biofilmes ou tapetes microbianos no substrato marinho, ou na microbiota de macrorganismos (STAL *et al*., 2012; GLÖCKNER *et al*., 2012). Independentemente de seu local de ocorrência, estes microrganismos desempenham diferentes papéis-chave na estruturação e equilíbrio das comunidades e dos ecossistemas marinhos como um todo (MUNN *et al*., 2019; LAIOLO *et al*., 2024).

A principal função atribuída a microrganismos marinhos é de mantenedores dos ciclos biogeoquímicos que ocorrem nos oceanos (FUHRMAN; CRAM; NEEDHAM, 2015; YORK, 2018). Por meio do seu metabolismo, estes organismos são responsáveis por grande parte da produção primária e sequestro de $CO_2$ nestes ambientes (CAVICCHIOLI *et al*., 2019; JIAO *et al*., 2024), reciclando importantes elementos como carbono, nitrogênio, fósforo e enxofre, assim como elementos menos abundantes como ferro, cobre e manganês, mantendo um balanço da presença destes componentes e, em última análise, controlando a dinâmica destes ecossistemas (STAL *et al*., 2012; SONG; ZHUANG; QUEIROZ, *et al*., 2023).

## 1.2 Impacto de atividades humanas nos ecossistemas marinhos

O impacto da ação humana nos ecossistemas pode ser observado por todo o globo. Devido ao descaso com o meio ambiente, um novo período geológico foi cunhado em nossa homenagem, o Antropoceno. Evidenciada estratigraficamente,

esta nova era geológica marca o período em que as mudanças ecológicas emergentes são decorrentes da ação antrópica (WATERS *et al*., 2016; WATERS; TURNER, 2022). Os ecossistemas marinhos sofrem diversos distúrbios pela ação humana. Seja direta ou indiretamente, atividades como pesca, mineração, agricultura e turismo causam grande pressão nesses ambientes. No entanto, a severidade do impacto dessas atividades nem sempre é proporcional à sua ação (NOGALES *et al*., 2011). A maioria dos distúrbios antrópicos nos ecossistemas marinhos permanece invisível aos nossos olhos, até que macrorganismos sejam afetados. Porém, muito antes deles, as comunidades microbianas e, desta forma, as cadeias tróficas, já percebem tais impactos e são significativamente afetadas (LABBATE *et al*., 2016; CHEN *et al*., 2019). Diversos contaminantes são encontrados nos oceanos oriundos da atividade antrópica. Metais pesados e polímeros, provenientes da mineração e da indústria petroquímica, respectivamente, provocam a contaminação das águas em diversos estratos, assim como das cadeias tróficas (DULEBA *et al*., 2018; KUPPUSAMY *et al*., 2019). Partículas plásticas e microplásticas são descartadas em toneladas anualmente nos oceanos (JAMBECK *et al*., 2015), acumulando-se nos organismos ao longo da cadeia trófica, servindo como fonte de toxinas e, eventualmente, até como vetores de transmissão de microrganismos patogênicos (CASABIANCA *et al*., 2019; AHMAD *et al*., 2020). Pesticidas e antimicrobianos, provenientes principalmente de atividades agroindustriais (MA *et al*., 2020), também são despejados permanentemente nos oceanos. Estes produtos impactam principalmente comunidades microbianas costeiras próximas a áreas de interface humana, alterando as comunidades e selecionando microrganismos (CHEN *et* al., 2019; HABIBI *et al*., 2022). Este impacto, no entanto, pode vir a se estender a comunidade naturais mais afastadas destes centros, podendo gerar uma pressão seletiva que promova a permanência de linhagens resistentes a estas moléculas, gerando uma consequente alteração da estrutura das comunidades microbianas (MICHAEL; DOMINEY-HOWES; LABBATE, 2014).

A carga de antimicrobianos liberada nos oceanos, mesmo que grande, é fragmentada. Devido ao seu grande volume, esses sistemas diluem as altas concentrações destas moléculas (LARSSON; FLACH, 2021). Estudos abordam que o perfil de resistência microbiana observada em ambientes ditos como intocáveis, como é o caso dos oceanos, apresentam genes de resistência homólogos muito distastes dos observados na área clínica (FONSECA; ANDRADE; VICENTE, 2018). Porém, como consequência do uso indiscriminado destes fármacos pode haver, de fato, uma

contribuição de pequenas doses de antimicrobianos que a longo prazo podem levar à adaptação, evolução e disseminação da resistência a estas moléculas para diferentes espécies bacterianas nestes ambientes (HATOSY; MARTINY, 2015; CHRISTAKI; MARCOU; TOFARIDES, 2020).

### 1.3 Bactérias resistentes a antibióticos em ambientes naturais

A resistência antimicrobiana pode ser um fator intrínseco. Determinados indivíduos, populações e/ou comunidades tendem a ter diferentes capacidades inerentes de resposta a estes fármacos (MARTI; VARIATZA; BALCAZAR, 2014; NADEEM *et al*., 2020). Da mesma forma, a resistência a antimicrobianos pode ser adquirida, por meio de mutações ou da obtenção de material genético exógeno, ou ainda adaptativa, em resposta a determinada sinalização ambiental (CHRISTAKI; MARCOU; TOFARIDES, 2020).

A resistência bacteriana a antimicrobianos é observada desde os primórdios da sua utilização. Cepas resistentes à penicilina e seus derivados, ou a outras moléculas, foram descritas desde então e estudadas quanto aos seus mecanismos de combate aos antimicrobianos (CHRISTAKI; MARCOU; TOFARIDES, 2020). Contudo, atualmente é observado que diferentes isolados bacterianos não são resistentes a apenas algumas moléculas antimicrobianas e sim a uma vasta gama, se não a todas (JINDAL; PANDYA; KHAN, 2015). A multirresistência é atualmente vista como um problema de saúde pública global. Neste contexto, a Organização Mundial da Saúde (OMS) publicou uma lista com os 12 patógenos prioritários, destacando-se entre eles *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e diferentes espécies da família *Enterobacteriaceae*, contra os quais o desenvolvimento de novos fármacos é necessário (WHO, 2017).

Independentemente do tipo de resistência a antimicrobianos, o uso desenfreado destes fármacos, em diferentes setores, vem proporcionando um aumento da velocidade de seleção de bactérias, fungos, vírus e parasitos (muitos deles patogênicos) com características associadas à capacidade de inativar tais moléculas, ou de escapar do seu mecanismo de ação (WHO, 2020; HOLMES *et al*., 2016). Além disso, a alta prevalência de linhagens resistentes a antimicrobianos contribuem com a transmissão de genes que conferem tais características, em diferentes comunidades microbianas (THAVASI *et al*., 2007; JANG *et al*., 2018).

A maioria dos estudos e medidas de contenção ainda se dedicam à caracterização da suscetibilidade a antimicrobianos de isolados de origem clínica e a

investigação do seu genoma para a elucidação dos mecanismos de resistência. Diferentemente, um número muito menor de estudos é voltado para relatar tais informações em linhagens ambientais (SUZUKI *et al*., 2017). Sendo assim, ainda pouco se sabe a respeito dos efeitos ecológicos dos antimicrobianos em comunidades microbianas de ambientes naturais, incluindo-se os ecossistemas marinhos (BILAL *et al*., 2020). Da mesma forma, a descrição genética desses microrganismos pode conter informações essenciais sobre o resistoma bacteriano e novos mecanismos de resistência selecionados ao longo do tempo (CUADRAT *et al*., 2020; ZHANG *et al*., 2022). Neste contexto, as bactérias mais comumente relacionadas à resistência a antimicrobianos em ambientes marinhos são pertencentes a diferentes famílias, tais como *Enterobacteriaceae*, *Pseudomonadaceae* e *Vibronaceae*, destacando-se entre elas as espécies *A. baumannii*, *Klebsiella pneumoniae*, *P. aeruginosa* e os gêneros *Vibrio* e *Salmonella* (RUBAN; GUNASEELAN, 2011; MATYAR, 2012), conhecidas clinicamente por apresentarem um elevado potencial de patogenicidade. Contudo, a característica de resistência a antimicrobianos também já foi observada em espécies bacterianas exclusivas de ambientes marinhos, como membros da família *Rhodobacteraceae* (HATOSY; MARTINY, 2015). Todos esses fatores afetam a ecologia marinha e geram consequências não apenas em comunidades locais, mas eventualmente a níveis globais, podendo vir a atingir também o ser humano.

De forma complementar, outras estratégias de sobrevivências que não são relacionadas a alterações genéticas podem ser observadas em microrganismos amboientais. A formação de biofilmes é uma destas estratégias. Observada em microrganismos, este mecanismo não está atrelado a modificações genéticas, mas influencia na expressão de um fenótipo de tolerância a diversos compostos (RATHER; GUPTA; MANDAL, 2021). O biofilme microbiano é caracterizado como um conjunto de células agregadas envoltas por uma matriz extracelular, composta por substâncias poliméricas extracelulares (EPS), que isola e protege estes microrganismos de adversidades do ambiente (DE CARVALHO, 2018). Na área clínica, pesquisas envolvendo biofilmes são de extrema relevância, visto que a matriz produzida permite a tolerância a altas doses de antimicrobianos e, devido ao agregado de microrganismos, favorece a transferência horizontal de genes na população (OLSEN, 2015; URUÉN *et al*., 2020). Já em pesquisas com isolados ambientais o foco é voltado, especialmente, para a avaliação da colonização de substratos (tanto artificiais quanto naturais) (ANTUNES; LEÃO; VASCONCELOS, 2018; DE CARVALHO, 2018), sendo a avaliação de tolerância a estressores presentes nestes ambientes deixada de lado.

### 1.4 Genes de resistência em microrganismos marinhos

Independentemente da sua classificação taxonômica, a observação de genes de resistência a antimicrobianos em bactérias oriundas de ambiente marinho vem se tornando cada vez mais comum, levando à estimativa de que os oceanos possam desempenhar um papel de reservatório de tais genes (MARTI; VARIATZA; BALCAZAR, 2014). Estudos mostram que nestes ambientes existe uma prevalência da presença de genes relacionados à resistência a tetraciclinas, sulfonamidas, βlactâmicos e quinolonas (DANG *et al*., 2008; JANG *et al*., 2018; MARTINY *et al*., 2022). Além disso, muitos destes encontram-se em material genético plasmidial, possivelmente proporcionando uma eficiente disseminação destes genes entre diferentes microrganismos marinhos (YANG *et al*., 2013; CUADRAT *et al*., 2020).

Os principais genes conhecidos como marcadores de resistência a antimicrobianos encontrados nestes ambientes são: *tet*A, *tet*B, *tet*D e *tet*M, relacionados à resistência a tetraciclinas (DI CESARE *et al*., 2013; YANG *et al*., 2013; TOMOVA *et al*., 2015; JANG *et al*., 2018; TAN *et al*., 2018); *sul*1 e *sul*2, à resistência a sulfonamidas (JANG *et al*., 2018; TAN *et al*., 2018); *bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{OXA-10}$, à resistência a β-lactâmicos (JANG *et al*., 2018; TAN *et al*., 2018; CARNEY *et al*., 2019; resistoma); e *qnr*A e *qnr*S, a quinolonas (YANG *et al*., 2013; TOMOVA *et al*., 2015; JANG *et al*., 2018; CARNEY *et al*., 2019). Estes estudos detectaram genes *tet* em isolados marinhos dos gêneros *Vibrio*, *Pseudomonas, Klebsiella, Acinetobacter, Marinomonas* e *Enterococcus*, além de outras espécies de diferentes filos bacterianos, tais como Proteobacteria, Cyanobacteria, Bacteroidetes e Verrucomicrobia (DANG *et al*., 2007; DI CESARE *et al*., 2013; YANG *et al*., 2013; TOMOVA *et al*., 2015; JANG *et al*., 2018; TAN *et al*., 2018). Isolados marinhos de *Vibrio* também foram caracterizados como portadores de genes *sul* (JANG *et al*., 2018; TAN *et al*., 2018). Genes *bla* e *qnr*, por sua vez, já foram detectados em isolados dos gêneros *Cetobacterium*, *Pseudomonas*, *Psychrosphera*, *Verrucomicrobia*, *Acetobacter*, *Cloacbacterium*, *Aeromonas*, *Acinetobacter* e *Bacteroides* cultivados a partir de amostras de ambiente marinho (MARAVIĆ *et al*., 2012; YANG *et al*., 2013; TOMOVA *et al*., 2015; JANG *et al*., 2018; TAN *et al*., 2018; CARNEY *et al*., 2019).

As praias e a costa marinha no geral representam importantes potenciais focos de bactérias resistentes a antimicrobianos, por serem áreas de interface dos ecossistemas oceânicos com a população humana. Neste sentido, promovem possíveis caminhos de exposição desta população a microrganismos resistentes e

patogênicos (DANG *et al*., 2007; MARAVIĆ *et al*., 2012; YANG *et al*., 2013; LUCZKIEWICZ *et al*., 2015; CARNEY *et al*., 2019). Mesmo assim, estudos mostram que, inclusive ambientes marinhos pouco manipulados pelo homem, como o Oceano Ártico, também já apresentam sinais de contaminação por antimicrobianos e da presença de genes de resistência a estes fármacos (TAN *et al*., 2018) e as comunidades nestas áreas tidas como “intocada” podem vir a servir de reservatórios de genes de resistência.

Neste contexto, a resistência a antimicrobianos não pode mais ser tratada como um problema encontrado apenas em unidades de saúde, visto que ambientes naturais podem ser grandes contribuintes para a aquisição e disseminação de genes de resistência (HERNANDO-AMADO *et al*., 2019). A avaliação da suscetibilidade a antimicrobianos em microrganismos de ambientes marinhos está relacionada ao conceito de saúde única, que propõe que este é um problema ecológico com consequências à saúde de animais humanos e não humanos, bem como ambiental (MCEWEN; COLLIGNON, 2018).

### 1.5 Potencial antimicrobiano de bactérias ambientais

Recentemente, os pesquisadores têm se voltado para os ambientes naturais com vista a encontrar e descrever novas potenciais moléculas de interesse às mais diversas áreas. Solos, recifes de corais, desertos, água e sedimentos marinhos são alguns dos pontos de estudo para novas moléculas antimicrobianas (MULLIS *et al*., 2019; SAYED *et al*., 2020). Tais pesquisas buscam diferentes propriedades nos seus microrganismos alvo estudados como: atividades antitumorais, anti-inflamatórias e antimicrobianas (WANG; MENG; WANG, 2020).

Na busca por novos agentes antimicrobianos, os microrganismos marinhos têm se mostrado fontes promissoras de novas moléculas para estudo (STINCONE; BRANDELLI, 2020; SRINIVASAN *et al*., 2021). Em destaque, espécies dos gêneros *Bacillus*, *Actinomyces*, *Streptomyces*, *Serratia* e *Pseudomonas* vêm ganhando evidência por se mostram grandes produtores de diferentes metabólitos (KAMJAM *et al*., 2017; STINCONE; BRANDELLI, 2020; LEE *et al*., 2023).

O gênero Pseudomonas apresenta a capacidade de produção de uma gama notável de metabólitos secundários (GROSS; LOPER, 2009), com propriedades antimicrobianas capazes de inibir bactérias resistentes a antibióticos, seus biofilmes, leveduras e até mesmo fungos filamentosos (HO *et al*., 2018; DOGHRI *et al*., 2020, ROMERO-GONZÁLEZ *et al*., 2023; THAKKER *et al*., 2023; SERAFIM *et al*., 2023; OVES *et al*., 2024). Dentre esses metabólitos, as fenazinas e sideróforos são bastante

investigadas quanto ao seu potencial de inibição de microrganismos (AL-GHAFRI *et al*., 2020; CASTALDI *et al*., 2021, HO *et al*., 2018; THAKKER *et al*., 2023; SERAFIM *et al*. 2023). A fenazina piocianina é a mais frequentemente mencionada (MORALES *et al*., 2013; HANIF *et al*., 2024; OVES *et al*., 2024), no entanto, o sideróforo pioquelina também foi identificado como um composto com propriedades antifúngicas produzido por certas espécies de Pseudomonas (GROSS; LOPER 2009; HO *et al*., 2018; VALYSHEV 2023).

De forma complementar, alguns estudos relatam que cepas de *Pseudomonas* resistentes a antimicrobianos geralmente expressam um amplo repertório de metabólitos com atividade antimicrobiana (HEMALA; ZHANG; MARGESIN, 2014; MOGROVEJO *et al*., 2020, PAUN *et al*., 2021, OVES *et al*., 2024). Neste contexto, algumas moléculas oriundas da produção de metabólitos secundários deste e de outros gêneros, podem se caracterizar como potenciais novos antimicrobianos e/ou biocidas, e minimizar o problema do crescente perfil de resistência observado entre espécies e cepas microbianas, principalmente em ambientes clínicos, mas também na agricultura e em ambientes naturais (BARBOSA *et al*., 2020).

### 1.6 Microrganismos de ambiente marinho profundo no Cone do Rio Grande

O Cone do Rio Grande (Bacia de Pelotas, RS) é um corpo geológico em formato de leque, associado a deposições de matéria orgânica, localizado na encosta continental da Bacia de Pelotas, Brasil. Ocupa uma área de 250.000 km² e possui uma espessura sedimentar de aproximadamente 7,5 km (MILLER *et al*., 2015).

