Universidade de Brasília

Faculdade de Medicina

Programa de Pós-graduação em Patologia Molecular

Osmel Fleitas Martínez

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Para minha família.
Especialmente, para meus pais.
Um filho nunca esquece.

.

“ Ser culto é a única maneira de ser livre”

José Martí

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Resumo

A resistência aos antimicrobianos tem sido reconhecida como umas das maiores ameaças para a saúde humana no século XXI, sendo capaz de causar até 10 milhões de mortes anuais para o ano 2050. A resistência desenvolvida pelas bactérias Gram-negativas tem atingido níveis alarmantes, tornando-se ameaçadora a emergência de bactérias resistentes a múltiplos antibióticos incluindo os de último recurso. *Klebsiella pneumoniae* constitui uma das bactérias Gram-negativas mais resistente aos antibióticos, causando milhões de infecções anuais segundo estimativas. Portanto, novos antimicrobianos efetivos contra este tipo de bactérias são desejados. Nesse sentido, os peptídeos antimicrobianos (PAMs) têm sido indicados como antimicrobianos promissores. Recentemente, o peptídeo PaDBS1R1 foi descrito como um antimicrobiano potente contra bactérias Gram-negativas, incluindo *K. pneumoniae* resistente aos carbapenêmicos. No entanto, pouco conhecimento existe sobre o modo de ação desse peptídeo, assim como, sobre como as bactérias respondem ao desafio com o mesmo. Deste modo, o presente trabalho, apoiado nas vantagens oferecidas pelas abordagens proteômicas, pretende apontar novos elementos que permitam compreender com maior profundidade a interação *K. pneumoniae*-PaDBS1R1. Frente ao desafio com PaDBS1R1, a *K. pneumoniae* sensível ao peptídeo parece remodelar a maquinaria metabólica bacteriana, assim como vários mecanismos defensivos que incluem a produção de cápsula polissacarídea, a modificação de lipopolissacarídeos (LPS), bombas de efluxo e vias de dobramento de proteínas de membrana externa. Por outro lado, PaDBS1R1 parece induzir danos no DNA bacteriano via estresse oxidativo, enquanto a bactéria responde ativando sistemas de reparo do DNA. A exposição continua a concentrações sub-inibitórias do peptídeo parece favorecer a emergência de bactérias resistentes ao mesmo. Por outro lado, a *K. pneumoniae* resistente a PaDBS1R1, parece remodelar também a maquinaria metabólica, assim como múltiplos sistemas defensivos. Particularmente, os sistemas regulatórios PhoPQ, CpxRA e ZraPSR parecem coordenar a reposta anti-PaDBS1R1 via modificações dos LPS e bombas de efluxo. Um elemento distintivo foi o incremento significativo na abundância de proteínas associadas a sistemas anti-estresse oxidativo, sugerindo que uma resposta anti-estresse oxidativo robusta é essencial para a resistência a PaDBS1R1.

Palavras chaves: PaDBS1R1, Peptídeos Antimicrobianos, *Klebsiella pneumoniae*, Proteômica, Resistência Microbiana

Abstract

Antimicrobial resistance is recognized as one of the greatest threats to human health in the 21st century, which could cause up to 10 million of annual deaths by 2050. The resistance developed by Gram-negative bacteria has reached alarming levels, being threatening the emergence of bacteria resistant to multiple antibiotics including those of last resort. *Klebsiella pneumoniae* is one of the most antibiotic-resistant Gram-negative bacteria, causing millions of annual infections according to estimates. Therefore, effective antimicrobials against this type of bacteria are desired. In this regard, antimicrobial peptides (AMPs) have been pointed as promising antimicrobials. Recently, the peptide PaDBS1R1 has been described as a potent antimicrobial against Gram-negative bacteria, including carbapenem-resistant *K. pneumoniae*. However, little knowledge exists about the mode of action of this peptide, as well as how bacteria respond to the challenge with it. Thus, the present work, based on the advantages offered by the proteomic approaches, intends to bring new elements that allow a deeper understanding of the *K. pneumoniae*-PaDBS1R1 interplay. Our results suggest that when challenged with PaDBS1R1, the peptide-sensitive *K. pneumoniae* appears to reshape the bacterial metabolic machinery as well as various defensive mechanisms. These mechanisms included polysaccharide capsule production, lipopolysaccharide (LPS) modification, efflux pumps, and outer membrane protein folding pathways. On the other hand, PaDBS1R1 appears to induce bacterial DNA damage via oxidative stress, while the bacterium responds by activating DNA repair systems. Continuous exposure to sub-inhibitory concentrations of the peptide appear to facilitate the emergence of peptide-resistant bacteria. Moreover, PaDBS1R1-resistant *K. pneumoniae* also appears to reshape the metabolic machinery as well as multiple defensive systems. Specifically, the regulatory systems PhoPQ, CpxRA and ZraPSR appear to coordinate the anti-PaDBS1R1 response via LPS modifications and efflux pumps. A distinctive element of PaDBS1R1-resistant *K. pneumoniae* response was the significant increase in the protein abundances associated with anti-oxidative stress systems, suggesting that a robust anti-stress oxidative response it is essential for PaDBS1R1 resistance.

Keywords: PaDBS1R1, Antimicrobial Peptides, *Klebsiella pneumoniae*, Proteomics, Antimicrobial Resistance

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Introdução

1. Resistência aos antimicrobianos como uma problemática

No início do século XX, com a descoberta e introdução dos compostos antimicrobianos no tratamento das doenças infeciosas, acreditava-se que estas doenças, que correspondiam a primeira causa de morte no mundo naquele momento, não representariam mais um perigo para a saúde humana (Saga and Yamaguchi, 2009; Zaffiri et al., 2012). No entanto, mais de um século depois, as doenças causadas por patógenos ainda constituem uma das principais ameaças à saúde humana (WHO, 2018b; Bloom and Cadarette, 2019). Múltiplos fatores têm contribuído a esta situação, entre os quais encontram-se a resistência dos patógenos aos compostos antimicrobianos (Miller-Petrie et al., 2017). A resistência aos antimicrobianos merece especial atenção, pois a propagação desta tem sido tal, que no Relatório Global sobre Vigilância da Resistência Antimicrobiana da Organização Mundial da Saúde (2014) foi declarado que : "*Uma era pós-antibiótico, na qual infecções comuns e ferimentos leves podem matar, longe de ser uma fantasia apocalítica tem virado uma possibilidade real no século XXI*" (WHO, 2014b).

Estima-se que anualmente os patógenos resistentes aos antimicrobianos causam a nível mundial aproximadamente 700 000 mortes e que estas podem chegar a 10 milhões no ano de 2050 (O'Neill, 2016). No entanto, existem questionamentos acerca da confiabilidade desta estimativa (de Kraker et al., 2016). Além disso, também é alarmante a estatística que associa 214 000 mortes anuais por septicemia neonatal a patógenos resistentes aos antimicrobianos, assim como a emergência de bactérias multidroga resistentes e pandroga resistentes as quais apresentam resistência até aos antibióticos de último recurso como carbapenêmicos, polimixinas, tigeciclina, daptomicina e vancomicina (Tran et al., 2015; Faron et al., 2016; Kumar, 2016; Laxminarayan et al., 2016; Mohapatra et al., 2018; Zhang et al., 2018; Moffatt et al., 2019; Mulani et al., 2019). A resistência aos antimicrobianos, não somente causa prejuízo à saúde por meio de doenças infecciosas, mas também compromete certos procedimentos médicos, como as cirurgias, transplante de órgãos, quimioterapia contra o câncer e tratamento de doenças imunossupressora (Jasovský et al., 2016; WHO, 2018a). Além disso, a resistência aos

antimicrobianos tem repercussão econômica. A estimativa de custos relacionados à resistência antimicrobiana, se as devidas ações não são tomadas rapidamente, são de 100 trilhões de dólares para o ano de 2050 (O'Neill, 2016; Piddock, 2016).

2. Resistência em bactérias Gram-negativas

A resistência aos antimicrobianos pode ser uma consequência evolutiva da pressão seletiva exercida por diferentes agentes antimicrobianos sobre os microrganismos. Por tanto, a mesma pode ser observada em diferentes tipos de patógenos (Amabile-Cuevas, 2010). A resistência bacteriana aos antimicrobianos se caracteriza por emergir com relativa facilidade, expandindo-se relativamente rápido e tendendo a persistir nas populações bacterianas, o que torna complexa e desafiadora a procura por soluções efetivas para conter a resistência das bactérias. Diversos fatores como o uso indiscriminado das drogas antibacterianas, a diversidade de habitats das bactérias, a plasticidade genética e variedade de mecanismos de propagação de genes de resistência, tem contribuído à essas características da resistência bacteriana (Amábile-Cuevas, 2010; Andersson and Hughes, 2014; Partridge et al., 2018).

As bactérias Gram-negativas têm-se tornado uma problemática em relação à resistência aos antimicrobianos. Estas bactérias apresentam uma fácil disseminação dos genes que determinam a resistência, tanto entre bactérias da mesma espécie, como entre espécies diferentes (Wyres and Holt, 2018). Em algumas destas bactérias, como por exemplo os membros da família *Enterobacteriaceae*, a disseminação pode ser silenciosa, devido a elas formarem parte da microbiota intestinal e, portanto, a colonização dos indivíduos pode acontecer sem sintomas, favorecendo para que estes indivíduos atuem como um reservatório (Vasoo et al., 2015).

A organização estrutural, mais especificamente a presença de uma membrana externa que atua como barreira impermeável aos antibióticos, torna difícil o desenvolvimento de antimicrobianos que consigam penetrar ao interior das bactérias Gram-negativas (Silver, 2016; Richter and Hergenrother, 2019). Além de funcionar como uma barreira à passagem dos antimicrobianos, a membrana externa providencia as bactérias Gram-negativas de mecanismos adicionais de resistência e propagação de genes de resistência mediante a formação de vesículas de membrana externa (Schwechheimer and Kuehn, 2015). As diferenças nas propriedades físico-químicas da membrana externa e da membrana citoplasmática fazem com que estas apresentem propriedades de tamisado

ortogonais dificultando ainda mais a penetração dos antimicrobianos ao interior da bactéria (Silver, 2016; Richter and Hergenrother, 2019). Tudo isto, em combinação com a presença de bombas de efluxos ``promíscuas´´ que expelem uma grande variedade de antimicrobianos ao meio extracelular fazem realmente desafiante o desenvolvimento de antimicrobianos efetivos contra as bactérias Gram-negativas (Blair et al., 2014).

O crescente problema da resistência bacteriana aos antibióticos tem obrigado à Organização Mundial da Saúde (OMS) a definir prioridades para a descoberta e desenvolvimento de novos antimicrobianos contra as bactérias resistentes. Nesse sentido, foram definidos três grupos de prioridades incluindo patógenos de prioridade crítica, elevada e média (Tacconelli et al., 2018). O grupo de patógenos que compuseram o nível de prioridade crítico, foram bactérias Gram-negativas, mostrando sua séria ameaça na atualidade. Especificamente no grupo de prioridade crítica, encontra-se as *Enterobacteriaceae* resistentes a carbapenemicos e cefalosporinas de terceira geração. Na atualidade as *Enterobacteriaceae* resistentes a carbapenêmicos são reconhecidas como uma séria ameaça à saúde humana pelos níveis de resistência desenvolvidos, assim como o aumento da mortalidade associada às infeções com esses patógenos resistentes (Logan and Weinstein, 2017). Nesse sentido um dos membros mais notórios dessa família é *Klebsiella pneumoniae.*

3. *Klebsiella pneumoniae*

Klebsiella pneumoniae consiste em uma bactéria Gram-negativa que pertence família *Enterobacteriaceae* e está associada a infecções adquiridas na comunidade (infecções do trato urinário, pneumonia e abscessos hepáticos), assim como a infeções nosocomiais (infeções sanguíneas e urinárias associadas a cateteres, pneumonia, infecção dos sítios cirúrgicos) (Shon et al., 2013; Paczosa and Mecsas, 2016; Calfee, 2017). Esta bactéria tem ganhado notoriedade por apresentar elevados níveis de resistência aos antibióticos, emergindo cepas multidroga resistentes e pan-droga resistentes, que causam infeções com elevada mortalidade (Kohler et al., 2017; Xu et al., 2017; Karakonstantis et al., 2019).

No início dos anos 1970, *Klebsiella pneumoniae* se estabeleceu no ambiente hospitalar e tornou-se um agente etiológico líder das infeções nosocomiais devido a sua capacidade de colonização, persistência e disseminação, assim como à facilidade de captar plasmídeos com genes de resistência a antibióticos (Tzouvelekis et al., 2012).

Posteriormente, se diversificou o espectro de resistência para antibióticos como aminoglicosídeos e β-lactâmicos, entre outros o que fez dos carbapenêmicos antimicrobianos importantes no tratamento das infecções causadas por *K. pneumoniae* (Tzouvelekis et al., 2012). No entanto, surgiram cepas de *K. pneumoniae* que expressavam carbapenemases, tornando-lhes resistentes aos carbapenêmicos. A produção de carbapenemases, assim como outros mecanismos, como bombas de efluxo e variações na permeabilidade, têm feito a resistência a carbapenêmicos problemática, sendo que a mesma tem se espalhado globalmente atingindo níveis alarmantes (Yigit et al., 2001; Tzouvelekis et al., 2012; Pitout et al., 2015).

Com esta situação, as polimixinas (colistina e polimixina B) passaram a ser as drogas para o tratamento das infeções por *K. pneumoniae* resistente aos carbapenêmicos, assim como outras bactérias Gram-negativas resistentes a múltiplas drogas. No entanto, a resistência a estes antimicrobianos tem sido reportada e as estatísticas mostram uma tendência ao aumento (Srinivas and Rivard, 2017; Li et al., 2019). Esta resistência às polimixinas era determinada basicamente por mutações nos genes presentes no cromossomo bacteriano, porém, recentemente, foi reportado na China cepas de *Escherichia coli* e *K. pneumoniae* que carregavam plasmídeos que portam o gene *mcr-1*, o qual confere resistência à colistina (Liu et al., 2016). Desde então, a presença de *mcr-1* tem sido descrita em isolados bacterianos de mais de 30 países em todos os continentes, assim como novas variantes do gene (*mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8* e *mcr-9*) (Carattoli et al., 2017; Yin et al., 2017; Dalmolin et al., 2018; Sun et al., 2018; Carroll et al., 2019).

Outra situação que foi alarmante, foi o aparecimento de isolados que apresentam a coexistência de *mcr-1* com genes de resistência a carbapenêmicos, o que favorece a emergência de cepas pan-resistentes (Wang et al., 2017; Dalmolin et al., 2018). De fato, já tem sido reportado a morte de pacientes por cepas de *K. pneumoniae* pan-droga resistentes. Em 13 de janeiro de 2017, os Centros para o Controle e Prevenção de Doenças reportaram a morte de uma mulher em Nevada, Estados Unidos, por uma cepa de *K. pneumoniae* resistente a todos antibióticos disponíveis no pais (Ofori-Asenso, 2017). Previamente, em Victoria, Austrália, no ano de 2016, já tinha sido reportada a morte de um homem por uma cepa de *Klebsiella pneumoniae* resistente, também, a todos os antibióticos disponíveis no país (Ofori-Asenso, 2017).

Particularmente no Brasil, *K. pneumoniae* constitui um dos principais agentes etiológicos das infecções primárias de corrente sanguínea associadas ao uso de cateter venoso central em unidades de terapia intensiva, sendo que uma porcentagem considerável desses isolados clínicos apresentam um fenótipo de resistência a β-lactâmicos e carbapenêmicos (ANVISA, 2016; 2017; 2019) . Também, tem sido reportado o isolamento de cepas de origem clinica resistentes à polimixina B e colistina, os quais são considerados antimicrobianos de último recurso (Sampaio and Gales, 2016; Aires et al., 2017).

4. Peptídeos antimicrobianos

Entre as alternativas que estão sendo exploradas como antimicrobianos contra as bactérias resistentes aos antibióticos encontra-se os peptídeos antimicrobianos (PAMs). Os PAMs são biomoléculas que de forma geral são catiônicas, estão compostas por sequências de aminoácidos que variam entre 10-50 resíduos e são de natureza anfipática apresentando uma certa proporção de resíduos de aminoácidos hidrofóbicos (Harris et al., 2009; Mahlapuu et al., 2016b). Estes peptídeos podem ser sintetizados via ribossomo ou por uma via independente do ribossomo, a qual implica complexos enzimáticos (Mahlapuu et al., 2016b; Moravej et al., 2018).

Existem diferentes critérios para a classificação dos PAMs, sendo o baseado na estrutura tridimensional um dos mais utilizados. Nessa acepção, os PAMs podem se classificar de forma geral nos grupos estruturais folhas β, α-hélice, αβ e estendido/ flexível (Wang, 2015; Kumar et al., 2018). Além da atividade antibacteriana, os PAMs podem apresentar uma diversidade de atividades biológicas que incluem atividade antiviral, antifúngica, antiparasitária, antiprotozoária, antibiofilme, anticâncer, entre outras (Wang et al., 2016; Felício et al., 2017; Giovati et al., 2018; Boas et al., 2019). Além disso, potencialmente poderiam ser usados como biomarcadores de doenças e são importantes na modulação do sistema imune (Hancock et al., 2016; Silva et al., 2018).

4.1. Mecanismos de ação antibacteriana dos peptídeos antimicrobianos

Os PAMs podem exercer sua atividade microbicida atuando sobre diferentes alvos bacterianos. Esses alvos moleculares podem se encontrar associados a membranas bacterianas ou no citoplasma (DNA, RNA ou proteínas), portanto estes peptídeos apresentam uma diversidade de mecanismos de ação que lhe permitem exercer a atividade antimicrobiana (Mahlapuu et al., 2016b; Kumar et al., 2018). A atividade antimicrobiana

dirigida aos alvos associados a membrana se baseia fundamentalmente (mas não exclusivamente) na permeabilização da membrana e no deslocamento dos componentes estruturais desta, afetando desta forma a integridade estrutural da membrana, assim como processos metabólicos associados à membrana. Enquanto aos alvos intracelulares, a atividade antimicrobiana recai fundamentalmente na inibição da síntese de proteínas e ácidos nucleicos, na inibição da divisão celular, assim como na inibição de proteases e chaperonas (Rashid et al., 2016; Le et al., 2017b).

A membrana citoplasmática bacteriana por si é um dos alvos mais notórios dos PAMs, provavelmente devido à composição química da mesma já que é enriquecida em fosfolipídios aniônicos (ex. fosfatidilglicerol, cardiolipina e fosfatidilserina) e apresenta um intenso potencial transmembrana negativo o que poderia favorecer a atração e interações eletrostáticas com os peptídeos (Yeaman and Yount, 2003; Mahlapuu et al., 2016b). Para atingir a membrana citoplasmática, os PAMs primeiramente devem transpassar componentes estruturais da superfície bacteriana que protegem à membrana citoplasmática. Esses componentes estruturais da superfície diferem dependendo do tipo de bactéria (Gram-positiva ou Gram-negativa).

No caso das bactérias Gram-positivas, a estrutura da superfície é uma parede celular constituída principalmente por ácidos lipoteicoicos os quais presentam um caráter aniônico e capas de peptidoglicano (40-80 nm) de estrutura porosa. No entanto, esta estrutura parece não interferir no caminho dos PAMs até a membrana citoplasmática (Malanovic and Lohner, 2016). Em referência às bactérias Gram-negativas, a estrutura da superfície compreende uma membrana externa assimétrica constituída na camada externa principalmente por lipopolissacarídeos (LPS) estabilizados por cátions Mg^{2+} e Ca^{2+} (Malanovic and Lohner, 2016). Tem sido proposto que os PAMs deslocam os cátions Mg^{2+} e Ca^{2+} que estabilizam os LPS, produzindo dessa forma, desestabilização da membrana externa que em conjunto com a acumulação de peptídeos na sua superfície faz com que se formem quebras transitórias na membrana externa permitindo a translocação dos PAMs (Hancock, 1997; Hancock and Chapple, 1999). Após os PAMs passarem a membrana externa das bactérias Gram-negativas, eles têm que traspassar a camada de peptidoglicano (7-8 nm) para poder atingir a membrana citoplasmática. Similarmente ao descrito para as bactérias Gram-positivas, o peptidoglicano parece não interferir no caminho dos PAMs até a membrana citoplasmática (Malanovic and Lohner, 2016).

Uma vez que os peptídeos passam as estruturas da superfície bacteriana e atingem a membrana citoplasmática, acontecem uma série de eventos moleculares na interação peptídeo-membrana citoplasmática que finalizam com a permeabilização da membrana (no caso de mecanismo membranolítico) e, consequentemente, há perda de íons e metabólitos o que leva a, desregulação osmótica, perda do potencial de membrana e uma destruição da membrana, provocando a morte da bactéria (Lee et al., 2016; Mahlapuu et al., 2016b). Esses eventos moleculares na interação peptídeo-membrana citoplasmática, basicamente compreendem a interação inicial do peptídeo com a membrana via interações eletrostáticas seguido da formação de estruturas secundárias dos peptídeos. Uma vez que esses peptídeos têm atingido uma concentração limiar se multimerizam e desencadeiam a permeabilização da membrana (Lee et al., 2016; Moravej et al., 2018). Nesse sentido vários modelos moleculares têm sido propostos para explicar o efeito dos PAMs sobre as membranas sendo os modelos de barril formador de poros, poro toroidal e carpete os mais estudados (Lee et al., 2016; Kumar et al., 2018) (Figura 1).

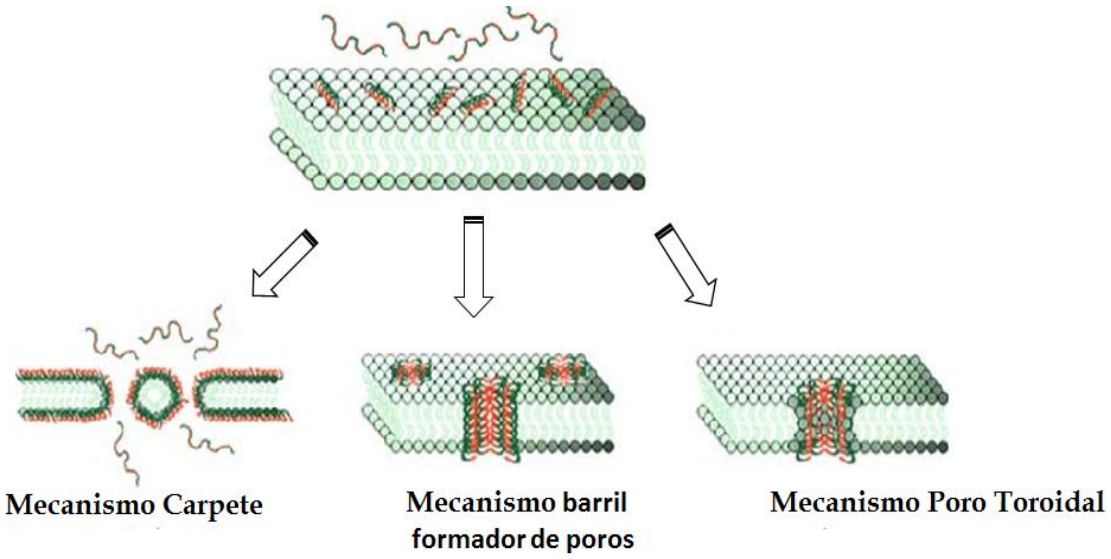

Figura 1. Interação dos peptídeos antimicrobianos com a membrana citoplasmática.

Figura adaptada de Huang et al. (2010).

No modelo barril formador de poros, após atingir a concentração limiar, os peptídeos multimerizam e se insertam na membrana formando poros revestido pelos peptídeos, onde a zonas hidrofóbicas estão interagindo com a parte alifática dos lipídeos da membrana, enquanto as zonas hidrofílicas estão para o interior do poro. No modelo poro toroidal, os peptídeos, após da interação inicial com a membrana e adopção da estrutura secundária, induzem a formação de curvatura na membrana, sendo que depois que os peptídeos atingem a concentração limiar e multimerizam o interior dos poros formados fica revestido por peptídeos e a cabeça polar dos fosfolipídios. No modelo carpete os peptídeos se colocam paralelos à membrana e produzem um efeito parecido aos dos detergentes com a consequente formação de micelas e destruição da membrana (Lee et al., 2016; Mahlapuu et al., 2016b).

O deslocamento de componentes estruturais da membrana também é outro dos mecanismos envolvidos na perturbação da membrana citoplasmática pelos PAMs. Inicialmente achava-se que a membrana dos procariontes era bem mais simples que a dos eucariontes, mas com a recente identificação de domínios funcionais de membranas nos primeiros, esta percepção mudou. Estes domínios apresentam uma composição lipídica e de proteínas características tornando eles componentes celulares especializados em certas funções como sinalização e secreção de proteínas (Bramkamp and Lopez, 2015b; Lopez and Koch, 2017). Por outro lado, é cada vez maior a literatura que mostra que a interação dos PAMs pode acontecer em sítios discretos da membrana, especificamente nestes domínios funcionais de membrana (Rashid et al., 2016). A interação dos peptídeos com estes domínios pode induzir a deslocação das proteínas e lipídeos presentes com a subsequente perda da estrutura do domínio assim com sua funcionalidade (Rashid et al., 2016).

Diferentes estudos sustentam este modo de ação dos peptídeos antimicrobianos sobre alvos associados às membranas. *Streptococcus pyogenes* contêm um domínio de membrana enriquecido em fosfolipídios aniônicos (denominado ExPortal) onde se encontra localizada a maquinaria proteica necessária para a secreção e maduração de fatores de virulência (Rosch and Caparon, 2004; Rosch et al., 2007). Tem sido demostrado que polimixina B e HNP-1 (*human neutrophil peptide*-1) interagem com ExPortal e provocam a redistribuição dos componentes do domínio o que afeta a organização estrutural do domínio e portanto sua funcionalidade (Vega and Caparon, 2012). Posteriormente, foi mostrado que cepas de *S. pyogenes* resistentes a polimixina B

mostravam uma grande estabilidade do ExPortal (Port et al., 2014). Similar a *S. pyogenes*, em *Enterococcus faecalis* a maquinaria molecular envolvida na secreção e montagem de fatores de virulência parece se localizar em microdomínios de membrana enriquecidos em lipídios aniônicos. Esses microdomínios podem ser alvos das β-defensinas humanas, as quais interagem com os microdomínios desintegrando-os e, consequentemente, deslocam a maquinaria proteica presente neles a qual é necessária para a secreção e montagem de fatores de virulência (Kandaswamy et al., 2013).

Em *Bacillus subtilis* a integração do peptídeo MP196 (enriquecido em triptofano e argininas) provocou a dispersão das proteínas MurG e citocromo C (proteínas periféricas de membrana) afetando a respiração celular e a biossínteses da parede celular (Wenzel et al., 2014a). Recentemente, também foi mostrado que os PAMs TC19, TC84 e BP2 induziram um estresse na membrana de *B. subtilis*, que provocou a deslocação de proteínas associadas a esta. Essas proteínas deslocadas estavam envolvidas na biossíntese da parede celular, na biossíntese da membrana, na produção de ATP, ciclo de Krebs e divisão celular. Além disso, foi observado que o peptídeo LL-37 também induzia deslocamento de proteínas associadas a membrana e que estavam envolvidas na biossíntese da parede celular (Omardien et al., 2018a).

Um estudo recente mostrou que em *B. subtilis* os peptídeos TC19, TC84 e BP2 afetavam o empacotamento dos lipídeos da membrana criando domínios de membrana com elevada fluidez os quais eram permeáveis. Adicionalmente, TC19, TC84 e BP2 e LL-37 também induziam a formação desses domínios de membrana em *Staphylococcus aureus* (Omardien et al., 2018b). Outro peptídeo que induz mudanças na fluidez da membrana é o peptídeo cíclico cWFW. Este peptídeo desencadeia alterações estruturais da membrana citoplasmática de *B. subtilis* diminuindo a fluidez da mesma e induzindo a formação de microdomínios nos quais são segregadas proteínas associada a membrana. Também aconteceu a desintegração de proteínas associadas à membrana. A deslocação das proteínas associadas a membrana afetou a biossínteses da parede celular assim como a regulação das enzimas autolíticas da parede celular, consequentemente a autólises bacteriana foi incrementada (Scheinpflug et al., 2017).

Um outro alvo dos peptídeos antimicrobianos associado a membrana é o lipídeo II, o qual constitui um intermediário fundamental na síntese da camada de peptidoglicano que compõe a parede celular (Breukink and de Kruijff, 2006). Nesse sentido, uma grande variedade de antimicrobianos de natureza peptídica incluindo glicopeptídeos,

lipoglicopeptídeos, depsipeptídeos, lantibioticos, defensinas, bacteriocinas e outros podem interagir e inibir o lipídeo II (Grein et al., 2019). Um mecanismo de inibição da síntese da parede celular independente da união ao lipídeo II tem sido proposto para lantibióticos como nisina e Pep 5. Neste, os peptídeos liberam enzimas autolíticas de sua associação com os ácidos teicoicos. Esta liberação ativa as enzimas para a lise da parede celular (Bierbaum and Sahl, 1987).