A região do Cone do Rio Grande vem sendo muito estudada devido à presença de hidratos naturais de gás e de emanações ativas de metano (KETZER *et al*., 2019; KETZER *et al*., 2020). Entre os anos 2011 e 2013, o Instituto do Petróleo e Recursos Naturais (IPR) da PUCRS realizou diversas missões oceanográficas através do projeto Conegás, com o objetivo de buscar hidratos de metano em sedimento marinho profundo. Nestas expedições também foram coletadas amostras de sedimento, água e metazoários marinhos, destinados a análises microbiológicas. Como resultados, foi descrita a ocorrência de comunidades quimiossintéticas associadas ao sedimento marinho desta região (GIONGO *et al*., 2016), bem como a estrutura das comunidades microbianas da coluna d'água (MEDINA-SILVA *et al*.,
2018A) e associada a poliquetas vestimentíferos do gênero *Escarpia* (MEDINASILVA *et al*., 2018B).

A partir de amostras de sedimento deste projeto foi também possível obter mais de 100 isolados microbianos - sendo a maioria de bactérias - por meio de diferentes tipos de cultivos. O potencial metabólico de oxidação de metano, assim como a classificação filogenética e taxonômica de parte desses microrganismos foi avaliada. Nestas análises foram encontrados representantes dos gêneros *Pseudomonas*, *Lysinibacillus*, *Bacillus*, *Brevibacillus* e *Paenibacillus* (PROENÇA *et al*., 2022). Além disso, alguns isolados de *Pseudomonas* e *Lysinibacillus* foram caracterizados como capazes de consumir uma ampla gama de fontes de carbono (PROENÇA *et al*., 2022) e de formar biofilmes em polietileno de alta densidade, com uma provável habilidade de degradar este polímero (OLIVEIRA *et al*., 2021). Estes dados indicam uma ampla adaptabilidade metabólica para estas linhagens de bactérias marinhas.

Alguns dos isolados caracterizados como *Pseudomonas* apresentam a capacidade de sintetizar moléculas fluorescentes, tendo sido caracterizados, em função desta propriedade, como produtores de sideróforos (moléculas orgânicas que atuam na captura do ferro do ambiente) (PROENÇA *et al*., 2022). Além disso, um destes isolados (denominado como *Pseudomonas* sp. MD195-PC81-125) teve seu genoma sequenciado, com a detecção preliminar, ainda não refinada, da presença de genes de resistência a antimicrobianos (PROENÇA *et al*., 2022). Este dado trouxe novos questionamentos sobre as comunidades microbianas de ambiente marinho da região do Cone do Rio Grande. Tais resultados abriram novas portas para a pesquisa destes isolados bacterianos, agora voltada para a avaliação da sua suscetibilidade a diferentes estressores ambientais, tais como antimicrobianos. Em se tratando de bactérias marinhas ainda pouco conhecidas, a prospecção de suas possíveis propriedades biotecnológicas (como potencial de ação antimicrobiana) foi também empregada.

## 2. OBJETIVOS

### 2.1 Objetivo Geral

Caracterizar fenotípica e geneticamente isolados bacterianos de sedimento marinho profundo do Cone de Rio Grande em relação à sua suscetibilidade a fármacos antimicrobianos e ao seu potencial de inibição de outros microrganismos

### 2.2 Objetivos Específicos

2.2.1 Recuperar isolados bacterianos de sedimento marinho dos estoques -80ºC.
2.2.2 Avaliar os isolados recuperados em relação à sua suscetibilidade a antimicrobianos em condição planctônica e à tolerância a estes fármacos em biofilme.
2.2.4 Obter o genoma completo de parte dos isolados resistentes a antimicrobianos.
2.2.5. Identificar genes de resistência a antimicrobianos presentes nos genomas dos isolados sequenciados.
2.2.5 Avaliar a expressão de genes de resistência destes isolados quando cultivados em condições moduladas, semelhantes ao ambiente natural.
2.2.6 Avaliar a habilidade dos isolados marinhos em inibir o crescimento de outros microrganismos.

## 3. CAPÍTULO 1

Manuscrito submetido ao periódico *Environmental Microbiology* (FI: 4,3; Qualis CAPES A1)

# High resistance and biofilm tolerance to antimicrobials of marine bacteria from Brazilian deep-sea sediment

**Running Title:** Deep-sea bacteria resistance and biofilm tolerance

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## DATA AVALABILITY STATEMENT

The dataset generated and analyzed during the current study are available in the National Center for Biotechnology Information (NCBI), under the accession number: BioProject PRJNA1143896, http://www.ncbi.nlm.nih.gov/bioproject/1143896

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**Abstract**

Bacterial resistance to antimicrobials is often addressed as an issue of human clinical interest, overlooking its profound implications for the natural environment. However, the oceans face daily contamination with antimicrobials from different activities, leading to the selection of resistant bacterial strains. This study evaluated the susceptibility to antibacterials of 15 bacteria from deep-sea sediments of the Pelotas Basin (Brazil). Isolates of *Pseudomonas* sp. (6), *Bacillus* sp. (5), *Brevibacillus* sp. (2) and *Paenibacillus* sp. (2) were evaluated and had antimicrobials' Minimal Inhibitory Concentration (MIC) determined. Susceptible isolates also had minimum biofilm inhibition (MBIC) and eradication (MBEC) concentrations determined for these drugs. Results detected two *Paenibacillus* resistant to clindamycin and one to erythromycin, and all *Pseudomonas* resistant to aztreonam at high concentrations (up to 1,024 μg/mL). The genome sequencing of *Pseudomonas* isolates led to the detection of *mexE* and *mexF* resistance genes. Only one third of biofilms were eradicated by antimicrobials, with MBIC values higher than their MICs. As far as we could screen, this is the first report on antimicrobial tolerance of marine bacterial biofilms, enhancing our knowledge about tolerance and resistance mechanisms in bacteria from deep marine sediments, specifically from a Brazilian oceanic site with a broad, yet unexplored biodiversity.

**Keywords:** Deep-sea sediment; Benthic bacteria; Biofilm tolerance; Microbial resistance; Antimicrobials.

# INTRODUCTION

Marine environments harbor a large portion of the planet's microorganisms, since more than 70% of the Earth's surface is covered by oceans (Glöckner et al. 2012). They are present in the most diverse forms (Glöckner et al. 2012) and play different key roles in structuring and balancing marine communities and ecosystems (Munn 2019; Laiolo et al. 2024).

Despite their importance in maintaining the Earth's biosphere balance, the oceans suffer numerous disturbances caused by human activities. Every year, several pollutants from industry, plastic and microplastic wastes, pesticides, and antimicrobials of various origins enter the marine waters (Jambeck et al 2015; Duleba et al. 2018; Ma et al. 2021). These xenobiotics can affect marine ecology and generate consequences not only in local ecosystems, but also at the global level, eventually reaching human populations (Chen et al. 2019; Habibi et al. 2022).

Antimicrobial multidrug resistance (AMR) is currently considered as a global public health problem (WHO 2023). However, most studies mainly focus on resistant microorganisms isolated from clinical sources, while microbial isolates from natural environments have only recently been studied in the context of AMR (Suzuki et al. 2017; Sweileh and Moh'd Mansour 2020). The rampant use of antimicrobial drugs in several sectors has accelerated the selection of resistant microorganisms (Holmes et al. 2016; Zhuang et al. 2021; WHO 2023). While the load of antimicrobials released into the oceans is substantial, it is diluted due to the large volume of these aquatic systems (Larsson and Flach 2021). Nevertheless, the long-term contribution of small doses of antimicrobials can lead to adaptation, gene expression changes, and the dissemination of resistance to these agents for different bacterial species in these environments (Kohanski et al. 2010; Hatsoy and Martiny

2015; Christaki et al. 2020; Xu et al 2023). So, antimicrobial resistance can no longer be treated as a problem found only in healthcare facilities, as natural environments can be major contributors to the acquisition and spread of resistance genes (Hernando-Amado et al. 2019). The detection of antimicrobial resistance genes (ARGs) in bacteria from marine environments

has increased, indicating their growing prevalence in these ecosystems (Li et al. 2020; Makkaew et al 2021; Castaño-Ortiz et al. 2023). In this context, marine environments are estimated to serve as important reservoirs for such genes (Marti et al. 2014). Moreover, beaches and the marine coastline in general may represent important potential foci of resistant microorganisms, as they are areas of interface between marine ecosystems and human populations (Dang et al. 2008a; Maravić et al. 2012; Yang et al. 2013; Luczkiewicz et al. 2015; Carney et al. 2019; Cuadrat et al. 2020). The prevalence of genes encoding resistance to tetracyclines, sulfonamides, β-lactams and quinolones has already been observed in coastal areas (Dang et al. 2008b; Jang et al. 2018). In addition, many of these genes are found in plasmids, possibly providing an efficient dissemination among different marine microorganisms (Yang et al. 2013; Cuadrat et al. 2020). Furthermore, most studies on antimicrobial resistance in marine environments are dedicated to coastal and pelagic microorganisms, whereas research with this focus on bacteria from deep-sea sediments is still scarce.

Evaluating antimicrobial susceptibility in microorganisms from marine environments aligns with the One Health concept, emphasizing the ecological nature of this issue and its repercussions for the health of both human and non-human animals, as well as the overall environment (McEwa and Collignon 2018). Therefore, this study aimed to evaluate the antimicrobial susceptibility to antibacterial drugs of *Pseudomonas* sp., *Bacillus* sp., *Brevibacillus* sp., and *Paenibacillus* sp. isolated from deep-sea sediment of a submarine fan at the southern Brazilian coast, in both planktonic and biofilm conditions.

## 2 EXPERIMENTAL PROCEDURES

### 2.1 Origin of bacterial isolates

Sediment samples were previously collected using piston corers within the Rio Grande Cone (RGC), a submarine fan described as a methane seepage site located in the Pelotas Basin (Brazil), in the Southwestern part of the Atlantic Ocean (Miller et al. 2015), during two oceanographic campaigns in 2011 and 2013 (Fig 1). Bacterial strains were isolated from

samples collected from 0 to 3 meters below the seabed, at sites with water depths ranging from1,800 to 2,500 meters. Brain Heart Infusion (BHI), yeast extract-peptone-dextrose (YPD), and nitrate mineral salts medium (NMS) were used for isolation and storage of bacterial strains at -80ºC with dimethyl sulfoxide (DMSO) or glycerol at 5% or 20% (v/v), respectively (Proenca et al. 2022).

A total of 15 bacterial isolates were recovered and used in this study. They were previously identified by rRNA 16S gene sequencing as belonging to the genus *Pseudomonas* (6), or to the genera *Bacillus* (5), *Brevibacillus* (2), and *Paenibacillus* (2), which are members of the order Bacillales (Table 1).

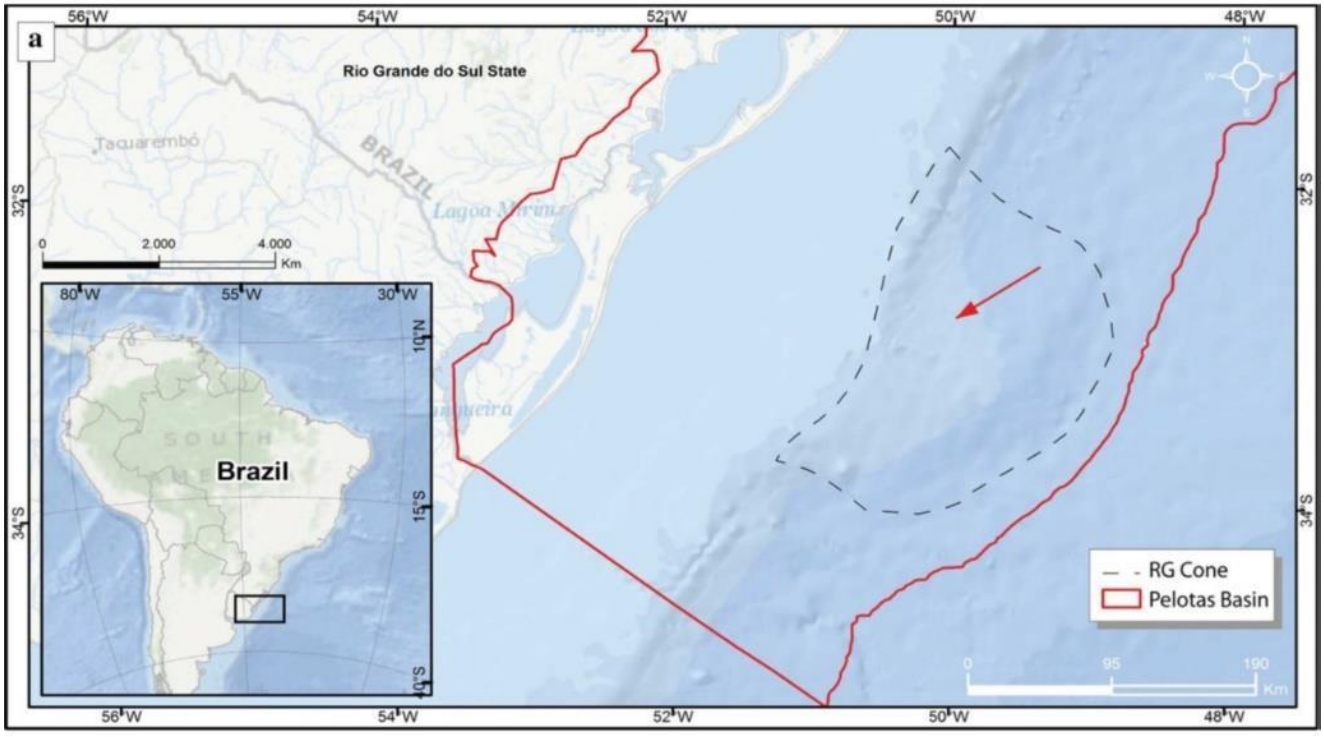


**Fig. 1-** Map of the Rio Grande Cone location, within the Pelotas Basin in the Southwestern Atlantic Ocean, Brazil (modified from Medina-Silva et. al 2018), the sampling site for deep-sea sediment bacteria used in this study.

**Table 1**- Taxonomic identification of isolates used in this study.

| Bacterial group | Isolates | Identification |
|---|---|---|
| ***Pseudomonas* genus** | MD330.6 | *Pseudomonas* sp. |
| | MD330.9 | *Pseudomonas* sp. |
| | MD330.10 | *Pseudomonas* sp. |
| | MD330.11 | *Pseudomonas* sp. |
| | MD332.6 | *Pseudomonas* sp. |
| | MD332.8 | *Pseudomonas* sp. |

| | | |
|---|---|---|
| **Bacillales order** | MET16 | *Paenibacillus* sp. |
| | MET17 | *Paenibacillus* sp. |
| | MR6 | *Brevibacillus* sp. |
| | MR16 | *Brevibacillus* sp. |
| | MR29 | *Bacillus* sp. |
| | MR38 | *Bacillus* sp. |
| | MR39 | *Bacillus* sp. |
| | MR46 | *Bacillus* sp. |
| | MR53 | *Bacillus* sp. |

2.2 Susceptibility to antimicrobials in planktonic condition

Antimicrobial susceptibility of the isolates was evaluated by disk diffusion and broth microdilution tests according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical & Laboratory Standards Institute (CLSI).

In disk diffusion, *Pseudomonas* isolates were tested against ceftazidime, cefepime, gentamicin, tobramycin, amikacin, aztreonam, ciprofloxacin, levofloxacin, imipenem, meropenem, and piperacillin-tazobactam; while Bacillales isolates were tested against ciprofloxacin, levofloxacin, imipenem, meropenem, erythromycin, clindamycin, and linezolid. A suspension of the isolates was prepared in sterile saline (0.85% NaCl) to a turbidity compatible with the 0.5 McFarland scale (~$1x10^8$ CFU/mL), then inoculated using a sterile swab onto cation-adjusted Mueller-Hinton (CAMH) plates, where antimicrobial discs were added and incubated at 25°C for 18-24 h to lately measure the inhibition zones. In broth microdilution, however, only those isolates that were resistant in the disk diffusion test were selected for this test. Fort this, CAMH broth with different antimicrobial concentrations and bacterial inoculum at a cell density of $1x10^8$ CFU/mL were added to 96-well plates and incubated at 25ºC for 24 h. In both tests *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus cereus* ATCC 33019 were used as reference strains.

Given that these are marine isolates, their optimal growth temperature differs from that recommended for these assays. Regarding this limitation, however, other studies have already used the same incubation time and temperature that we used for both the disk diffusion (Miller

et al. 2003; Smith and Kronvall 2015; Wamala et al. 2018) and broth microdilution tests (Smith et al. 2018) when evaluating the susceptibility of environmental isolates to antimicrobials, with little variation and no significant divergence in the results.