Embora que a atividade perturbadora de membrana seja o mecanismo de ação antimicrobiano mais estudado para os PAMs, outros mecanismos têm sido descritos. Os PAMs podem translocar a membrana citoplasmática e ganhar acesso ao interior celular onde participam inibindo uma série de processos celulares. Um desses processos inibidos pelos PAMs é a biossínteses de ácidos nucleicos. A inibição da biossíntese dos ácidos nucleicos compreende a inibição da replicação e da transcrição e basicamente acontece mediante a união direta dos peptídeos às moléculas de DNA ou RNA ou mediante a inibição de enzimas que participam nestes processos (Le et al., 2017b).

Uma das consequências da união do peptídeo ao DNA pode ser a formação de um complexo estável que dificulta o desenrolamento do DNA, um requisito importante para acontecer a replicação e a transcrição. Nesse sentido, foi mostrado que o peptídeo indolicidina pode se unir a dupla hélice do DNA, envolvendo-a, dificultando seu desenrolamento (Ghosh et al., 2014).

Uma outra consequência da união dos PAMs ao DNA poderia ser a produção de danos na molécula de DNA. Recentemente, foi observado que a indolicidina e uma variante sintética desta (In-58), induziam uma resposta SOS em *Escherichia coli* MG1655 (bactéria repórter, pcolD´::lux) sugerindo que os peptídeos poderiam produzir danos no DNA e/ou inibir a replicação (Vasilchenko et al., 2017). Adicionalmente, tem sido mostrado que a união de LL-37 ao DNA em *Pseudomonas aeruginosa* induz mutagêneses via a polimerase DinB. O estudo sugere um mecanismo ação no qual a união de LL-37 ao DNA bloqueia a replicação mediada pela polimerase PolIII o qual induze a replicação de translesão dependente de DinB (Limoli et al., 2014).

Como previamente exposto, outro dos alvos na inibição da biossíntese do DNA e RNA são as enzimas envoltas nesses processos (Le et al., 2017b). Nesse sentido, tem sido mostrado que a microcina B17 estabiliza um complexo covalente entre o DNA e a enzima DNA girase interferindo com a replicação (Heddle et al., 2001; Pierrat and

Maxwell, 2005). Outros peptídeos que participam da inibição da síntese de ácidos nucleicos são a microcina J25 e capistruina, os quais participam especificamente na inibição da transcrição. O alvo desses peptídeos é a RNA polimerase dependente de DNA, sendo que ambos peptídeos se unem ao canal secundário da enzima afetando o dobramento da enzima, especificamente o dobramento do *trigger-loop* que é necessário para uma catalise eficiente. No caso de microcina J25 a união interfere com o fluxo de substrato ao sitio catalítico enquanto o capistruina parece exercer seu efeito inibitório via bloqueio do dobramento do *trigger-loop* (Adelman et al., 2004; Braffman et al., 2019).

Outro dos processos intracelulares, que é alvo dos PAMs, é a biossínteses de proteínas. Especialmente, PAMs enriquecidos no amino ácido prolina apresentam uma grande atividade inibitória da biossíntese de proteínas. Estes peptídeos podem ser transportados ao interior da bactéria mediante transportadores específicos (SmbA e YjiL-MdtM) e uma vez translocados unem-se a seu alvo no citoplasma bacteriano, sendo este alvo o ribossomo, o qual constitui uma organela essencial na síntese de proteínas (Krizsan et al., 2015; Wilson et al., 2015). Estes peptídeos parecem inibir a transição do início da tradução para a elongação, pois eles se posicionam de jeito tal no ribossomo que através de seu N-terminal interferem com a acomodação do aminoacil-RNAt (Gagnon et al., 2016; Mardirossian et al., 2018). Outro mecanismo diferente de inibição da tradução que implica também a união ao ribossomo e é mediada por PAMs enriquecidos em prolina foi descrito por Florin *et al.,* 2017. Estes autores mostraram que Api-137 um derivado do peptídeo antimicrobiano natural apidaecina 1b inibe a síntese proteínas na etapa de terminação da tradução. O peptídeo une-se ao ribossomo uma vez que é liberado o polipeptídio recém-sintetizado e apressa os fatores de liberação RF-1 e RF-2 (necessários para a terminação da tradução) neste sítio, isto trai como consequência que o pool celular destes fatores de liberação diminui e não podem ser utilizados por outros ribossomos onde acontece a síntese dos polipeptídios, mas não podem ser liberados dos ribossomos (Florin et al., 2017).

Além dos ribossomos, os PAMs enriquecidos em prolina podem interferir com as proteínas chaperonas, principalmente DnaK, uma das chaperonas mais representativa (Rozgonyi et al., 2009). Esta chaperona participa no dobramento co-traducional e pós-traducional de uma grande quantidade de proteínas tanto citoplasmáticas como de outras localizações celulares e tem um papel central na rede molecular de chaperonas (Calloni et al., 2012). Em um estudo, Chesnokova *et al.,* 2004 mostraram que o peptídeo L-

pyrrhocoricina (proveniente de inseto) atuava como um inibidor competitivo para DnaK pois unia-se ao sítio de união ao substrato na proteína e impedia a união dos outros substratos (Chesnokova et al., 2004). Posteriormente, Liebscher e Roujeinikova, 2009 mediante cristalografia de raios-X mostraram que peptídeos derivados de pyrrhocoricina atuam como inibidores competitivos já que se unem ao sítio de união dos substratos, além de atuar como inibidores alostéricos (Liebscher and Roujeinikova, 2009). Outro estudo mostrou que um peptídeo derivado de oncocina (PAM enriquecido em prolina) tinha como alvo a DnaK e se unia a esta proteína mediante o sitio de união a substratos (Knappe et al., 2011).

A inibição de proteases dos patógenos é outro dos mecanismos de ação descrito para os peptídeos antimicrobianos. Gusman *et al.*, 2001 mostraram que a histatina 5 inibia competitivamente à enzima clostripaina de *Clostridium histolyticum* e presentava uma atividade inibitória mais potente que o leupeptina (Gusman et al., 2001a). Além disso, a histatina tem mostrado atividade inibitória sobre as enzimas Arg-gingipaina e Lys-gingipaina (podem estar associadas a membrana) de *Porphyromonas gingivalis* (Gusman et al., 2001b). Outro peptídeo antimicrobiano com atividade inibitória de peptidases é o eNAP-2, este peptídeo mostrou atividade inibitória da enzima subtilisina A de *Bacillus licheniformes* e proteinase K do fungo *Tritirachium album* (Couto et al., 1993).

Por outro lado, os PAMs também têm sido associados à inibição da divisão celular. Estudos inicias com os peptídeos microcina J25 e diptericina mostraram que o desafio de *E. coli* com eles produzia mudanças na morfologia das células, as quais adquiriam um aspecto filamentoso sugerindo que os peptídeos provocavam uma inibição da divisão celular (Ishikawa et al., 1992; Salomon and Farías, 1992). Posteriormente, outros estudos mostraram que indolicidina e α-defensina 5 humana oxidada produziam efeitos similares em *E. coli* sugerindo que também produzem inibição da divisão celular (Subbalakshmi and Sitaram, 1998; Chileveru et al., 2015). Especificamente, a α-defensina 5 humana oxidada entrava no citoplasma bacteriano e se localizava no sítio de divisão celular e polos celulares. Além da elongação da bactéria, a α-defensina 5 humana oxidada também induziu formação de borbulhas (no sítio de divisão celular e polos) e aglomerados. Esses efeitos foram observados em outras bactérias como *P. aeruginosa*, *K. pneumoniae* e *Acinetobacter baumannii* (Chileveru et al., 2015). Adicionalmente, tem sido mostrado que mediante sinalização através do sistema de dois-componentes PhoPQ, os PAMs podem induzir o bloqueio da divisão celular e a formação de uma morfologia

filamentosa em *E. coli*. A ativação de PhoPQ promove a produção da proteína QueE, a qual via interação com componentes do divisoma parece bloquear a septação (Yadavalli et al., 2016).

Recentemente, baseados em microscopia de fluorescência de super-resolução Zhu e colaboradores (2019) mostraram que a entrada do peptídeo LL-37, no citoplasma de *E. coli*, provoca uma rigidificação do mesmo. Nesse sentido, a entrada de uma grande quantidade de moléculas de LL-37 no citoplasma após a permeabilização da membrana citoplasmática faz com que provavelmente se forme uma rede de interações eletrostática entre o DNA e os poliribossomos (os quais tem caráter polianiônico e normalmente se repelem) mediada pelo o peptídeo. Os efeitos dessa pseudo-reticulação entre o DNA cromossomal e os poliribossomos é a rigidificação do citoplasma, o qual perturba a difusão dos componentes intracelulares assim como a homeostase intracelular (Zhu et al., 2019). Um outro estudo também sugeriu que os PAMs podem afetar a homeostase intracelular bacteriana, já que os peptídeos poderiam provocar a floculação de poliâniones citoplasmáticos afetando-se dessa forma a organização estrutural intracitoplasmática, a difusão dos componentes citoplasmáticos assim como os processos bioquímicos que acontecem no citoplasma (Chongsiriwatana et al., 2017).

4.2. Peptídeos antimicrobianos como agentes terapêuticos promissores

Os PAMs têm emergido como agentes antimicrobianos promissores para introduzir-se na terapêutica. Estas biomoléculas são elementos centrais do sistema imune inato e constituem um dos mecanismos de defesa mais antigos conhecidos. Em alguns organismos o sistema imune inato constitui a única fonte de defesa sugerindo isto que os mecanismos de defesas baseados em peptídeos antimicrobianos são efetivos (Dutta and Das, 2016). A atividade antimicrobiana destes peptídeos é exercida de maneira rápida, na faixa $\leq \mu M$ e é de amplo espectro (Mahlapuu et al., 2016b; Moravej et al., 2018). Essa atividade microbicida de amplo espectro os fazem atrativos para o tratamento de infeções polimicrobianas (Mahlapuu et al., 2016b).

Os PAMs podem exercer sua atividade microbicida mediante vários mecanismos de ação, podem atuar sobre múltiplos alvos e afetar simultaneamente vários processos bioquímicos o que em teoria limitaria o desenvolvimento de resistência antimicrobiana pelos patógenos (Mahlapuu et al., 2016b; Chongsiriwatana et al., 2017; Moravej et al., 2018; Zhu et al., 2019). Nesse sentido, estudos de evolução de resistência sugerem que a

possibilidade de surgimento de resistência aos PAMs é menor que aos antibióticos convencionais, além de que bactérias resistentes aos antibióticos tendem ser sensíveis aos PAMs (Lázár et al., 2018; Spohn et al., 2019). Outros elementos que suportam uma menor probabilidade de emergência de resistência aos PAMs, em comparação aos antibióticos convencionais, é que aparentemente os PAMs são menos propensos a ativar mecanismos que promovem diversidade genética como a resposta SOS, resposta ao estresse controlada por RpoS ou recombinação do DNA (Rodríguez-Rojas et al., 2014; Rodríguez-Rojas et al., 2018). Adicionalmente, em comparação aos antibióticos, as propriedades farmacodinâmicas dos PAMs têm sido associadas com uma menor probabilidade de emergência de resistência (Yu et al., 2018).

Outro fator a favor dos PAMs como agentes terapêuticos, poderia ser que a atividade microbicida deles aparentemente afeta marginalmente a microbiota intestinal humana, já que as bactérias que a compõem mostram elevada resistência aos PAMs (Cullen et al., 2015; Kintses et al., 2019). Além disso, aparentemente a transferência horizontal genes de resistência aos PAMs de espécies bacterianas da microbiota intestinal para bactéria susceptíveis parece ser limitada (Kintses et al., 2019; Spohn et al., 2019). Adicionalmente, os PAMs apresentam atividade moduladora do sistema imune, podendo potencializar desta forma a resposta imune. Além disso, os PAMs também apresentam atividade anti-inflamatória, antitumoral, angiogênica, facilitam a cicatrização de feridas, podem neutralizar endo-toxinas bacterianas e atuar sinergicamente com os antibióticos (Dutta and Das, 2016; Piotrowska et al., 2017; Moravej et al., 2018).

Apesar das potencialidades dos PAMs como agentes terapêuticos, ainda existem questões a resolver para que haja uma implementação efetiva dos mesmos na prática clínica. Essas limitações estão vinculadas à estabilidade e biodisponibilidade dos PAMs *in vivo*, a citotoxicidade assim como ao custo de produção (Kang et al., 2014; Piotrowska et al., 2017). Além disso, embora a probabilidade de emergência de resistências para os PAMs seja menor que para os antibióticos, as bactérias podem desenvolver mecanismos de resistência contra os PAMs (Maria-Neto et al., 2015; Joo et al., 2016a).

4.3 PaDBS1R1

O peptídeo PaDBS1R1, é um peptídeo antimicrobiano obtido mediante desenhado racional usando o algoritmo *Joker* desenvolvido por Porto et al. (2018). Para o desenho do peptídeo foi usada como sequência primaria um fragmento da proteína do ribossomo

L39E da arqueia hipertermófila *Pyrobaculum aerophilum* (Porto et al., 2018; Irazazabal et al., 2019). Alguma das características físico-químicas de PaDBS1R1 incluem hidrofobicidade de 0.60, um momento hidrofóbico de 1.60, propensão para a formação de hélice de 0.46 e carga liquida de 5^+ (Irazazabal, 2016). PaDBDS1R1 e sua variante amidada no C-terminal (PaDBS1R1-NH_2) se mostraram como potentes peptídeos antimicrobianos com valores de concentração inibitória mínima (MIC) na faixa dos µM para bactérias Gram-negativas e Gram-positivas, incluindo isolados clínicos de *K. pneumoniae* resistente a carbapenêmicos e *S. aureus* resistente a meticilina (Irazazabal et al., 2019). Interessantemente, PaDBS1R1 também mostrou uma atividade bactericida rápida contra bactérias Gram-negativas (*E. coli*) em comparação a bactérias Gram-positivas (*S. aureus*) (Irazazabal et al., 2019). O peptídeo PaDBS1R1 parece exercer uma atividade membranolítica já que permeabiliza, despolariza e deforma as membranas bacterianas (Irazazabal et al., 2019).

Ensaios com vesículas lipídicas sugeriram que PaDBS1R1 apresenta uma maior preferência de interação pelas vesículas que simulavam a membrana de bactérias Gram-negativas das que simulavam as membranas das bactérias Gram-positivas e células humanas. Experimentos de dicroísmo circular e ressonância magnética nuclear, sugeriram que PaDBS1R1 adotava uma estrutura secundária de α-hélice anfipática (Figura 2). Nesse sentido, tem sido proposto que PaDBS1R1 em contato com a membrana adota uma estrutura α-hélice anfipática que medeia interações eletrostática e hidrofóbicas do peptídeo com a membrana e a subsequente inserção do peptídeo na membrana desencadeando a permeabilização e despolarização da mesma (Irazazabal et al., 2019).

A aparente atividade microbicida preferencial contra bactérias Gram-negativas de PaDBS1R1 faz dele um peptídeo promissor, já que como exposto previamente, o desenvolvimento de antimicrobianos efetivos contra bactérias Gram-negativas é desafiador. Portanto, é necessário aprofundar sobre diferentes aspectos de PaDBS1R1 visando a implementação do mesmo como opção terapêutica contra infeções bacterianas num futuro. É a atividade membranolítica o único mecanismo bactericida desenvolvido pelo peptídeo? É efetivo o uso do PaDBS1R1 *in vivo*? Para quais tipo de infecções poderia ser efetivo PaDBS1R1? Pode ser usado PaDBS1R1 como adjuvante numa terapia combinada com antibióticos? Podem as bactérias desenvolver resistência a PaDBS1R1? Em caso de desenvolvimento de resistência a PaDBS1R1, quais mecanismos poderiam estar mediando a resistência ao peptídeo? A resistência a PaDBS1R1 implica resistência

cruzada a outros PAMs e/ou antibióticos? Podem bactérias resistentes a PaDBS1R1 se tornar resistentes a componentes do sistema imune? Dar respostas as essas assim como a outras questões, poderia marcar o caminho de futuras pesquisas.

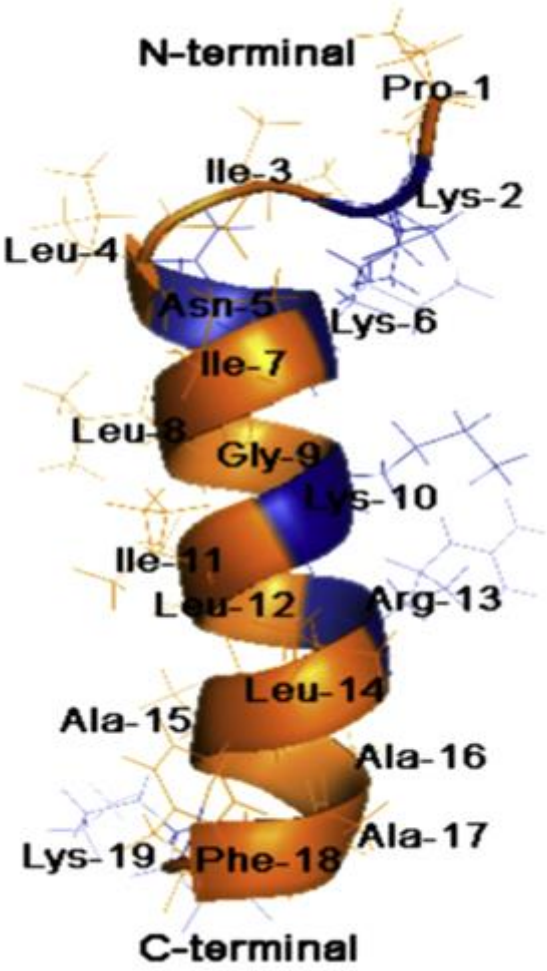

Figura 2. Estrutura de PaDBS1R1.
Adaptada de Irazazabal et al. (2019).

4.4. Mecanismos de resistência a peptídeos antimicrobianos

Como foi descrito anteriormente um dos fatores que faz os PAMs antimicrobianos promissores é que o desenvolvimento de resistência a eles parece ser menos provável que aos antibióticos. No entanto, os PAMs não são imunes ao desenvolvimento de resistência pelos patógenos. Nesse sentido, foram reportados vários mecanismos usados pelas bactérias para evadir a atividade microbicida dos PAMs (Maria-Neto et al., 2015; Joo et al., 2016a). Um fato de preocupação associado à resistência aos PAMs, é que esta poderia favorecer a resistência cruzada aos antibióticos assim como a efetores do sistema imune

inato (Andersson et al., 2016; Fleitas and Franco, 2016b). Portanto, compreender como as bactérias resistem a atividade dos PAMs, se torna de suma importância para o desenvolvimento de PAMs mais efetivos, assim como para o desenho de estratégias terapêuticas eficientes.

As estratégias de resistência aos PAMs usada pelas bactérias compreendem desde mecanismos ao nível celular (bactéria) até mecanismos ao nível de população bacteriana (Fleitas et al., 2016; Wu and Tan, 2019). Basicamente os mecanismos de resistência compreendem a produção de peptidases, sequestro e neutralização dos PAMs, extrusão dos PAMs mediante bombas de efluxo, remodelagem da superfície celular, estabilização osmótica e formação de biofilmes (Figura 3).

4.4.1. Degradação proteolítica dos PAMs

A degradação proteolítica dos PAMs acontece principalmente por proteases que são secretadas no meio extracelular ou proteases que são inseridas na superfície bacteriana (diretamente ou indiretamente mediante a formação de complexos proteicos). A essência deste mecanismo consiste em inativar os peptídeos antes que consigam atingir a membrana citoplasmática do patógeno (Fleitas et al., 2016). Em referência a isto, mostraram-se interessantes as estratégias desenvolvidas por *Streptococcus pyogenes*, baseadas em proteases, que atuam como forma de evasão à ação de peptídeos efetores do sistema imune inato. *S. pyogenes* mediante a expressão da proteína GRAB, associa a sua superfície a molécula do hospedeiro α-2-macroglobulina. Esta proteína do hospedeiro serve como ponto de enclave para a peptidase bacteriana SpeB, a qual produto da interação com α-2-macroglobulina fica associada na superfície bacteriana, além de mudar sua especificidade proteolítica de substratos de grande tamanho (proteínas) para substratos pequenos como os peptídeos. Nesse contexto, atividade proteolítica de SpeB foi maior quando estava formando o complexo proteico (com α-2-macroglobulina) em comparação a quando não estava formando complexo, além de proteger à bactéria da ação de LL-37 (Nyberg et al., 2004).

Outra estratégia desenvolvida por *S. pyogenes* consiste na secreção da proteína Ska a qual forma complexos proteicos Ska-plasminogênio ou Ska-fibrinogênio-plasminogênio. Estes complexos proteicos rendem a protease ativa plasmina a qual pode-se associar à superfície bacteriana mediante receptores de plasminogénio e fibrinogénio. A presença de plasmina na superfície bacteriana protege a bactéria de LL-37 (Hollands

et al., 2012). Além de LL-37, a presença de plasmina na superfície de *S. pyogenes* poderia protegê-lo contra outros efetores da resposta imune inata como C3-b e histonas (Ly et al., 2014; Nitzsche et al., 2016).

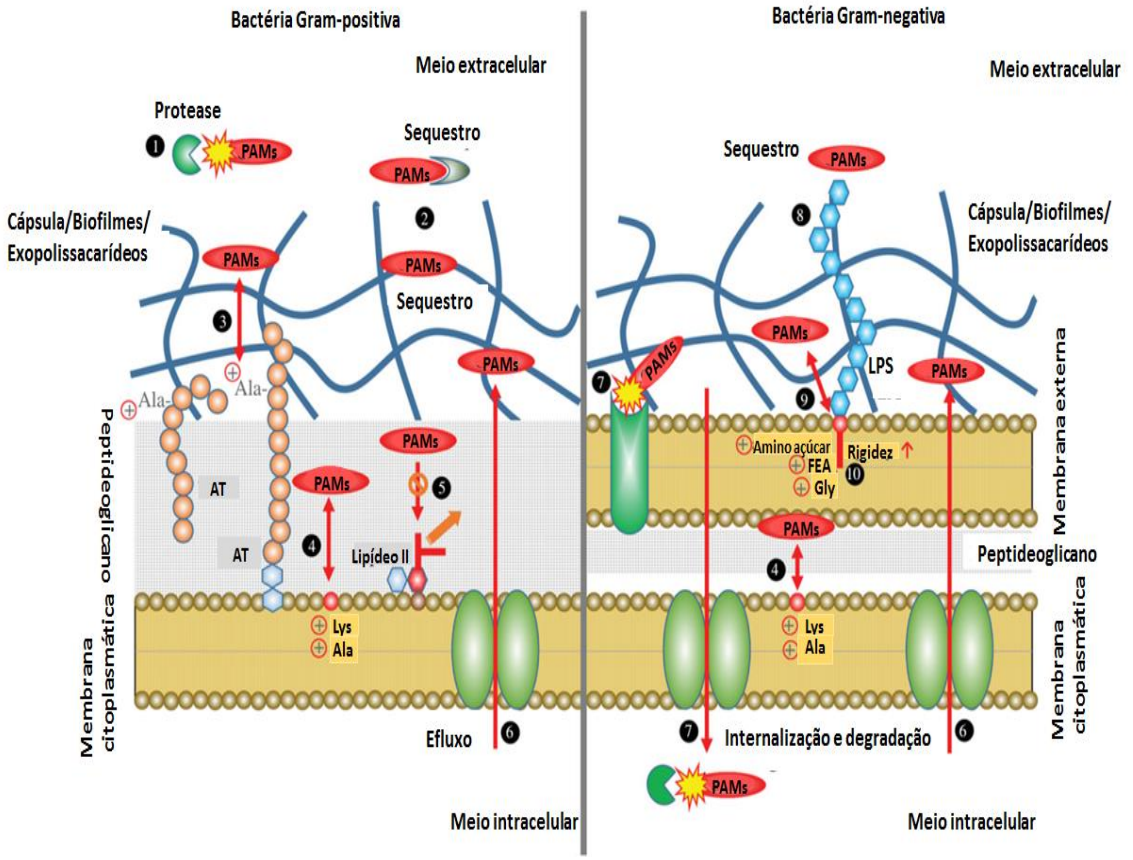

Figura 3. Mecanismos de resistência a peptídeos antimicrobianos desenvolvidos por bactérias Gram-positivas e Gram-negativas.

1) Degradação proteolítica dos peptídeos antimicrobianos (PAMs) por proteases secretadas. 2) Sequestração dos PAMs por proteínas extracelulares ou matriz extracelular. 3) Repulsão eletrostática dos PAMs pelos ácidos teicoico (AT) modificados com alanina (Ala). 4) Repulsão eletrostática dos PAMs por fosfolipídeos de membrana citoplasmática modificados com lisina (Lys) e alanina. 5) Afetação da união dos PAMs ao lipídeo II mediante modificação deste. 6) Expulsão dos PAMs para o meio extracelular mediante bombas de efluxo. 7) Degradação proteolítica mediante proteases associadas à membrana externa ou proteases intracelulares. 8) Sequestração dos PAMs mediante os lipopolissacarídeos (LPS). 9) Repulsão eletrostática dos PAMs mediante LPS modificados com amino açucares, fosfoetanolamina (FEA) e glicina (Gly). 10) Aumento da rigidez da membrana externa mediante a acilação dos LPS. Figura adaptada de Joo *et al.* (2016).

Além de secretar diretamente as peptidases no meio extracelular as bactérias também podem secretar as peptidases associadas a vesículas de membrana externa.

Recentemente, Urashima *et al.,* 2017 mostraram que o desafio de *E. coli* enterrohemorrágica com LL-37 induzia a liberação de vesículas de membrana externa que continham associada a protease OmpT e mediavam a proteção contra o peptídeo (Urashima et al., 2017). Outras evidências de peptidases associadas a vesículas de membrana externa participando na resposta de resistência contra PAMs foi aportada pelos estudos de Kulkarni *et al.,* 2015 e Rompikuntal *et al.,* 2015. Os primeiros mostraram que a cepa *E.coli* MG 1655 secretava vesículas de membrana externa que continham várias proteases que mediavam a degradação de melitina. Estas vesículas além de proteger a *E.coli* MG 1655 também podiam proteger a *P. aeruginosa* NCTC6751 e *Acinetobacter radioresistens* MMC5. Enquanto os segundos mostraram que as vesículas de membrana externas secretadas por *Vibrio cholerae* podiam conter a protease PrtV e participavam na resistência contra LL-37 (Kulkarni et al., 2015; Rompikuntal et al., 2015).

Embora a proteção mediada por peptidases se baseie principalmente em proteases secretada ou associada a superfície, a degradação intracelular de PAMs também tem sido observada. Nesse sentido, *Haemophilus influenzae* acopla a internalização de PAMs mediante o transportador Sap ABC com proteólise intracelular destes (Shelton et al., 2011). Destaca-se que a proteólise intracelular dos PAMs poderia proteger à bactéria dos peptídeos que exercem sua atividade antimicrobiana intracelularmente.

4.4.2. Sequestro e neutralização dos peptídeos antimicrobianos

O sequestro e neutralização dos PAMs pode estar mediado por biomoléculas ou por estruturas celulares. Igual aos mecanismos baseados em proteólise, estes impedem que os peptídeos atinjam seus alvos celulares (Fleitas et al., 2016). A neutralização mediada por biomoléculas acontece no meio extracelular ou na superfície bacteriana, e pode implicar na interação direta do peptídeo antimicrobiano com moléculas bacterianas ou na interação com moléculas do hospedeiro (Fleitas et al., 2016).

Em referência à secreção de biomoléculas neutralizadoras dos PAMs, tem sido mostrado que *S. pyogenes* secreta ao meio extracelular as proteínas SIC e DRS, as quais podem interagir e capturar diretamente os PAMs (Frick et al., 2003; Fernie-King et al., 2007). Adicionalmente, SIC poderia também proteger *S. pyogenes* da atividade lítica das histonas extracelulares (Westman et al., 2018). *S. pyogenes* também secreta a proteína sHIP que interage e neutraliza a atividade microbicida da glicoproteína enriquecida em histidina assim como de um peptídeo antibacteriano derivada desta (Wisniewska et al.,

2014). *Streptococcus dysgalactiae* subsp. *equisimilisa* produz uma proteína homologa a SIC e DRS, a proteína DrsG, a qual é secretada e pode interagir e unir LL-37 (Smyth et al., 2014). Por outro lado, *S. aureus* secreta a proteína estafiloquinase a qual interage e neutraliza os peptídeos α-defensinas e mCRAMP (Jin et al., 2004; Braff et al., 2007). Interessantemente, *Chlamydia trachomatis* produze uma proteína (Pgp3) codificada por plasmídeo, a qual interage e neutraliza a atividade bactericida de LL-37 (Hou et al., 2015). Algumas evidências recentes sugerem que a secreção da proteína BcnA (*bacterial lipocalin protein*) também poderia mediar a proteção contra os PAMs através da união destes (El-Halfawy et al., 2017; Naguib and Valvano, 2018).