2.3 Antimicrobial susceptibility testing of biofilms

Biofilm formation ability was determined by the crystal violet staining technique on 96-well plates, using the protocol adapted from Ceri et al. 1999. The isolates were cultured in BHI broth at 25ºC for 18 h, and then an aliquot of each one was transferred in triplicate to the wells of the polystyrene plates along with 200 µL of BHI broth and incubated at 25ºC for 48 h. After that, the wells were washed twice with phosphate-buffered saline (PBS – NaCl, 8 g/L; KCl, 0.2 g/L; $Na_2HPO_4$, 1.44 g/L; and $KH_2PO_4$, 0.24 g/L) and stained with 0.1% crystal violet for 5 min. Subsequently, the biofilms were washed with 96% ethanol for 10 to 15 min, and the absorbance of the removed violet stain was measured using a SpectraMax® microplate reader at 570 nm. Isolates were classified according to Stepanović et al. (2000) as: non-biofilm forming ($OD \leq ODc$), weak biofilm formers ($ODc < OD \leq 2ODc$), moderate biofilm formers ($ODc < OD \leq 2ODc$), and strong biofilm formers ($4ODc < OD$), where ODc is the optical cutoff density determined as the mean OD plus three standard deviations from the negative control.

For the biofilm susceptibility test, ciprofloxacin and tobramycin were selected for *Pseudomonas* isolates, and ciprofloxacin and vancomycin for Bacillales isolates, since these strains are susceptible to such antimicrobials. For this test, 96-well plates were used with 200 µL of CAMH broth and 2 µL of 0.5 scale McFarland bacterial suspensions, which were then incubated at 25ºC for 48 h. Afterwards, the wells were washed twice with PBS and 200 µL of CAMH broth containing different antimicrobial concentrations were added and incubated again at 25ºC for 24 h. The concentrations used were based on the EUCAST cut-off point for planktonic assays, from which six concentrations above and two concentrations below were used.

After the incubation period a first visual result was determined, the minimum biofilm inhibitory concentration (MBIC), characterized by the first concentration where microbial growth is no longer visible. The same plates had their wells washed again with PBS, and fresh CAMH broth was added and subjected to an ultrasonic bath for 5 min. A 100 μL aliquot of each treatment was then serially diluted and plated (10 μL) on CAMH plates and incubated at 25°C for 24 h. Subsequently a second result was determined, the minimum biofilm eradication concentration (MBEC), through the counting of the colonies formed, for these antimicrobials (Ceri et al. 1999). Furthermore, non-eradicated biofilms, even at maximum concentrations, had their colonies counted and estimated in CFU/mL, for comparison with untreated biofilms.

2.4 Whole genome sequencing and bioinformatic analysis

A total of five out of six *Pseudomonas* sp. isolates were subjected to whole genome sequencing (WGS) in this work, due to their interesting antimicrobial resistance profile. For isolate MD330.6, the whole genome had already been extracted and deposited at the National Center for Biotechnology Information (NCBI), accession number (BioProject PRJNA616366), from a previous work (Proença et al. 2022). DNA was extracted using the FastDNA Spin Kit for soil (MP Biomedicals), and DNA purity and concentration were assessed using Nanodrop and Qubit measurements. The library was prepared, and De Novo sequencing was performed by Illumina plataform (Novogene). Raw read quality was determined with FasQC, trimmed with Trimmomatic and assembled with St. Petersburg *genome* assembler (SPAdes) (Bankevich et al. 2012; Prjibelski et al. 2020) through Galaxy interface. The search for antimicrobial resistance genes was performed using the Comprehensive Antibiotic Resistance Database (CARD) and MEGARes (Alcock et al. 2023; Bonin et al. 2023), and the phylogenetic analysis was carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2021) and IQ-Tree, employing the maximum likelihood method TPM3+F+R2 based on 1000 replications, ultrafast bootstrap (Minh et al. 2020). All

genomes were submitted to the NCBI database under the accession numbers (BioProject PRJNA1143896).

2.5 Statistical analysis

The parametric Student t-test was used to compare the MIC and MBIC values of antimicrobials for each isolate, and CFU/mL counts of non-eradicated biofilms with controls. *P* values <0.05 were considered statistically significant. GraphPad Prism version 8.0.1 was used for data analysis.

RESULTS

3.1 Antimicrobial resistance profile

The results of the disk diffusion test indicated that the two *Paenibacillus* isolates MET16 and MET17 were resistant to clindamycin, and MET17 was also resistant to erythromycin. In the broth microdilution test, the MIC values of erythromycin and clindamycin were determined to be 1 and 2 μg/mL, respectively. All other Bacillales isolates were susceptible to all antimicrobials tested. Moreover, all six *Pseudomonas* isolates were resistant to aztreonam, and one isolate (MD330.9) was also resistant to ceftazidime. The MIC of ceftazidime was determined to be 128 μg/mL for *Pseudomonas* MD330.9, while the MIC value (128 μg/mL) of aztreonam was determined for only two *Pseudomonas* isolates (MD330.6 and MD330.9), as for the other four isolates (MD330.10, MD330.11, MD332.6, MD332.8) the maximum concentration used in this study (1,024 μg/mL) was still permissive (Table 2).

**Table 2-** Antimicrobial susceptibility of the 15 isolates in planktonic condition. For the disk diffusion test, the antibiotic acronyms to which the isolates were resistant are given. The minimum inhibitory concentrations (MIC) of these drugs are indicated for the isolates that showed resistance in the disk diffusion test.

| **Identification** | **Isolate** | **Antimicrobial** | **MIC (µg/mL)** | | | |
|---|---|---|---|---|---|---|
| | | | *ATM* | *CAZ* | *ERY* | *CLIN* |
| *Pseudomonas* sp. | MD330.6 | ATM | 128 | - | - | - |
| | MD330.9 | ATM, CAZ | 128 | 128 | - | - |
| | MD330.10 | ATM | >1024 | - | - | - |
| | MD330.11 | ATM | >1024 | - | - | - |
| | MD332.06 | ATM | >1024 | - | - | - |
| | MD332.08 | ATM | >1024 | - | - | - |
| *Paenibacillus* sp. | MET16 | CLI | - | - | - | 1 |
| | MET17 | CLI, ERY | - | - | 1 | 2 |
| *Brevibacillus sp.* | MR6 | | - | - | - | - |
| | MR16 | | - | - | - | - |
| *Bacillus* sp. | MR29 | | - | - | - | - |
| | MR38 | | - | - | - | - |
| | MR39 | | - | - | - | - |
| | MR46 | | - | - | - | - |
| | MR53 | | - | - | - | - |

ATM- aztreonam, CAZ- ceftazidime, CLI- clindamycin, ERY- erythromycin.

## 3.2 Determination of biofilm forming ability

Among all the isolates studied, six were classified as non-biofilm forming, two as weak, four as moderate, and three as strong biofilm formers. When comparing the taxonomic groups, some differences could be observed. The *Pseudomonas* isolates were non-formers (two), weak (two) or moderate (two). Regarding the order Bacillales, non-forming (four) and moderate (two) isolates were also present, however, this group presented no weak formers and included three strong biofilm-forming *Bacillus* sp. (Table 3).

**Table 3-** Classification of the intensity of the biofilm formed by all 15 isolates, according to Stepanović et al. (2000).

| Identification | Isolate | Absorbance value (570 nm)* | Classification |
|---|---|---|---|
| *Pseudomonas* sp. | MD330.6 | 0.390 | Weak |
| | MD330.9 | 0.154 | Non-former |
| | MD330.10 | 0.700 | Moderate |
| | MD330.11 | 0.604 | Moderate |
| | MD332.6 | 0.211 | Non-former |
| | MD332.8 | 0.425 | Weak |
| Bacillales | MET16 | 0.140 | Non-former |
| | MET17 | 0.132 | Non-former |
| | MR6 | 0.625 | Moderate |
| | MR16 | 0.202 | Non-former |
| | MR29 | 0.618 | Moderate |
| | MR38 | 0.159 | Non-former |
| | MR39 | 0.771 | Strong |
| | MR46 | 0.779 | Strong |
| | MD53 | 1.133 | Strong |

*Average value of biofilm triplicates

3.3 Antimicrobial susceptibility testing under biofilm condition

A total of 30 biofilms were treated with antimicrobials, 12 from *Pseudomonas* and 18 from Bacillales. encompassing all our isolates, even those classified as weak or non-biofilm formers. The MBIC values were 2-11 times higher than MIC values for most isolates (93.3%). A significant difference was observed when comparing MIC and MBIC values for all treatments (*P* value <0.05), except for ciprofloxacin on *Bacillus* isolates MR46 and MR53, for which the MBIC values were below the minimum concentration tested and therefore could not be determined and compared. Ciprofloxacin was the antimicrobial that presented the highest differences between MIC and MBIC values, with six out of 15 biofilms with MBIC value for this drug that was >128 μg/mL), the maximum concentration tested (Table 4).

In the MBEC assays, only one-third of the biofilms were successfully eradicated, including nine were from Bacillales isolates and one from the *Pseudomonas* MD330.6. When

considering biofilms of the order Bacillales, ciprofloxacin was more efficient in eradicating biofilms (6 isolates) when compared to vancomycin (3 isolates). Moreover, only two *Bacillus* isolates (MR46 and MR53) were eradicated by both antimicrobials. The *Pseudomonas* isolate MD330.6 was eradicated, but only at the maximum concentration of tobramycin. In addition, eradication was not observed when this isolate was treated with ciprofloxacin. The other 20 biofilms (11 of *Pseudomonas* and nine of Bacillales) were not eradicated even at the maximum concentrations used (32 μg/mL for tobramycin, 128 μg/mL for ciprofloxacin, and 64 μg/mL for vancomycin (Table 4).

The CFU/mL counts of the non-eradicated biofilms were compared with those of the untreated controls (Fig. 2). Significant reductions in cell counts were observed for most isolates (17 out of 20 non-eradicated biofilms). These reductions ranged from $10^1$ (MD332.8 with ciprofloxacin; and MR16 and MR29 with vancomycin) to a maximum of $10^4$ CFU/mL (MD330.11 with tobramycin). Conversely, biofilms of isolates MET17, MR16, MR38 presented no significant differences in CFU/mL compared to their controls when treated with ciprofloxacin, indicating their high tolerance to this antimicrobial, which only occurs in the biofilm state.

**Table 4**- Minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) of ciprofloxacin, tobramycin, and vancomycin for the bacterial isolates treated with these antimicrobials.

| **Bacteria** | **Isolate** | **Antimicrobials** (μg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Ciprofloxacin | | | Tobramycin | | | Vancomycin | | |
| | | MIC | MBIC | MBEC | MIC | MBIC | MBEC | MIC | MBIC | MBEC |
| *Pseudomonas* sp. | MD330.6 | 0.062 | >128* | >128 | 0.25 | 16* | 32 | NT | NT | NT |
| | MD330.9 | 0.062 | 1* | >128 | 0.25 | 8* | >32 | NT | NT | NT |
| | MD330.10 | 0.031 | >128* | >128 | 0.25 | 16* | >32 | NT | NT | NT |
| | MD330.11 | 0.062 | >128* | >128 | 0.25 | 16* | >32 | NT | NT | NT |
| | MD332.6 | 0.062 | >128* | >128 | 0.25 | 32* | >32 | NT | NT | NT |
| | MD332.8 | 0.25 | >128* | >128 | 0.25 | 32* | >32 | NT | NT | NT |
| Bacillales | MET16 | 0.125 | 64* | 128 | NT | NT | NT | 0.25 | 4* | >64 |
| | MET17 | 0.062 | >128* | >128 | NT | NT | NT | 0.25 | 0.5* | >64 |
| | MR6 | 0.031 | 128* | 128 | NT | NT | NT | 0.125 | 2* | >64 |
| | MR16 | 0.031 | 4* | >128 | NT | NT | NT | 0.125 | 0.5* | >64 |
| | M29 | 0.031 | 4* | 128 | NT | NT | NT | 0.125 | 4* | >64 |
| | MR38 | 0.031 | 16* | >128 | NT | NT | NT | 1 | 1* | 16 |
| | MR39 | 0.062 | 8* | 64 | NT | NT | NT | 0.125 | 4* | >64 |
| | MR46 | 0.031 | <0.5 | 64 | NT | NT | NT | 0.125 | 1* | 8 |
| | MR53 | 0.031 | <0.5 | 128 | NT | NT | NT | 0.125 | 0.5* | 32 |

Values proceeded by "<" : visual growth in biofilm condition (MBIC test) was not observed in the lowest concentration applied.

Values proceeded by " >" : the maximum concentrations tested of these antimicrobials were not able to inhibit (MBIC) or eradicate (MBEC) biofilms.

"*" indicates significant differences between MIC and MBIC values (Student *t* test, $P < 0.05$).

"NT": biofilms that were not treated with the respective antimicrobial.

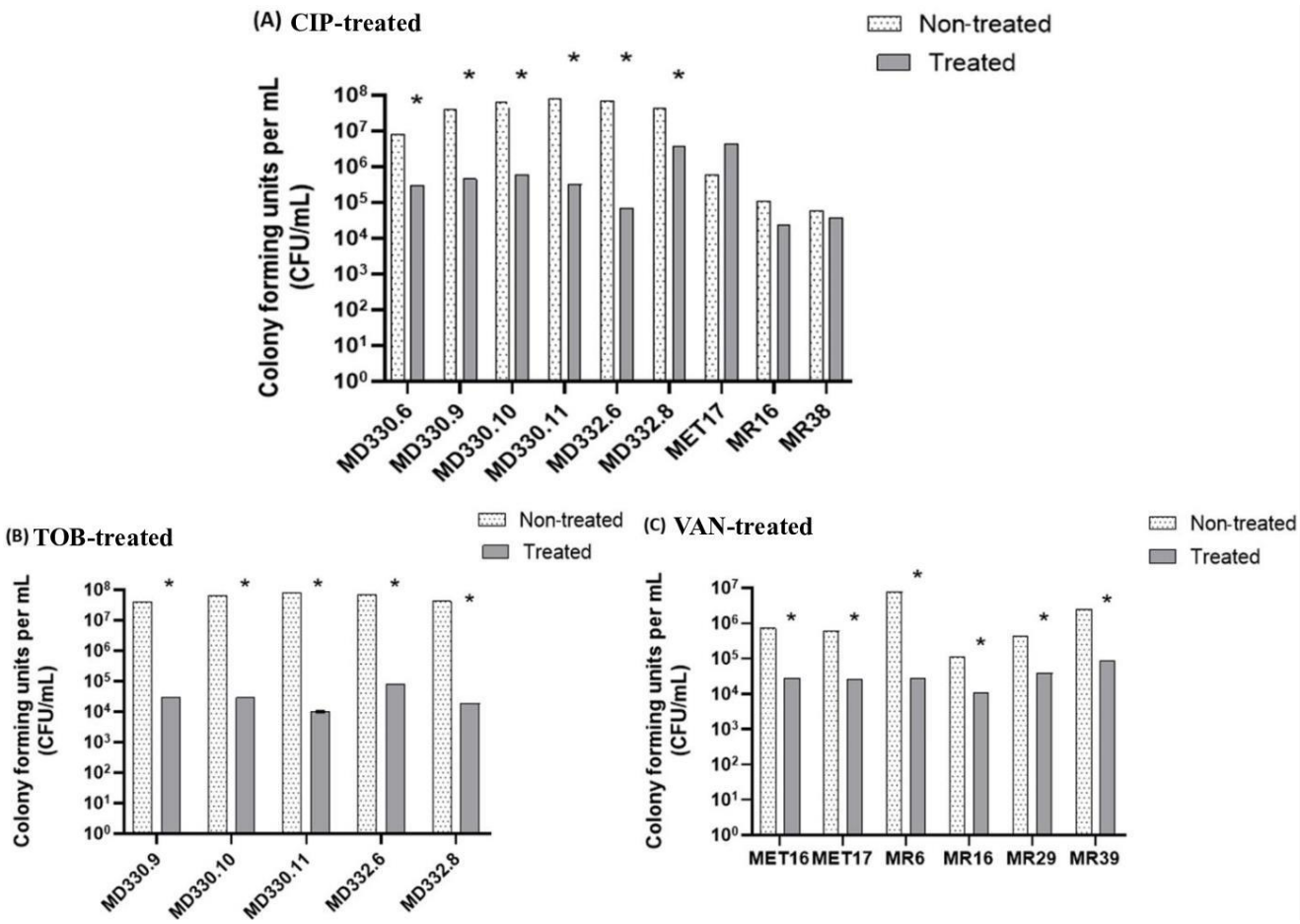


**Fig. 2-** Average values of CFU/mL counts from isolates' biofilms that were not eradicated when treated with the maximum concentrations tested of: (A) ciprofloxacin (CIP); (B) tobramycin (TOB); (C) vancomycin (VAN). (*) indicates statistically significant differences between treated and non-treated biofilms (Student *t* test, $P < 0.05$).

### 3.4 Phylogenetic and genotypic analyses

Regarding the *Pseudomonas* sp. genomes evaluated, five were used for antimicrobial resistance gene mining and gene comparisons. Due to the low quality of isolate MD330.9 genome sequences it was not possible to include it this analysis.

Using the CARD and MEGARes database, it was possible to identify in all five isolates the presence of the genes *mexE* and *mexF*, which confer resistance to different antimicrobials – possibly including aztreonam - through the expression of efflux pumps. As *mex* genes have already been described in *Pseudomonas* (mostly of clinical origin), a phylogenetic comparison between our *mex* genes and those of some isolates deposited at NCBI was performed. Regarding *mexE*, it is possible to observe one clade that contains three of our marine isolates (MD330.10, MD330.11, MD332.6), which are grouped together with a *P. rhodesiae* strain, based on high bootstrap values. The isolate MD330.6 remained in a sister clade, phylogenetically closer to other three *Pseudomonas* species. For MD332.8, the only isolate with a divergent high branch length, this relationship remains unresolved, as the program could not differentiate it from the external group and find a defined position in the phylogeny. For this

reason, the outgroup was rooted in the phylogenetic tree (Fig 3A). For *mexF* phylogeny a lower number of gene sequences were possible to retrieve from NCBI, and some differences in isolates' relationships were detected when compared to *mexE*. *mexF* analysis grouped MD330.10, MD330.11 and MD332.6 together again, in this case within an exclusive clade. Nevertheless, differently from *mexE*, MD332.8 was located within a sister clade with one *Pseudomonas paraeruginosa* and one *P. aeruginosa.* However, its high branch length is maintained in *mexF* phylogeny, indicating that although MD332.8 has grouped with two *Pseudomonas*, its *mexF* sequence presents important differences comparing to the sequences of this gene of such strains. Moreover, in this phylogeny MD330.6 was the isolate whose relationship was unresolved (Fig 3B).

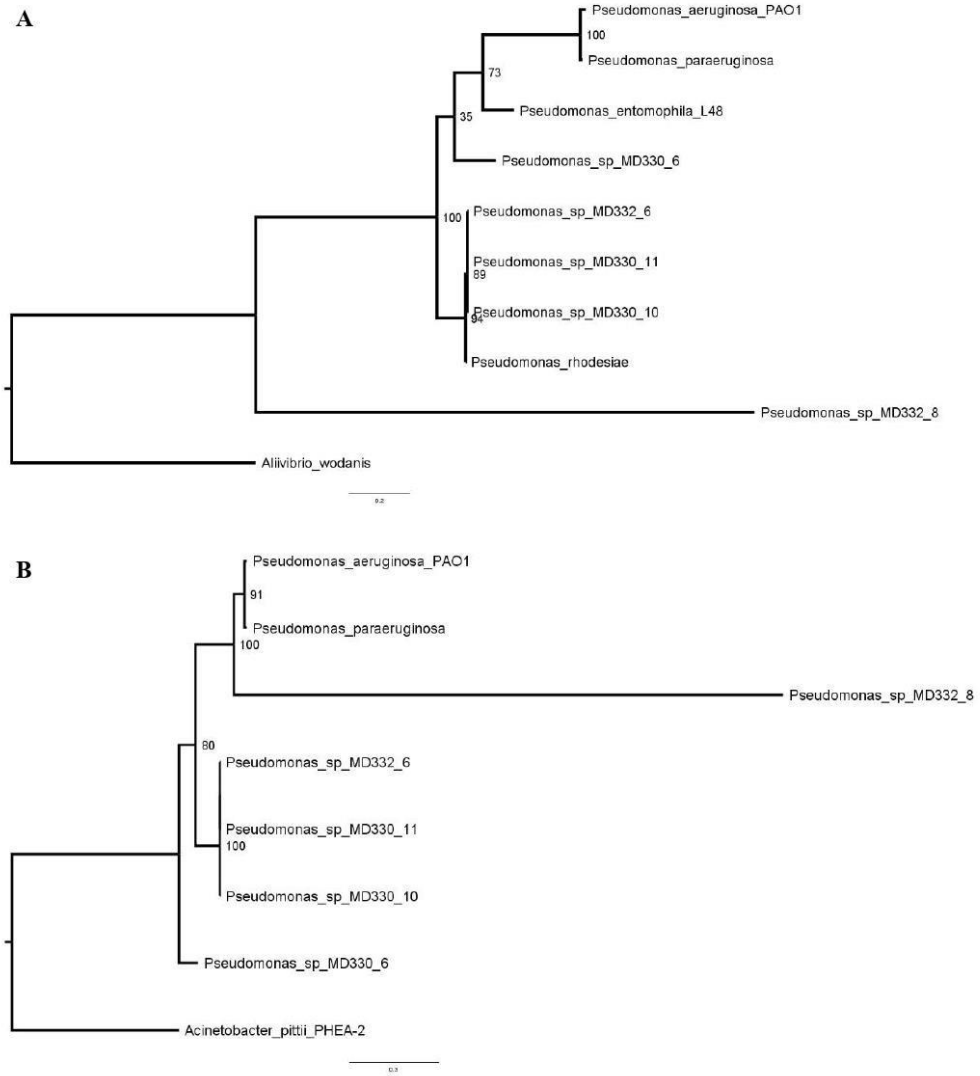


**Fig.3-** Phylogenetic analysis of five marine *Pseudomonas* isolates (MD330.10, MD330.11, MD330.6, MD332.6 and MD332.8) performed with reference genome sequences obtained from the Genbank database, using the maximum likelihood method. (A) Phylogenetic tree of the *mexE* gene. Outgroups is: *Aliivibrio wodanis*; (B). Phylogenetic tree of the *mexF* gene. the outgroup is: *Acinetobacter pittii*. Bootstraps ratios based on 1000 replications are indicated at branch points.

## DISCUSSION

Antimicrobial resistance is no longer limited to healthcare facilities, as natural environments can play an important role in the acquisition and dissemination of resistance genes. In this context, this study aimed to evaluate the susceptibility to antibacterial drugs in both planktonic and biofilm conditions and the presence of resistance genes through genomic sequencing of bacterial isolates from deep marine sediments from the southern Brazilian coast.

This study obtained a total of eight isolates (six *Pseudomonas* sp. and two *Paenibacillus* sp.) resistant to at least one antimicrobial tested. Regarding the resistance profile of the *Paenibacillus* MET16 and MET 17 isolates, the resistance observed for lincosamides and macrolides has already been reported in the literature for this bacterial group (Bacillales order) in other oceanic regions (Barbosa et al. 2014; Dash et al. 2017; Joshi et al. 2021; Furlan et al. 2021). A similar mechanism of action of clindamycin and erythromycin may be responsible for resistance to these classes of antimicrobials in MET17 (Roberts 2011).

Resistance to aztreonam has been observed in *P. aeruginosa* strains of clinical origin, primarily due to mutations in operons related to the overexpression of efflux pumps (Masuda et al. 2000; Sobel et al. 2005; Jorth et al. 2017). Likewise, but at a much lower frequency, *Pseudomonas* isolates from various environmental sources have also been reported to be resistant to this antimicrobial (Luczkiewicz et al. 2015; Braz et al. 2016). In this context, the high MIC values observed for aztreonam in our six *Pseudomonas* isolates motivated the sequencing of these bacteria's genomes. This analysis allowed to detect the presence of the *mexE* and *mexF* genes in our isolates, which provided new information about the *mexEFOprN* operon in aztreonam-resistant *Pseudomonas* from natural environments. Moreover, variability in the sequences of both the *mexE* and *mexF* genes was observed among these aztreonam-resistant marine isolates, with distinct similarities of their sequences to those of different *Pseudomonas* strains. Likewise, the presence of long branch lengths in one isolate (MD332.8), indicate that despite some similarities with other strains our isolates still present their own genetic singularities, at least for these genes. These data may indicate that *mexE* and *mexF* genes may contribute to antimicrobial resistance in addition to their sequence variability and strain origin, which includes remote environments. However, resistance to different antimicrobials may be mediated by the same mechanisms in a wide variety of isolates, including those from marine samples. Moreover, genes related to metal resistance that also confer resistance to antimicrobials (cross-resistance), or even a genetic marker for both in the same mobile genetic elements, such as plasmids (co- resistance) (Pal et al. 2017; Squadrone 2020) can induce co-selection between antimicrobial and metal resistance in these environments.

It was reported that some operons that express efflux pumps respond to different stress conditions, which may lead to an overexpression of respective genes, indicating that these resistance-nodulation-cell division (RND) efflux pumps may not be exclusively used for antimicrobials (Kumar and Schweizer 2011). In this context, the presence of the operon *mexEF-oprN* in our isolates may indicate these genes may play functions distinct from antimicrobial resistance in marine environment. This is consistent with the environment of origin of our marine isolates, which is constantly under significant impact from navigation activities – occurring in southern Brazil (Patos Lagoon Estuary) and Uruguay (Plata River Basin) - and surrounding anthropogenic influences, including the presence of metals (da Silva et al. 2023). This scenario creates a challenging environment for at least some taxa occurring within the marine communities, which may have led to the selection of microbial strategies - as pump efflux mechanisms - that deal with the occurrence of these toxic xenobiotics (Roberts 2011).

Research evaluating antimicrobial resistance in the environment has witnessed an increasing interest, however there are still no standardized methods to measure the susceptibility of biofilms (Malone et al. 2017; Coenye 2023). Different parameters are employed, among which MBIC and MBEC are the main allies in determining the behavior of microbial biofilms treated with antimicrobial drugs (Coenye 2023). In addition, there are no studies detected so far that evaluate the resistance behavior of biofilms of environmental microorganisms in forms other than planktonic (Sweileh and Moh'd Mansour 2020). So, as far as we could screen, our results bring the first report comparing MIC and MBIC values, and evaluating MBIC and MBEC parameters for environmental microorganisms, at least for deep-sea bacteria.

From the same marine environment, we detected bacterial isolates belonging to two distinct taxonomic groups that showed high tolerance to different classes of antimicrobials under biofilm conditions. Our MBIC and MBEC data (compared to the respective MIC values) demonstrated the high biofilm tolerance of our isolates, which may be related to different mechanisms, such as the efficiency of the extracellular matrix to protect them against the tested antimicrobials (Gilbert et al. 2002). The Rio Grande Cone region is geologically characterized as a submarine fan, described as a methane cold seep site with occurrences of methane hydrates (Miller et al. 2015).

Due to global warming, this region faces the widespread dissociation of methane hydrates into gas, resulting in intense methane flux, and massive methane releases through the sea sediment (Ketzer et al. 2020). Thus, the ability to form protective biofilms may have been selected to at least cope with these high methane concentrations. In this context, our results reinforce that bacterial cells tolerate more harmful agents in biofilm than in their planktonic condition (Gilbert et al. 2002; Chen et al. 2020). However, when relating MBEC (and MBIC)

values were correlated with to biofilm intensity as determined by crystal violet staining, no correlation between the intensity and antimicrobial tolerance was observed. Some isolates classified as weak or non-biofilm formers (such as MD330.9, MET17 and MR16) had their biofilms unaffected even at maximum antimicrobial concentrations, while other isolates classified as strong biofilm formers (such as MR46 and MR53) were eradicated, not necessarily at the highest antimicrobial concentrations. The crystal violet staining method, commonly used to assess biofilm formation, can produce varying ratings of biofilm intensity due to particular features of the biofilm, as well as to external factors related to the method, which can affect staining penetration and lead to divergent interpretations (Kragh et al. 2019; Coenye 2023). Therefore, our further biofilm analysis included all isolates, independently the crystal violet results, which is particularly relevant considering that we analyzed the impact of stress factors (such as antimicrobials) on biofilms (Kragh et al. 2019).

A pattern of high tolerance of our *Pseudomonas* sp. biofilms to two classes of antimicrobials - a fluoroquinolone and an aminoglycoside – was observed, which was different from the result they presented in the MIC test. A previous study indicated that ciprofloxacin was more efficient than tobramycin in eradicating clinical biofilms of *P. aeruginosa* (Preston et al. 1996). Nevertheless, this aspect differs from our results because one isolate (MD330.9) was successfully eradicated by tobramycin, while the biofilms treated with ciprofloxacin tolerated the maximum concentration applied and were not eradicated by this drug. However, there is evidence that the effect of this antimicrobial may be time dependent, with longer exposure leading to a more effective eradication of biofilms (Haagensen et al. 2017; Chen et al. 2020).

Biofilms of the Bacillales isolates were more susceptible to ciprofloxacin than those of *Pseudomonas* isolates. Only three isolates (MET17, MR16, MR38) were not eradicated by this antimicrobial. However, we highlight the results of the CFU/mL counts of MET16 and MET17 biofilms, as they did not show significant differences in relation to the untreated control, with a slight (not significant) increase for MET17. Furthermore, a greater number of biofilms were not eradicated when treated with vancomycin. Among these, MET16 and MET17 were observed again, indicating that the biofilms of our *Paenibacillus* isolates showed a good tolerance response to these two classes of antimicrobials (a glycopeptide and fluoroquinolone). Again, considering that there is a great variability among studies regarding the antibiotic exposure time for biofilms (24 h to 2 weeks) (Chen et al. 2020), as well as the lack of standardized methods to evaluate them, it is not possible to rule out that our MBEC data could be different with an exposure time longer than the 24 h used.

Marine ecosystems face daily contamination from human activities. Antimicrobials disposal from different sectors, even at subinhibitory doses, can impact microbial communities

(Nogales et al. 2011) and potentially select for resistant/tolerant bacteria. While environmental resistant bacteria may not belong to pathogenic species, they may serve as reservoirs of antibiotic resistance genes that may be transferred to pathogens in the marine environment (Xu et al. 2023), posing as yet unmeasured risks to human populations. Even though detected in a small number of isolates, the observed bacterial features of resistance and biofilm tolerance to antimicrobials are relevant, as they are not commonly reported for bacteria from natural environments. As all isolates analyzed in this study are from deep marine sediments, our data contribute to the understanding of the selection and occurrence of resistant bacteria in these extreme natural environments that are part of the oceanic benthic realm, the least studied marine zone regarding microbial life (Laiolo et al. 2024). Furthermore, this study evaluates phenotypic characteristics of bacteria from a Brazilian oceanic site with a poorly described microbial diversity (Proenca et al. 2022), providing unprecedented insights into deep-sea bacteria from Brazilian marine sites and shedding light on the impact of microbial resistance and tolerance to antimicrobials in marine environments. These findings are not only relevant to assess the conservation of marine microbial communities, which are fundamental to ecosystems and human health (Munn 2019), but also align with the principles of One Health and Global Health (Hernando-Amado et al. 2019).

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AUTHORS CONTRIBUTIONS

ASD and RMS were involved in study conception. RMS provided project management. ASD designed and performed experiments and wrote the manuscript. ASD, RMS and SDO analyzed data and provided data interpretation. RMS and SDO provided funding support. YDPFB analyzed the bioinformatics data. HF provided funding and technical support for genomic sequencing and analysis. All authors were involved in revisions and approval of the manuscript.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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**TABLE AND FIGURE LEGENDS**

**Table 1**- Taxonomic identification of isolates used in this study.

**Table 2-** Antimicrobial susceptibility of the 15 isolates. For the disk diffusion test, the antibiotic acronyms to which the isolates were resistant are given. The minimum inhibitory concentrations (MIC) of these drugs are indicated for the isolates that showed resistance in the disk diffusion test.

**Table 3-** Classification of the intensity of the biofilm formed by all 15 isolates, according to Stepanović et al. (2000).

**Table 4**- Minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) of ciprofloxacin, tobramycin, and vancomycin for the bacterial isolates treated with these antimicrobials.

**Fig. 1-** Map of the Rio Grande Cone location, within the Pelotas Basin in the Southwestern Atlantic Ocean, Brazil (modified from Medina-Silva et. al 2018), the sampling site for deepsea sediment bacteria used in this study.

**Fig. 2-** Average values of CFU/mL counts from isolates' biofilms that were not eradicated when treated with the maximum concentrations tested of: (A) ciprofloxacin (CIP); (B) tobramycin (TOB); (C) vancomycin (VAN). (*) indicates statistically significant differences between treated and non-treated biofilms (Student *t* test, $P < 0.05$).

**Fig.3-** Phylogenetic analysis of five marine *Pseudomonas* isolates (MD330.10, MD330.11, MD330.6, MD332.6 and MD332.8) performed with reference genome sequences obtained from the Genbank database, using the maximum likelihood method. (A) Phylogenetic tree of the *mexE* gene. Outgroups is: *Aliivibrio wodanis*; (B). Phylogenetic tree of the *mexF* gene. the outgroup is: *Acinetobacter pittii*. Bootstraps ratios based on 1000 replications are indicated at branch points.

## 4. CAPÍTULO 2

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# Evaluation of *mexE* and *mexF* gene expression in marine *Pseudomonas* sp. through modulation of culture conditions

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**Keywords**: Gene expression; Microbial resistance; Antimicrobials; Efflux pump

**Abstract**

The detection of antimicrobial resistance genes (ARGs) in bacteria from marine environments has increased significantly. The genus *Pseudomonas* has been described in this environment to be resistant to a variety of antimicrobial classes, and the presence of ARGs has been observed mainly in efflux pump related genes such as *ampC, oprD, mexT*. Besides these observations, the evidence of the effective expression levels of ARGs under original environmental conditions is an information that is still scarce. This study aimed to evaluate the levels of expression of the *mexE* and *mexF* genes (from to *mexEF-OprN* operon) by RT-PCR of six aztreonam-resistant marine *Pseudomonas* sp. when exposed to culture conditions simulating their original environment. Despite the presence of *mexE* and *mexF* genes in the genomes of these isolates and the high aztreonam resistance observed *in vitro*, for isolates MD330.10, MD330.11, MD332.6, and MD332.8 no significant variation was observed in the expression of these genes among the culture medium conditions (Small - S, Medium – M and Large - L) under both temperatures tested (8ºC and 20ºC). Considering data from S, M and L conditions as one, isolate MD332.6 was the only one that showed significant difference in the expression of *mexF* between growth temperatures. Also, isolates MD330.11 and 332.6 showed significant difference in the expression level of the *mexF* and *mexE* genes at 8º C. For isolates MD330.9 and MD330.6, these genes presented no detectable expression, or had expression detected only under certain culture conditions, respectively. Our results suggest that nutrient modulation seems not to be a factor that regulates the expression of these genes, while temperature may influence their expression in at least some of these *Pseudomonas* isolates.