Proteínas inseridas na superfície bacteriana podem ser liberadas no meio extracelular mediante a ação de peptidases, uma vez liberadas, estas proteínas unem-se e neutralizam os PAMs. Exemplo disto é a liberação das proteínas H e M1 da superfície de *S. pyogenes* mediante a ação de proteases secretadas pelos neutrófilos. Uma vez liberadas no meio extracelular, estas proteínas podem unir e neutralizar peptídeos derivados da β-2-glicoproteína I (Nilsson et al., 2008). Outra estratégia desenvolvida pelas bactérias consiste na manipulação de moléculas do hospedeiro para se defender, neste caso as bactérias promovem a liberação de moléculas do hospedeiro com capacidade para se unir aos peptídeos antimicrobianos. Exemplo disto são *P. aeruginosa* e *E. faecalis*, as quais secretam peptidases que degradam os proteoglicanos do hospedeiro liberando glicosaminoglicanos, os quais pelo seu caráter aniônico podem unir-se aos PAMs catiônicos (Schmidtchen et al., 2001).

A secreção de biopolímeros aniônicos também pode ser uma estratégia para sequestrar e neutralizar os PAMs. Nesse sentido, foi observado que um mutante de *Staphylococcus epidermidis* deficiente para a produção de poli-ϒ-DL-ácido glutâmico, foi mais sensível a LL-37 e β-defensina-3 que a cepa selvagem (Kocianova et al., 2005). Por outro lado, patógenos como *P. aeruginosa, Inquilinus limosus* e o complexo *Burkholdelia cepacia* produzem exopolissacarídeos aniônicos que podem unir e inibir vários ortólogos de LL-37 (Foschiatti et al., 2009). Outro estudo mostrou que exopolissacarídeos aniônicos produzidos por *P. aeruginosa, K. pneumoniae* e o complexo *Burkholdelia cepacia* inibiam a atividade microbicida de LL-37, β-defensina-3 humana, SMAP-29, PG-1 e Bac7 (1-35). Foi sugerido que a liberação de exopolissacarídeos aniônicos poderia facilitar a persistência desses patógenos nos pulmões dos pacientes com fibrose cística (Benincasa et al., 2009).

Proteínas localizadas na superfície bacteriana também podem mediar proteção mediante o sequestro e neutralização dos PAMs. Exemplo disto, constitui a proteína de superfície M1 de *S. pyogenes*, a qual pode sequestrar o peptídeo LL-37 assim como o precursor deste (hCAP18), evitando dessa forma a geração do peptídeo ativo (Lauth et al., 2009; LaRock et al., 2015). M1 também pode unir outros peptídeos como KR-20, KR-12 e mCRAMP (LaRock et al., 2015). Outra forma de proteção mediada por M1 (também pela proteína H), é o sequestro na superfície bacteriana da β-2-glicoproteína I, o que impede a degradação da mesma por proteases derivadas de neutrófilos, e portanto, evita a liberação de peptídeos com atividade antimicrobiana (Nilsson et al., 2008). Uma estratégia bem interessante, é a desenvolvida por *Streptococcus* grupo G, que mediante a expressão de uma proteína de união a albumina cobre a sua superfície da proteína albumina humana, evitando deste jeito a ação bactericida de MIG/CXCL9, a qual fica entrapada pela albumina na superfície bacteriana (Egesten et al., 2011).

A união e neutralização de peptídeos antimicrobianos por estruturas celulares, pode ser mediado principalmente por vesículas de membrana externa secretadas e por cápsulas bacterianas. Um estudo pioneiro de Manning e Kuehn. (2011), mostrou que o desafio de *E. coli* com polimixina B e colistina, induziu a produção e secreção de vesículas de membrana externa, as quais mediavam a proteção contra esses antimicrobianos. O mecanismo de proteção, possivelmente era baseado na união dos antimicrobianos aos LPS constituintes da superfície das vesículas (Manning and Kuehn, 2011). Posteriormente, outro estudo mostrou que proteínas presentes nas vesículas de membrana externa também podem mediar a união aos PAMs. Especificamente, foi demostrado que vesículas secretadas por *V. cholerae* desafiada com polimixina B, tinham a proteína Bap1 associada, a qual mediava a proteção contra LL-37 mediante a captura do peptídeo (Duperthuy et al., 2013). Por outro lado, as vesículas de membrana externa podem sequestrar os PAMs por interação destes com a membrana das vesículas (Kulkarni et al., 2015).

Embora, a formação de vesículas tem sido mais associada a bactérias Gram-negativas, também tem sido descrita para bactérias Gram-positivas. Nesse sentido, foi mostrado que LL-37 induz a formação de partículas semelhantes a vesículas por *S. pyogenes*, e que o peptídeo se encontrava associado a estas vesículas (Uhlmann et al., 2016).

A resistência a PAMs mediada pela cápsula pode acontecer mediante o sequestro do peptídeo na superfície ou mediante a liberação de material capsular impedindo que o mesmo possa atingir as membranas bacterianas. Cepas mutantes de *K. pneumoniae* para a produção de cápsula, foram mais sensíveis que as cepas selvagens à ação de *human neutrophil defensin*-1(HNP-1), β-defensina-1, lactoferrina, protamina e polimixina B (Campos et al., 2004). Além disto, foi notado que entre isolados clínicos, aqueles que produziam maior quantidade de cápsula eram mais resistentes à polimixina B (Campos et al., 2004). O desafio com polimixina B e HNP-1 de *K. pneumoniae* K2, *Streptococcus pneumoniae* sorotipo 3 e *P. aeruginosa* produziu a liberação de material capsular, o qual podia unir-se aos peptídeos antimicrobianos e neutralizar sua atividade microbicida. Esta forma de ação foi comprovada para cápsulas aniônicas, enquanto as neutras ou catiônicas, não apresentavam este tipo de atividade (Llobet et al., 2008). Adicionalmente, a produção de cápsula tem sido vinculada à resistência aos PAMs em *E. coli*, *Neisseria meningitidis* e *A. baumannii* (Spinosa et al., 2007; Jones et al., 2009; Thomassin et al., 2013; Geisinger and Isberg, 2015; Chin et al., 2018).

4.4.3. Remodelagem da superfície celular

Como previamente foi exposto, a membrana citoplasmática das bactérias constitui um dos principais alvos dos peptídeos antimicrobianos. Tanto em bactérias Gram-negativas como em Gram-positivas, os peptídeos têm que interagir e transpassar as estruturas da superfície celular para poder atingir a membrana citoplasmática. Portanto, as bactérias modificam as características físico-químicas destas estruturas de superfície visando impedir que os PAMs atinjam a membrana citoplasmática e/ou o citoplasma bacteriano.

4.4.3.1 Modificação da membrana externa das bactérias Gram-negativas

A organização estrutural da superfície das bactérias Gram-negativas consiste em dois sistemas de membranas, uma membrana externa e uma interna (ou citoplasmática), as quais estão separadas fisicamente por um espaço periplasmático, onde encontra-se uma camada de peptidoglicano (Schwechheimer and Kuehn, 2015). Portanto, nas bactérias Gram-negativas a membrana externa constitui uma barreira de primeira ordem para o passo dos PAMs. Esta membrana se caracteriza por uma bicamada lipídica assimétrica, sendo a camada interna da membrana enriquecida em fosfolipídios, enquanto a camada externa é enriquecida em LPS (Schwechheimer and Kuehn, 2015). Assim, modificado os

LPS, as bactérias Gram-negativas conseguem reforçar a membrana externa como barreira protetora contra os PAMs.

Essas modificações nos LPS, basicamente visam diminuir o caráter aniônico destas biomoléculas. Nesse âmbito, uma estratégia usada é a eliminação dos grupos fosfatos do dissacarídeo de glucosamina que compõe o lipídeo A. As enzimas fosfatasses do lipídeo A LpxE e LpxF, são as encarregadas de hidrolisar esses grupos fosfatos nas posições 1C´ e 4C´, respetivamente. A desfosforilação do lipídeo A parece mediar a proteção contra vários PAMs incluindo polimixina B, LL-37, P-113 e β-defensina-2 (Wang et al., 2007; Ingram et al., 2010; Cullen et al., 2011). Outra forma de reduzir a carrega negativa dos LPS é mediante a adição de agentes catiônicos na molécula. Nesse sentido, mediante a atividade catalítica de fosfoetanolamina transferases (EptA, EptB e EptC), as bactérias podem adicionar fosfoetanolamina nas posições 1C´e/ou 4C´ do dissacarídeo de glucosamina (EptA), no Kdo (ácido 3-deoxi-D-manno-octulosonico) (EptB) e na heptose I (EptC) (Reynolds et al., 2005; Salazar et al., 2017)(Figura 4).

Em adição à fosfoetanolamina transferases (EptA, EptB e EptC) codificadas por genes cromossômicos, as bactérias possuem fosfoetanolamina transferases codificadas por genes plasmídeais (ex. MCR-1, MCR-2, MCR-3, MCR-4 e MCR-5), as quais mediam a resistência as polimixinas (Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Fukuda et al., 2018). Adicionalmente, a modificação do lipídeo A dos LPS com 4-amino-4-deoxi-L-arabinose também influência nas propriedades eletrostática dos LPS, conferindo a estes um caráter menos aniônico e consequentemente aprovisionando as bactérias de resistências contra uma variedade de PAMs (Figura 4) (Anaya-López et al., 2013; Cole and Nizet, 2016). Na modificação via 4-amino-4-deoxi-L-arabinose, estão envolvidas várias enzimas codificadas pelo operon *arnBCADTEF* (Raetz et al., 2007). Estas enzimas transformam o UDP-ácido glucorônico em 4-amino-4-deoxi-L-arabinose, a qual posteriormente, é adicionada via ArnT aos LPS nas posições 1C´e/ou 4C´ do dissacarídeo de glucosamina do lípideo A (Raetz et al., 2007). Também tem sido descrito a adição de 4-amino-4-deoxi-L-arabinose no núcleo Kdo dos LPS (Vinogradov et al., 2006; Hamad et al., 2012).

A adição de glucosamina ao lipídeo A tem sido vinculada à resistência a polimixina B, colistina, LL-37, indolicidina, HHC-10 e CP28 em *Bordetella pertussis*, enquanto em *Bordetella bronchiseptica* tem sido vinculada à resistência a polimixina B e β-defensina-1 porcina (Rolin et al., 2014; Shah et al., 2014). Por outro lado, a adição de

galactosamina ao lipídeo A intervém na resistência a colistina em isolados clínicos, assim como de cepas de laboratório de *A. baumannii* (Pelletier et al., 2013).

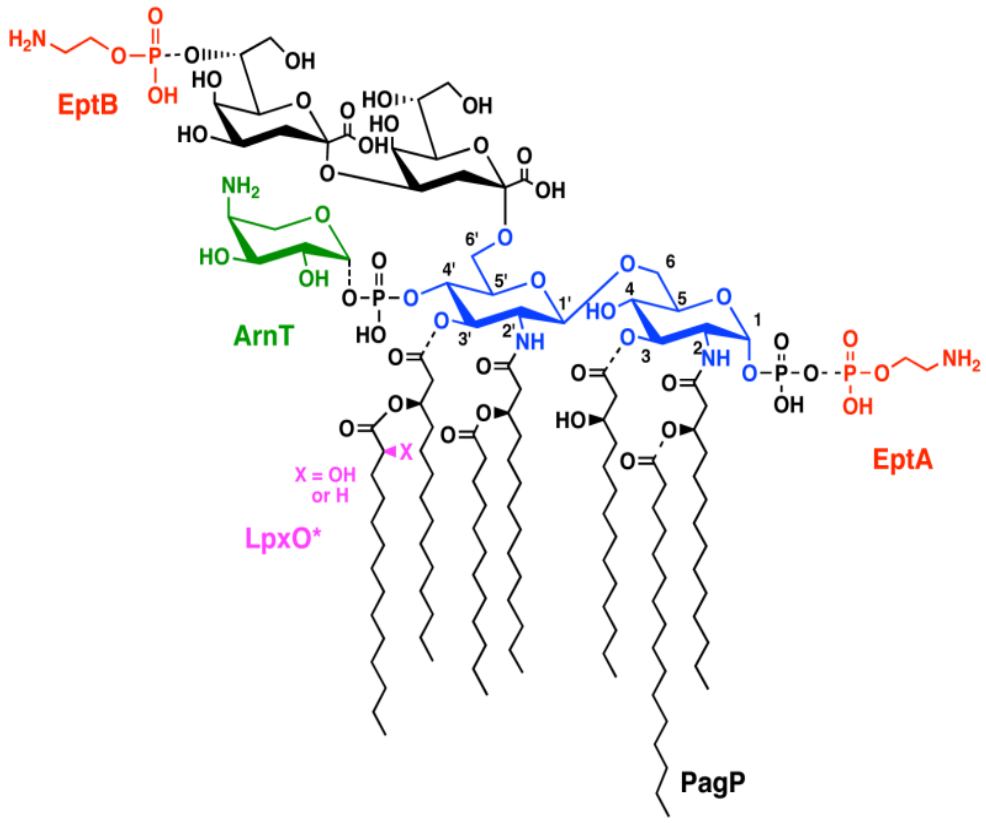

Figura 4. Modificações na estrutura do Kdo2-lipídeo A dos lipopolissacarídeos que estão associadas à resistência aos peptídeos antimicrobianos.

Adaptada de Raetz et al. (2007).

Outro elemento estrutural dos LPS que pode ser modificado visando resistência aos antimicrobianos são as cadeias de ácidos graxos do lipídeo A. A adição de glicina ou dipeptídeo de glicina a uma das cadeia acil secundária do lipídeo A, foi descrito em *Vibrio cholerae* El Tor e está envolvido na resistência a polimixina B. O desafio com polimixina B ativa o sistema de dois componentes VprAB, o qual ativa o operon *almEFG* que codifica para as proteínas envolvidas nesta modificação específica (Hankins et al., 2012;

Herrera et al., 2014). Outra modificação do lipídeo A é a adição de cadeias aciladas mas, diferentemente das outras modificações descritas acima, esta não visa transformar a carga da molécula de LPS. A adição de cadeias aciladas parece influenciar na fluidez da membrana externa. Nesse sentido, tem sido observado que uma cepa de *K. pneumoniae* mutante para o gene *lpxM*, o qual codifica para a enzima (LpxM) responsável pela acilação secundária do lipídeo A, é mais susceptível polimixina B, colistina, CP-28 e C18G, além de sofrer atenuação *in vivo* durante a infecção de camundongos BALB/c (Clements et al., 2007). Tem sido sugerido, que produto da não acilação secundária do lipídeo A nesta cepa, as interações laterais entre as moléculas de LPS são mais fracas o que produz uma maior fluidez na membrana externa, facilitando a inserção do N-terminal das polimixinas (Velkov et al., 2013). Por outo lado, uma proteína homóloga de LpxM em *A. baumannii*, também parece mediar a resistência aos PAMs (Boll et al., 2015). Ademais, a proteína homóloga a LpxM em *V. cholerae* (MsbB) parece estar envolvida na resistência a LL-37, mCRAMP, magainina 2 e polimixina B, mas o efeito protetor varia entre cepas (Matson et al., 2010). Similarmente, foi mostrado que em *V. cholerae* a enzima LpxN a qual transfere grupos 3-hidroxi-lauratos ao lipídeo A está envolvida na resistência à polimixina B (Hankins et al., 2011).

Uma outra modificação dos ácidos graxos do lipídeo A dos LPS que tem sido associada à resistência aos PAMs, é a hidroxilação (catalisada por LpxO) (Figura 4). Em *K. pneumoniae*, a hidroxilação do miristato do lípideo A, catalisada pela enzima LpxO, da como produto moléculas de LPS que contém 2-hidroxi-miristato. Essas espécies modificadas de LPS, foram essenciais para a virulência de *K. pneumoniae* num modelo animal, assim como, para a resistência aos PAMs (Llobet et al., 2015). De maneira similar, recentemente foi mostrado que a produção de moléculas de LPS modificadas com 2-hidroxi-laurato (catalisada por LpxO) é fundamental para a resistência de *A. baumannii.* aos PAMs (Bartholomew et al., 2019). A adição de palmitato ao lipídeo A pela enzima PagP (Figura 4), é outra modificação dos LPS que tem sido vinculada à resistência aos PAMs em *Bordetella parapertussis* e *P. aeruginosa* (Thaipisuttikul et al., 2014; Hittle et al., 2015). Também em *Salmonella* e *Legionella*, a adição de palmitato ao lipídeo A está envolvido na resistência aos PAMs (Guo et al., 1998; Robey et al., 2001). Em *Salmonella Typhimurium* o palmitato também pode ser adicionado ao fosfatidilglicerol da cara interna da membrana externa. Esse processo envolve o sistema de dois componentes PhoPQ e a enzima PagP. O aumento de fosfatidilglicerol modificado com palmitato na

membrana externa aumenta o nível hidrofóbico e de saturação da mesma, mediando isto, a resistência aos PAMs (Dalebroux et al., 2014).

4.4.3.2 Modificação da parede celular em bactérias Gram-positivas

Diferentemente das bactérias Gram-negativas, as bactérias Gram-positivas não apresentam uma membrana externa. A estrutura de superfície mais externa está constituída por uma parede celular que consiste em múltiplas camadas de peptidoglicanos (40-80 nm). Essas camadas de peptidoglicanos podem ter insertado diretamente o ácido teicoico, além de ter associado o ácido lipoteicoico, o qual está insertado na membrana(Malanovic and Lohner, 2016). Estes glicopolímeros da superfície apresentam um caráter aniônico, portanto constituem um ponto de interação e acumulação de PAMs na superfície da bactéria (Malanovic and Lohner, 2016).

Então, as estratégias de resistência desenvolvidas pelas bactérias Gram-positivas a nível de superfície celular visam a redução do caráter aniônico desses glicopolímeros constituintes. Basicamente, a estratégia se baseia na esterificação do grupo carbóxilo da D-alanina com as unidades monoméricas dos glicopolímeros, fazendo com que fique exposto o grupo amino do aminoácido o qual contém carga positiva, este processo acontece pela ação de várias proteínas codificadas pelo operon *dlt(X)ABCD* (Neuhaus and Baddiley, 2003; Kamar et al., 2017). A adição de D-alanina aos ácidos teicoico e lipoteicoico, tem sido reportada em diferentes gêneros de bactérias Gram-positivas incluindo *Staphylococcus*, *Listeria*, *Enterococcus*, *Bacillus*, *Clostridium*, *Streptococcus* e *Lactobacillus* e medeia a proteção contra uma grande variedade de PAMs como colistina, nisina, polimixina B, magainina 2, HNP 1-3, β-defensina-2, gallidermina, mCRAMP, protegrinas derivadas de animais, tachyplesina, indolicidina, cecoprina B, bactofencina A, vancomicina, daptomicina, protamina e LL-37 (Bertsche et al., 2013; O'Shea et al., 2013; Fleitas et al., 2016; Omardien et al., 2016). A atividade do operon *dlt(X)ABCD* parece apresentar um papel importante na resistência das bactérias ao sistema imune inato de insetos (Khattar et al., 2009; Kamar et al., 2017).

Por outro lado, cogita-se um mecanismo alternativo de resistência mediada pela adição de D-alanina aos ácidos teicoico e lipoteicoico. É proposto que em *Streptococcus* do grupo B este mecanismo media a resistência mediante modificações da rigidez e permeabilidade da parede celular (Saar-Dover et al., 2012). Também, em *Listeria*

monocytogenes, a modificação dos ácidos teicoicos com L-rhamnose parece proteger contra os PAMs através da diminuição da permeabilidade da parede celular aos peptídeos (Carvalho et al., 2015).

4.4.4. Modificação da membrana citoplasmática

Além de modificar as estruturas de superfície externas, as bactérias modificam também suas membranas citoplasmáticas. Estas modificações estão focadas principalmente em seus fosfolipídeos aniônicos (fosfatidilglicerol e cardiolipina) visando, assim como nas modificações das estruturas externas, a repulsão dos PAMs. Nesse sentido, interessantemente a modificação de fosfolipídios da membrana com aminoácidos (ex. lisina, alanina e arginina) constitui um mecanismo de resistência contra os PAMs que é utilizado por múltiplas bactérias como *S. aureus*, *P. aeruginosa*, *L. monocytogenes*, *Mycobacterium tuberculosis, Enterococcus faecalis*, *Enterococcus faecium* e *B. subtilis* (Roy, 2009; Nawrocki et al., 2014; Fields and Roy, 2018). Esses microrganismos, ativam enzimas aminoacil-fosfatidilglicerol sintetases que usam principalmente como substratos a lisina-tRNA e/ou alanina-tRNA para produzir lipídios modificados com lisina ou alanina, os quais são posteriormente expostos na membrana citoplasmática mudando as propriedades eletrostáticas desta e consequentemente afetando a união dos PAMs (Roy, 2009; Fields and Roy, 2018).

Outras modificações dos componentes das membranas visam mudanças na fluidez da mesma. Em *E. faecalis* uma diminuição no conteúdo de lipídeos ramificados pôde favorecer a rigidez da membrana, o qual contribuiu em conjunto com o aumento de cargas positivas na membrana à resistência aos PAMs (Kumariya et al., 2015). Previamente, o aumento na rigidez da membrana já havia sido associado à resistência a daptomicina em *E. faecalis* e *E. faecium* (Mishra et al., 2012). Também em *L. monocytogenes* e *S. aureus* a rigidez da membrana parece estar envolvida na resistência a nisina no caso do primeiro e de magainina 2 e gramicidina no segundo (Mazzotta and Montville, 1997; Shireen et al., 2013). Apesar de para algumas bactérias o aumento na rigidez da membrana media a resistência a certos PAMs, para outras um aumento na fluidez da membrana é o que media a resistência. Nesse sentido, tem sido mostrado que uma cepa de *S. aureus* resistente a pediocina, apresentava uma membrana mais fluida que a cepa sensível. Esta fluidez impedia a oligomerização do peptídeo na membrana celular (Lather et al., 2015). Além da resistência a pediocina, um incremento na fluidez da membrana em *S. aureus* parece

estar associado à resistência a tPMP, HNP-1, polimixina B, daptomicina e telavacina (Bayer et al., 2000; Mishra et al., 2011; Mishra and Bayer, 2013; Song et al., 2013).

A redistribuição de domínios lipídicos na membrana citoplasmática, constitui outro mecanismo de resistência contra peptídeos antimicrobianos. Em *E. faecalis* a resistência a daptomicina pode acontecer mediante a diminuição do conteúdo de fosfatidilglicerol em conjunto com a redistribuição dos domínios de cardiolipina. Isto provocava a união do antimicrobiano numa localização longe do septo de divisão celular, o qual constitui o principal alvo deste (Tran et al., 2013). Adicionalmente, tem sido mostrado que a interferência com a redistribuição de domínios de cardiolipina pode tornar a *E. faecalis* sensível a uma variedade de PAMs incluindo LL-37, β-defensina-3 e RP-1 (Reyes et al., 2014). Recentemente, foi demostrado que mutações no gene *cls2*, o qual codifica para a cardiolipina sintase, estavam associadas à resistência à daptomicina em *S. aureus* (Jiang et al., 2019). O efeito dessas mutações foi um incremento no conteúdo de cardiolipina na membrana, assim como uma diminuição do fosfatidilglicerol. Esse incremento nos níveis de cardiolipina, foi associado a um incremento na espessura da membrana, o qual interfere com a penetração da daptomicina (Jiang et al., 2019). Um outro efeito protetor sugerido para a cardiolipina contra certos PAMs, é que a cardiolipina promove curvatura negativa na membrana o que poderia contrariar o efeito de peptídeos que promovem curvatura positiva na membrana (Poger et al., 2018). Por outro lado, a resistência à daptomicina assim como a HNP-1 em *Streptococcus mitis/oralis* foi associada a uma perda do conteúdo de fosfatidilglicerol, cardiolipina e microdomínios de fosfolipídeos aniônicos na membrana citoplasmática. Essas alterações na composição da membrana foram produto de mutações de perda de função no gene *cdsA*, o qual codifica para a enzima CdsA (phosphatidate cytidylyltransferase) encarregada de produzir o intermediário metabólico citidina difosfato-diacilglicerol, o qual é essencial para a síntese de fosfatidilglicerol e cardiolipina (Mishra et al., 2017).

4.4.5. Estabilização osmótica

A estabilização osmótica constitui um dos mecanismos de resistência aos PAMs. Este se baseia no aumento da síntese e secreção de aminoácidos que conferem estabilização osmótica quando a bactéria é desafiada com peptídeos estressores de membrana (Wenzel et al., 2014a). Isto foi suportado pelas mudanças observadas na expressão de certas proteínas, assim como nos níveis de certos aminoácidos intracelulares

e extracelulares quando *B. subtilis* foi desafiado com o hexapeptídeo MP196. Especificamente, aconteceu um aumento da abundância de enzimas envolvidas no anabolismo de aminoácidos, assim como uma diminuição no pool intracelular dos aminoácidos glutamina/glutamato, asparagina/ ácido aspártico, lisina e prolina, além de um aumento nos níveis extracelulares de glutamina/glutamato, asparagina/ ácido aspártico, arginina, lisina e prolina (Wenzel et al., 2014a). A resposta de estabilização osmótica foi observada também quando a bactéria foi desafiada com gramicidina S, gramicidina A, aureina 2.2 e nisina (Wenzel et al., 2014a).

4.4.6. Extrusão dos peptídeos antimicrobianos

A extrusão dos PAMs pode ser mediada principalmente pelas bombas de efluxo. Nesse sentido, várias bombas de efluxos têm sido associadas à resposta de resistência aos PAMs (Tabela 1, adaptada de Fleitas et al. (2016)).

Tabela 1. Bombas de efluxo associadas à resistência a peptídeos antimicrobianos

Bomba de efluxo	Bactéria	PAMs	Referências
MtrC-MtrD-MtrE	*Neisseria meningitidis*	Polimixina B LL-37 PG-1	(Tzeng et al., 2005)
MtrC-MtrD-MtrE	*Neisseria gonorrhoeae*	TP-1 PC-8 PG-1 LL-37 CRAMP-38	(Shafer et al., 1998; Warner et al., 2008)
MtrC-MtrD-MtrE	*Haemophilus ducreyi*	LL-37 β-defensina-3	(Rinker et al., 2011)
QacA	*S. aureus*	tPMP-1	(Kupferwasser et al., 1999)
RosA	*Yersinia enterocolitica*	Polimixina B Cecropina P1 Melitina	(Bengoechea and Skurnik, 2000)
AcrAB	*K. pneumoniae*	HNP-1 β-defensina-1 β-defensina-2	(Padilla et al., 2010)

		Polimixina B	
KpnGH	*K. pneumoniae*	Polimixina B	(Srinivasan et al., 2014)
KpnEF	*K. pneumoniae*	Colistina	(Srinivasan and Rajamohan, 2013)
MexAB-OprM	*P. aeruginosa*	Colistina	(Pamp et al., 2008)
MeFE/Mel	*S. pneumoniae*	LL-37	(Zähner et al., 2010)
CmeABC **CmeDEF**	*Campylobacter jejuni*	Polimixina B	(Akiba et al., 2005)
AcrAB-TolC **EmrAB-TolC**	*E. coli*	Protamina	(Weatherspoon-Griffin et al., 2014)

Além de sua função de ejetar os PAMs das células bacterianas, as bombas de efluxos podem atuar também na detecção dos peptídeos. Nas bactérias Gram-positivas existem módulos especializados na detecção e detoxificação de peptídeos que consistem de um sistema de dois componente acoplado a um transportador ABC (Falord et al., 2012; Dintner et al., 2014). Evidências experimentais sugerem que nestas estruturas especializadas, os transportadores podem unir diretamente os peptídeos e interagir com a proteína histidina quinase do sistema de regulador de dois componentes para formar um complexo de sinalização. Este complexo de sinalização permite a expressão de mais transportador, assim como de outros elementos envolvidos na resistência como os genes *mprF* e operon *dlt* (Falord et al., 2012; Dintner et al., 2014; Grubaugh et al., 2018; Jiang et al., 2019).

Recentemente, um mecanismo de extrusão de PAMs independente de bombas de efluxo foi proposto para *V. cholerae* (Matson and Saul-McBeth, 2019). O mecanismo proposto, se baseia no reconhecimento dos PAMs pelo sistema de dois componentes VC1638/1639, o que desencadeia a produção da proteína SipA, a qual no periplasma se une aos PAMs e os direciona para a porina de membrana externa OmpA, a qual

provavelmente media a expulsão dos PAMs (Matson and Saul-McBeth, 2019). Previamente, um mecanismo similar tinha sido sugerido em *Salmonella enterica* serotipo Typhimurium, para explicar a resistência aos PAMs mediada pelas proteínas YdeI e OmpD. Nesse modelo, se cogitou que YdeI poderia unir os PAMs no periplasma, perto do poro da proteína OmpD, criando dessa forma um gradiente de PAMs que facilitaria a difusão dos mesmos para o exterior através de OmpD (Pilonieta et al., 2009).