# 1. INTRODUCTION

Bacterial resistance to antimicrobials has been observed since the early days of the discovery of penicillin and its derivatives (Christaki et al. 2020). However, due to the indiscriminate use of antimicrobials over decades, a high prevalence of multidrug-resistant or even pandrug-resistant bacteria can now be observed (O'Brien 2002; Jindal et al. 2015). Antimicrobial multidrug resistance (AMR) is currently considered a global public health problem (WHO 2023), although most studies have focused on antimicrobial resistance in a clinical context, while only recently have microbial isolates from natural environments received attention in the context of AMR (Suzuki et al. 2017; Sweileh and Moh'd Mansour 2020).

The detection of antimicrobial resistance genes (ARGs) in bacteria from marine environments has increased, indicating their growing prevalence in these ecosystems (Li et al. 2020; Makkaew et al. 2021; Castaño-Ortiz et al. 2023). The marine environment may serve as an important reservoir for such genes (Marti et al. 2014), and the beaches and the marine coastline in general may represent important potential foci of resistant microorganisms, as they are areas of interface between marine ecosystems and human populations (Dang et al. 2008; Maravić et al. 2012; Yang et al. 2013; Luczkiewicz et al. 2015; Carney et al. 2019; Cuadrat et al. 2020).

The bacterial species most related to antimicrobial resistance collected from marine environments belong to the families *Enterobacteriaceae*, *Pseudomonadaceae* and *Vibronaceae* (Rubans and Gunaseelan 2011; Matyra 2012). The genus *Pseudomonas* has been described to be resistant to beta-lactam, aminoglycoside, tetracycline and fluoroquinolone classes, presenting resistance genes mostly related to efflux pumps, such as *AmpC*, *OprD*, *MexT*, but also genes from the *bla* and *tet* groups (Luczkiewicz et al. 2015; Teixeira et al. 2016).

In *Pseudomonas aeruginosa*, the presence of a Resistance Nodulation Division (RND)-type efflux system, encompassing four main genetically distinct operons (*mexAB-OprM, mexCD-OprJ, mexEF-Opr-N* and *mexXY-OprA*) and their regulators, each capable of conferring resistance to different classes of antimicrobials has been well established (Köhler et at. 1997; Köhler et al. 1999; Poole 2004). Bacterial resistance to aztreonam is observed in *P. aeruginosa* strains of clinical origin, primarily due to mutations in these operons associated with overexpression of the efflux pump (Masuda et al. 2000; Sobel et al. 2005; Jorth et al. 2017). Likewise, but at a much lower frequency, *Pseudomonas* isolates from different environmental origins have also been reported to be resistant to this antimicrobial (Luczkiewicz et al. 2015; Braz et al. 2016). The high expression of efflux pumps related to these operons in *Pseudomonas* is due to multiple gene mutations acting in combination (Jorth et al. 2017). The most frequently observed mutations are related to *MexAB-OprM* through its regulators *mexR*, *nalC*, and *nalD*

(Sobel et al. 2005), which has specificity for aztreonam. In contrast, *mexEF-OprN* has not been reported to confer resistance specifically to aztreonam but is active against other beta-lactam antimicrobials such as imipenem (Köhler et al. 2001). Additionally, one study observed mutations in core components of *mexEF-OprN* and its positive transcriptional regulator *mexT* in *P. aeruginosa* strains undergoing *in vitro* selection for aztreonam resistance, suggesting that these mutations may be somehow beneficial during aztreonam selection (Jorth et al. 2017).

Despite the extensive description of these operons in the genus *Pseudomonas*, little information is available regarding the presence of these genes in isolates of non-clinical origin. Furthermore, resistance genes have been increasingly detected in environmental *Pseudomonas* isolates, but their expression levels under original environmental conditions are still scarce. In this context, this study aimed to evaluate the levels of gene expression of the *mexE* and *mexF* genes (from the *mexEF-OprN* operon) of six aztreonam-resistant marine *Pseudomonas* sp. isolates from deep-sea sediments of the Cone do Rio Grande region, Pelotas Basin (Brazil), when exposed to different culture conditions simulating their original environment.

## 2. MATERIAL AND METHODS

### 2.1 Origin of bacterial isolates

Bacterial strains were isolated from sediment samples collected 0 to 3 meters below the seabed at the Rio Grande Cone (RGC) between 2011 and 2013 at sites with water depths ranging from 1,800 to 2,500 meters. The RGC is a methane seepage region located at the Pelotas Basin (Brazil), in the Southwestern portion of the Atlantic Ocean (Miller et al. 2015). A culture of each isolate in nitrogen mineral salts (NMS) medium was then stored at -80ºC with dimethylsulfoxide (DMSO) or glycerol at 5% or 20% (v/v), respectively (Proenca et al. 2022). A total of six isolates previously identified as *Pseudomonas* sp. were recovered in Brain Heart Infusion (BHI) broth and used in this study. These isolates were selected based on a previous study that reported their outstanding antimicrobial resistance profile to aztreonam combined with the detection of the *mexE* and *mexF* genes in their genomes (Dias et al.

2024A) (Table 1).

**Table 1-** Marine *Pseudomonas* sp. isolates used in this study and their previously detected aztreonam Minimum Inhibitory Concentration (MIC) values (Dias et al., 2024A).

| Isolate | MIC (µg/mL) |
|---|---|
| MD330.6 | 128 |
| MD330.9 | 128 |
| MD330.10 | >1024 |
| MD330.11 | >1024 |
| MD332.6 | >1024 |
| MD332.8 | >1024 |

2.2 Modulation of bacterial culture conditions

The *Pseudomonas* isolates were cultured in artificial seawater (AWS) according to Kester et al. (1967), adjusted to a salinity of 35 practical salinity units (PSU) (Table 2). This medium was adjusted to three modulation conditions, identified as S, M and L for "small", "medium" and "large" concentrations of nutrients (phosphorus, carbon and nitrogen), respectively (Table 3). Cultures were performed in triplicate under these three different conditions and two temperatures (8º C and 20º C). These culture conditions were based on the availability of nutrients throughout the depth variation, as well as the average temperature of the southwestern Atlantic oceanic waters (Suzuki et al. 2015). The experiments were carried out at the Center of Ecology and Evolution in Microbial Model Systems at Linnaeus University, Kalmar, Sweden.

Isolates grown at 20º C served as controls and as parameters for RT-PCR comparisons. Isolates were grown overnight on BHI agar plates from which 0.5 McFarland scale (~$1x10^8$ CFU/mL) was prepared in sterile saline (0.85% NaCl). 100 µL of each scale were inoculated in triplicate into 15 mL Falcon tubes containing 8 mL of the culture media. The three culture medium conditions were incubated at their respective temperatures of 8º C and 20º C, and the optical density (OD) of the tubes was measured daily until it reached 0.4 nm. When the OD of the tubes was reached, their contents were transferred to 1.5 mL microtubes and centrifuged at 20,000 rpm for 20 min to harvest the bacterial pellets. RNAlater was added to the microtubes containing the pellets and these were stored at -80º C. The QIAGEN Rneasy mini kit was employed for total RNA extraction. The cDNA from the samples was obtained using the QuantiTect Reverse Transcription kit (QIAGEN).

**Table 2**- Composition of artificial seawater medium at a salinity of 35 practical salinity units according to Kester et al. (1967)

| **Gravimetric salts** | **g/L** |
|---|---|
| NaCl | 29.926 |
| $Na_2SO_4$ | 4.008 |
| KCl | 0.677 |
| KBr | 0.098 |
| $H_3BO_3$ | 0.026 |
| NaF | 0.003 |
| **Volumetric salts** | **g/L** |
| $MgCl_2$ x $6H_20$ | 10.831 |
| $CaCl_2$ x $2H_2O$ | 1.519 |
| $SrCl_2$ x $6H_20$ | 0.024 |

**Table 3-** Concentration of modulated elements (P, C and N) employed for artificial seawater bacterial cultures performed under the different nutrient conditions (S – small; M – medium, and L- large).

| **Medium** | **Elements modulated (in µM)** | | |
|---|---|---|---|
| | P | C | N |
| S | 0.2 | 20 | 0.5 |
| M | 2 | 80 | 50 |
| L | 20 | 200 | 500 |

## 2.3 Antimicrobial resistance genes and primer design

In previous studies, the complete genome of these isolates was sequenced (Proenca et al. 2022, Dias et al. 2024A) and the presence of the *mexE* and *mexF* genes belonging to the *mexEF-OprN* operon was detected (Dias et al. 2024A). The sequences of these genes were isolated, and primers were designed using the Eurofins genomics program available at the website (https://eurofinsgenomics.eu/en/ecom/tools/qpcr-assay-design/) and are shown in Table 4.

**Table 4-** Primer sequences employed for the RT-PCR analysis of *mexE* and *mexF* genes.

| Target gene | Primer sequence (5'→3') | Product length (bp) |
|---|---|---|
| *mexE1*(Fw) | TCGCCTTTCTTGACCAAAGC | 83 |
| *mexE2*(Rev) | GAAACCGTACAGATTCGTCCAC | |
| *F1*(Fw) | ATGCTSTACCTGTCCAACTACG | 89 |
| *mexF2*(Rev | ATGCCGAACAAYTGCACRTC | |

bp- base pairs

## 2.4 RT-PCR analysis

Relative gene expression from RT-PCR data was calculated according to Jensen et al. (2010). This approach evaluates RT-PCR data by run-internal mini standard curves (RIMS) and direct real-time relative quantitative PCR (drqPCR) (RIMS-based drqPCR). In this method, average expression values from one internal group are used as a control to calculate gene expression levels of all samples. Comparison of the expression rates are made through the equation $2^{-\Delta\Delta Cq}$. In this equation, $C_q$ is the quantification cycle (that indicates the position of the amplification curve with respect to the cycle axis), which is directly related to the starting concentration of the target, while $\Delta\Delta C_q$ values are the differences in $C_q$ variations. In this case $\Delta\Delta Cq = \Delta Cq$ (samples exposed to 8ºC) – $\Delta Cq$ (samples exposed to 20º C). The resulting data

are illustrated in log scale. This and other similar methods (like Matz et al. 2013) have been used in previous studies to evaluate gene expression by RT-PCR without normalizing external genes employed in the usual methodology that is based on comparative ΔCt values (Cui et al. 2020; Wang et al. 2022; Groot et al. 2022; Jung et al. 2023). In this study, data from 20º C cultures of each isolate were used as internal controls.

The cDNA samples were synthesized from all RNA samples using Qiagen's QuantiTect Reverse Transcription kit. PCR reactions were performed using the PowerUp™ SYBR™ Green Master Mix. Each sample was run in 4 replicates using 2 µL cDNA template, 5 µL PowerUp™ SYBR™ Green Master Mix, 0.5 uM of each primer and 2 µL UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™) to a final reaction volume of 10 µL. Reactions were run on the Roche LightCycler® 480 Instrument with the following thermocycling settings: 1 cycle of 50°C for 2 min and 95°C for 2 min; 40 cycles of 95°C for 15s followed by 60°C (for *mexE*) and 59º (for *mexF*) for 1 min. Melt curve analysis was also performed, running 1 cycle of 95°C for 15s, 60°C for 1 min and 95°C for 15s.

2.5 Statistical analysis

The two-way ANOVA and Sidak multiple comparison tests were applied to evaluate the differences in the expression rate of *mexE* and *mexF* genes in the three culture conditions for each isolate separately. Additionally, these tests were used to compare the expression rates among isolates under the two temperatures tested (8º C and 20º C), independent of the culture condition. The parametric Student's *t*-test was applied to evaluate, for each isolate, the influence of temperature on *mexE* and *mexF* expression individually, and to verify if the expression levels of these genes differed from each other, at each temperature, regardless of the culture condition. *P* values <0.05 were considered statistically significant. GraphPad Prism version 8.0.1 was used for data analysis.

RESULTS AND DISCUSSION

The indiscriminate use of antimicrobials may contribute to the long-term adaptation, evolution, and dissemination of resistance to these molecules among different bacterial species in natural environments (Hatsoy and Martiny 2015; Christaki et al. 2020), and the prevalence of antimicrobial-resistant strains can contribute to the transmission of genes conferring such characteristics in different microbial communities (Thavasi et al. 2007; Jang et al. 2018). Resistance to aztreonam in clinical settings has been well described and characterized by the presence of operon-regulated efflux pumps (Pool 2011; Kunz et al. 2022), with emphasis on the

*mexAB-oprM* operon (Nakae et al. 1999; Pan et al. 2016; Ma et al. 2021). However, despite evidence for the presence of this and other similar operons in bacteria of the genus *Pseudomonas* isolated from sources other than the clinical environment, little is known about the presence of these genes in the environment and their effective expression (Yu Pan et al. 2012; Braz et al. 2016). Our previous study reported a high level of resistance to aztreonam and the presence of the *mexE* and *mexF* genes in the genome of six deep-sea *Pseudomonas* sp. isolates (Dias et al. 2024A). In this study, we evaluated the gene expression levels of the *mexE* and *mexF* genes (from the *mexEF-OprN* multidrug efflux complex operon) of these six marine *Pseudomonas* sp. isolates.

The RT-PCR results for isolate MD330.6 did not detect gene expression for some of the culture conditions. For MD330.9 no gene expression was detected at all for both genes, making it not possible to evaluate these data. For isolates MD330.10, MD330.11, MD332.6 and MD332.8 and MD330.6, no significant variation was observed in the expression of these genes among the culture medium conditions (S, M, L) under both temperatures (Figure 1). Differently from our results, Lin et al. (2018) reported a significant decrease in the expression levels of the *mexCD-OprJ* operon in *Pseudomonas aeruginosa* when exposed to low levels of nutrients. Moreover, fetar et al. (2011) reported that the expression of the efflux pumps coded by the we studied (*mexEF-OprN*) is induced by environmental stressors. However, the lack of differences observed in for both *mexE* and *mexF* genes among our different culture conditions for all isolates may show that the challenging medium conditions applied are not the main environmental trigger for the variation in the expression of these genes in our *Pseudomonas* isolates. Considering this lack of difference, we used all data from these three medium conditions together to compare the gene expression levels between growth temperatures and between *mexE* and *mexF* genes for each isolate, and among isolates, for those that presented gene expression detected in all conditions (MD330.10, MD330.11, MD332.6 and MD332.8).

The individual results were variable among isolates. Regarding growth temperatures, MD332.6 was the only isolate that presented a significant difference, which was a reduction in *mexF* expression levels at 8ºC (Figure 2). For the other isolates, although without significant differences, this seemed to be a general tendency. The permissive temperature of 20ºC is expected to induce higher levels of gene expression than 8ºC due to high metabolism rates observed between 20 and 30ºC for environmental *Pseudomonas* strains (Moreno and Rojo 2014).

Comparing the expression levels between genes, isolate MD332.6 presented the *mexF* expression rates significantly higher than *mexE*, but only at 8º C, which was also observed for isolate MD330.11 (Figure 3). This challenging temperature is close to what is observed in deep-

sea sediment, the original habitat of our marine isolates. So, under the culture conditions we employed, this result may indicate that for at least some *Pseudomonas* strains from such environments, *mexF* protein seems to be more demanded than the protein coded by *mexE*. Fonseca et al. (2011) compared the transcriptome and proteome profiles of *P. putida* KT2440 growing in a complex medium at 30°C and at 10°C and reported that the expression of several genes involved in the transport and assimilation of less-preferred compounds, such as branched-chain amino acids, aromatic amino acids or glucose, was higher at 10°C than at 30°C (Fonseca et al. 2011). The gene *mexF* is responsible for encoding a cell membrane protein transporter, while *mexE* encodes the periplasmic membrane-fusion protein (Köhler et al. 1997). Considering that low temperatures are described as influencing the fluidity of *Pseudomonas* cell membrane (Moreno and Rojo 2014), it is also possible that *mexF* gene may present higher relative expression levels compared to *mexE* to maintain the bacterial viability under this stressful condition. Moreover, the permeability of *Pseudomonas* cell wall to several compounds increases as temperature falls (Kropinski et al. 1987; Kumar et al. 2002). In this context, proteomic and transcriptomic techniques in this bacterial genus have detected significant changes in the levels of several cell wall and cell membrane proteins according to temperature, including porins, transporters and surface proteins (Knight et al. 2010; Fonseca et al. 2011; Jagannadham and Chowdhury 2012).