4.4.7. Resistência coletiva

O arranjo das bactérias em populações tem uma influência direta na resistência aos antimicrobianos. A resistência coletiva faz referência à resposta coordenada desenvolvida pela população de bactérias como um todo (Vega and Gore, 2014; Meredith et al., 2015). Mecanismos de resistência aos PAMs previamente expostos como degradação dos PAMs por proteases secretadas ou associada à superfície bacteriana, assim como, o sequestro dos PAMs por biomoléculas ou estruturas celulares, potencialmente podem atuar como mecanismos de resistência coletiva (Llobet et al., 2008; Manning and Kuehn, 2011; Fleitas et al., 2016; Malhotra et al., 2018; Wu and Tan, 2019). Um outro mecanismo de resistência coletiva contra os PAMs (estudado especificamente com LL-37), pode ser a absorção dos peptídeos por uma subpopulação de células que é preferencialmente permeabilizada e subsequentemente morta. Essa subpopulação de células mortas, absorve os peptídeos provocando uma diminuição na concentração ativa dos mesmos, e, portanto, facilita o crescimento das outras bactérias da população (Wu and Tan, 2019). Previamente, tinha sido observado uma espécie de "cooperação passiva" em populações isogênicas de *E. coli*, onde existia uma heterogeneidade de absorção do peptídeo LL-37. Observou-se que nessas populações existia uma subpopulação de células cujo crescimento era inibido, mas absorbia uma grande quantidade do peptídeo, diminuindo desse jeito a concentração ativa de LL-37, e consequentemente, facilitando o crescimento do resto da população (Snoussi et al., 2018). A absorção heterogênea de peptídeos também tem sido observada na resposta de *S. mitis/oralis* ao desafio com daptomicina, onde existe uma hiperacumulação do peptídeo numa subpopulação de bactérias (Mishra et al., 2017; Tran et al., 2019).

O crescimento em biofilmes representa um exemplo de resistência coletiva aos PAMs. Nos biofilmes convergem diferentes mecanismos de resistência simultaneamente permitindo de forma efetiva a persistência das bactérias. Por exemplo, biofilmes criados

por *P. aureginosa* se caracterizam por uma matriz do exopolissacarídeo alginato, a qual pode formar estruturas hidrofóbicas semelhantes a "bolsos" onde os peptídeos são sequestrados e tendem a polimerizar antes de atingir a membranas bacterianas (Chan et al., 2004; Chan et al., 2005). No caso de *Staphylococcus epidermidi*s, a matriz do biofilme está composta pelo exopolissacarídeo PIA (*exopolysaccharide intercellular adhesin*), o qual é de natureza catiônica, portanto pode repelir aos PAMs catiônicos, assim como, sequestrar aos PAMs aniônicos (Vuong et al., 2004; Yasir et al., 2018). Adicionalmente, na matriz dos biofilmes, também há presença de DNA extracelular.

Foi mostrado que em biofilmes de *P. aeruginosa*, o DNA extracelular pode atuar como agente sequestrador de cátions favorecendo dessa forma a aparição de microambientes deficientes em cátions. Isto induz nas bactérias a ativação de sistemas de modificação de LPS (Mulcahy et al., 2008). Além disso, o DNA extracelular também poderia ativar a formação poliaminas (ex. espermidina), a quais podem se associar com a membrana externa das bactérias estabilizando-a e interferindo com a união dos PAMs (Johnson et al., 2012; Lewenza, 2013). Adicionalmente, o DNA extracelular também poderia sequestrar os PAMs, evitando que atinjam as bactérias (Lewenza, 2013). Por outro lado, nos biofilmes há presença de subpopulações de bactérias heterogêneas metabolicamente. Nesse sentido, foi observado que em biofilmes de *P. aeruginosa* as células que eram mais ativas metabolicamente expressavam os genes *mexAB-oprM*, conferindo tolerância à colistina (Pamp et al., 2008).

4.5. Abordagens experimentais nos estudos de resistência a peptídeos antimicrobianos

As pesquisas que visam estudar a resistência aos peptídeos antimicrobianos têm crescido nos últimos anos. Este aumento das pesquisas nesta área tem vindo acompanhada da evolução nas abordagens experimentais, permitindo-nos conhecer com uma maior profundidade as bases moleculares da resistência. Uma abordagem experimental amplamente usada para o estudo da resistência aos PAMs, recai no uso de cepas mutantes em genes específicos visando elucidar mediante ensaios funcionais se o produto codificado por esses genes está envolvido ou não diretamente na resistência aos PAMs (Weatherspoon-Griffin et al., 2014; Andersson et al., 2016; Paulsen et al., 2016). O acúmulo de conhecimento baseados nos resultados experimentais no decorrer destes anos, tem mostrado que múltiplos mecanismos de resistência podem estar envolvidos na resposta das bactérias aos PAMs, e que, esta resposta envolve vários processos

bioquímicos. Portanto, abordagens experimentais que permitam explorar a resposta de resistência de uma forma mais abrangente poderiam ser de grande utilidade para compreender a complexidade da referida resposta com uma maior profundidade.

Nesse sentido, um grande progresso veio com os avanços tecnológicos nos métodos de sequenciamento de ácidos nucleicos. O sequenciamento de alto desempenho de ácidos nucleicos, tem permitido estudar a grande escala as mutações no genoma das cepas resistentes, assim como os níveis de expressão dos genes envolvidos na resistência. Isto aporta informação valiosa sobre a montagem de uma resposta de resistências a um nível mais sistêmico, permitindo conhecer as possíveis vias metabólicas e redes moleculares que estão envolvidas direta ou indiretamente na resposta. Este tipo de abordagem, baseada no sequenciamento de alto desempenho de ácidos nucleicos se torna particularmente interessante nos estudos de evolução da resistência permitindo conhecer a nível molecular a dinâmica da emergência, espalhamento e persistência dos mutantes resistentes (Schürch and Schaik, 2017).

Embora, os estudos da resistência baseados nas análises do genoma e transcriptoma sejam um pulo significativo na compreensão das bases moleculares da mesma, estes apresentam certas limitações, pois não são analisadas diretamente as proteínas codificadas pelos genes envolvidos na resposta. Estes estudos se baseiam na inferência sobre as proteínas envolvidas na resposta de resistência a partir dos dados obtidos do genoma e transcriptoma. Este tipo de inferência pode se tornar complicada, pois vários eventos moleculares com diferentes mecanismos de regulação acontecem para tornar a informação genética contida em um gene em uma proteína funcional. Como exemplo disso, é que não necessariamente há uma correlação entre os níveis de expressão de um transcrito e os níveis da proteína correspondente, o qual pode estar influenciado por mecanismos de regulação pós-transcrição e pós-tradução. Além disso, podem acontecer modificações pós-tradução nas proteínas, o que pode influenciar na sua localização celular, tempo de vida, interação com outras proteínas e função (Haider and Pal, 2013; Pérez-Llarena and Bou, 2016). Pode ser neste cenário onde os estudos de proteômica têm um protagonismo indiscutível.

A proteômica visa o estudo do proteoma, sendo este o conjunto de proteínas sintetizadas por uma célula, um tecido, órgão ou organismo. Semelhante aos estudos baseados em sequenciamento de alto desempenho de ácidos nucleicos, os estudos de proteômica fazem inferências de vias metabólicas e redes moleculares que poderiam estar

envolvidas na resposta de resistência a partir das mudanças nos níveis de proteínas (Park et al., 2016; Pérez-Llarena and Bou, 2016). Vários estudos têm evidenciado as utilidades das abordagens proteômica para elucidar mecanismo de ação dos peptídeos antimicrobianos, assim como os mecanismos de resistência desenvolvidos pelas bactérias (Wenzel et al., 2014a; Martinez et al., 2016; Cardoso et al., 2017).

Justificativa

A resistência aos antimicrobianos tem-se tornado possivelmente uma das maiores ameaças para a vida humana neste século. A resistência desenvolvida pelas bactérias tem alcançado níveis tão alarmantes, que a possibilidade de voltar à época pré-antibiótico é cada vez mais real. Sem nenhuma dúvida, este panorama constitui um desafio para a comunidade científica, a qual tem se focado na procura de novas estratégias e agentes terapêuticos que permitam fazer frente à crescente problemática da resistência. Entre estes novos agentes terapêuticos, os peptídeos antimicrobianos têm emergido como um dos mais promissores, pois em princípio, a possibilidade de as bactérias desenvolverem resistência a eles pode ser baixa em comparação aos antibióticos. No entanto, atualmente têm sido observados diversos mecanismos pelos quais as bactérias resistem à ação dos peptídeos antimicrobianos. Portanto, para desenvolver peptídeos antimicrobianos mais eficientes, é necessário, conhecer com profundidade as bases moleculares da resistência desenvolvida a eles. Nesse sentido, uma abordagem proteômica permite compreender as redes moleculares envolvida nesta reposta de resistência aos peptídeos antimicrobianos assim como os mecanismos regulatórios. Além disso, um enfoque proteômico também aporta informação valiosa para elucidar novos mecanismos de ação dos peptídeos antimicrobianos.

Objetivos

Caracterizar a resposta de resistência desenvolvida por *Klebsiella pneumoniae* ao peptídeo antimicrobiano PaDBS1R1 através de uma perspectiva proteômica.

Objetivo Específicos

- Identificar mediante análises de proteômica quantitativa os mecanismos defensivos que possam estar envolvidos na resposta anti-PaDBS1R1 desenvolvida por *Klebsiella pneumoniae* sensível ao peptídeo.
- Desenvolver a resistência de *Klebsiella pneumoniae* ao peptídeo antimicrobiano PaDBS1R1.
- Identificar a partir de análises de proteômica quantitativa os mecanismos de resistência que possam estar envolvidos na resposta anti-PaDBS1R1 desenvolvida por *Klebsiella pneumoniae* resistente ao peptídeo.

Capítulo I

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Resumo

O aumento da resistência aos compostos antimicrobianos pelos microrganismos impôs novos desafios para a comunidade científica, levando à necessidade de descoberta, desenho e produção de novos compostos antimicrobianos. Nesse contexto, entre os novos compostos antimicrobianos investigados intensamente encontra-se os peptídeos antimicrobianos (PAMs), os quais representam uma alternativa esperançosa para enfrentar o problema da resistência antimicrobiana. Consequentemente, o número de novos PAMs sintéticos relatados aumentou nos últimos anos. Entre eles, o peptídeo PaDBS1R1 parece ser um agente antimicrobiano promissor, pois exerce uma potente atividade antibacteriana contra vários patógenos, incluindo *Klebsiella pneumoniae* resistente a múltiplas drogas. No entanto, existe um conhecimento escasso sobre como os patógenos respondem a um desafio com PaDBS1R1 ou sobre como esse peptídeo exerce sua atividade antimicrobiana. Portanto, o entendimento desses aspectos é vital para conseguir a implementação bem-sucedida de PaDBS1R1 como agente terapêutico no futuro. Nesse sentido, para o estudo do sistema patógenos-PAMs tem-se implementado várias abordagens microbiológicas, bioquímicas e biofísicas, incluindo também abordagens ômicas de alto desempenho. Assim, o presente estudo explorou uma abordagem proteômica quantitativa para obter novas informações sobre a resposta de *K. pneumoniae* frente ao desafio com PaDBS1R1. Nossos resultados sugerem que *K. pneumoniae* remodelou sua maquinaria metabólica em resposta ao desafio com uma concentração sub-inibitória de PaDBS1R1. *K. pneumoniae* parece desenvolver uma resposta anti-PaDBS1R1 complexa, que envolve vários mecanismos de defesa, incluindo produção de cápsula, modificação dos lipopolissacarídeos (LPS), bombas de efluxo e ativação do sistema de resposta ao estresse do fator sigma E (σ^E); além de promover a ocorrência de danos no DNA bacteriano. A exposição contínua a concentrações sub-inibitórias do peptídeo promoveu o surgimento de bactérias com susceptibilidade reduzida ao mesmo.

Palavras chaves: PaDBS1R1, *Klebsiella pneumoniae*, proteômica, peptídeos antimicrobianos, resistência

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Corresponding Author: Dr. Octavio L. Franco,

Corresponding Author's Institution: Universidade Catolica de Brasilia / Universidad Católica de Dom Bosco

First Author: Osmel Fleitas

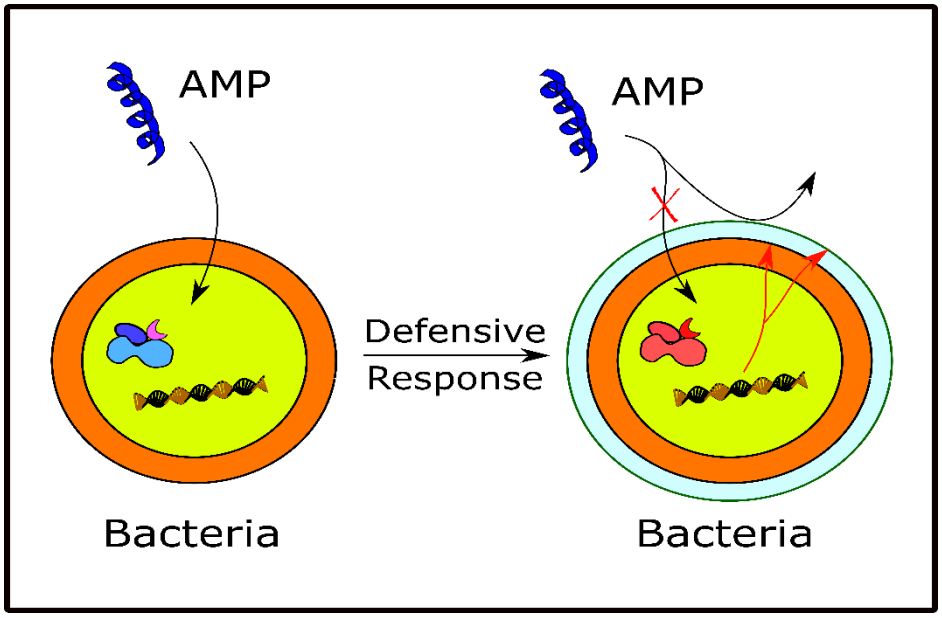
Order of Authors: Osmel Fleitas; William Porto, PhD; Osmar Silva, PhD; Wagner Fontes, PhD; Mariana Castro, PhD; Marcelo Sousa, PhD; Carlos Ricart, PhD; Marcelo Ramada, PhD; Octávio Franco, PhD

Abstract: The rise of resistance to antimicrobial compounds by microbes has imposed new challenges on the scientific community, leading to the need for the discovery, design and production of novel antimicrobial compounds. In this regard, among the intensively investigated novel antimicrobial compounds are the antimicrobial peptides (AMPs). Among these, the AMP PaDBS1R1 appears to be a promising antimicrobial agent as it exerted potent antibacterial activity against several bacterial pathogens, including antibiotic-resistant Klebsiella pneumoniae. However, there is scarce knowledge about how the pathogens respond to a PaDBS1R1 challenge or concerning how this peptide exerts its antimicrobial activity. Therefore, the present study explored a label-free quantitative shotgun proteomics approach to gain new insight into the response of K. pneumoniae to the novel AMP PaDBS1R1. Our results suggest that K. pneumoniae remodeled its metabolic machinery when challenged with a sub-inhibitory concentration of PaDBS1R1. Furthermore, the bacteria appear to build up a complex anti-PaDBS1R1 response, which involves several mechanisms of defense including capsule production, lipopolysaccharide (LPS) modification, efflux pumps and sigma factor E (σE)-stress response system activation. Moreover, PaDBS1R1 appears to promote bacterial DNA damages, and continuous exposure to sub-inhibitory concentrations of the peptide favored the emergence of bacteria with reduced susceptibility to PaDBS1R1.

Suggested Reviewers: Robert Ulrich
rulrich@bhsai.org

David Tirrell
tirrell@caltech.edu

Graphical Abstract



Highlights

- PaDBS1R1 challenge promoted changes in *Klebsiella pneumoniae* metabolic machinery
- *Klebsiella pneumoniae* mounts an anti-PaDBS1R1 response that comprises multiple defense mechanisms
- PaDBS1R1 challenge might promote bacterial genome damage
- Sub-inhibitory concentrations of PaDBS1R1 facilitate the emergence of PaDBS1R1-resistant bacteria

Klebsiella pneumoniae proteome changes under antimicrobial peptide PaDBS1R1-induced stress indicates the assembly of a complex defensive response

Osmel F. Martínez [1,2], William F. Porto [3,4], Osmar N. Silva [3], Wagner Fontes [1,5], Mariana S. Castro [1,5], Marcelo V. Sousa [1,5], Carlos A. O. Ricart [1,5], Marcelo H. S. Ramada [6,7], Octávio L. Franco [1,2,3 *]

[1]Programa de Pós-Graduação em Patologia Molecular, Universidade de Brasília, Brasília, Brazil.

[2]Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil.

[3]S-Inova Biotech, Programa de Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, Brazil.

[4]Porto Reports, Brasília, Brazil.

[5]Laboratório de Bioquímica e Química de Proteínas, Universidade de Brasília, Brasília, Brazil.

[6]Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil.

[7]Programa de Pós-Graduação em Gerontologia, Universidade Católica de Brasília, Brasília, Brazil.

*Corresponding author: Octávio Luiz Franco: Phone number: +55 61 34487167/ +55 61 34487220, Fax number: + 55 61 33474797, ocfranco@gmail.com

Abstract

The rise of resistance to antimicrobial compounds by microbes has imposed new challenges on the scientific community, leading to the need for the discovery, design and production of novel antimicrobial compounds. In this regard, among the intensively investigated novel antimicrobial compounds are the antimicrobial peptides (AMPs), which signify a hopeful alternative strategy with which to face the microbial resistance problem. Accordingly, the number of novel synthetic AMPs reported has increased in recent years. Among these, the AMP PaDBS1R1 appears to be a promising antimicrobial agent as it exerted potent antibacterial activity against several bacterial pathogens, including antibiotic-resistant *Klebsiella pneumoniae*. However, there is scarce knowledge about how the pathogens respond to a PaDBS1R1 challenge or concerning how this peptide exerts its antimicrobial activity. Therefore, the understanding of these aspects is vital to achieve the successful implementation of PaDBS1R1 as a new therapeutic agent in the future. In this regard, many microbiological, biochemical and biophysical approaches have been implemented to study the pathogen-AMP system including high-throughput omics approaches. Therefore, the present study explored a label-free quantitative shotgun proteomics approach to gain new insight into the response of *K. pneumoniae* to the novel AMP PaDBS1R1. Our results suggest that *K. pneumoniae* remodeled its metabolic machinery when challenged with a sub-inhibitory concentration of PaDBS1R1. Furthermore, the bacteria appear to build up a complex anti-PaDBS1R1 response which involves several mechanisms of defense including capsule production, lipopolysaccharide (LPS) modification, efflux pumps and sigma factor E (σ^E)–stress response system activation. Moreover, PaDBS1R1 appears to promote bacterial DNA damages, and continuous exposure to sub-inhibitory concentrations of the peptide favored the emergence of bacteria with reduced susceptibility to PaDBS1R1.

Keywords: PaDBS1R1, *Klebsiella pneumoniae*, proteomics, antimicrobial peptides, resistance

Significance

Resistance to antibiotics by bacterial pathogens constitutes a challenge for the scientific community, which has been searching for new antimicrobial compounds as an alternative to conventional antibiotics. Among the new explored antimicrobial compounds are the antimicrobial peptides (AMPs), which arise as a promising alternative. In this regard, the AMP PaDBS1R1 was newly announced as a powerful AMP with broad-spectrum antimicrobial activity. However, there is scarce knowledge about the action mechanism(s) of this peptide or about the anti-PaDBS1R1 response produced by the bacterium. The present study tries to shed light on the PaDBS1R1-*Klebsiella pneumoniae* interplay from a proteomics perspective. Our results suggest that *K. pneumoniae* assembles a complex response against the PaDBS1R1 challenge. This anti-PaDBS1R1 response involves a remodeling of the bacterial metabolic machinery as well as the involvement of several protective mechanisms. Moreover, we revealed that the continuously challenged with sub-inhibitory concentrations of PaDBS1R1 supported the appearance of PaDBS1R1-resistant bacteria. This study provides much-needed information for the understanding of the bacterial response against PaDBS1R1, which could be relevant for the development of future studies that aim to deepen the knowledge about PaDBS1R1-bacteria interplay.

Introduction

Bacterial resistance to antimicrobial drugs is of extreme concern to human health due to the mortality and morbidity increase associated with drug-resistant pathogen infections (CDC, 2017; Tacconelli and Pezzani, 2019). It has been estimated that by 2050 the number of deaths caused by antimicrobial-resistant pathogens could exceed the number of deaths per year currently caused by cancer (O'Neill et al., 2016; Tagliabue and Rappuoli, 2018). Furthermore, the spread of multi-drug resistant bacteria that overcome the last-resort antibiotics and the possibility of cross-resistance have made it even harder to find solutions to the bacterial resistance phenomenon (Napier et al., 2013; Dobias et al., 2017; Jeannot et al., 2017; Wang et al., 2018). This has moved the scientific community toward the search for alternatives to conventional antimicrobials. In this regard, among the explored alternatives, the isolation and design of AMPs stands out (Felício et al., 2017; Cardoso et al., 2018a; Porto et al., 2018).

AMPs are small bioactive amphipathic biomolecules that usually carry a net positive charge. These biomolecules are ubiquitously produced in prokaryotic and eukaryotic organisms and exert potent antimicrobial activity, performing a crucial function in the innate immune system of multicellular organisms (Mahlapuu et al., 2016a; Moravej et al., 2018). It is accepted that the main mechanism of action of AMPs to exert their antibacterial activity is based on the permeabilization and disruption of the bacterial cytoplasmic membrane (Sierra et al., 2017; Moravej et al., 2018). However, other mechanisms of action may involve the AMPs' translocation to the bacterial cytoplasm, where they interfere with replication, transcription, translation, cell division and protein folding (Le et al., 2017a). This multiplicity of action mechanisms and bacterial targets of AMPs, along with their pharmacodynamic properties, suggests that evolution of resistance toward AMPs could be less probable than resistance observed toward antibiotics (Mahlapuu et al., 2016a; Yu et al., 2018). This makes AMPs an attractive option by which to fight bacterial pathogens. Accordingly, several AMPs have been submitted to clinical trials (E Greber and Dawgul, 2017; Naafs, 2018).

Despite the potential of AMPs, bacterial pathogens have developed strategies to overcome their antimicrobial activity (Maria-Neto et al., 2015; Nuri et al., 2015). Therefore, the design of novel optimized AMPs is necessary to magnify their utility as antimicrobial agents. In this regard, a greater understanding of the AMPs' structure-

activity relationship and physicochemical properties, as well as the development of molecular and bioinformatics tools, has allowed the rational design of novel synthetic AMPs with enhanced antimicrobial activity and structural stability (de Castro and Franco, 2015; Porto et al., 2018; Torres et al., 2019). In fact, synthetic AMPs constitute a high proportion among the AMPs that are currently under pre-clinical and clinical trials (Naafs, 2018; Kang et al., 2019).

Recently, Irazazabal and co-workers (2019) using computational, microbiological and biophysical methods reported the peptide PaDBS1R1 as a potent synthetic AMP (Irazazabal et al., 2019). The peptide PaDBS1R1 was obtained by rational design via the Joker algorithm, using the ribosomal protein L39E from *Pyrobaculum aerophilum* as the parental template (Porto et al., 2018). This AMP exerted broad-spectrum microbicide activity in the low μM range, probably via a membrane-lytic mechanism. Specifically, the amidated derivative of PaDBS1R1 was seen to be active against carbapenem-resistant *K. pneumoniae* (Irazazabal et al., 2019). This antibacterial activity against resistant *K. pneumoniae* is encouraging, given the fact that nowadays *Enterobacteriaceae* family members constitute frequent agents in nosocomial infections as well as in the propagation of resistance genes, which facilitate the emergence of multidrug-resistant pathogens (Tacconelli et al., 2018; Temkin et al., 2018; Wyres and Holt, 2018).

Several biophysical and computational approaches have been used to gain insight into AMPs' structure and their mechanisms of action (Porcelli et al., 2013; Avci et al., 2018; Cardoso et al., 2018c). In addition, high-throughput omics approaches (e.g. genomics, transcriptomics and proteomics) also constitute suitable and complementary options to acquire understanding about how AMPs act and how pathogens respond to these antimicrobial compounds (Franco, 2015; Martinez et al., 2016; Cardoso et al., 2018b).

Particularly, proteomics has proven to be a useful strategy to elucidate AMPs' targets and action mechanisms. In this regard, two-dimensional gel electrophoresis coupled to liquid chromatography-mass spectrometry (2D-LC-MS/MS) and gel-free LC-MS/MS approaches have been used as complementary methods to elucidate the action mechanisms of the AMP MP196 and its derivatives (Wenzel et al., 2014b; Wenzel et al., 2016). *Escherichia coli* proteome microarrays enabled the identification of intracellular targets of the AMPs bactenecin-7, lactoferricin B, proline-arginine-rich peptide (PR-39), and a hybrid peptide of pleurocidin and dermaseptin (P-Der) (Ho et al., 2016).

Furthermore, another study focused on the *E. coli*-membrane-associated proteome when the bacteria was challenged with the macrocyclic peptide JB-95, which suggested that JB-95 might interfere with the β-barrel assembly machinery (BAM) (Urfer et al., 2016).

Proteomic approaches have also been valuable in the comprehension of bacterial resistance mechanisms to AMPs (Wenzel et al., 2014b; Martinez et al., 2016; Cardoso et al., 2017). In this regard, Wenzel et al. 2014 combined proteome and amino acid pool analysis to identify the osmotic stabilization via release of osmoprotective amino acids as a new bacterial defense mechanism against membrane–disturbing peptides (Wenzel et al., 2014b). Moreover, Schmidt et al. 2016 utilized proteomic approaches to make available evidence that suggests that changes in the abundance levels of fimbrial complex and the enzyme isochorismatase hydrolase (YcaC) might be involved in the resistance of *E. coli* to the peptide apidaecin 1b (Schmidt et al., 2016). Other proteomic studies have highlighted that the resistance response to AMPs is a complex one, in which the activation of several defense mechanisms and the remodeling of the bacterial metabolic machinery are involved (Maria-Neto et al., 2012; Urfer et al., 2016; Cardoso et al., 2017).

Hence, based on the benefits that proteomic approaches provide to study AMPs, in this research we aim to gain new insights into the mode of action of PaDBS1R1 and the response developed by *K. pneumoniae* against the PaDBS1R1 challenge through a label-free quantitative shotgun proteomic approach. For this purpose, the differentially abundant proteins (DAPs) were identified between an experimental target group (PaDBS1R1-challenged bacteria) and an experimental control group (PaDBS1R1 non-challenged bacteria). For this, *K. pneumoniae* was grown in a nutrient-rich medium and challenged with a sub-inhibitory concentration of PaDBS1R1. Subsequently, total proteins were extracted and analyzed via liquid chromatography coupled to mass spectrometry. The functional profile of the DAPs suggested that in response to the PaDBS1R1 challenge, *K. pneumoniae* restructured the molecular machinery associated with metabolic energy production and with biomolecular biosynthesis processes. Moreover, the bacteria appear to activate several anti-PaDBS1R1 defense mechanisms, including capsule production, lipopolysaccharide (LPS) modification, efflux pumps and sigma factor E (σ^E)–stress response system. Additionally, it was observed that continuous exposure to sub-inhibitory concentrations of PaDBS1R1 promoted the raise of bacteria with diminished sensitivity susceptibility to the peptide.

Material and methods

PaDBS1R1 peptide

The AMP PaDBS1R1 (PKILNKILGKILRLAAAFK) was purchased from AminoTech P&D (Brazil) at 95% purity. The homogeneity of the synthesized peptide (theoretical monoisotopic mass $(M+H)^+$: 2107.3841) was checked by MALDI-TOF analysis using an Autoflex Speed spectrometer (Bruker Daltonics, GmbH, Leipzig) (Supplemental Figure 1). Briefly, PaDBS1R1 was mixed with 3 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 $mg.mL^{-1}$ CHCA in 50% acetonitrile (ACN)/0.3 % trifluoroacetic acid (TFA)) and 1 µL of the mixture was deposited on the stainless steel sample plate and left to dry at room temperature. Subsequently, the spectrum of PaDBS1R1 was obtained using the RP 700-3500 method (reflector positive ion mode) with laser energy of 20% and 3000 laser shots on random position at the sample spot. The obtained mass spectrum was processed using the software flexAnalysis 3.4 (Bruker Daltonics).

Bacteria

In the present study the bacterium used for the proteomic analysis was *K. pneumoniae* subsp. *pneumoniae* ATCC 13883 (American Type Culture Collection) adapted to Lysogeny Broth (LB) medium (designed as *K. pneumoniae* NP). Briefly, overnight cultures of *K. pneumoniae* ATCC 13883 (5 mL, 37 °C, without agitation) were adjusted to ~ $1x10^8$ CFU. mL^{-1} and transferred to 100 µL of fresh LB medium to a final concentration of ~ $1x10^6$ CFU. mL^{-1} and incubated for 24h (37 °C, 80 rpm). After 24 h of growth, the cultures were transferred (1 µL) to 100 µL of fresh LB medium and incubated under the same conditions (24h, 37 °C, 80 rpm). This procedure was performed repeatedly during 24 transfers in LB medium. To obtain the bacteria with diminished sensitivity to PaDBS1R1 (designed as *K. pneumoniae* P) from the strain *K. pneumoniae* subsp. *pneumoniae* ATCC 13883, the same procedure as described above was performed, but the LB medium was supplemented with sub-inhibitory concentrations of PaDBS1R1, which were increased by 1.5 fold after four transfers. The initial concentration of PaDBS1R1 was 7.6 µM, which represented half of the minimum inhibitory concentration

(MIC) of PaDBS1R1 for parental *K. pneumoniae* subsp. *pneumoniae* ATCC 13883 strain. The other subsequent concentrations of PaDBS1R1 supplemented in LB medium were 11.4; 17.1; 25.7; 38.5 and 57.7 µM.

At the end of the 24 transfers in LB medium, the cultures of both *K. pneumoniae* NP and *K. pneumoniae* P were checked by MALDI-TOF to discard contamination by other microorganisms using the protocol described by Alatoom et al. 2011, with some modifications (Alatoom et al., 2011). Briefly, several colonies were isolated from LB agar plates of both *K. pneumoniae* NP and *K. pneumoniae* P. The isolated colonies were individually dissolved in 300 µL of Milli Q H_2O and vortexed by 10 s. Next, 900 µL of ethanol were added and the samples were centrifuged at 13, 000 g for 2 min. Subsequently, the pellets were collected and dissolved in 30 µL of formic acid (70% v/v) with the subsequent addition of 30 µL of acetonitrile and vortexed for 10 s. Afterwards, the samples were centrifuged at 13, 000 g for 2 min and the supernatants were collected. The collected supernatants were analyzed by MALDI-TOF (microFlex, Bruker Daltonics) using the MBT method (linear positive, 2000-20000 m/z) and the obtained spectra were compared with the Biotyper library using the Biotyper 3 software (Bruker Daltonics).

Minimum inhibitory concentration assay

The minimum inhibitory concentrations (MIC) of PaDBS1R1 for *K. pneumoniae* ATCC 13883, *K. pneumoniae* NP and *K. pneumoniae* P were determined by broth microdilution assay in a 96-well microtiter plate as described by Wiegand et al. 2008 with modifications (Wiegand et al., 2008). Briefly, for each tested bacterium three colonies were selected, inoculated in 5 mL of LB medium and incubated for 3 - 4 h (37 °C, 225 rpm). After that, the bacterial concentration was adjusted to ~ $1x10^8$ CFU. mL^{-1} and diluted 10^{-2} times (inoculum). Subsequently, 50 µL of the inoculum was transferred to microtiter plate wells previously filled with 50 µL of LB medium (supplemented with PaDBS1R1), yielding a final assay volume of 100 µL, final bacterial concentration of ~ 5 $x10^5$ CFU. mL^{-1} and final peptide concentrations from 0.24–121.6 µM (two-fold diluted). The microtiter plate was incubated for 24 h, at 37 °C and under continuous agitation in the microplate reader Bio-tek PowerWave, and the bacterial growth was checked by computing the OD 595 nm (software Gen5 2.00). The MIC was reported as the lowest peptide concentration (µM) that inhibited bacterial growth, considering the average value from at least three independent experiments performed in triplicate. The

growth control was the inoculated bacteria in LB medium without being supplemented with PaDBS1R1. The bacterial growth curves were created using the GraphPad Prism 6 software.

Protein extraction

K. pneumoniae NP was grown in LB medium (10 mL) for 8 h at 37 °C and 80 rpm in two experimental conditions (target- and control- conditions). Under the target condition, bacteria (four biological replicates) were grown in LB medium supplemented with a sub-inhibitory concentration of PaDBS1R1 (5.8 µM) whereas, under the control condition, bacteria (four biological replicates) were grown in LB medium without being supplemented with PaDBS1R1. After bacterial growth for 8 h, the total proteins from both target- and control-groups were isolated using the MPLEx method (*the metabolite, protein, lipid extraction protocol*) described by Burnum-Johnson et al. 2017, but with some modifications (Burnum-Johnson et al., 2017). Briefly, bacterial cultures were centrifuged at 11, 000 g for 10 min at 4 °C. Subsequently, the pellets were reconstituted in 2 mL of NH_4HCO_3 (50 mM) and centrifuged again under the same conditions. The obtained pellets were reconstituted in 150 µL of NH_4HCO_3 (50 mM) and 600 µL of MPLEx solution [$CHCl_3$/MeOH: 2/1 (v/v)] was added. Next, the samples were vortexed for 10 s, left on ice for 5 min and vortexed again. Afterwards, the samples were centrifuged at 13, 000 g for 10 min at 4 °C and the protein-contained interphase was collected (two phases separated by a protein disc are formed after centrifugation). The collected protein disc was reconstituted in 200 µL of cold methanol (-20 °C) and centrifuged at 13, 000 g for 10 min at 4 °C. Subsequently, the collected pellet was dissolved in 500 µL of NH_4HCO_3 (50 mM) and sonicated for 30 s. Later, the protein concentration was determined by Qubit assay (Invitrogen) and 500 µg of total proteins of each sample were dried by Speed-Vac.

Sample preparation for LC-MS/MS

The total protein pellet (500 µg) was dissolved in 30 µL of RapidGest solution (0.1 % w/v in NH_4HCO_3 (50 mM)) and DTT was supplemented to a final concentration of 5 mM. The samples were incubated at 60 °C for 30 min and after being cooled at room temperature, iodoacetamide was added to a final concentration of 10 mM with the subsequent incubation in the dark for 30 min. Afterwards, DTT was added (10 mM final concentration) and incubated for 15 min in the dark. Next, 5 µg of trypsin (Trypsin Gold,

Promega, USA) was added to each sample and incubated for 15 h at 37 °C. After that time, 2 µg of trypsin was additionally supplemented to each sample and incubated for 9 h at 37 °C. At the end of the trypsinization step, the samples were supplemented with TFA to a final concentration of 0.5% (v/v) and incubated for 30 min at 37 °C. Subsequently, the samples were centrifuged at 13,000 g for 10 min at 4 °C and the supernatants were collected and dried by Speed-Vac. Successively, the dried peptide samples were dissolved in 20 µL of 0.1%TFA (v/v) and desalted by Zip-Tip following the manufacturer's recommendations. Later, the desalted peptide samples were quantified by Qubit assay and dried by Speed-Vac.

LC-MS/MS

The dried tryptic peptide samples were reconstituted in 0.1% formic acid and injected (1µg of peptides per injection) in a chromatography system (Dionex Ultimate 3000 RSLCnano UPLC, Thermo, USA) coupled to Orbitrap Elite mass spectrometer (Thermo, USA). The peptide mixtures were separated by a 2-35 % acetonitrile linear gradient in 0.1% formic acid over 155 min (flow rate 230 $nL.min^{-1}$) using a trap column (3 cm x inner diameter 100 µm, 5 µm of C18 particle size, ReprosilPur, Dr. Maich, GmbH) connected in line with an analytical column (24 cm x ID 75 µm, 3 µm of C18 particle size, ReprosilPur, Dr. Maich, GmbH). The chromatographed peptides were directly eluted into an Orbitrap Elite mass spectrometer, which was operated in data-dependent acquisition (DDA). The full scan MS spectra (m/z 300-1650) were acquired in the Orbitrap analyser at a resolution of 120, 000. The 15 most intense ions with intensity over 3000 were fragmented at the HCD collision cell and the generated fragment ions were analysed by Orbitrap analyser yielding the respective MS/MS spectrum at a resolution of 15, 000.

The raw MS spectra data were processed with Progenesis QI for proteomics (Nonlinear dynamics, UK). In this regard, the alignment of the runs was performed and the raw abundance of the precursor ions was calculated from the extracted ion chromatogram (XIC) and normalised via the Progenesis default method (normalise to all proteins). The MS/MS spectra from peptide ions with significantly ($p < 0.05$) normalised abundance differences between the target and control groups were exported to the Peaks software version 7.0 (BSI, USA) for protein identification. The protein identification was performed using the UniprotKB *Klebsiella pneumoniae* ID. 573 proteome (downloaded in April 2018). The search parameters included trypsin as protease allowing up to two

missed cleavages, cysteine carbamidomethylation as a fixed modification, whereas methionine oxidation was selected as variable modification. The mass tolerance values for precursor and fragment ions were 10 ppm and 0.05 Da, respectively. Furthermore, the false discovery rate (FDR) for peptide-spectrum matches was 1%. Subsequently, the identified proteins with confidence scores (-10lgP ≥ 20), FDR < 1% and at least one unique peptide were exported back to the Progenesis software and quantitated using the normalized abundance of non-conflicting peptides assigned to each protein. The proteins with significantly normalised abundance difference between the target and control groups ($q < 0.05$) and fold change (FC) ≥ 2 or ≤ 0.5 were considered as differentially abundant proteins (DAPs) and were selected for further analysis. All the raw dataset from LC-MS/MS analysis are available at FTP host = massive.ucsd.edu, User = LBQP_Osmel_NPspxNPcpprot; Password = lbqp1cbsp9.

Data analysis

To perform functional profile and pathway enrichment analysis of relevant identified proteins based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), the DAPs were mapped to the proteome of the KEGG-annotated *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700721 / MGH 78578 strain using the Blast2GO tool (Conesa et al., 2005). Next, for the functional profile of the DAPs based on the KEGG orthology database, the KEGG ortholog (KO) identifiers were assigned using the KofamKOALA tool (Aramaki et al., 2019). Moreover, the Network-based Visualization for Omics (NeVomics) tool was used for KEGG- and Gene Ontology (GO)-enrichment analysis of DAPs (Zúñiga-León et al., 2018).

Results

Identification of differentially abundant proteins in the PaDBS1R1-challenged *Klebsiella pneumoniae* NP and their functional profile based on KEGG Orthology

To expose the proteome changes of *K. pneumoniae* NP under PaDBS1R1-induced stress conditions, the bacteria were challenged with 5.8 µM of PaDBS1R1. This concentration is sub-inhibitory as the MIC of PaDBS1R1 for *K. pneumoniae* NP was 11.4 ± 3.8 µM (Supplemental Figure 2). After data processing, 366 proteins were identified as differentially abundant proteins (DAPs) (FC ≥ 2 or ≤0.5, $q < 0.05$) between the target and control groups. Among the DAPs, 117 proteins were up-regulated (FC ≥ 2, $q < 0.05$), whereas 249 proteins were down-regulated (FC ≤ 0.5, $q < 0.05$) (Supplemental Table 1).

The functional profile of DAPs based on KEGG Orthology (KO) displayed 219 proteins distributed in five biological classes (Figure 1A, Supplemental Table 1). These biological classes included cellular processes, environmental information processing, genetic information processing, human diseases, and metabolism. Particularly, metabolism was the most represented biological class, since 159 DAPs were associated with this category (Figure 1A, Supplemental Table 1).

A further profile of the proteins associated with the category of metabolism into metabolic sub-categories yielded 11 metabolic sub-categories. These metabolic sub-categories included amino acid metabolism, metabolism of other amino acids, xenobiotics biodegradation and metabolism, metabolism of terpenoids and polyketides, lipid metabolism, glycan biosynthesis and metabolism, carbohydrate metabolism, nucleotide metabolism, metabolism of cofactors and vitamins, biosynthesis of other secondary metabolites and energy metabolism (Figure 1B). The proteins associated with each subcategory and the exclusive proteins of each sub-category are summarized in Figure 1B and Supplemental Table 1.

KEGG pathway enrichment analysis of differentially abundant proteins in the PaDBS1R1-challenged *Klebsiella pneumoniae* NP

KEGG pathway enrichment analysis using the Nevomics tool for up-regulated DAPs showed enrichment for glycerolipid metabolism, propanoate metabolism, inositol phosphate metabolism, and metabolic pathways (Figure 2). The main up-regulated DAPs associated with these pathways identified by Nevomics software are described in

Supplemental Table 2. Additionally, similar enrichment analysis for down-regulated DAPs did not detect enrichment of KEGG pathways.

Gene ontology enrichment analysis of differentially abundant proteins in the PaDBS1R1-challenged *Klebsiella pneumoniae* NP

The GO enrichment analysis of up-regulated DAPs using the Nevomics tool detected enrichment for three biological processes including acetyl-CoA biosynthetic process, inositol catabolic process, and nucleotide-excision repair (Figure 3A, Supplemental Table 3). In the category of molecular function, three functions were enriched, including helicase activity, oxidoreductase activity, acting on CH-OH group of donors, and excinuclease ABC activity (Figure 3B, Supplemental Table 3). Nevertheless, the analysis for cellular component category showed two enriched components that included the excinuclease repair complex and the cytoplasm (Figure 3C, Supplemental Table 3).

On the other hand, the analysis of GO for down-regulated DAPs showed enrichment for the biological processes gluconeogenesis, tricarboxylic acid cycle (TCA), histidine catabolic process to glutamate and formate, histidine catabolic process to glutamate and formamide, translation, sulfate assimilation, hydrogen sulphide biosynthetic process, *de novo* CTP biosynthetic process, *de novo* UMP biosynthetic process, glycine betaine biosynthetic process from choline, tRNA wobble uridine modification, and iron-sulfur cluster assembly (Figure 4A, Supplemental Table 4). Furthermore, the analysis of the molecular function category displayed enrichment for structural constituent of ribosome, metal ion binding, ATP binding, succinate-CoA ligase (ADP-forming)-activity, sulfate adenylyltransferase (ATP) activity, L-serine-ammonia–lyase activity, betaine-aldehyde dehydrogenase activity, protein disulphide oxidoreductase activity, N2-acetyl-L-ornithine:2-oxoglutarate 5-aminotransferase activity, succinate dehydrogenase (ubiquinone) activity, chaperone binding, succinyldiaminopimelate transaminase activity, RNA polymerase binding, and flavin adenine dinucleotide binding (Figure 4B). The DAPs associated with these molecular functions are summarized in Supplemental Table 4. Moreover, in the category of cellular component were enriched the ribosome, periplasmic space and cytoplasm components (Figure 4C, Supplemental Table 4).

Emergence of *Klebsiella pneumoniae* with reduced susceptibility to PaDBS1R1

As previously described, the GO enrichment analysis for up-regulated DAPs showed enrichment for the nucleotide-excision repair system (Figure 3, Supplemental Table 3), suggesting that the challenge of the bacteria with sub-inhibitory concentrations of PaDBS1R1 could promote DNA damage, which potentially could facilitate the emergence of bacteria with reduced susceptibility to PaDBS1R1. To verify if the prolonged challenge of *K. pneumoniae* with sub-inhibitory concentrations of PaDBS1R1 promotes the emergence of bacteria with reduced susceptibility to the peptide, the parental *K. pneumoniae* ATCC 13883 strain was subjected to continuous challenge with sub-inhibitory concentrations of PaDBS1R1 during 24 culture transfers. After 24 culture transfers in LB medium supplemented with sub-inhibitory concentrations of PaDBS1R1 the challenged bacteria (denominated as *K. pneumoniae* P) showed a four-fold higher MIC for PaDBS1R1 than parental strain (*K. pneumoniae* ATCC 13883) (Table 1, Supplemental Figures 3 and 4). This suggests that continued exposure to sub-inhibitory concentrations of PaDBS1R1 promoted the emergence of bacteria with reduced susceptibility to the peptide.

Table 1. Minimum inhibitory concentration (MIC) of PaDBS1R1 against *Klebsiella pneumoniae* strains

Bacteria	Initial MIC (μM) *	Final MIC (μM)**
***K. pneumoniae* ATCC 13883**	15.2	15.2
***K. pneumoniae* P**	15.2	60.8

* MIC of PaDBS1R1 before bacteria were successively transferred in LB medium

** MIC of PaDBS1R1 after bacteria were successively transferred (24 transfers) in LB medium. The parental *K. pneumoniae* ATCC 13883 strain was not successively transferred in LB medium, whereas *K. pneumoniae* P was derived from *K. pneumoniae* ATCC 13883 via successive transfers (24 transfers) in LB medium supplemented with increased concentrations of PaDBS1R1.

Discussion

Recently, the AMP PaDBS1R1 and its C-terminal amidated variant (PKILNKILGKILRLAAAFK-NH_2) were reported as powerful peptides with antimicrobial activity against several bacteria including ATCC strains of *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *K. pneumoniae*, as well as clinical isolates of methicillin-resistant *S. aureus* and carbapenem-resistant *K. pneumoniae* (Irazazabal et al., 2019). These preliminary and promising results pave the way for intensifying the investigations into this novel synthetic AMP, of which little is known. Following this, in the present study, we explored a label-free quantitative shotgun proteomic approach to shed light on the interplay of PaDBS1R1 and *K. pneumoniae*.

PaDBS1R1 challenge promotes changes in the *Klebsiella pneumoniae* metabolic machinery

Our results suggest that the challenge of *K. pneumoniae* with sub-inhibitory concentrations of PaDBS1R1 affect the proteome associated with bacterial metabolic processes. Previous proteomic studies that focused on the response of *E. coli* to the challenge with the membrane-active AMP magainin-I also showed changes in the metabolism-associated proteome in response to AMP-induced stress (Maria-Neto et al., 2012; Cardoso et al., 2017). Moreover, other works dedicated to studying the effect on *Bacillus subtilis* of the small AMP MP196 and its derivatives also showed changes in the metabolism-associated proteome of *B. subtilis* in response to AMP-induced stress (Wenzel et al., 2014b; Wenzel et al., 2016). Therefore, the readjustment of the metabolic machinery appears to be a key element in the bacterial response against AMP-induced stress.

The PaDBS1R1-challenged *K. pneumoniae* appears to remodel its metabolic machinery so that it equalizes the activity of pathways associated with metabolic energy production with anabolic pathways. In this regard, we observed that significant down-regulation of enzymes associated with the TCA cycle (key process in metabolic energy production) was coupled to significant down-regulation of proteins associated with the translational machinery as well as other biosynthetic processes (e.g. gluconeogenesis, *de novo* CTP- and UMP-biosynthesis) (Figure 4, Supplemental Tables 1 and 4), suggesting

that challenged bacteria could respond to the PaDBS1R1-induced stress via decreasing its metabolic activity. In this context, alternative routes could be operating for metabolic energy production. Indeed, we observed enrichment for the acetyl-CoA biosynthetic process, with the associated enzymes phosphate acetyltransferase (Pta) and acetate kinase (AckA) (Figure 3A, Supplemental Table 3). The enzymes Pta and AckA constitute the Pta-AckA system, which could perform the ATP biosynthesis from acetyl-CoA via substrate-level phosphorylation (Wolfe, 2005). In line with this, enrichment was observed for the inositol catabolic process (Figures 2 and 3A, Supplemental Tables 2 and 3), which putatively allows the bacteria to use external inositol-contained compounds to obtaining acetyl-CoA (Berman and Magasanik, 1966a; b; Anderson and Magasanik, 1971). Then, ATP production via Pta-AckA system could be strategic for metabolic energy production in the PaDBS1R1–challenged bacteria.

Klebsiella pneumoniae mounts an anti-PaDBS1R1 response that comprise multiple defense mechanisms

In the challenged bacteria, proteins associated with carbohydrate uptake systems were also up-regulated including the components ManZ and ManX of mannose PTS permease MaXYZ (Supplemental Table 1). This mannose transporter is involved in the uptake of mannose as well as of other sugars, such as N-acetylglucosamine, D-glucosamine, fructose and glucose (Erni et al., 1987; Postma et al., 1993). The parental bacterium used in this study was *K. pneumoniae* ATCC 13883, which is capsular serotype K3 (ATCC, 2019). The capsule of *K. pneumoniae* serotype K3 is composed of a pyruvate acetal-containing pentasaccharide as the basic unit, which contains mannose, galactose, and D-galacturonic acid (3: 1: 1) (Dutton et al., 1986). Then, mannose constitutes a major *K. pneumoniae* capsular component in capsular serotype K3 strains and capsule production is a key mechanism of defense against AMPs (Campos et al., 2004; Llobet et al., 2008). In line with this, the significantly up-regulated abundance of transporter YjiY and threonine/serine:H^+ symporter (TdcC) as well as the enzyme TdcB (Supplemental Table 1), could also be linked with the supply of pyruvate for capsule production. The transporter YjiY is a high-affinity pyruvate transporter that mediates the pyruvate uptake, whereas the threonine/serine:H^+ symporter (TdcC) and the enzyme TdcB have been linked to serine uptake and degradation yielding pyruvate (Shizuta et al., 1969; Kristoficova et al., 2018).

Moreover, we observed a up-regulation of the proteins RcsF, BamA, BamB and Wza (Supplemental Tables 1and 5). The lipoprotein RcsF is a component of the Rcs stress response system, whereas BamA and BamB are components of the β-barrel assembly machinery (BAM), and Wza translocates the capsular polysaccharides toward the bacterial surface (Yuan et al., 2013; Wall et al., 2018; Ricci and Silhavy, 2019). The Rcs system is activated by bacterial envelope stress (Laloux and Collet, 2017). In this system, RcsF acts by sensing the envelope stress, and its interaction with BamA appears to be important to sense envelope stress and triggers the activation of Rcs system (Cho et al., 2014; Laloux and Collet, 2017). Among the cues that trigger the Rcs system activation are the AMPs, among which polymyxin B is an activator of the Rcs system in *K. pneumoniae* (Farris et al., 2010; Llobet et al., 2011; Audrain et al., 2013). The activated Rcs system controls the expression of several genes, including the capsular polysaccharide synthesis (*cps*) gene cluster, which encodes the molecular machinery responsible for biosynthesis and export of capsular polysaccharides (Majdalani and Gottesman, 2005; Whitfield, 2006; Wall et al., 2018). Therefore, our proteomic data suggest that PaDBS1R1-induced stress could activate the Rcs system with the subsequent triggering of capsule production as a strategic defense mechanism against PaDBS1R1 (Figure 5). Capsule production has been identified as a key mechanism through which *K. pneumoniae* resists the action of antimicrobials like polymyxin B, human neutrophil α-defensin 1 (HNP-1), β-defensin 1, lactoferrin and protamine sulfate (Campos et al., 2004; Llobet et al., 2008; Llobet et al., 2011).

The production of 4-amino-4-deoxy-L-Arabinose (L-Ara4N)-modified lipid A is one of the key mechanisms used by *K. pneumoniae* to resist AMPs (Helander et al., 1996; Cannatelli et al., 2013). In line with this, we observed an increased abundance (although FC< 2) of the proteins UDP-4-amino-4-deoxy-L-arabinose aminotransferase (ArnB), undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase (ArnC), and bifunctional polymyxin resistance protein (ArnA) that are associated with lipid A modification via L-Ara4N (Supplemental Table 5), while the response regulator protein PhoP from the two-component PhoPQ regulatory system displayed a marginally increased abundance (Supplemental Table 5). However, proteins such as LpxC, LpxL and KdsB that are associated with LPS biosynthesis showed significantly down-regulated abundance levels (Supplemental Table 1). Therefore, there could be an imbalance between the molecular machinery involved in the LPS production and the one associated

with LPS modification, which could limit the supply of LPS precursor molecules to be modified with L-Ara4N. So, lipid A modification via L-Ara4N might be involved in the response to PaDBS1R1, but may not be the main mechanism to overcome the PaDBS1R1 action (Figure 5). The two-component PhoPQ system appears to be involved in AMPs recognition, with the subsequent triggering of anti-AMP response that includes the activation of LPS modification machinery (e.g. addition of L-Ara4N and/or phosphoethanolamine to LPS) (Bader et al., 2005; Merighi et al., 2005; Richards et al., 2012; Shprung et al., 2012).

Moreover, Llobet et al. 2011 showed that *K. pneumoniae* responds to polymyxin B challenge, activating capsule production and lipid A modification with L-Ara4N systems (Llobet et al., 2011). In this regard, our proteomic data suggest that, in response to PaDBS1R1 challenge, *K. pneumoniae* also activates the capsule production and the lipid A modification with L-Ara4N systems (Figure 5). An interesting find by Llobet et al. 2011 was that there is cross-talk among the Rcs and PhoPQ systems (Llobet et al., 2011).

On the other hand, among the up-regulated DAPs zinc metalloprotease YaeL (RseP) was identified (Supplemental Table 1). This protease is involved in the activation cascade of sigma factor E (σ^E)–stress response system (Barchinger and Ades, 2013; Grabowicz and Silhavy, 2017). Once the σ^E –stress response system is activated in response to bacterial envelope stress, the σ^E is released into the bacterial cytoplasm allowing σ^E -modulon transcription with consequent envelope stress relief (Barchinger and Ades, 2013). The response raised after σ^E –stress system activation basically reduces the OMP biosynthesis, up-regulates the OMP folding pathways (through the increased abundance of the BAM machinery, periplasmic chaperones and periplasmic proteases), and promotes LPS biosynthesis and transport (Grabowicz and Silhavy, 2017; Konovalova et al., 2018). In this regard, as previously discussed, the LPS biosynthesis appears to be down-regulated; however, proteins associated with the BAM complex (BamA, BamB and BamD) showed up-regulated abundance levels (Supplemental Tables 1 and 5). Furthermore, the uncharacterized protein YgiM and the protein PlsB also displayed up-regulated abundance levels (Supplemental Table 1). Both *ygiM* and *plsB* genes are part of the σ^E –modulon (Dartigalongue et al., 2001; Rhodius et al., 2005). Therefore, the possibility that the σ^E –stress response system is activated and operates mainly via the BAM complex in the PaDBS1R1-challenged bacteria cannot be ruled out (Figure 5).

Previous studies have shown that σ^E –stress response system could be involved in the bacterial response against AMPs (Crouch et al., 2005; Mathur et al., 2007; Audrain et al., 2013; Woods and McBride, 2017).

In addition to capsular polysaccharide production, LPS modification, and σ^E – stress response, we also detected the proteins MacA and AcrB with increased abundance (Supplemental Tables 1and 5). This suggests that the MacAB-TolC and AcrAB-TolC efflux pump systems could be operating in the challenged bacteria (Figure 5). The AcrAB-TolC system is involved in the resistance response against several AMPs (Warner and Levy, 2010; Weatherspoon-Griffin et al., 2014; De Majumdar et al., 2015; Cardoso et al., 2017). Particularly in *K. pneumoniae*, it appears to be involved in resistance against polymyxin B, human neutrophil defensin 1, human β-defensin-1, and human β-defensin-2 (Padilla et al., 2010). Moreover, the MacAB-TolC system appears to be involved in resistance to bacitracin and colistin (Crow et al., 2017). Additionally, both systems facilitate the resistance to a broad range of antibiotics and promote bacterial virulence (Bogomolnaya et al., 2013; Li et al., 2015; Greene et al., 2018).

The assembly of a complex response involving several anti-AMP mechanisms is in agreement with the observations of previous studies that used ``OMICs´´ approaches to understand the bacterial anti-AMP response. These studies reported the arrangement of complex bacterial responses in which several anti-AMP mechanisms appear to operate actively (Maria-Neto et al., 2012; Cardoso et al., 2017; Cardoso et al., 2018b).

PaDBS1R1 challenge might promote bacterial genome damage

The GO enrichment analysis of the up-regulated DAPs shows nucleotide-excision repair, excinuclease ABC activity and excinuclease repair complex as enriched terms (Figure 3, Supplemental Table 3). Specifically, the up-regulated DAPs UvrA and UvrB were associated with the exinuclease repair complex (Figure 3, Supplemental Table 3). These proteins form the $UvrA_2B_2$ complex, which is part of the nucleotide-excision repair system (NER) (Kamarthapu and Nudler, 2015; Deaconescu and Suhanovsky, 2017). Furthermore, we observed an increased abundance of DNA polymerase I (PolA), DNA ligase (LigA), and XseB, which also are linked to the NER system (Supplemental Tables 3 and 5) (Husain et al., 1985; Morita et al., 2010; Adebali et al., 2017). Moreover, subunit NrdB of the ribonucleotide-diphosphate reductase was significantly up-regulated (Supplemental Table 1). Ribonucleotide-diphosphate reductase is involved in the supply

of deoxynucleotides, which could be used by the repair machinery (Torrents, 2014). Then, this abundance pattern of proteins associated with the NER repair system suggests that PaDBS1R1 might promote bacterial genome damage.