Regarding the comparison of the expression rate of these genes among all four isolates under the two temperatures, no significant differences were observed (Figure 4). This indicates that besides the observed significant difference of the expression *mexF* gene for one isolate (MD332.6) and between *mexE* and *mexF* at 8ºC for two isolates (MD330.11 and MD332.6), when we compare all four *Pseudomonas,* they seem to present a similar behavior in terms of expression of both genes under 20ºC and 8ºC.

Our data provides new information on the *mexEF-OprN* operon in isolates of the genus *Pseudomonas* from environmental samples, in this case from deep-sea sediments of the southern coast of Brazil. Likewise, our results contribute to the understanding of the expression levels of these genes under growth conditions more similar to their natural environment, showing that the variations in nutrients concentration applied may not interfere directly on the expression of *mexE* and *mexF* genes, although for some isolates the environmental temperature may influence stress response that depends on the efflux pumps coded by *mexEF-OprN* operon.

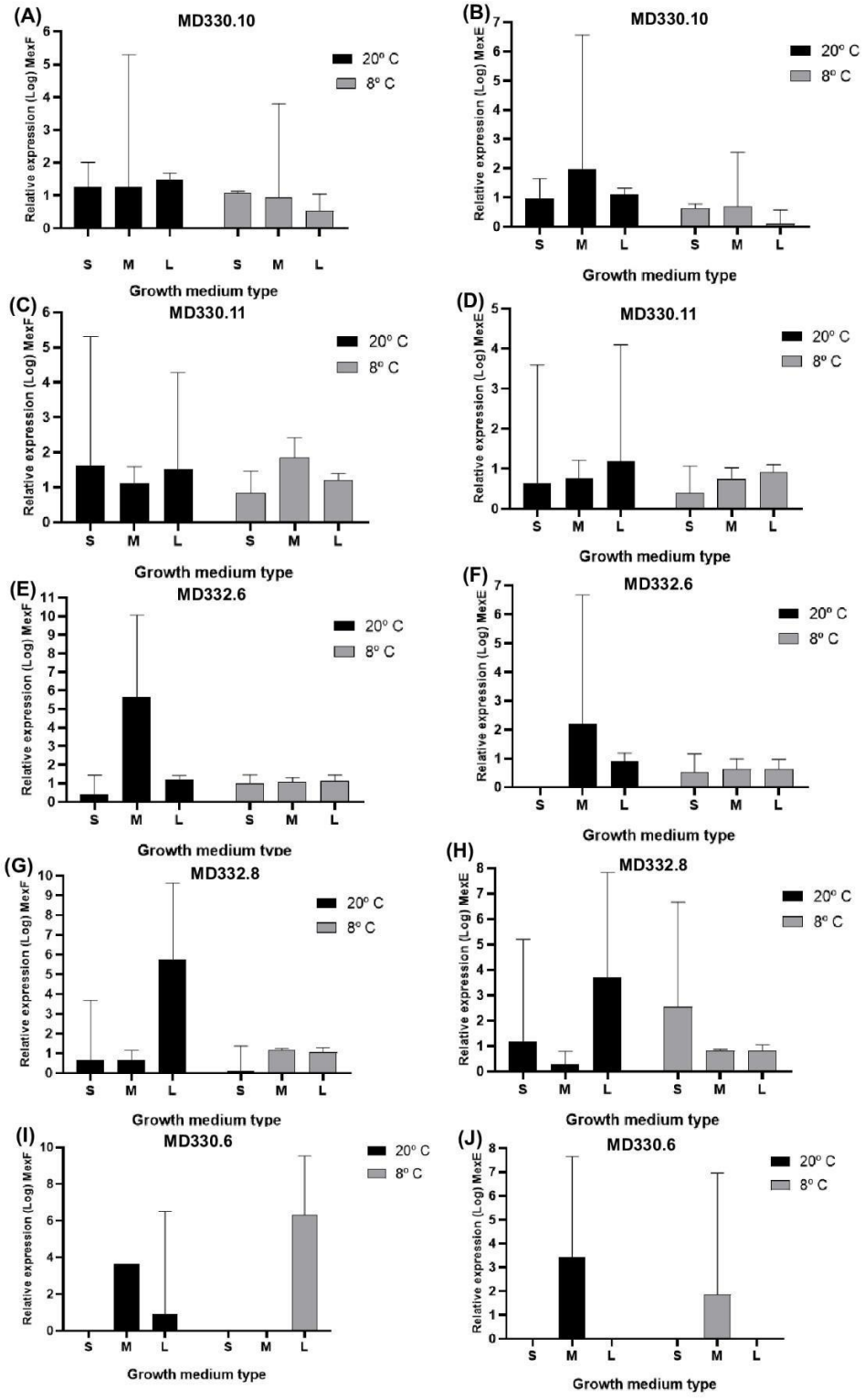


Fig 1- Relative expression (in Log) of *mexF* and *mexE* genes in five marine *Pseudomonas* isolates cultures, at the three growths conditions (S, M, L), under the two temperatures tested (20º C and 8ºC). (A) *mexF* gene from MD330.10; (B) *mexE* gene from MD330.10; (C) *mexF* gene from MD330.11; (D) *mexE* gene from MD330.11; (E) *mexF* gene from MD332.6; (F) *mexE* gene from MD332.6; (G) *mexF* gene from MD332.8; (H) *mexE* gene from MD332.8.; (I) *mexF* gene from MD330.6; (J) *mexE* gene from MD330.6.The results are average values of 12 replicates. No significant differences were detected by two-way ANOVA and Sidak multiple comparison tests ($p<0,05$).

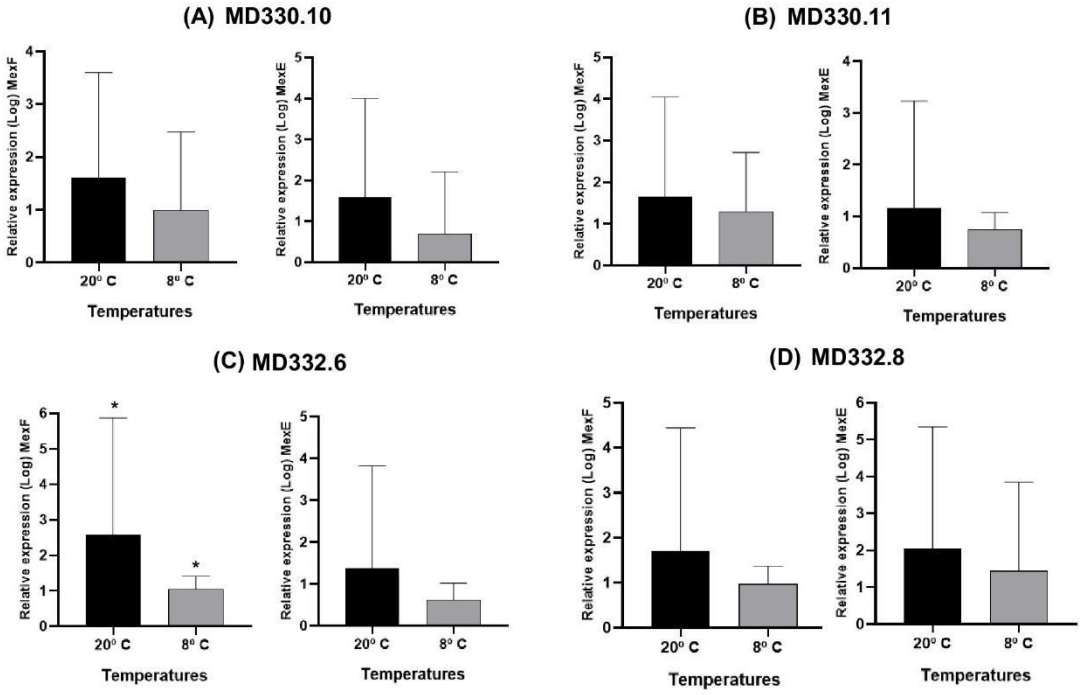


Fig 2- Comparison between growth temperatures (20º C and 8ºC) on relative expression levels (in Log) of both *mexF* and *mexE* genes, for each of four marine *Pseudomonas* isolates, combining data of all culture conditions performed (average values of 36 replicates). Isolates MD330.10 (A), MD330.11 (B); MD332.6 (C) and MD332.8 (D). (*) indicates statistically significant differences ($p<0,05$) tested by Student t-test.

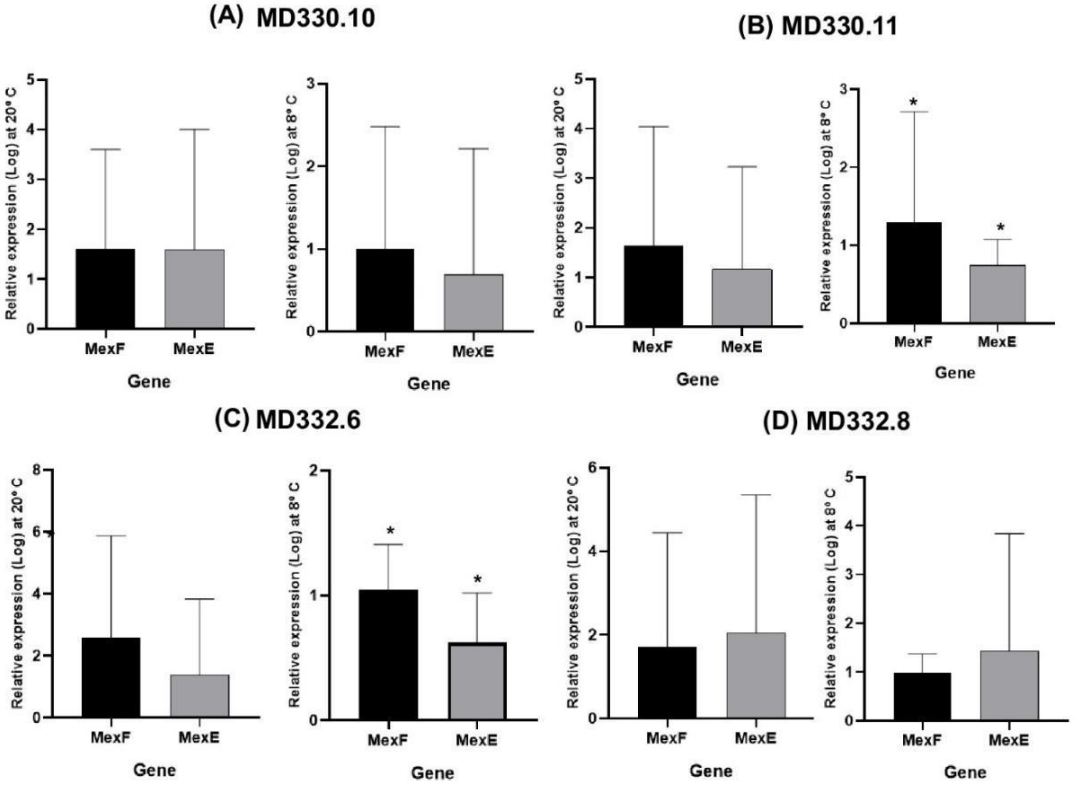


Fig 3- Comparison of relative expression levels (in Log) between *mexF* and *mexE* genes for each of four marine *Pseudomonas* isolates, grown under 20ºC or 8ºC, combining data of all culture conditions performed (average

values of 36 replicates). Isolates MD330.10 (A), MD330.11 (B); MD332.6 (C) and MD332.8 (D). (*) indicates statistically significant differences (p<0,05) tested by Student t-test.

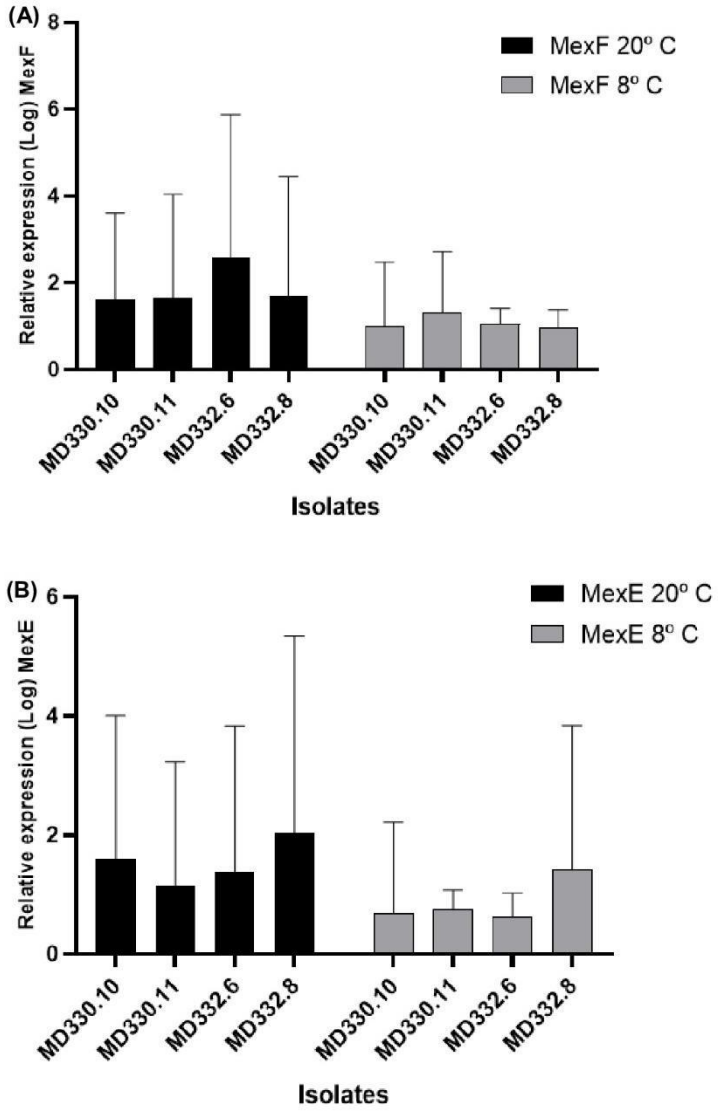


Fig 4- Relative expression (in Log) of *mexF* (A) and *mexE* (B) genes among four marine *Pseudomonas* isolates, under the two growth temperatures (20º C and 8ºC), combining data of all culture conditions performed (average values of 36 replicates). No significant differences were detected by the Student t-test (p<0,05).

## CONCLUSION

In this work, we modulated the growth conditions of six *Pseudomonas* sp. isolates, altering the availability of essential nutrients for their growth (P, C and N) as well as their incubation temperature, with the aim of evaluate the expression levels of the *mexE* and *mexF* genes belonging to the *mexEF-OprN* operon, previously detected in these isolates. The results indicated that the modulation of culture media and temperature altered the expression of *mexF* for one isolate ate 8º C and the proportion in the expression rate of this gene also at 8º C for two isolates, expressing *mexF* at a higher rate than *mexE*. Although, for the others isolates there was no significant variation at the expression levels of *mexE* and *mexF*, suggesting that the

expression of this efflux system in the tested *Pseudomonas* sp. isolates is not directly influenced by the tested environmental conditions, but that they may contribute to their change. These findings suggest that the expression of these efflux system genes are likely triggered by other mechanisms beyond the sole influence of nutrients and temperature.


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## COMPETING INTEREST INFORMATION

The authors declare no conflict of interest.

## AUTHORS CONTRIBUTIONS

ASD, RMS and HF were involved in study conception. RMS and HF provided project management. ASD designed and performed experiments and wrote the manuscript. LBC performed experiments. ASD, RMS and SDO analyzed data and provided data interpretation. HF provided funding support. All authors were involved in revisions and approval of the manuscript.

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## 5. CAPÍTULO 3

## - Manuscrito a ser submetido ao periódico

## *Applied and Environmental Microbiology* (FI: 3.9; Qualis CAPES A1)

# Antifungal activity of deep-sea marine *Pseudomonas* sp.

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**Keywords:** Marine bacteria; Microbial bioactives; Antifungal activity; Marine Biotechnology

**Abstract**

Marine microorganisms are a promising source of novel and bioactive compounds with diverse applications. The genus *Pseudomonas* is one of the most described producers of secondary metabolites with antimicrobial properties. This work explored the antimicrobial potential of six antibiotic-resistant and siderophore-producing *Pseudomonas* sp., isolated from deep-sea sediment against clinically relevant microorganisms and filamentous fungi. Genomic analysis using the antiSMASH program detected gene clusters related to the biosynthesis of the siderophore pyochelin. The total siderophore production rate was measured by the reaction with Chrome Azuro S (CAS). The competition assays showed that among the *Pseudomonas* isolates, one inhibited *Staphylococcus aureus* and five inhibited *Candida albicans*. This yeast species was also inhibited in co-culture with some *Pseudomonas* and when exposed to their supernatants. Moreover, four isolates were able to inhibit or reduce the growth of filamentous fungal species in the antibiosis assay. The results highlighted the isolate MD330.9, since it inhibited *S. aureus*, *C. albicans* and all seven filamentous fungi and had the highest siderophore production among *Pseudomonas* isolates. Our data corroborate other reports showing that *Pseudomonas* siderophores can inhibit a diversity of fungal species and provide new information regarding the potential antifungal ability of marine siderophoreproducing *Pseudomonas*.