The NER system allows bacteria to check and repair DNA damage at the genome scale via the global genome repair (GGR) sub-pathway and at transcription level via the transcription-coupled repair (TCR) sub-pathway (Morita et al., 2010; Kamarthapu and Nudler, 2015; Deaconescu and Suhanovsky, 2017). With regard to the TCR sub-pathway, a significant down-regulation in the protein Mfd abundance was observed (Supplemental Table 1), whereas the protein Rho showed significantly increased abundance (Supplemental Table 1). Particularly, the protein Rho was associated with the enriched molecular function of helicase activity (Figure 3B, Supplemental Table 3). Recently, it has been proposed that protein Rho could act synergistically with Mdf and could also facilitate the recognition of DNA lesion by the GGR sub-pathway (Jain et al., 2019). Therefore, in the PaDBS1R1-challenged bacteria, the Mdf down-regulated abundance may be compensated by the significantly increased abundance of Rho.

In addition to components of the NER repair system, we also detected a significantly up-regulated abundance of RsmA (KsgA) (Supplemental Table 1). The protein RsmA is a methyltransferase mainly involved in the biogenesis of the small subunit of ribosome (Connolly et al., 2008). However, the GO enrichment analysis for down-regulated DAPs suggests the repression of the translational machinery (Figure 4). Interestingly, RsmA has also been linked to DNA protection against oxidative stress, since it holds DNA glycosylase activity, which removes cytosine mispairing with oxidized thymine from DNA (Zhang-Akiyama et al., 2009).

The fact that proteins associated with DNA repair systems were up-regulated suggests that PaDBS1R1 might promote DNA damages. How could PaDBS1R1 promote this putative DNA damage?

In this regard, it has been shown that membrane damage via AMP activity facilitates the entry of Fe^{2+} and Fe^{3+} to the bacterial cytoplasm. Once in the cytoplasm, the Fe^{3+} is reduced to Fe^{2+} which in the presence of reactive oxygen species (ROS) (e.g. H_2O_2) produces, via Fenton reaction, reactive hydroxyl radicals, which are DNA-damaging agents that promote mutations (Rodríguez-Rojas et al., 2015). In line with this, we observed that in PaDBS1R-treated bacteria there was a significantly increased

abundance of Fe^{2+}-storage protein ferritin (Supplemental Table 1), which could be a strategy to limit the availability of Fe^{2+} to react with H_2O_2 but also could be a sign of risen intracellular Fe^{2+} levels (Figure 5). Moreover, among up-regulated DAPs were identified proteins like CydB, YieF, MacA, and Tpx, which are associated with anti-oxidative stress response, suggesting that PaDBS1R1-challenged bacteria are under oxidative stress (Supplemental Table 1) (Ackerley et al., 2004a; Ackerley et al., 2004b; Cha et al., 2004; Imlay, 2008; Bogomolnaya et al., 2013; Al-Attar et al., 2016).

Furthermore, in the challenged bacteria there was a significantly increased abundance of FrdA, which constitutes the FAD-cofactored subunit of the FrdABCD complex (Supplemental Table 1). In the presence of O_2, the FAD cofactor of this enzymatic complex is prone to oxidation, yielding ROS like H_2O_2 and superoxide (Imlay, 1995; Korshunov and Imlay, 2010). Another enzyme with increased abundance in the challenged bacteria and that has been associated with ROS production is the respiratory NADH dehydrogenase Ndh (Supplemental Table 5) (Messner and Imlay, 1999; Seaver and Imlay, 2004). Then, taking into account these elements and given the facts that LB medium is an iron-rich medium and that PaDBS1R1 disturbs bacterial membrane, it is possible that PaDBS1R1 promotes DNA damage through the favoring of iron entrance into the bacterial cytoplasm with subsequent hydroxyl radical formation (Figure 5). The NER repair system has been linked to ROS-induced DNA damage repair (McKibbin et al., 2013; Melis et al., 2013). Moreover, the protein RsmA (KsgA), as previously discussed, is involved in the protection against the mutagenic effect of oxidized thymine species, which are formed via hydroxyl radicals (Zhang-Akiyama et al., 2009). Based on all these elements, we hypothesized that PaDBS1R1, in addition to membrane damage, also promotes the production of toxic species (e.g. hydroxyl radicals) that provoke DNA damage.

Given the fact that hydroxyl radicals are mutagenic, we envisioned that the prolonged challenge of *K. pneumoniae* with sub-inhibitory concentrations of PaDBS1R1 might promote the emergence of bacteria with reduced susceptibility to PaDBS1R1. In this regard, we observed that after challenging the parental *K. pneumoniae* ATCC 13883 strain with sub-inhibitory concentrations of PaDBS1R1 during 24 culture transfers, the bacterium is less susceptible to the peptide, since a four-fold increase in the MIC was detected for PaDBS1R1 (Table 1, Supplemental Figure 4). Therefore, the use of improper

concentrations of PaDBS1R1 (i.e. sub-MIC concentrations) might promote the emergence of PaDBS1R1-resistant bacteria.

Conclusions

Our data suggest that in response to the antimicrobial peptide PaDBS1R1 challenge, *K. pneumoniae* raises a multifaceted response that involves activation of several protective systems as well as the remodeling of bacterial metabolic machinery. Moreover, sub-inhibitory concentrations of PaDBS1R1 appear to stimulate the overexpression of efflux pump systems, which are linked to multi-drug resistance and pathogen virulence. Additionally, sub-inhibitory concentrations of PaDBS1R1 might also promote the production of toxic molecular species with mutagenic potential, which could facilitate the emergence of PaDBS1R1-resistant bacteria. Although the assays in this study were performed under *in vitro* conditions, which differ from *in vivo* conditions, the fact that sub-inhibitory concentrations of PaDBS1R1 stimulate the emergence of bacteria with decreased susceptibility to the AMP, as well as the activation of mechanism associated with antibiotic resistance, constitutes a warning to be careful in the design of future therapeutic schemes that could involve PaDBS1R1.

Conflict of interest Statement

The authors declare that the research was conducted in the absence of any potential conflict of interest.

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FIGURE CAPTIONS

Figure 1. Functional profile of *Klebsiella pneumoniae* NP differentially abundant proteins based on KEGG Orthology. A) Distribution of the differentially abundant proteins through the KEGG biological categories. B) Distribution of the differentially abundant proteins associated with metabolism category into metabolic subcategories. The values in the boxes represent the number of proteins associated with the category or subcategory. The values in parenthesis represent the number of proteins exclusive of the category or subcategory. Exclusive proteins make reference to proteins that are only associated with a particular category or subcategory.

Figure 2. Enriched KEGG pathways detected by Nevomics for up-regulated differentially abundant proteins. The enriched KEGG pathways are graphically represented as a network clustered by colors. Each colored bar in the bar plot represents an enriched pathway with its respective –log10 (p-value). The black line in the bar plot represents the –log10 (p-value) of 0.05, which was selected as the reference p-value. The blue line in the bar plot represents the –log10 (adjusted p-value) for each enriched pathway. The number at the end of each bar corresponds to the number of proteins distinguished as associated with each pathway by Nevomics tool. The color of each node in the network is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein.

Figure 3. Enriched Gene Ontology terms for up-regulated differentially abundant proteins. A) Biological process. B) Molecular function. C) Cellular component. The enriched GO terms are represented in chord plots clustered by colours. Each colour is associated with an enriched GO term and links the proteins related to the respective GO term. The colour of each segment associated with a protein identifier is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein. The values in parenthesis denote the number of proteins detected as associated with each category by the Nevomics tool.

Figure 4. Enriched Gene Ontology terms for down-regulated differentially abundant proteins. A) Biological process. B) Molecular function. C) Cellular component. The enriched biological process and cellular component categories are represented as chord plots clustered by colours. Each colour is associated with a specific enriched GO term

and links the proteins related to the respective GO term. The colour of each segment associated to a protein identifier is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein. The values in parenthesis denote the number of proteins detected as associated with each term by Nevomics tool. The molecular function category is graphically represented as a network clustered by colors. Each coloured bar in the bar plot represents an enriched GO term with its respective –log10 (p-value). The black line in the bar plot represents the –log10 (p-value) of 0.05, which was selected as the reference p-value. The blue line in the bar plot represents the –log10 (adjusted p-value) for each enriched GO term. The number at the end of each bar corresponds to the number of proteins detected as associated with each GO term by Nevomics tool. The colour of each node in the network is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein.

Figure 5. Schematic representation of the main processes that putatively take place in the PaDBS1R1-challenged *Klebsiella pneumoniae* NP according to its proteome changes. 1) Sub-inhibitory concentrations of PaDBS1R1 could provoke transient bacterial membrane perturbations that could facilitate the uncontrolled influx of extracellular Fe^{2+}. 2) The increased abundance of the FAD-cofactored subunit FrdA of the FrdABCD complex in the presence of O_2 might promote the production of H_2O_2. 3) The Fe^{2+} and H_2O_2 react via Fenton reaction producing hydroxyl radicals (ȮH), which produce DNA damages and mutations. 4) Bacteria activate the NER machinery and protein RsmA to perform DNA repair. 5) The protein FtnA might sequester Fe^{2+} cations to reduce their availability for hydroxyl radical production. 6) The PaDBS1R1-induced outer membrane perturbations might be detected by RcsF, which activates the Rcs phosphorelay system with the consequent polysaccharide capsule production. 7) The PaDBS1R1-induced stress could also activate the σ^E –stress response system that stimulates the expression of the β-barrel assembly machinery (BAM). 8) The two-component system PhoPQ could be involved in PaDBS1R1 sensing and subsequent activation of the operon *arnBCADTEF* that mediates the modification of LPS with L-Ara4N. 9) The AcrAB-TolC and MacAB-TolC efflux pump systems could be activated in response to PaDBS1R1-induced stress.

Supplemental Figure 1. MALDI spectrum of the peptide PaDBS1R1.

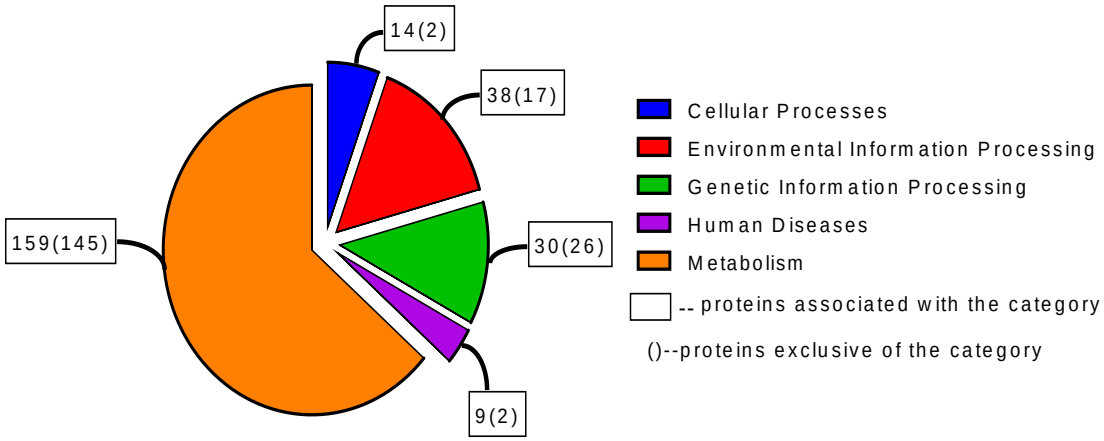
Supplemental Figure 2. *Klebsiella pneumoniae* NP growth curves at different PaDBS1R1 concentrations. A) Experiment 1. B) Experiment 2. C) Experiment 3. D) Experiment 4. The minimum inhibitory concentration (MIC) of PaDBS1R1 that inhibit *K. pneumoniae* NP growth in each independent experiment is showed in bold and signaled with an asterisk (*). The MIC was reported as the lowest peptide concentration (µM) that inhibited the bacterial growth, considering the average value from the independent experiments.

Supplemental Figure 3. *Klebsiella pneumoniae* ATCC 13883 growth curves at different PaDBS1R1 concentrations. A) Experiment 1. B) Experiment 2. C) Experiment 3. The minimum inhibitory concentration (MIC) of PaDBS1R1 that inhibits the *K. pneumoniae* ATCC 13883 growth in each independent experiment is showed in bold and signaled with an asterisk (*). The MIC was reported as the lowest peptide concentration (µM) that inhibited the bacterial growth, considering the average value from the independent experiments.

Supplemental Figure 4. *Klebsiella pneumoniae* P growth curves at different PaDBS1R1 concentrations. A) Experiment 1. B) Experiment 2. C) Experiment 3. D) Experiment 4. The minimum inhibitory concentration (MIC) of PaDBS1R1 that inhibits the *K. pneumoniae* P growth in each independent experiment is showed in bold and signaled with an asterisk (*). The MIC was reported as the lowest peptide concentration (µM) that inhibited the bacterial growth, considering the average value from the independent experiments.

Figure 1

A


14(2)
38(17)
30(26)
9(2)
159(145)
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Human Diseases
Metabolism
-- proteins associated with the category
()--proteins exclusive of the category

B

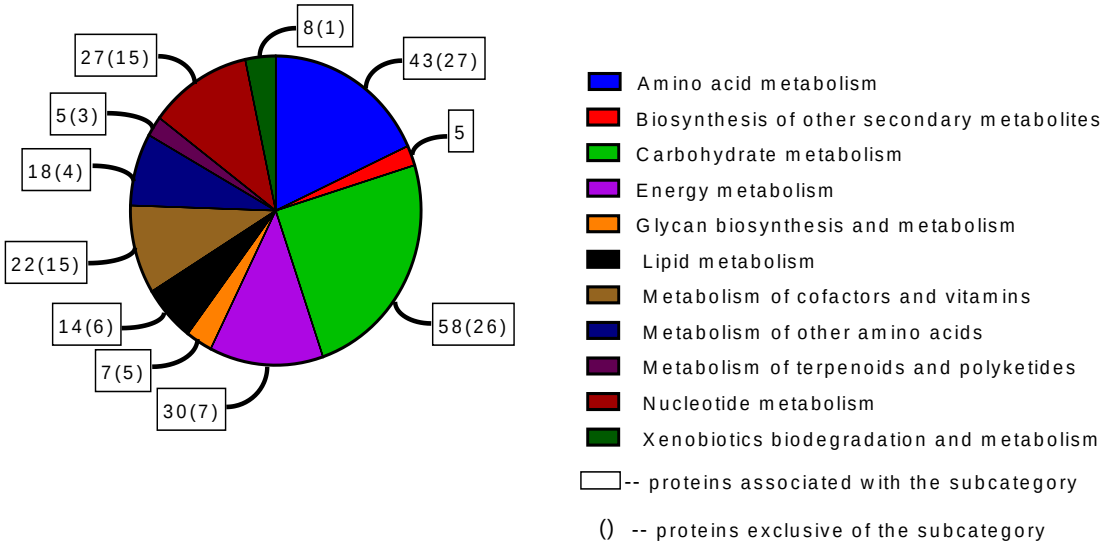
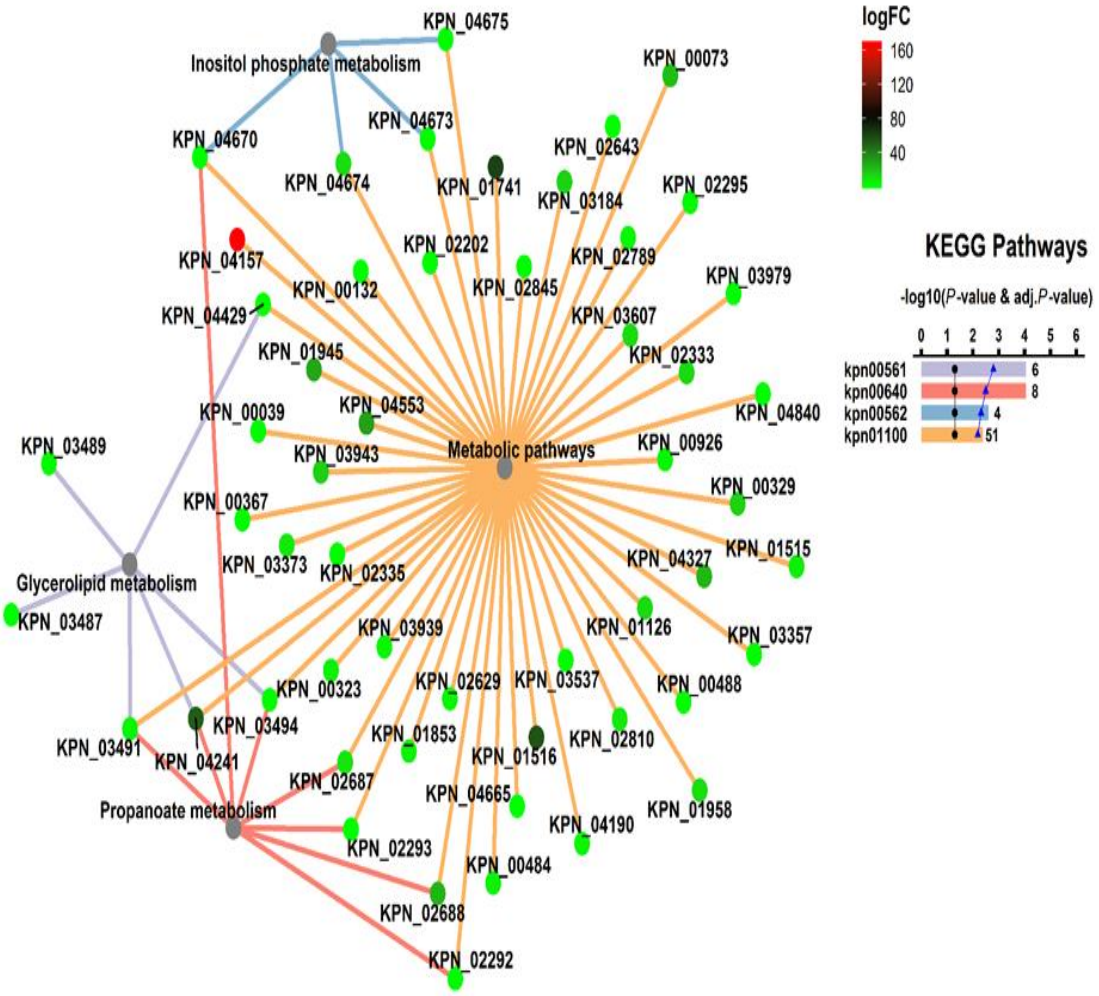

8(1)
43(27)
5
58(26)
30(7)
7(5)
14(6)
22(15)
18(4)
5(3)
27(15)
Amino acid metabolism
Biosynthesis of other secondary metabolites
Carbohydrate metabolism
Energy metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Metabolism of cofactors and vitamins
Metabolism of other amino acids
Metabolism of terpenoids and polyketides
Nucleotide metabolism
Xenobiotics biodegradation and metabolism
-- proteins associated with the subcategory
() -- proteins exclusive of the subcategory

Figure 2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120
80
40
KEGG Pathways
-log10(P-value & adj.P-value)
0 1 2 3 4 5 6
kpn00561 6
kpn00640 8
kpn00562 4
kpn01100 51
Inositol phosphate metabolism
Glycerolipid metabolism
Propanoate metabolism
Metabolic pathways
KPN_04675
KPN_00073
KPN_04673
KPN_04670
KPN_02643
KPN_04674
KPN_01741
KPN_03184
KPN_02295
KPN_04157
KPN_02202
KPN_02789
KPN_03979
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KPN_04241
KPN_01516
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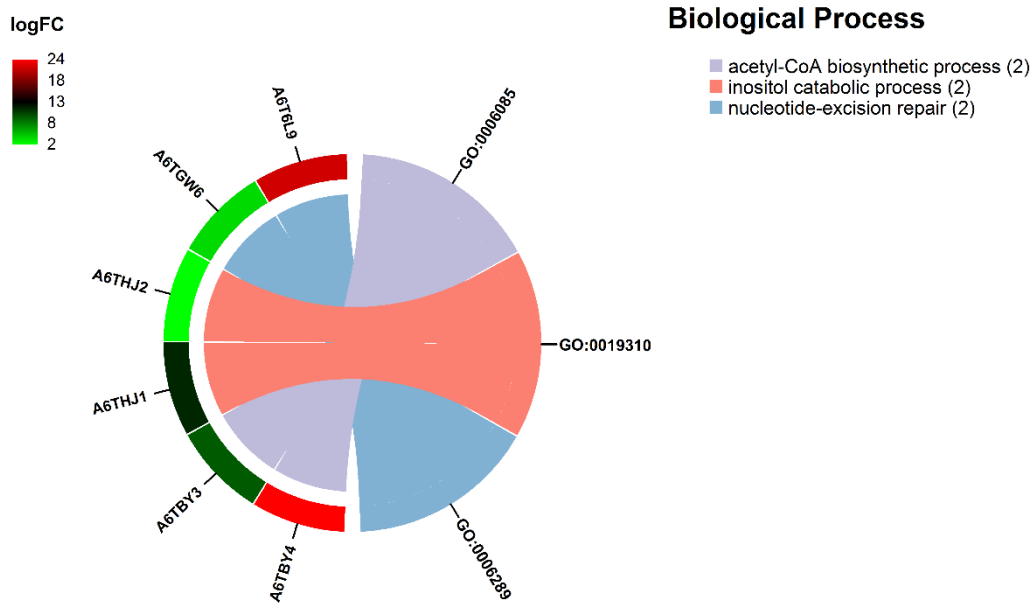
KPN_02688
KPN_02292

Figure 3

A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13
8
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A6TGW6
A6THJ2
A6THJ1
A6TBY3
A6TBY4
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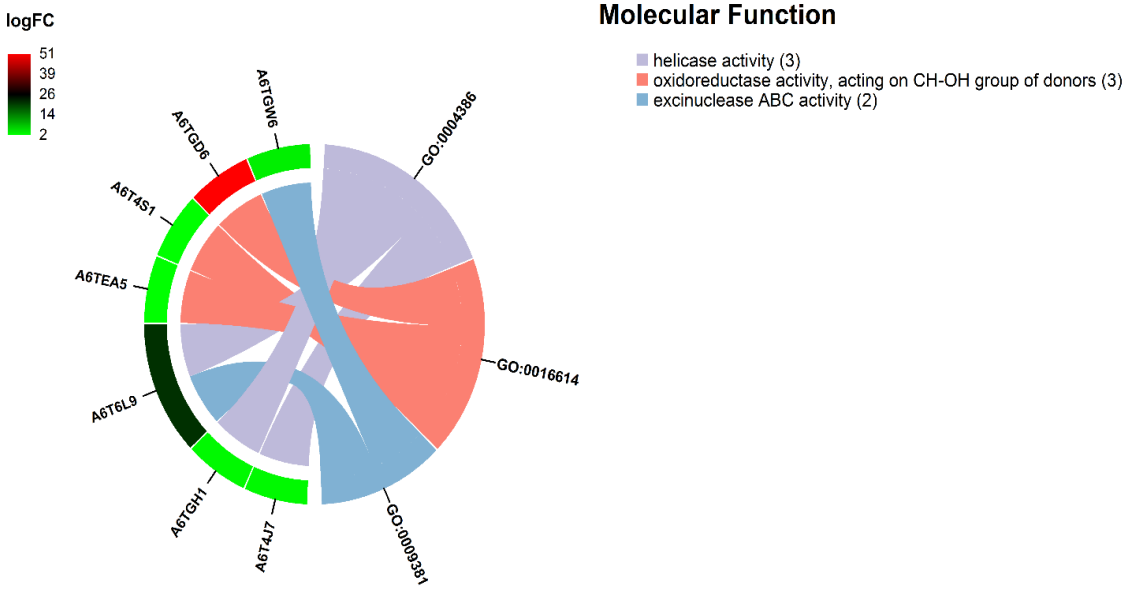
GO:0019310
GO:0006289
Biological Process
acetyl-CoA biosynthetic process (2)
inositol catabolic process (2)
nucleotide-excision repair (2)

B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39
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A6TGD6
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A6TEA5
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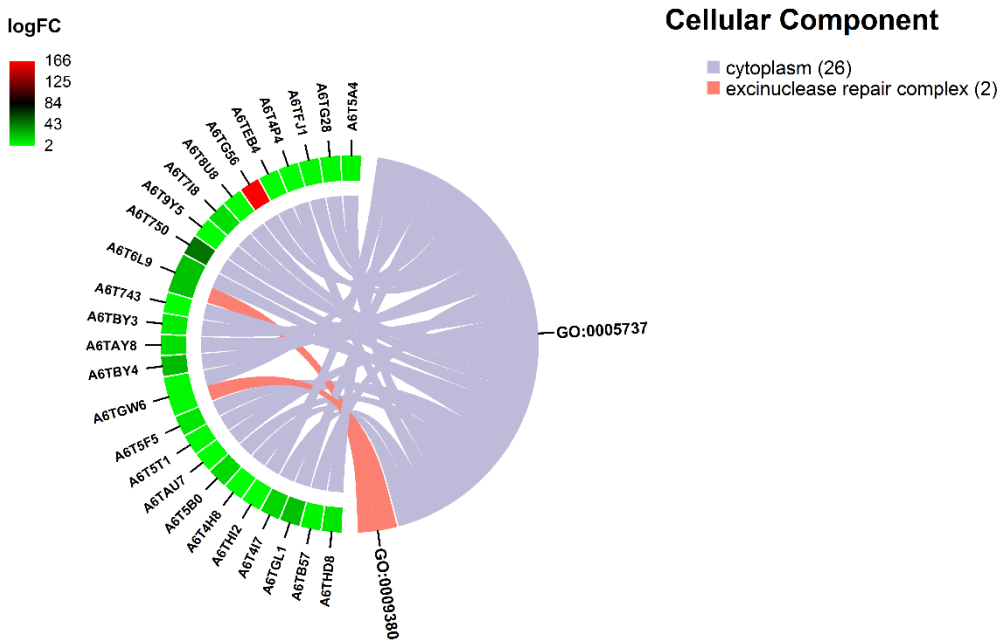
A6TGH1
A6T4J7
GO:0004386
GO:0016614
GO:0009381
Molecular Function
helicase activity (3)
oxidoreductase activity, acting on CH-OH group of donors (3)
excinuclease ABC activity (2)

Figure 3

C


logFC
166
125
84
43
2
Cellular Component
cytoplasm (26)
excinuclease repair complex (2)
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A6TFJ1
A6T4P4
A6TEB4
A6TG56
A6T8U8
A6T7I8
A6T9Y5
A6T750
A6T6L9
A6T743
A6TBY3
A6TAY8
A6TBY4
A6TGW6
A6T5F5
A6T5T1
A6TAU7
A6T5B0
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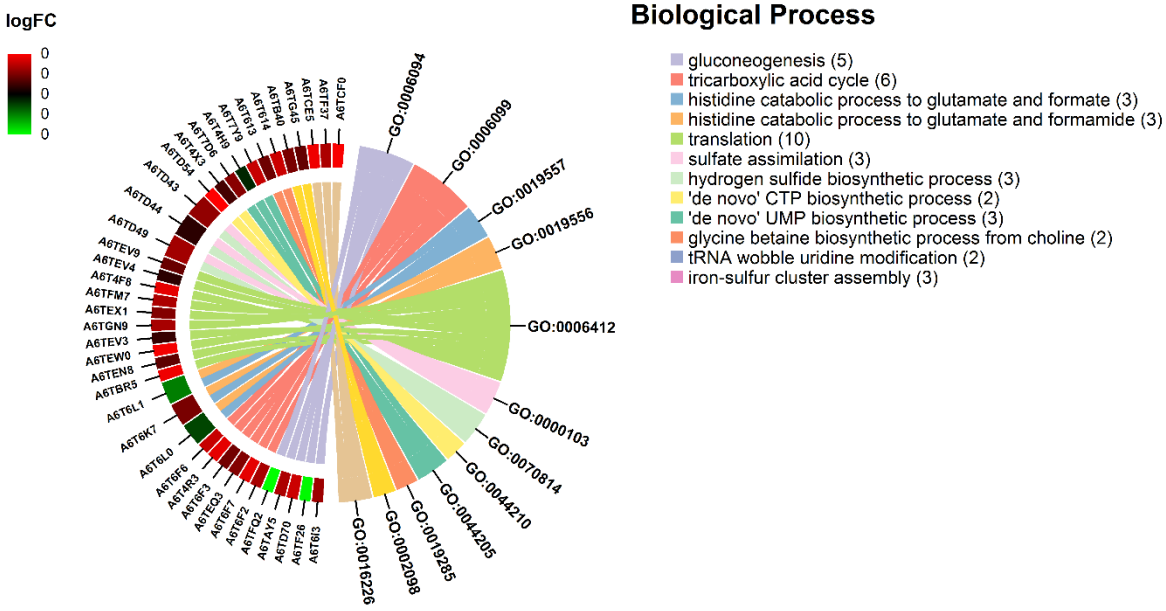
GO:0009380