## 1. INTRODUCTION

The use of natural potential of microorganisms for human benefit is a practice with a long history and documented evidence around the world. In ancient Egyptian and Chinese cultures, mold was used in wound treatment (Pelczar et al. 1993, Ravina 2011), even before the concept of microorganisms was established. Recently, researchers have turned to natural environments with a view to finding and describing new potential molecules of interest to the most diverse areas. Soils, coral reefs, deserts, water, and marine sediment represent a number of sources for the prospecting of microbial species or metabolites with biotechnological properties (Mullis et al. 2019, Sayed 2020). Such studies seek to identify different properties in the target microorganisms, including antitumor, antibacterial, antibiofilm, and antifungal activity potential (Wang et al. 2020). The search for new microbial metabolites with antimicrobial and/or biocontrol properties is driven by the emergence of resistance among microbial species and strains, particularly in clinical settings but also in natural environments (Barbosa et al. 2020). These strains have the potential to cause significant impacts on natural biota in aquatic and terrestrial environments, threatening biodiversity, as well as on human health (Mahmood et al. 2016). In the pursue for new antimicrobial agents, marine microorganisms have proven to be a promising source of new molecules for study (Stincone and Brandelli 2020). In this context, marine strains from *Bacillus*, *Actinomycetes*, *Streptomycetes*, *Serratia*, and *Pseudomonas* are reported to be major producers of different metabolites, including antimicrobials (Kamjam et al. 2017, Stincone and Brandelli 2020, Lee et al. 2023).

*Pseudomonas* is a ubiquitous and diverse genus of Gammaproteobacteria, comprising 344 validly named species (List of Prokaryotic Names with Standing in Nomenclature https://lpsn.dsmz.de/genus/pseudomonas), excluding subspecies and synonymous species. They are well known for their capacity to utilize a striking variety of organic compounds as energy sources, resistance to a wide range of antimicrobial compounds, and production of a remarkable array of secondary metabolites (Gross and Loper 2009). There are several reports of different species, such as *P. aeruginosa* and *P. fluorescens*, capable of producing metabolites with antimicrobial properties that can inhibit antibiotic-resistant bacteria and their biofilms (Doghri et al. 2020, Romero-González et al. 2023). Other studies indicate that some *Pseudomonas* strains also inhibit *Candida* species (Morales et al. 2013, Hanif et al. 2024, Oves et al. 2024) and phytopathogen fungal strains through the production of different compounds, like phenazines and siderophores (Al-Ghafri et al. 2020, Castaldi et al. 2021, Ho et al., 2018, Thakker et al. 2023, Serafim et al. 2023). Among such metabolites, the phenazine pyocyanin is the most frequently highlighted (Morales et al. 2013, Hanif et al. 2024, Oves et al. 2024). Nevertheless, the siderophore pyochelin has also been identified as a compound with antifungal

properties produced by certain *Pseudomonas* species (Gross and Loper 2009, Ho et al. 2018, Valyshev 2023). Moreover, research indicates that antimicrobialresistant *Pseudomonas* strains often express a wide repertoire of metabolites with antimicrobial activity (Hemala et al. 2014, Mogrovejo et al. 2020, Paun et al. 2021, Oves et al. 2024).

Considering the critical need for novel compounds that can act as antimicrobials for human and animal health, agriculture, or industry, this study focused on exploring the potential of six siderophore-producing and antibiotic-resistant *Pseudomonas* sp. isolated from deep-sea sediment of the Rio Grande Cone region (Pelotas Basin, RS) in terms of their ability to inhibit the growth of clinically relevant bacteria, as well as different fungal species.

## 2. MATERIAL AND METHODS

### 2.1 Origin of bacterial isolates

Bacterial strains were isolated from sediment samples collected 0 to 3 meters below the seabed at the Rio Grande Cone (RGC) in sites with 1,800 to 2,500 meters of water depth, between 2011 and 2013. The RGC is a methane seepage region located at the Pelotas Basin (Brazil), in the Southwestern portion of the Atlantic Ocean (Miller et al. 2015). The isolates were then stored at -80ºC with dimethylsulfoxide (DMSO) or glycerol in the proportion of 5% or 20% (v/v), respectively (Proenca et al. 2022). A total of six isolates previously identified as *Pseudomonas* sp. were recovered in BHI broth cultures and used in this study. These isolates were selected based on their ability to produce siderophores (Proenca et al. 2022) and their established antimicrobial resistance profile (Dias et al. 2024) (Table 1).

**Table 1-** Antimicrobial susceptibility and minimal inhibitory concentration (MIC) values of marine *Pseudomonas* sp. isolates.

| **Isolate** | **Disk diffusion** | **MIC (µg/mL)** | |
|---|---|---|---|
| | | *ATM* | *CAZ* |
| MD330.6 | ATM | 128 | - |
| MD330.9 | ATM, CAZ | 128 | 128 |
| MD330.10 | ATM | >1024 | - |
| MD330.11 | ATM | >1024 | - |
| MD332.6 | ATM | >1024 | - |
| MD332.8 | ATM | >1024 | - |

ATM- aztreonam, CAZ- ceftazidime.

2.2 Competition test

The competition test, adapted from Marinho et al (2009), was carried out to initially assess the potential of six marine *Pseudomonas* sp. strains to inhibit the growth of different types of microorganisms. *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25992, and *Candida albicans* ATCC 18804 were used as target microorganisms. The cultures of these microorganisms were made suspended in a sterile 0.85% saline at a turbidity level compatible with the 0.5 McFarland scale (~$1x10^8$ CFU/mL), and then inoculated onto Mueller-Hinton (MH) agar plates using swabs. Subsequently, 10 µL of each *Pseudomonas* isolate was dripped in triplicate onto the plates containing one of the target microorganisms. The plates were incubated for 24 h at 25º C to observe and measure the induction of inhibition halos.

2.3 Co-cultivation with *C. albicans*

The co-cultivation assays were adapted from Vazquez-Rodriguez et al (2018). Only the isolates that demonstrated some ability to inhibit *C. albicans* in the competition test were selected. Each marine isolate was co-cultured with *C. albicans* ATCC 18804. From overnight Tryptic Soy Agar (TSA) cultures, cell suspensions were prepared in sterile 0.85% saline for each marine isolate and *C. albicans* strains, and the optical densities (OD) measured until they reached a value between 0.8 and 1.2 nm. Co-cultures were prepared in Erlenmeyer flasks containing 50 mL of Tryptic Soy Broth (TSB), combining 0.5 mL of a marine isolate suspension with 0.5 mL of *C. albicans* suspension, and incubated at 25 ºC under 140 rpm for 48 h. As a positive control, the microorganisms were individually inoculated in 50 mL TSB cultures. After the incubation period, a serial dilution was carried out to $10^{-7}$, from which 100 µL aliquots were removed and spread on TSA plates, followed by incubation at 25 ºC for 48 h. Colony forming units per mL (CFU/mL) were then estimated for each microorganism in the co-cultures and controls by counting the bacterial and yeast colonies.

2.4 *C. albicans* growth inhibition by *Pseudomonas* supernatants

In this experiment, which was adapted from Doghri et al (2020), TSB overnight cultures of all marine isolates and *C. albicans* ATCC 18804 were used to prepare cell suspensions in sterile 0.85% saline at an OD between 0.8 and 1.2 nm. A 0.5 mL volume of each suspension was then inoculated into Erlenmeyer flasks containing 50 mL of TSB and incubated at 25º C for 48 h under 140 rpm. Three groups of test cultures were prepared: (i) solely with *C. albicans*; (ii) solely with a marine isolate; and (iii) a combination of one marine isolate with *C. albicans* in equal volumes (0.5 mL of each suspension). After the incubation period, the contents of the Erlenmeyer flasks were transferred to sterile falcon tubes and centrifuged at 5,000 *g* for 20 min.

They were then filtered twice, using 0.22 µm sterile filters and syringes, to obtain the pure supernatant of the cultures.

In a 96-well plate, 50 µL of supernatant were added to 50 µL of TSB in eight replicates. A volume of 2 µL of the *C. albicans* cell suspension (0.5 McFarland scale) was inoculated into these wells. Triplicates of sterile TSB and pure supernatants were also included as negative controls. The microplate was incubated at 25º C for 48 h in the SpectraMax 190 Multimode Microplate Reader device, with OD readings at 650 nm every 15 min, with shaking before and after each measure, to monitor and generate yeast growth curves.

2.5 Standard antibiosis test against filamentous fungi

To analyze the antifungal activity of the six marine *Pseudomonas* on filamentous fungi, the antibiosis test adapted from Melo and Valarini (1995) was carried out with seven strains of different fungal species: *Alternaria solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Macrophomina phaseolina, Cylindroclodium* sp. (kindly provided by the PUCRS Plant Biotechnology Laboratory), *Aspergillus brasiliensis* ATCC 9642, and *Penicillium funiculosum* ATCC 11797.

For this test, 10 µL of bacterial suspensions on a 0.5 McFarland scale were inoculated through a continuous line made in the center of Potato Dextrose Agar (PDA) and incubated at 25º C for 24 h. After this period, a square (1x1cm) of each fungal mycelium was inoculated on their respective plates, at one end, 1 cm from the edge of the plate. As a negative control, sterile 0.85% saline was used instead of the bacterial culture. Plates were incubated at 25º C and observed every 3 days for a maximum period of 15 days. Subsequently, mycelial and bacterial growth was analyzed, and the extent of the inhibition zone was evaluated using the ImageJ v.1.8.0 program (Schneider et al. 2012). The percentage of fungal growth inhibition (PGI) was calculated using the formula (Melo et al. 2016):

PGI (%) = KR-R1/KR x 100 Where:

KR = the distance (in mm) from the point of inoculation to the colony margin on the cornel plate

R1 = the distance of fungal growth from the point of inoculation to the colony margin on the treated plates in the direction of the antagonist

If the fungus manages to cross the line made with bacteria, it is classified as a noninhibitory result.

2.6 Genomic analysis of secondary metabolites production

Five of the *Pseudomonas* isolates had their complete genomes sequenced in previous studies (Proenca et al. 2022, Dias et al. 2024A). For the genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in these bacterial genomes, the antiSMASH 6.0 program (Blin et al. 2021) was used. This program integrates and cross-links with a large number of *in silico* secondary metabolite analysis tools. To identify the potential genetic clusters for secondary metabolite biosynthesis in our marine isolates were analyzed their genomes using antiSMASH in restricted detection mode (Blin et al. 2021).

2.7 Siderophores production

The six marine isolates were previously identified as siderophore producers in Luria Bertani-Chrome Azurol S (LB-CAS) agar, which is a qualitative test (Proenca et al. 2022). To evaluate the concentration of total siderophore production by these isolates, a Chrome Azurol S (CAS) solution colorimetric reaction test was performed (Schwyn and Neilands 1987, Pérez-Miranda et al. 2007). The isolates were grown in TSB overnight and centrifuged for 20 min at 2,500 g. The colorimetric reaction mixture was prepared in 96-well plates in quintuplicate by combining 100 µL of the supernatant with 100 µL of CAS solution. The mixture was incubated for 20 min at room temperature and the absorbance was measured using SpectraMax at 630 nm. TSB without inoculum was used as a negative control. Siderophore production was estimated according to the color change of the CAS solution from blue to yellow (Pérez-Miranda et al. 2007) using the following equation:

$$\text{Siderophore unit (Us\%)} = [(Ar - As) / Ar] \times 100$$

Where:

Ar: absorbance related to the sample (CAS + medium without inoculum) As: absorbance of each sample (CAS + sample)

2.8 Statistical Analysis

The parametric Student t-test was used to compare the inhibition areas of the isolates in the antibiosis test with their controls, and the *C. albicans* growth inhibition rate by bacterial supernatants. *P* values <0.05 were considered statistically significant. Two-way ANOVA and Sidak multiple comparison tests were applied for CFU counts from the *C. albicans* cocultivation test. GraphPad Prism version 8.0.1 was used for data analysis.

RESULTS

3.1 Competition test against unicellular species

Regarding these initial cultures, it was observed that five *Pseudomonas* sp. isolates (MD 330.9, MD330.10, MD330.11, MD332.6, and MD332.8) showed some ability to inhibit *C. albicans*. Among them, MD330.9 was the one that presented the largest mean inhibition diameter measurement (14 mm), while MD332.8 induced the smallest one (10.7 mm). For *S. aureus*, only MD330.9 showed a slight inhibitory capacity, forming a measurable halo (13.9 mm), but with slight bacterial growth in the background, indicating a slowing of *S. aureus* growth but not complete inhibition (Figure 1). For *E. coli*, however, none of the six marine isolates showed any inhibitory potential.

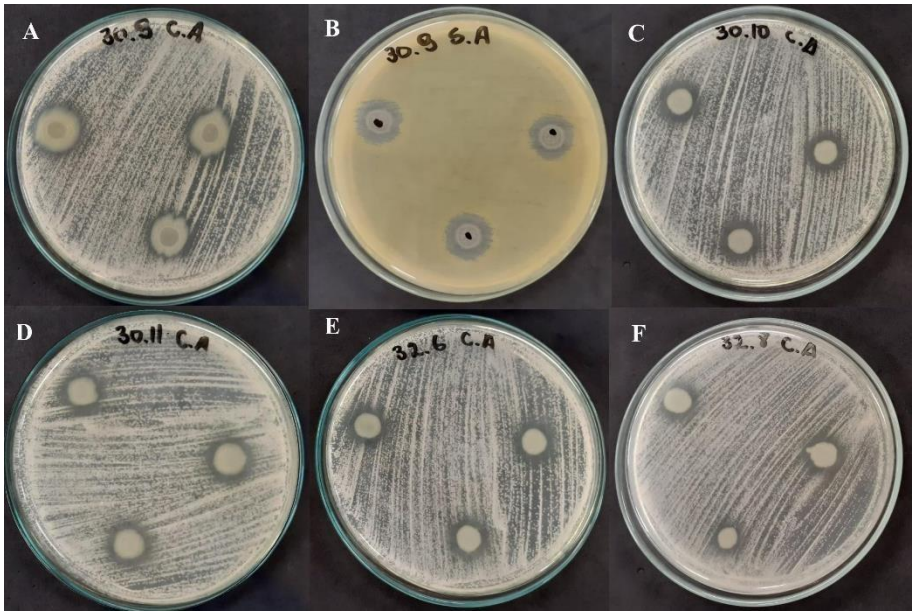


**Fig. 1-** Competition test plates against unicellular species of those isolates that showed inhibitory activity. (A) Isolate MD330.9 against *Candida albicans*; (B) Isolate MD330.9 against *Staphylococcus aureus*; (C) Isolate MD330.10 against *C. albicans*; (D) Isolate MD330.11 against *C. albicans*; (E) Isolate MD332.6 against *C. albicans*; (F) Isolate MD332.8 against *C. albicans*.

3.2 Co-cultivation test with *C. albicans*

In this test, five of the six *Pseudomonas* sp. isolates (MD330.9, MD330.10, MD330.11, MD332.6, and MD332.8) were individually co-cultured with *C. albicans* and the CFU numbers of each microorganism were counted and tested by the ANOVA and Sidak (post-hoc) tests. The results, presented in Figure 2, showed the absence of growth of *C. albicans* when co-cultured with each of these marine *Pseudomonas*, indicating an anti-*C. albicans* potential for all tested isolates. Regarding the bacterial isolates, there was no significant difference in variation of CFU counts when comparing their controls to co-cultures with *C. albicans* (Figure 2).

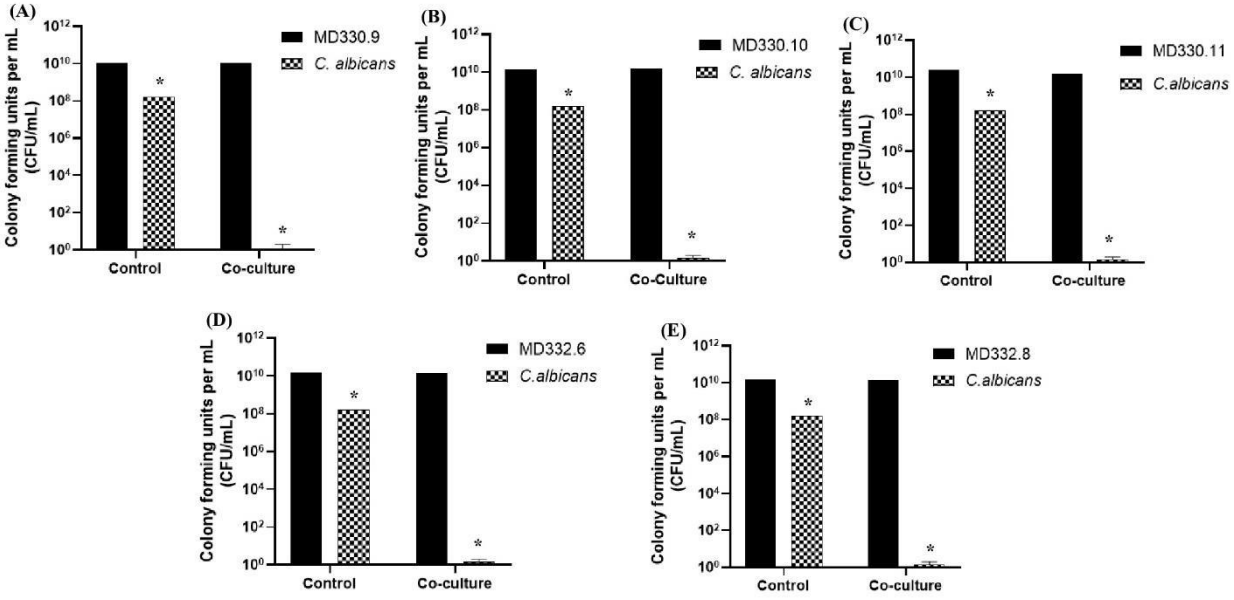


**Figure 2-** Result of CFU/mL counts from co-cultivation test of each *Pseudomonas* sp. strain with *Candida albicans* and their controls. (A) Isolate MD330.9; (B) Isolate MD330.10; (C) Isolate MD330.11; (D) Isolate MD332.6; (E) Isolate MD332.8; (*) indicate cultures that significantly differed from each other (*P* value <0.05).