Figure 4

A


logFC
0
0
0
0
0
Biological Process
gluconeogenesis (5)
tricarboxylic acid cycle (6)
histidine catabolic process to glutamate and formate (3)
histidine catabolic process to glutamate and formamide (3)
translation (10)
sulfate assimilation (3)
hydrogen sulfide biosynthetic process (3)
'de novo' CTP biosynthetic process (2)
'de novo' UMP biosynthetic process (3)
glycine betaine biosynthetic process from choline (2)
tRNA wobble uridine modification (2)
iron-sulfur cluster assembly (3)
GO:0006094
GO:0006099
GO:0019557
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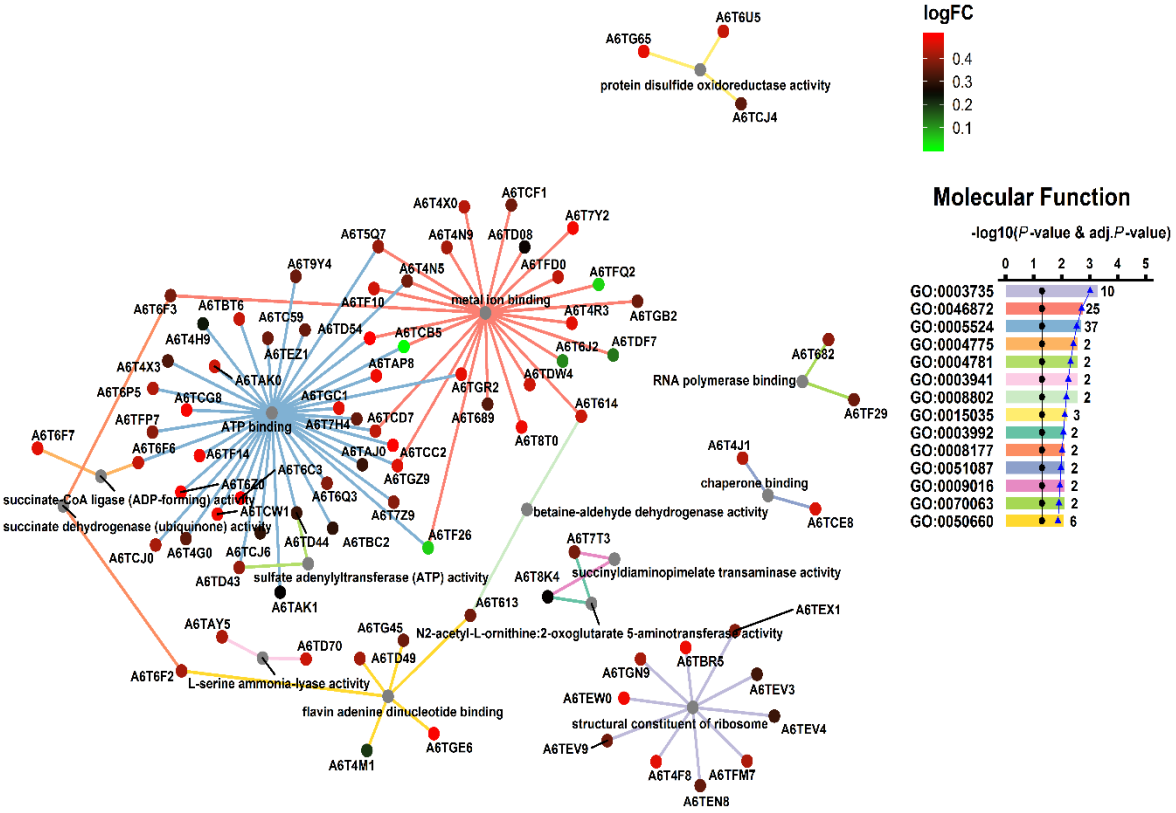
GO:0044205
GO:0019285
GO:0002098
GO:0016226

B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0.4
0.3
0.2
0.1
protein disulfide oxidoreductase activity
metal ion binding
ATP binding
RNA polymerase binding
chaperone binding
betaine-aldehyde dehydrogenase activity
succinate-CoA ligase (ADP-forming) activity
succinate dehydrogenase (ubiquinone) activity
sulfate adenylyltransferase (ATP) activity
succinyldiaminopimelate transaminase activity
N2-acetyl-L-ornithine:2-oxoglutarate 5-aminotransferase activity
L-serine ammonia-lyase activity
flavin adenine dinucleotide binding
structural constituent of ribosome
Molecular Function
-log10(P-value & adj.P-value)
0 1 2 3 4 5
GO:0003735 10
GO:0046872 25
GO:0005524 37
GO:0004775 2
GO:0004781 2
GO:0003941 2
GO:0008802 2
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GO:0050660 6

Figure 4

C

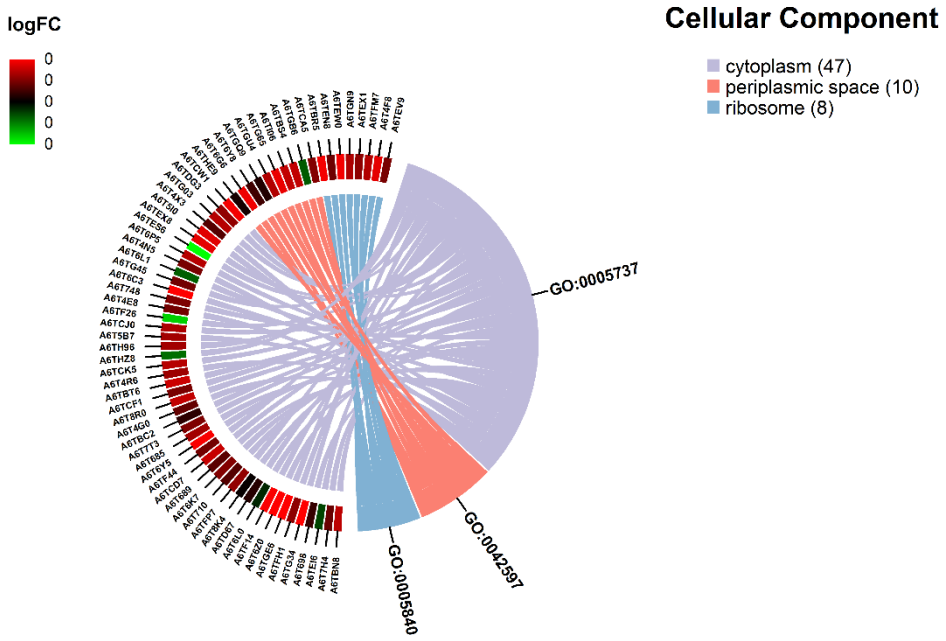
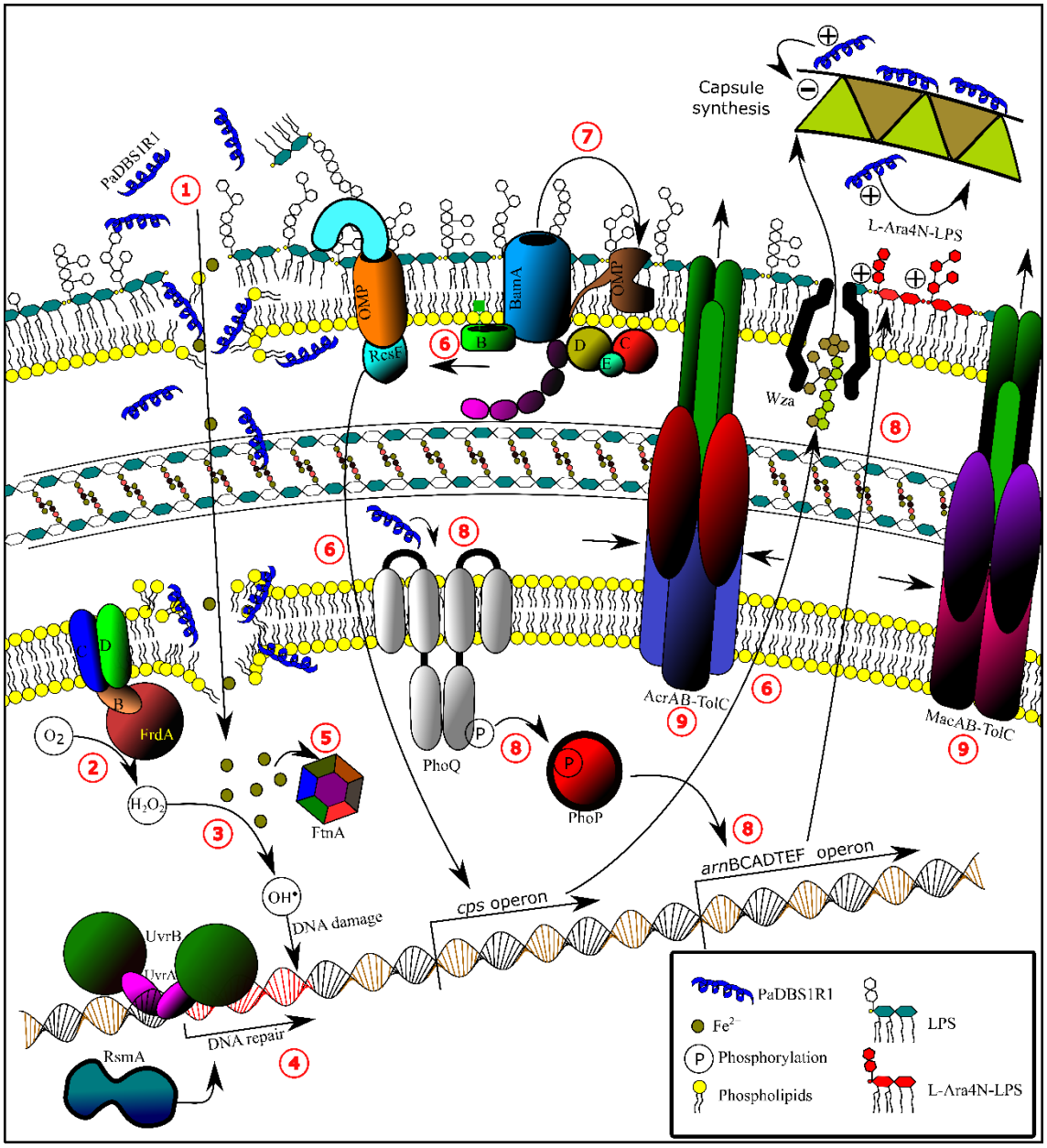
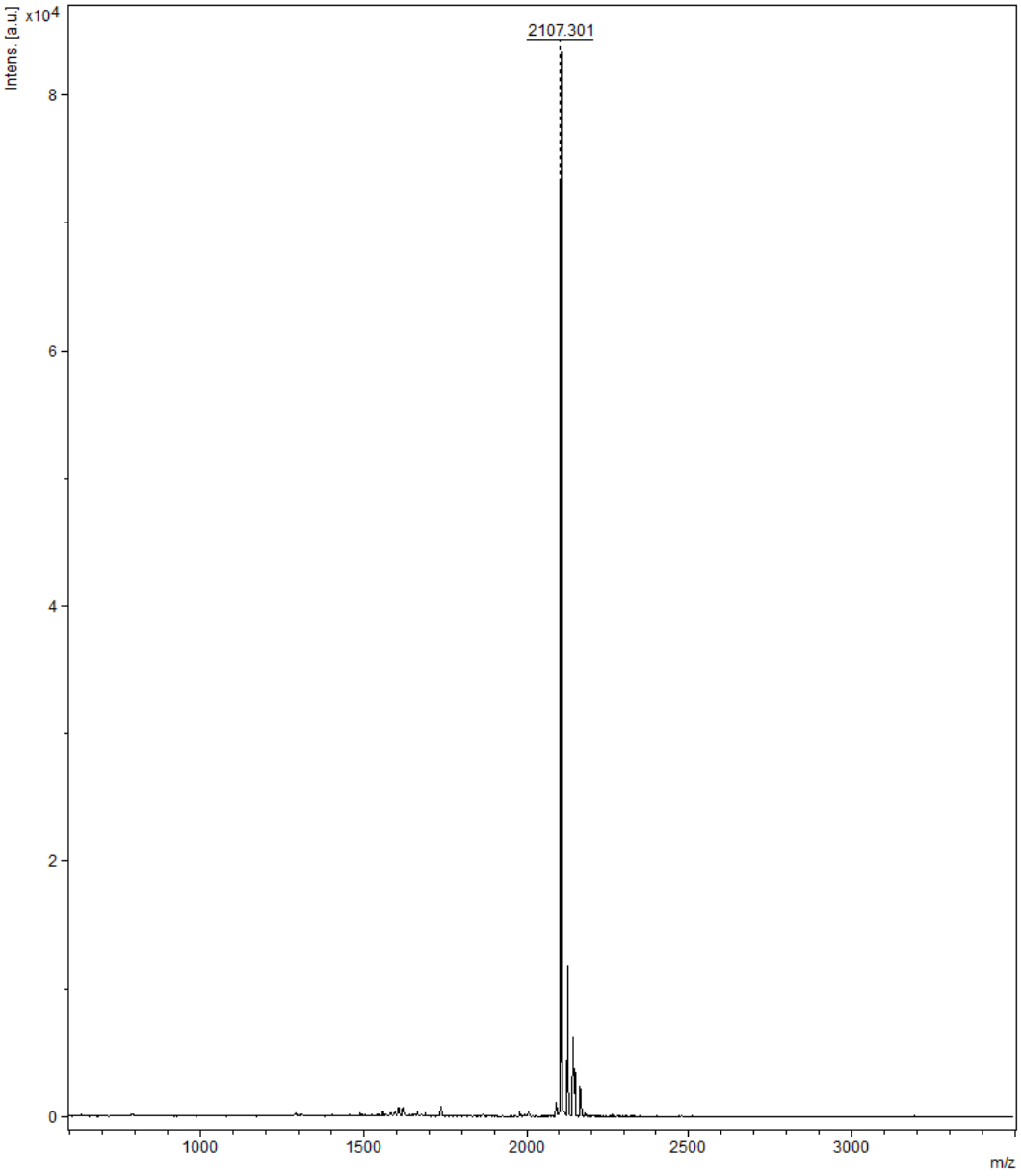

logFC
0
0
0
0
0
Cellular Component
cytoplasm (47)
periplasmic space (10)
ribosome (8)
GO:0005737
GO:0042597
GO:0005840

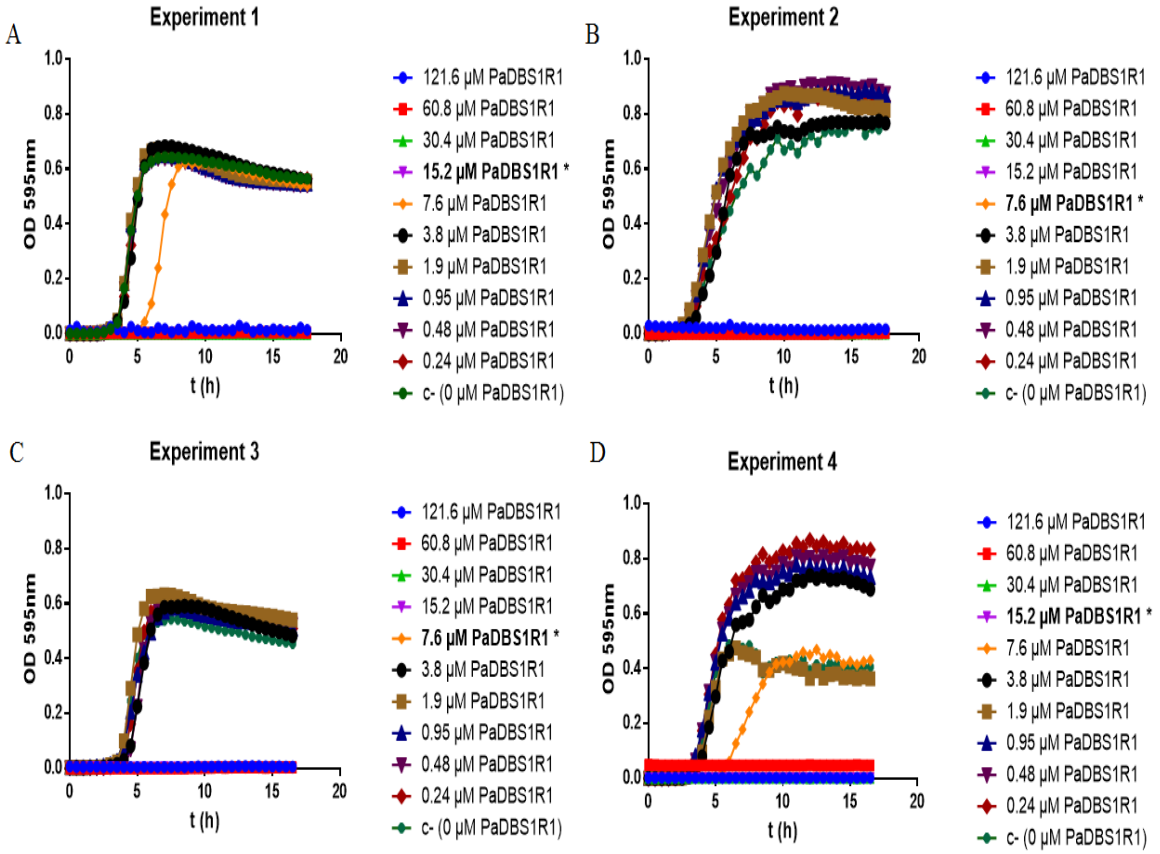
Figure 5


Capsule synthesis
L-Ara4N-LPS
PaDBS1R1
OMP
RcsF
BamA
OMP
B
D
C
E
Wza
AcrAB-TolC
MacAB-TolC
PhoQ
PhoP
P
C
D
B
FrdA
O2
H2O2
FtnA
OH•
DNA damage
UvrB
UvrA
DNA repair
RsmA
cps operon
arnBCADTEF operon
PaDBS1R1
Fe2-
Phosphorylation
Phospholipids
LPS
L-Ara4N-LPS

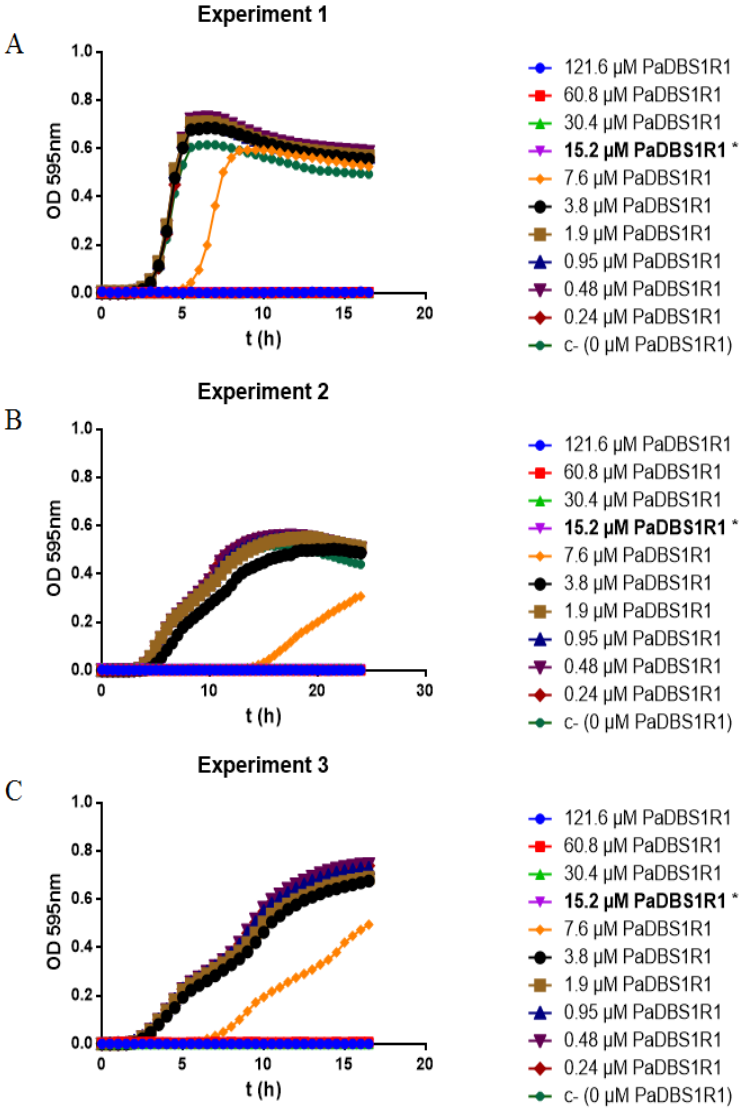
Supplemental Figure 1



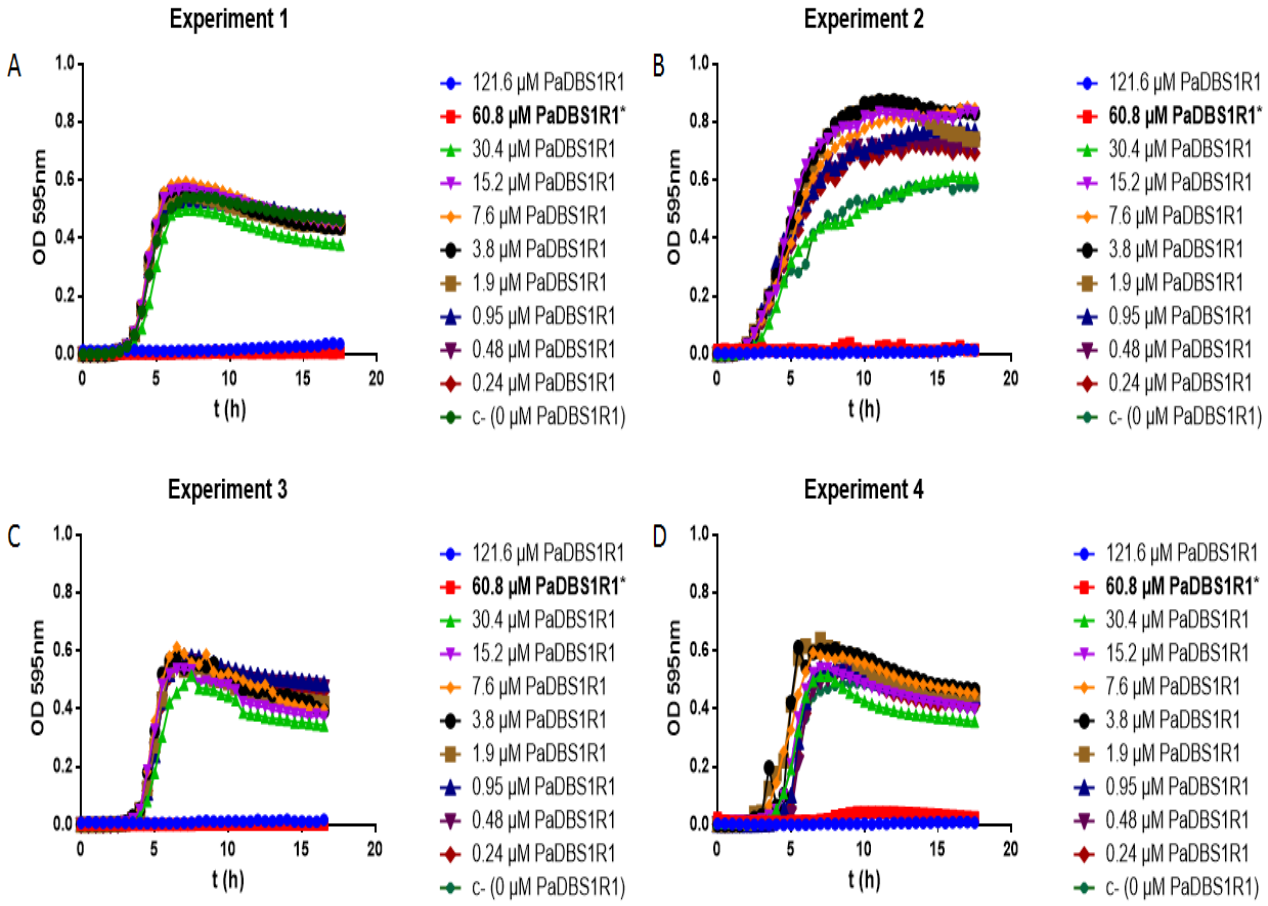
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Capítulo II

Artigo que será submetido na Frontiers in Microbiology

Resumo

O aumento acelerado da resistência desenvolvida por patógenos contra os compostos antimicrobianos ameaça mergulhar-nos em uma era pós-antibiótico em um futuro não muito distante. Isso pode ter consequências desastrosas para a saúde humana e animal. Portanto, terapias alternativas estão sob intensa pesquisa, incluindo a descoberta e o desenho de novos compostos antimicrobianos. Nesse sentido, os peptídeos antimicrobianos (PAMs) têm sido sugeridos como uma opção promissora a ser explorada. No entanto, as bactérias têm desenvolvido uma serie de mecanismos para resistir a atividade microbicida dos PAMs. A compreensão sobre como as bactérias resistem a atividade dos PAMs é obrigatória para o planejamento de estratégias terapêuticas que envolvem essas biomoléculas. Com base nisso, no presente estudo, foi explorado a resposta desenvolvida por *Klebsiella pneumoniae* resistente ao PAM PaDBS1R1 por meio de uma abordagem de proteômica quantitativa. Nossos resultados sugerem que vários sistemas reguladores (sistemas de dois componentes PhoPQ, CpxRA e ZraPSR) orquestraram a resposta anti-PaDBS1R1. Especificamente, as modificações dos lipopolissacarídeo (LPS) parecem ser uma peça chave na resposta de resistência, uma vez que foram regulados positivamente enzimas codificadas por componentes do operon *arnBCADTEF* e a enzima LpxO. Além disso, outros fatores associados à resistência também foram regulados positivamente, incluindo a bomba de effluxo AcrAB-TolC e as proteínas DsbA e PpiA. Curiosamente, foi observado que a *K. pneumoniae* resistente a PaDBS1R1 aumentou a abundância de componentes do sistema de reparação de fosfolipídios LpLT/Aas, bem como um inibidor da lisozima C, o qual fortalece a bactéria resistente com mecanismos de resistência a efetores da resposta imune inata do hospedeiro.

P.D. As figuras do artigo se encontram anexadas com uma maior resolução no final do artigo

Proteomic approach of *Klebsiella pneumoniae* resistance response to the antimicrobial peptide PaDBS1R1

Osmel F. Martínez [1,2], William F. Porto [3,4], Osmar N. Silva [3], Wagner Fontes [1,5], Mariana S. Castro [1,5], Marcelo V. Sousa [1,5], Carlos A. O. Ricart [1,5], Marcelo H. S. Ramada [6,7], Octávio L. Franco [1,2,3 *]

[1]Programa de Pós-Graduação em Patologia Molecular, Universidade de Brasília, Brasília, Brazil.

[2]Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil.

[3]S-Inova Biotech, Programa de Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, Brazil.

[4]Porto Reports, Brasília, Brazil.

[5]Laboratório de Bioquímica e Química de Proteínas, Universidade de Brasília, Brasília, Brazil.

[6]Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil.

[7]Programa de Pós-Graduação em Gerontologia, Universidade Católica de Brasília, Brasília, Brazil.

*Corresponding author: Octávio Luiz Franco: Phone number: +55 61 34487167/ +55 61 34487220, Fax number: + 55 61 33474797, ocfranco@gmail.com

Abstract

The accelerated rising of pathogens resistance against antimicrobial compounds up to unprecedented levels threaten to plunge us into a post-antibiotic era in the not too distant future. This could have disastrous consequences for human and animal health. Therefore, alternative therapies are under intense research including the discovery and design of novel antimicrobial compounds. In this regard, the antimicrobial peptides (AMPs) have been envisioned as a promising option to be explored since they are less prone to resistance than conventional antibiotics. However, the bacteria have developed an array of mechanisms to overcome the AMPs microbicide activity. Hence, the comprehension about how bacteria resist the AMPs activity is mandatory to the planning of therapeutic strategies that involve these biomolecules. Based on this, in the present study we explored via a label-free quantitative shotgun proteomic approach the response performed by *Klebsiella pneumoniae* resistant to the AMP PaDBS1R1. Our results suggest that several regulatory systems (PhoPQ, CpxRA and ZraPSR two-component systems) orchestrate the anti-PaDBS1R1 response. Specifically, lipopolysaccharide (LPS) modifications appear to be a key piece in the resistance response since enzymes encoded by components of the *arnBCADTEF* operon and the enzyme LpxO were up-regulated. Moreover, other resistance-associated factors were also up-regulated including the efflux pump AcrAB-TolC and the proteins DsbA and PpiA. Interestingly, we observed that PaDBS1R1-resistant *K. pneumoniae* up-regulated components of the phospholipid-repair system LpLT/Aas as well as a C-lysozyme inhibitor, fortifying the resistant *K. pneumoniae* with mechanisms of resistance to the host innate immune system effectors.