### 3.3 Inhibition of *C. albicans* by bacterial supernatants

The results showed that the supernatants of all five *Pseudomonas* tested (MD330.9, MD330.10. MD330.11, MD332.6 and MD332.8) were able to significantly inhibit or reduce the growth of *C. albicans*. The MD330.9 supernatant was able to completely inhibit the growth of *C. albicans* (Supplementary material S1). In the presence of MD330.10, MD330.11, MD332.6, and MD332.8 supernatants, *C. albicans* growth parameters were significantly decreased, when compared to those from the control growth curves (Table 2).

**Table 2-** Parameters of *C. albicans* growth curves with and without marine *Pseudomonas* sp. supernatants. Mean values of: Log phase start time (in hours), time of reaching maximum OD value (in hours), and maximum OD value reached along 48 hours.

| ***Pseudomonas* sp. isolate** | ***C. albicans* control** | | | ***C. albicans* + supernatant** | | |
|---|---|---|---|---|---|---|
| | **Log phase start time** | **Time of max. OD** | **Max. OD** | **Log phase start time** | **Time of max. OD** | **Max. OD** |
| MD330.9 | 24 h 22 min | 48 h | 0.78 | -* | -* | 0* |
| MD330.10 | 19 h 20 min | 48 h | 0.92 | 27 h 39 min* | 48 h | 0.38* |
| MD330.11 | 15 h 37 min | 48 h | 1.05 | 21 h 58 min* | 46 h 22 min* | 0.2* |
| MD332.6 | 12 h 13 min | 48 h | 1.08 | 20 h 5 min* | 41 h 40 min* | 0.29* |
| MD332.8 | 17 h 18 min | 48 h | 1.03 | 20 h 13 min* | 48 h | 0.37* |

(*) indicate statistically significant differences (*P* value <0.05) compared to values from *C. albicans* controls; OD- optical density

3.4 Antibiosis test against filamentous fungi

The results of *C. albicans* inhibition by marine *Pseudomonas* sp. and their supernatants led us to investigate the inhibition potential of these isolates against filamentous fungal species. In this test, the inhibition potential was calculated (as a percentage) by comparing the fungal growth area in the presence of marine bacteria compared to the control without bacteria.

The results in Table 3 showed that one isolate (MD330.9) had the ability to inhibit five of the seven fungi tested. Its highest and lowest inhibition potentials were observed against *Cylindroclodium* sp. (86.3%) and *P. funiculosum* (33.7%), respectively. Moreover, *Cylindroclodium* sp. was the only fungus significantly inhibited by *Pseudomonas* isolates MD330.11 (53.8%) and MD332.6 (47.8%). Regarding the isolate MD332.8, despite reducing *A. brasiliensis* growth, it was not significantly different from the control, while MD330.10 did not inhibit any of the fungi tested (Table 3).

**Table 3-** Percentage growth inhibition (PGI%) of the fungal species in the presence of the *Pseudomonas* sp. supernatant.

| **Fungi** | ***Pseudomonas* sp. isolates** | | | | |
|---|---|---|---|---|---|
| | PGI (%) | | | | |
| | MD330.9 | MD330.10 | MD330.11 | MD332.6 | MD332.8 |
| *Alternaria solani* | 78.9%* | - | - | - | - |
| *Sclerotinia sclerotiorum* | 80%* | - | - | - | - |
| *Aspergillus brasiliensis* | 44.3% | - | - | - | 14.3% |
| *Fusarium oxysporum* | 72.5%* | - | - | - | - |
| *Macrophomina phaseolina* | 74%* | - | - | - | - |
| *Penicillium funiculosum* | 33.7% | - | - | - | - |
| *Cylindroclodium* sp. | 86.3%* | - | 53.8%* | 47.8%* | - |

(*) indicate statistically significant values (*P* value <0.05). (-) indicates those isolates in which the fungus was able to grow above the bacterial line, being classified as a non-inhibitory result.

## 3.4 Siderophore gene clusters in *Pseudomonas* sp. genomes

The results of the antiSMASH program indicate that the five *Pseudomonas* isolates present genetic clusters responsible for siderophore production, more specifically pyochelin, with more than 85% similarity to *Pseudomonas aeruginosa* PAO1 and *Burkholderia cepacia* ATCC 25416. Pyochelin is a molecule commonly produced by *Pseudomonas* and is responsible for chelating and assimilating iron through membrane transposers (Kümmerli 2023). It has also been shown to have to antifungal activity against phytopathogens (Buysens et al. 1996, Ho et al. 2018).

## 3.5 Siderophore production

The results confirmed that all five *Pseudomonas* sp. isolates produce siderophores and allowed a quantitative analysis, through the measurement of their siderophore unit production. Moreover, the highest Us% rates were detected in the isolates MD330.9, MD330.10, and MD330.11 isolates (Table 4).

**Table 4-** Siderophore production by marine *Pseudomonas* sp. isolates.

| **Isolate** | **As** | **Ar** | **Us%** |
|---|---|---|---|
| MD330.9 | 0.490 | 3.356 | 85.3 |
| MD330.10 | 1.006 | 3.499 | 71.2 |
| MD330.11 | 0.793 | 3.499 | 77.3 |
| MD332.6 | 1.862 | 3.499 | 46.7 |
| MD332.8 | 1.511 | 3.499 | 56.8 |

(As) Absorbance of each sample; (Ar) absorbance related to the sample; (Us%) Siderophore unit production

DISCUSSION

The increase in global population and the need to expand agricultural production to meet food demand (Tudi et al. 2021), as well as the increasing use of agrochemicals and antimicrobials in urban and rural activities (Meftaul et al. 2020), have resulted in a meaningful presence of these compounds in the natural environment (Sharma et al. 2019). This situation promotes an increase in the resistance to these biocidal products in microorganisms of clinical and agricultural interest, making the detection of new molecules and the development of new strategies for controlling undesired resistant microorganisms an urgent and highly relevant issue.

Marine microorganisms are a promising source of novel and bioactive compounds with diverse applications. These microbes can produce a wide array of secondary metabolites with potential such as antimicrobial, anticancer, anti-inflammatory used in pharmaceutical, cosmetic, and industrial fields (Wang et al. 2020). The genus *Pseudomonas* is one of the most described among producers of secondary metabolites of interest in marine environments (Stincone and Brandelli 2020, Lee et al. 2023). These compounds can be produced constitutively or induced by special conditions, such as co-cultures (Marmann et al. 2014, Wakefield et al. 2017, Selegato and Castro-Gamboa 2023). In our experiments, marine *Pseudomonas* sp. isolates triggered different responses in other microbial species. Isolate MD330.9 presented the ability to inhibit *S. aureus*, and most isolates (including MD330.9) inhibited the growth of eukaryotic (fungal) microorganisms, especially *C. albicans*. The cocultivation strategy allows the promotion of gene clusters related to the production of secondary metabolites and thus the expression of potential metabolites, some of which are usually not detected in monocultures. So, co-cultures may promote the recognition of undescribed microbial compounds, including antimicrobial molecules (Harwani et al. 2018, Peng et al. 2021).

The production of siderophores by the genus *Pseudomonas* has been well described (Kümmerli 2023). Siderophores are iron-chelating compounds that are regulated by environmental factors and help microorganisms to acquire iron and essential nutrients from the environment (Cornelis 2010, Hider and Kong 2010). Regarding the siderophore production, our isolates were already classified as siderophore producers by the LB-CAS agar method (Proenca et al. 2022). However, with the method of Pérez-Miranda et al. (2007) used in this study, it was possible to quantify the production of siderophores by our isolates, showing that MD330.9, MD330.10 and MD330.11 were highlighted as major producers. The genus *Pseudomonas* produces several types of siderophores, such as pyoverdine, pyochelin, enantio-pyochelin, achromobactin, pseudomonine, corrugatin and (thio)quinolobactin (Cornelis 2010, Ho et al. 2018, Schalck et al. 2020). The use of siderophores is being investigated in both clinical

(Swayambhu et al. 2021) and environmental settings, mainly as plant growth promoters and potential inhibitors of fungal growth (Ong et al. 2017, Ho et al. 2018). The efficacy of siderophores produced by *Pseudomonas* against phytopathogenic fungal species has been already described (Buysens et al. 1996, Matthij et al. 2007, Deshwal 2012, Solanki et al. 2014, Abo-Zaid et al. 2023). Regarding our *Pseudomonas* isolates, besides being siderophore producers, the results provided by the antiSMASH program sugest that they could specifically produce pyochelin, which is a siderophore produced by the condensation of salicylic acid with two cysteine molecules that has a lower affinity for iron than pyoverdine (Cox et al. 1988, Ghssein and Ezzeddine 2022). However, this information still needs to be confirmed through other, more specific analyses. Moreover, all fungi (unicellular or filamentous) tested in this study were inhibited by at least one of our *Pseudomonas* sp. isolates.

The marine isolate MD330.9 showed the most outstanding result, with the ability to inhibit *S. aureus*, *C. albicans* and all filamentous fungi. It was also the isolate with the highest efficiency of siderophore production. Moreover, it is the only *Pseudomonas* isolate that is resistant to two antimicrobials (aztreonam and ceftazidime). The other five isolates, which are only resistant to aztreonam, produced lower levels of siderophores and inhibited up to two fungal species (including *C. albicans*). This result reinforces what previous studies have reported on antimicrobial-resistant *Pseudomonas* strains, which have a wide repertoire of metabolites with antimicrobial activity (Hemala et al. 2014, Mogrovejo et al. 2020, Paun et al. 2021, Oves et al. 2024).

In contrast with the findings for MD330.9, the other five isolates did not show a direct relationship between siderophores production rate and ability to inhibit filamentous fungal growth. Some isolates with high siderophore production rates, like MD330.10, did not show antibiosis capacity against the filamentous fungi tested, while others with low siderophore productions rates, such as MD332.8, were able to reduce fungal growth. As discussed previously, co-cultivation may trigger a diversity of responses involving the production of secondary metabolites that may inhibit some microorganisms (Harwani et al. 2018). The genomic evaluation performed by antiSMASH focuses on gene clusters involved in secondary metabolite biosynthesis (Blin et al. 2021). Nevertheless, it is possible that our marine *Pseudomonas* sp. isolates promote an antifungal response in co-culture that cannot be predicted by this analysis. So, the pattern of fungal inhibition observed for at least some *Pseudomonas* sp. isolates may not be solely due to siderophore production.

In this work we detected five isolates of the genus *Pseudomonas* as putative producers of the siderophore pyochelin, with the ability of *in vitro* inhibiting unicellular (*C. albicans*) and multicellular fungi. Our data provide unprecedented information regarding the antifungal ability

of antibiotic-resistant, siderophore-producing *Pseudomonas* sp. strains from deep-sea sediments. As these marine strains are not related to pathogenic species (Dias et al., 2024) and can be easily cultured under low-cost conditions, our results indicate that they represent an interesting source for prospecting molecules of both clinical and agricultural interest.


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## COMPETING INTEREST INFORMATION

The authors declare no conflict of interest.

## AUTHORS CONTRIBUTIONS

ASD and RMS were involved in study conception. RMS provided project management. ASD designed and performed experiments and wrote the manuscript GFC and LMR designed and performed experiments. ASD, RMS and SDO analyzed data and provided data interpretation. RMS and SDO provided funding support. All authors were involved in revisions and approval of the manuscript.

## DATA AVALABILITY

The dataset analyzed during the current study are available in the National Center for Biotechnology Information (NCBI), under the accession number: BioProject PRJNA1143896,

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## 6 CONSIDERAÇÕES FINAIS

Os oceanos enfrentam contaminação diária por diferentes poluentes devido resultantes de diversas atividades humanas, o que inclui altas cargas de antimicrobianos (MA *et al*., 2020). A detecção de genes de resistência antimicrobiana (ARGs) em bactérias marinhas tem aumentado significativamente (MAKKAEW *et al*., 2021; CASTAÑO-ORTIZ *et al*., 2023). O uso excessivo de antimicrobianos em vários setores vem acelerando a seleção de microrganismos resistentes (HOLMES *et al*., 2016; ZHUANG *et al*., 2021; OMS, 2023). Tal situação pode chegar em comunidades naturais, levando ao favorecimento de linhagens resistentes e causando a alteração da estrutura das comunidades microbianas (MICHAEL; DOMINEY-HOWES; LABBATE, 2014).

A multirresistência a antimicrobianos (AMR) é atualmente reconhecida como um problema global de saúde pública, no entendo, apenas recentemente pesquisas sobre resistência antimicrobiana em ambientes naturais tem ganhado destaque (SUZUKI *et al*., 2017; SWEILEH; MOH'D MANSOUR, 2020). Simultaneamente, o estudo desses ambientes está crescendo com o objetivo de encontrar e descrever novas moléculas de interesse biotecnológico, especialmente devido ao seu alto potencial na produção de antimicrobianos e outras substâncias valiosas, observado em diversos gêneros microbianos (KAMJAM *et al*., 2017; STINCONE; BRANDELLI, 2020, LEE *et al*., 2023).

Os dados obtidos neste trabalho revelaram a presença de oito bactérias resistentes a diferentes classes de antimicrobianos, assim como evidenciaram a capacidade de tolerância a antimicrobianos por estes isolados quando em condição de biofilme, não sendo possível erradicar dois terços dos biofilmes investigados, tanto em isolados da ordem Bacillalles quanto do gênero *Pseudomonoas*.

O elevado perfil de resistência a aztreonam pelos isolado de *Pseudomonas* foi investigado mais a fundo a partir do sequenciamento genômico destas cepas. Por meio do programa de internacionalização de alunos CAPES-Print foi possível realizar uma parceria com o Centro de Ecologia e Evolução em Sistemas Microbianos Modelos da Universidade de Linnaeus, na Suécia, em colaboração com a professora Hanna Farnelid. Durante este período estes isolados bacterianos foram sequenciados, o que levou à detecção da presença de dois genes de resistência, *mexE* e *mexF*, pertencentes ao operon *mexEF-OprN*, relacionados à expressão de um mecanismo de bomba de efluxo descrito para bactérias do gênero *Pseudomonas*. Avaliando estes

genes foi possível observa que eles apresentam certas semelhanças com genes presentes em outros gêneros de *Pseudomonas*, mas que certos isolados também apresentam variações genéticas exclusivas. Através desta parecia também foi possível investigar a expressão destes genes em condições mais semelhantes ao ambiente natural de onde estas bactérias foram isoladas. No entanto a modulação de nutrientes essenciais para o crescimento destes microrganismos, bem como da temperatura de cultivo, não apresentou diferenças significativas entre si para a maioria dos isolados, levando a conclusão de que estes fatores não são os principais responsáveis por regular diretamente a expressão destes genes nestas bactérias.

Da mesma forma que investigamos o perfil de resistência nos isolados de *Pseudomonas*, a avaliação da sua habilidade antimicrobiana revelou a capacidade destes isolados de inibir o crescimento de um patógeno bacteriano, mas principalmente de fungos de importância clínica e agrícola. A investigação do genoma dos isolados bacterianos revelou a presença de um cluster gênico responsável pela produção de sideróforo, mais especificamente de Piochelina, descrita na literatura como inibidora de diversos organismos eucariotos e procariotos. Além disso, uma alta taxa de produção de sideróforos totais foi detectada para certos isolados. Nestes aspectos o isolado MD330.9 se destacou, por ter sido a única *Pseudomonas* resistente a dois antibióticos, ter apresentado a maior taxa de produção de sideróforos, assim como a capacidade de inibir microrganismos bacterianos, fungos unicelulares e filamentosos. Neste sentido, os dados revelaram um interessante potencial de ação antimicrobiana destas bactérias marinhas, que poderá ser explorado futuramente, seja na área de micologia clínica como para o controle de fungos fitopatógenos na agricultura.

Nossos dados trazem informações novas e relevantes quanto ao perfil de resistência microbiana em ambiente marinho, assim como o potencial de produção de moléculas antimicrobianas por estes isolados. Estes resultados contribuem para o entendimento do impacto antrópico em ecossistemas naturais, especialmente de ambiente marinho profundo, uma área ainda pouco investigada. Além disso, o estudo se encaixa no contexto dos princípios de Saúde Única (“One Health”), investigando aspectos importantes de comunidades microbianas de ecossistemas naturais, que são fundamentais tanto para o equilíbrio e qualidade do meio ambiente, quanto para a saúde da população humana.

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