Keywords: PaDBS1R1, *Klebsiella pneumoniae*, resistance, proteomics, antimicrobial peptides

Introduction

The resistance to antimicrobials compounds by pathogens has become a tangible threat to human health, turning real the possibility to enter in a post-antibiotic era where the antibiotics will not be more helpful (WHO, 2014a). This possesses serious implications for human health since the number of infections and deaths caused by antibiotic-resistant pathogens will increase. Currently, it is estimated that around 700 000 people die each year by infection with antimicrobial-resistant pathogens, but if no actions are implemented to stop the spread of resistance, the projections are about 10 millions of deaths by 2050 (O'Neill et al., 2016). Additionally, the antibiotic resistance might jeopardize medical procedures including organ transplantation, surgery, cancer chemotherapy and immunosuppressive disease treatment (WHO, 2018a).

Among the antimicrobial-resistant pathogens, bacteria are one of the most prone to develop resistance (Amabile-Cuevas, 2010). Particularly, the prevalence of antimicrobial resistance in Gram-negative bacteria has increased in recent years to such extent that has been stated that it constitutes a crisis that is going out of control (Rossolini et al., 2014). Gram-negative bacteria are predominant in the World Health Organization (WHO) groups of critical and high priority to guide the development of new antimicrobial compounds (WHO, 2017). Specifically, the carbapenem-resistant *Enterobacteriaceae* are included among the critical priority group, since they have been recognised as a serious threat due to their worldwide spread as well as the morbidity and mortality rates associated with the infections caused by them (Iredell et al., 2016; Logan and Weinstein, 2017; Cui et al., 2019). In this regard, currently, *Klebsiella pneumoniae* is one of the most representative members of the carbapenem-resistant *Enterobacteriaceae* since high mortality rates are associated with carbapenem-resistant *K. pneumoniae* infections (Karaiskos and Giamarellou, 2014; Pitout et al., 2015; Kohler et al., 2017; Xu et al., 2017). Furthermore, *K. pneumoniae* is frequently found as an etiologic agent in infections caused by pan-drug resistant bacteria associated to high mortality rates (Karakonstantis et al., 2019). Therefore, research focused on understanding the molecular bases of the resistance developed by *K. pneumoniae* against antimicrobials and exploration of novel effective antimicrobials against this pathogen is a priority.

In line with the above exposed, novel therapeutic strategies against multidrug-resistant *K. pneumoniae* have been explored, including antimicrobial combination, phage

therapy, nanoparticles, vaccine and antimicrobial peptides (AMPs) (Chhibber et al., 2017; Seeberger et al., 2017; van der Weide et al., 2017; Corbellino et al., 2019; Mulani et al., 2019). Particularly, many AMPs have shown potent antibacterial and anti-biofilm activity *in vitro* against *K. pneumoniae* (van der Weide et al., 2017; Lin et al., 2018a; Agbale et al., 2019; Cardoso et al., 2019; Fensterseifer et al., 2019; Irazazabal et al., 2019; van der Weide et al., 2019). Moreover, some AMPs exerted good activity either when used alone or in combination with other antimicrobials at *in vivo* studies (Tan et al., 2015; Liu et al., 2017b; Otvos Jr et al., 2018). The AMPs have been envisioned as promising therapeutic agents because, in addition to their potent microbicide activity, these peptides also exert immunomodulatory activity, are less prone to resistance development than conventional antibiotics and antibiotic-resistant bacteria tend to develop collateral sensitivity to them (Rodríguez-Rojas et al., 2014; Hancock et al., 2016; Lázár et al., 2018; Rodríguez-Rojas et al., 2018; Yu et al., 2018; Spohn et al., 2019). However, the bacteria have implemented resistance mechanisms to evade the AMPs microbicide activity (Maria-Neto et al., 2015; Andersson et al., 2016; Joo et al., 2016a). The strategies used by *K. pneumoniae* to circumvent AMPs activity are based mainly on capsule production, lipopolysaccharides (LPS) chemical modifications, efflux pumps and biofilm formation (Llobet et al., 2008; Padilla et al., 2010; Llobet et al., 2011; Llobet et al., 2015; Benincasa et al., 2016; Bellich et al., 2018). Therefore, understanding how bacterial pathogens develop resistance to AMPs is vital for the development of AMPs with therapeutic potential.

Recently, Izazarabal et al. (2019) reported the peptide PaDBS1R1 and its C-terminal amidated derivative as AMPs with improved microbicide activity against a broad range of bacteria including the carbapenem-resistant *K. pneumoniae* (Irazazabal et al., 2019). Subsequently, we used a quantitative shotgun proteomic approach to explore the PaDBS1R1-*K. pneumoniae* interplay and found that sub-inhibitory concentrations of PaDBS1R1 could promote bacterial genome damage since DNA repair systems were up-regulated in the challenged bacteria (Fleitas et al., submitted). Therefore, the use of sub-inhibitory concentrations of PaDBS1R1 could make bacteria prone to the development of resistance against it. In this regard, we observed that when *K. pneumoniae* was challenged continuously with sub-inhibitory concentrations of PaDBS1R1 reduced sensitivity against the peptide was developed (Fleitas et al., submitted). To investigate how PaDBS1R1-resistant *K. pneumoniae* overcomes the PaDBS1R1 microbicide activity, we used a label-free quantitative shotgun proteomic approach in the present study.

Material and Methods

Peptide

The peptide PaDBS1R1 (PKILNKILGKILRLAAAFK) was acquired from AminoTech P&D (Brazil) at 95% purity. The homogeneity of the synthesized peptide was confirmed by MALDI-TOF analysis using the RP 700-3500 method (reflector positive ion mode) in an Autoflex Speed spectrometer (Bruker Daltonics, GmbH, Leipzig) (Supplemental Figure 1).

Bacteria

For proteomic analysis were used PaDBS1R1-sensitive and PaDBS1R1-resistant bacteria obtained by Fleitas et al (submitted). Specifically, it was analyzed a PaDBS1R1-sensitive *Klebsiella pneumoniae* subp. *pneumoniae* ATCC 13883 adapted to Lysogeny Broth (LB) medium (designed as *Klebsiella pneumoniae* NP) and a PaDBS1R1-resistant *Klebsiella pneumonia*e subp. *pneumoniae* ATCC 13883 (designed as *Klebsiella pneumoniae* P).

Protein extraction

For total protein extraction, the PaDBS1R1-resistant *K. pneumoniae* (four biological replicates) and PaDBS1R1-sensitive *K. pneumoniae* (four biological replicates) were grown in 10 mL of LB medium supplemented with 5.8 µM of PaDBS1R1during 8 h at 37 °C and 80 rpm. Subsequently, the bacterial cultures were centrifuged at 11, 000 g for 10 min at 4 °C. Afterward, the bacterial pellets were collected and the total proteins from them were extracted following the MPLEx (*the metabolite, protein, lipid extraction protocol*) methodology described by Burnum-Johnson et al. 2017 but with modifications (Burnum-Johnson et al., 2017). Briefly, bacterial pellets were solubilized in 50 mM of NH_4HCO_3 (2 mL) and centrifuged at 11, 000 g for 10 min at 4 °C. Subsequently, the obtained pellets were reconstituted in 50 mM of NH_4HCO_3 (150 µL) and 600 µL of MPLEx solution [$CHCl_3$/MeOH: 2/1 (v/v)] was added. Afterward, the samples were mixed by vortex for 10 s, leaving on ice for 5 min and vortexed again. Next, the samples were centrifuged at 13, 000 g for 10 min at 4 °C allowing obtain protein-enriched interphase that was collected. To the collected protein-enriched interphase was

added 200 µL of cold methanol (-20 °C) and centrifuged at 13, 000 g for 10 min at 4 °C. Successively, the pellet was reconstituted in 50 mM of NH_4HCO_3 (500 µL) and sonicated by 30 s. After that, using the Qubit assay (Invitrogen), the protein concentration was determined and 500 µg of total proteins of each sample were dried by Speed-Vac.

Sample preparation for LC-MS/MS

For the trypsinization of the extracted proteins, the dried protein samples (500 µg) were dissolved in 30 µL of RapidGest solution (0.1 % w/v in 50 mM NH_4HCO_3). Subsequently, the solubilized samples were supplemented with DTT to a final concentration of 5 mM and incubated at 60 °C for 30 min. Next, the samples were cooled at room temperature. After that, iodoacetamide was added to a final concentration of 10 mM and samples were incubated in the dark for 30 min. Next, it was added DTT again (10 mM final concentration) and samples were incubated for 15 min in the dark. Then, to each sample were added 5 µg of trypsin (Trypsin Gold, Promega, USA), followed by incubation for 15 h at 37 °C. After this incubation step, were additionally added 2 µg of trypsin to each sample, followed by another incubation for 9 h at 37 °C. Subsequently, the trypsin-mediated proteolysis was stopped by the addition of TFA to a final concentration of 0.5% (v/v) and samples were incubated for 30 min at 37 °C. Then, the samples were centrifuged at 13,000 g for 10 min at 4 °C and the peptide-enriched supernatants were collected and dried by Speed-Vac. Consecutively, the dried samples were reconstituted in 20 µL of 0.1%TFA (v/v) and desalted by Zip-Tip (Merck Millipore, Germany) following the manufacture's recommendations. Later, the peptide concentrations in the desalted samples were calculated by Qubit assay (Invitrogen). Then the quantified samples were dried by Speed-Vac.

LC-MS/MS

The trypsinized protein samples were solubilized in 0.1% formic acid and injected (1µg of peptides per injection) in a chromatography system (Dionex Ultimate 3000 RSLCnano UPLC, Thermo, USA) coupled to Orbitrap Elite mass spectrometer (Thermo, USA). Initially, the tryptic peptide mixtures were separated by a linear gradient (2-35 % acetonitrile in 0.1% formic acid) over 155 min (flow rate 230 $nL.min^{-1}$) using an analytical column (24 cm x ID 75 µm, 3 µm of C18 particle size, ReprosilPur, Dr. Maich, GmbH). The separated peptides were directly eluted into the Orbitrap Elite mass spectrometer that was operated in data-dependent acquisition (DDA) mode. In the

Orbitrap analyser were acquired the full scan MS spectra (m/z 300-1650) at a resolution of 120, 000. Moreover, in the HCD collision cell were fragmented the 15 most intense ions (intensity over 3000) and the produced fragment ions were examined by Orbitrap analyser yielding the respective MS/MS spectrum at a resolution of 15, 000.

The software Progenesis QI for proteomics (Nonlinear Dynamics, UK) was used to process the raw MS spectra data. In brief, the runs were alignment and from the extracted ion chromatogram (XIC) were calculated the precursor ions raw abundances. Subsequently, the precursor ions raw abundances were normalised to all proteins (default method). For protein identification, the MS/MS spectra from peptide ions with significantly normalised abundance changes ($p < 0.05$) were exported to the Peaks software version 7.0 (BSI, USA). In this regard, it was used the UniprotKB *Klebsiella pneumoniae* ID. 573 (downloaded in April 2018) as the protein sequence database. The selected search parameters included cysteine carbamidomethylation as a fixed modification and methionine oxidation as variable modification, whereas trypsin was selected as protease permitting up to two missed cleavages. The mass tolerance for precursor and fragment ions were 10 ppm and 0.05 Da, respectively. The false discovery rate (FDR) for peptide-spectrum matches was 1%. Next, the identified proteins with at least one unique peptide, confidence scores ($-10\lg P \geq 20$) and FDR < 1% were returned to the Progenesis software to be quantitated via the normalized abundance of non-conflicting peptides assigned to each protein. It was considered as differentially abundant proteins (DAPs), those presented significantly normalised abundance changes ($q < 0.05$) with fold changes (FC) ≥ 2 or ≤ 0.5. The raw dataset from LC-MS/MS analysis including protein quantitation and identification are available at ftp://massive.ucsd.edu/ MSV000082334, PorteomeXchange ID = PXD009673.

Data analysis

The software Blast2GO (Conesa et al., 2005), was used to maps the identified DAPs to the Kyoto Encyclopedia of Genes and Genomes (KEGG)-annotated *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700721 / MGH 78578 strain. The functional profile of the mapped DAPs based on the KEGG orthology database was performed with the KofamKOALA tool (Aramaki et al., 2019). The KEGG- and Gene Ontology (GO)-enrichment analysis of DAPs were performed with the Network-based Visualization for Omics (NeVomics) tool (Zúñiga-León et al., 2018).

Results

Identification of differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae* and their functional profile based on KEGG Orthology

To identify from a proteomic perspective, the possible(s) molecular mechanism(s) used by PaDBS1R1-resistant bacteria to overcome the PaDBS1R1 antimicrobial activity, both PaDBS1R1-sensitive and -resistant bacteria were challenged with 5.8 µM of PaDBS1R1. This peptide concentration represents a sub-inhibitory concentration as the previous determined minimum inhibitory concentrations (MICs) for PaDBS1R1-sensitive *K. pneumoniae* and PaDBS1R1-resistant *K. pneumoniae* were 11.4 (± 3.8) and 60.8 µM, respectively (Fleitas et al., submitted). Comparison of proteins abundance between PaDBS1R1-resistant bacteria and PaDBS1R1-sensitive bacteria revealed 295 proteins as differentially abundant proteins (DAPs) ($FC \geq 2$ or ≤ 0.5, $q < 0.05$). Of these DAPs, 225 proteins were up-regulated ($FC \geq 2$, $q < 0.05$), whereas 70 proteins were down-regulated ($FC \leq 0.5$, $q < 0.05$) (Supplemental Table 1).

The KEGG Orthology (KO)-based functional profile of DAPs showed 169 proteins distributed in the biological classes: cellular processes, environmental information processing, genetic information processing, human diseases, and metabolism (Figure 1A, Supplemental Table 1). Most of the DAPs (123 proteins) were associated with the metabolism class (Figure 1A, Supplemental Table 1). Specifically, these metabolism-associated DAPs were distributed in the metabolic subcategories: carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, amino acid metabolism, metabolism of other amino acids, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, biosynthesis of other secondary metabolites and xenobiotics biodegradation and metabolism (Figure 1B).

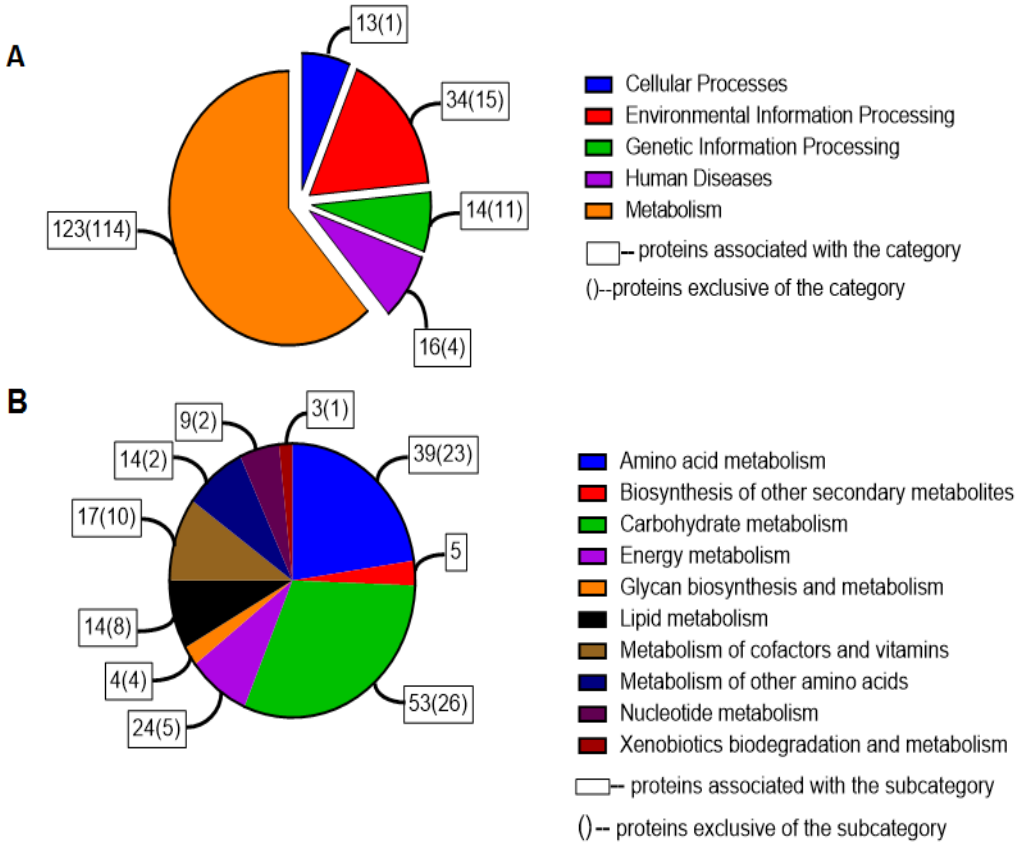


Figure 1. KEGG Orthology-based functional profile of the differentially abundant proteins in PaDBS1R1-resistant *Klebsiella pneumoniae*. A) Distribution of the differentially abundant proteins through the KEGG biological categories. B) Distribution of the metabolism-associated differentially abundant proteins in metabolic subcategories. The values in the boxes represent the number of proteins associated with the category or subcategory. The values in parenthesis represent the number of proteins exclusive of the category or subcategory. Exclusive proteins refer to proteins that are only associated with a particular category or subcategory.

KEGG pathways enrichment analysis of differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*

The KEGG pathway-based enrichment analysis for up-regulated DAPs employing the Nevomics tool displayed enrichment of three pathways, which included cationic antimicrobial peptide (CAMP) resistance, histidine metabolism, and alanine, aspartate and glutamate metabolism (Figure 2A, Supplemental Table 2). Moreover, the enrichment analysis for down-regulated DAPs resulted in the enrichment of glycolysis/gluconeogenesis, microbial metabolism in diverse environments, propanoate

metabolism and glycerolipid metabolism (Figure 2B, Supplemental Table 3). The main up-regulated and down-regulated DAPs associated with the KEGG enriched pathways are described in Supplemental Tables 2 and 3, respectively.

Gene ontology enrichment analysis of up-regulated differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*

The analysis of gene ontology (GO) enrichment for up-regulated DAPs showed enrichment of twenty biological processes in the PaDBS1R1-resistant *K. pneumoniae* (Figure 3A). The enriched biological processes encompassed catabolic pathways as histidine catabolic process to glutamate and formamide, histidine catabolic process to glutamate and formate, tricarboxylic acid cycle and L-fucose catabolic process. Moreover, were also enriched anabolic processes that included the lipopolysaccharide biosynthetic process, lipid A biosynthetic process, 4-amino-4-deoxy-alpha-L-arabinopyranosyl undecaprenyl phosphate biosynthetic process, translation, hydrogen sulfide biosynthetic process, cysteine biosynthetic process, arginine biosynthetic process, aromatic amino acid family biosynthetic process, chorismate biosynthetic process, gluconeogenesis and lysine biosynthetic process via diaminopimelate. Other enriched processes comprised sulfate assimilation, pentose phosphate shunt, response to antibiotic, protein folding and peptide transport (Figure 3A, Supplemental Table 4). Furthermore, four molecular functions were enriched including sulfite reductase (NADPH) activity, L-serine ammonia-lyase activity, succinyldiaminopimelate transaminase activity and succinate dehydrogenase (ubiquinone) reductase activity (Figure 3B, Supplemental Table 4). Additionally, the periplasmic space was detected by Nevomics tool as enriched cellular component in the challenged resistant bacteria (Figure 3C, Supplemental Table 4).

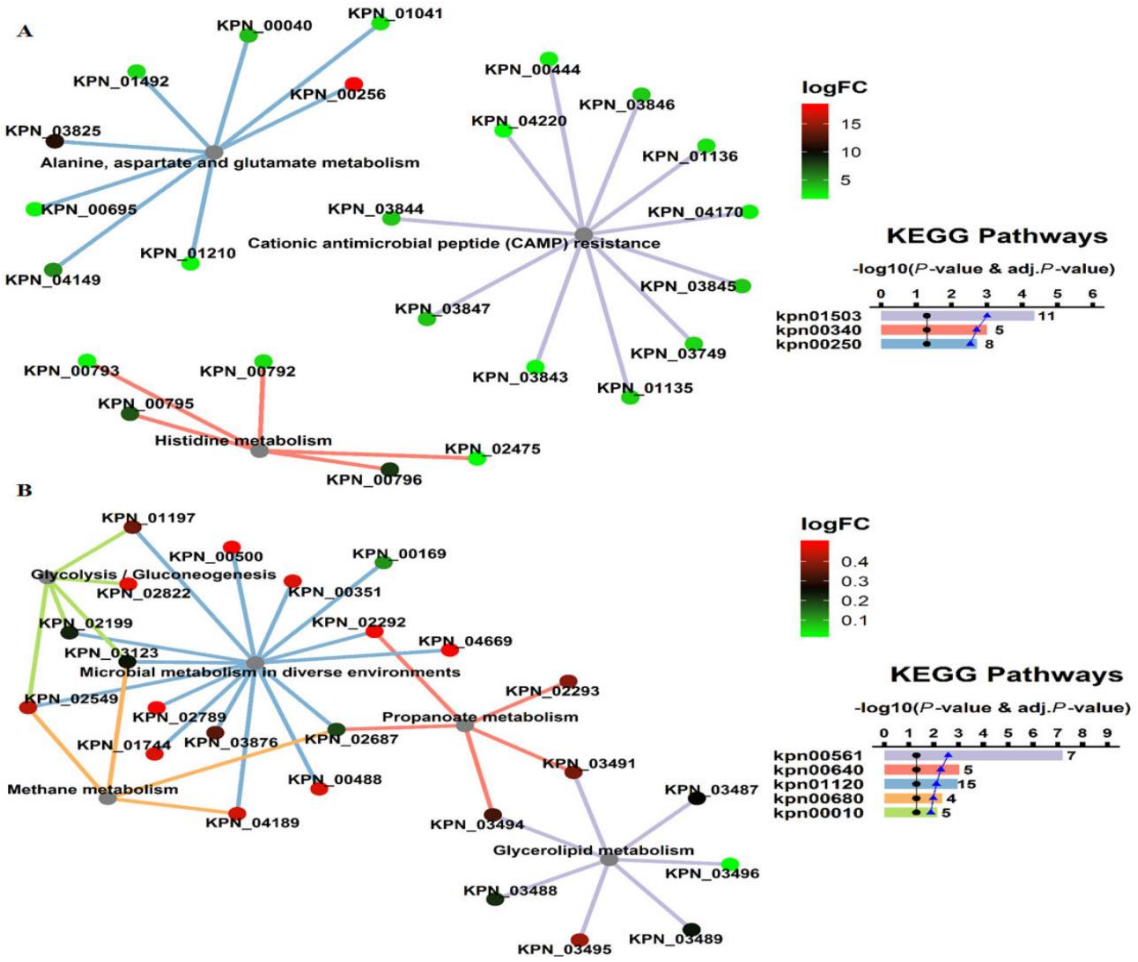


Figure 2. Enriched KEGG pathways in the PaDBS1R1-resistant *Klebsiella pneumoniae* detected by Nevomics software. A) Enriched KEGG pathways for up-regulated differentially abundant proteins. B) Enriched KEGG pathways for down-regulated differentially abundant proteins. The enriched KEGG pathways are displayed as a network clustered by colors. The bar plot contains colored bars that correspond to an enriched pathway with its respective –log10 (p-value). The reference p-value (0.05) is represented as the –log10 (0.05) by the black line in the bar plot, whereas the blue line represents the –log10 (adjusted p-value) for each enriched pathway. At the end of each bar in the bar plot, it is specified the number of proteins detected as associated with the respective pathway by Nevomics tool. The color of each node in the network is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein.

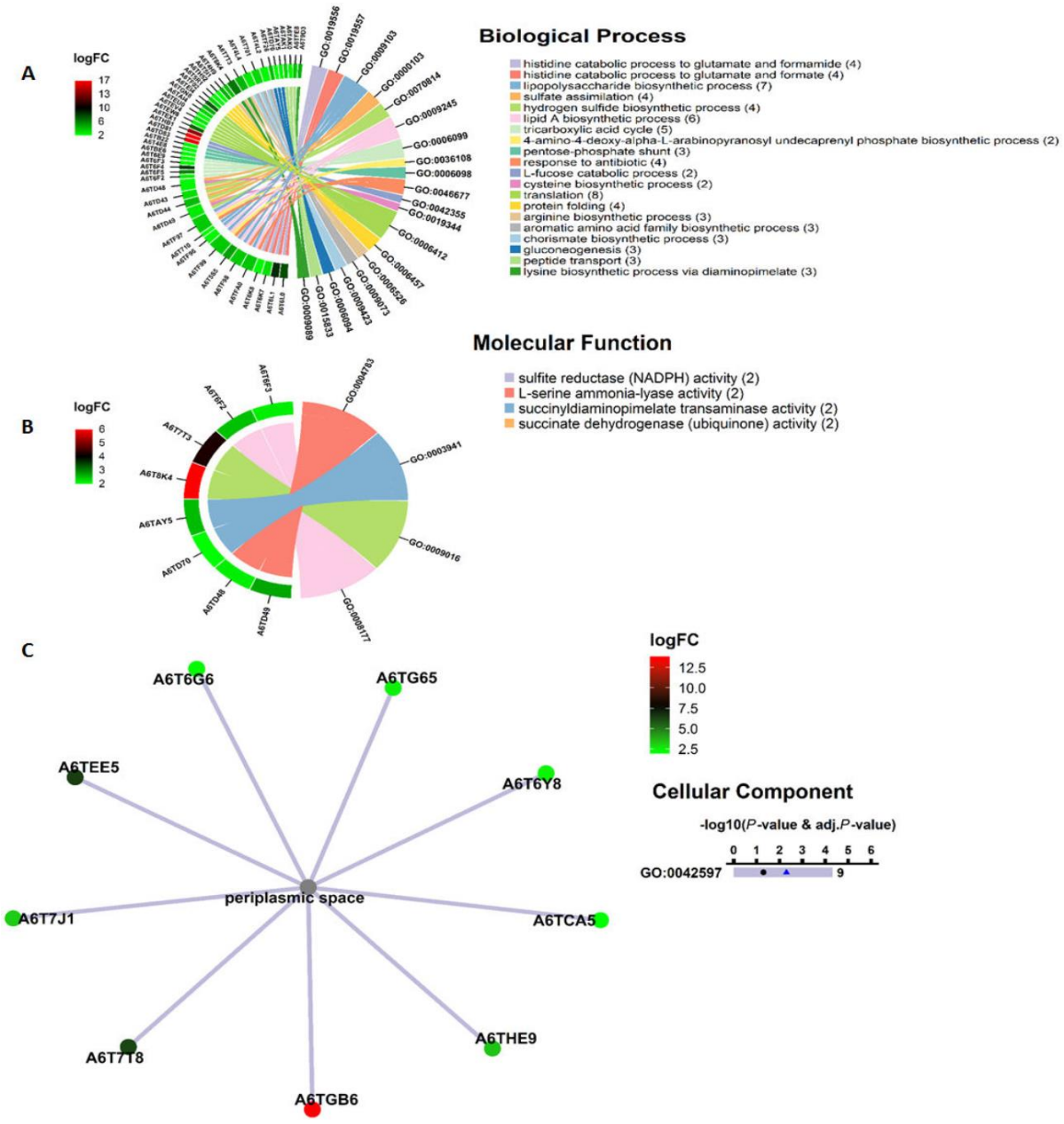


Figure 3. Enriched Gene Ontology terms detected by Nevomics for up-regulated differentially abundant proteins. A) Biological process. B) Molecular function. C) Cellular component. The enriched GO terms associated with the biological process (A) and molecular function (B) categories are represented in chord plots clustered by colors. Each enriched term and its associated proteins are linked by a specific color. Each protein identifier is associated with a colored segment, whose color is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein. The numbers in parenthesis specify the number of proteins detected by the Nevomics tool as associated with each term. The enriched GO terms associated with the cellular component category (C) are displayed as a network

clustered by colors. The bar plot contains colored bars that correspond to an enriched GO term with its respective –log10 (p-value). The reference p-value (0.05) is represented as the –log10 (0.05) by the black line in the bar plot, whereas the blue line represents the –log10 (adjusted p-value) for each enriched term. At the end of each bar in the bar plot, it is specified the number of proteins detected as associated with the respective GO term by Nevomics tool. The color of each node in the network is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein.

Gene ontology enrichment analysis of down-regulated differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*

The GO enriched analysis for down-regulated DAPs displayed enrichment of three biological process (Figure 4A). Specifically, were enriched the cell adhesion, glycerol metabolic process and cellular amino acid metabolic process (Figure 4A, Supplemental Table 5). Moreover, in the category of molecular function were detected by Nevomics as enriched eight molecular functions (Figure 4B). These molecular functions involved molybdopterin cofactor binding, molybdenum ion binding, dimethyl sulfoxide reductase activity, glycerone kinase activity, nucleoside transmembrane transporter activity, formate dehydrogenase (NAD^+) activity, 4 iron, 4 sulfur cluster binding and metalloendopeptidase activity (Figure 4B, Supplemental Table 5). Furthermore, cellular component analysis showed enrichment for pilus (Figure 4C, Supplemental Table 5).

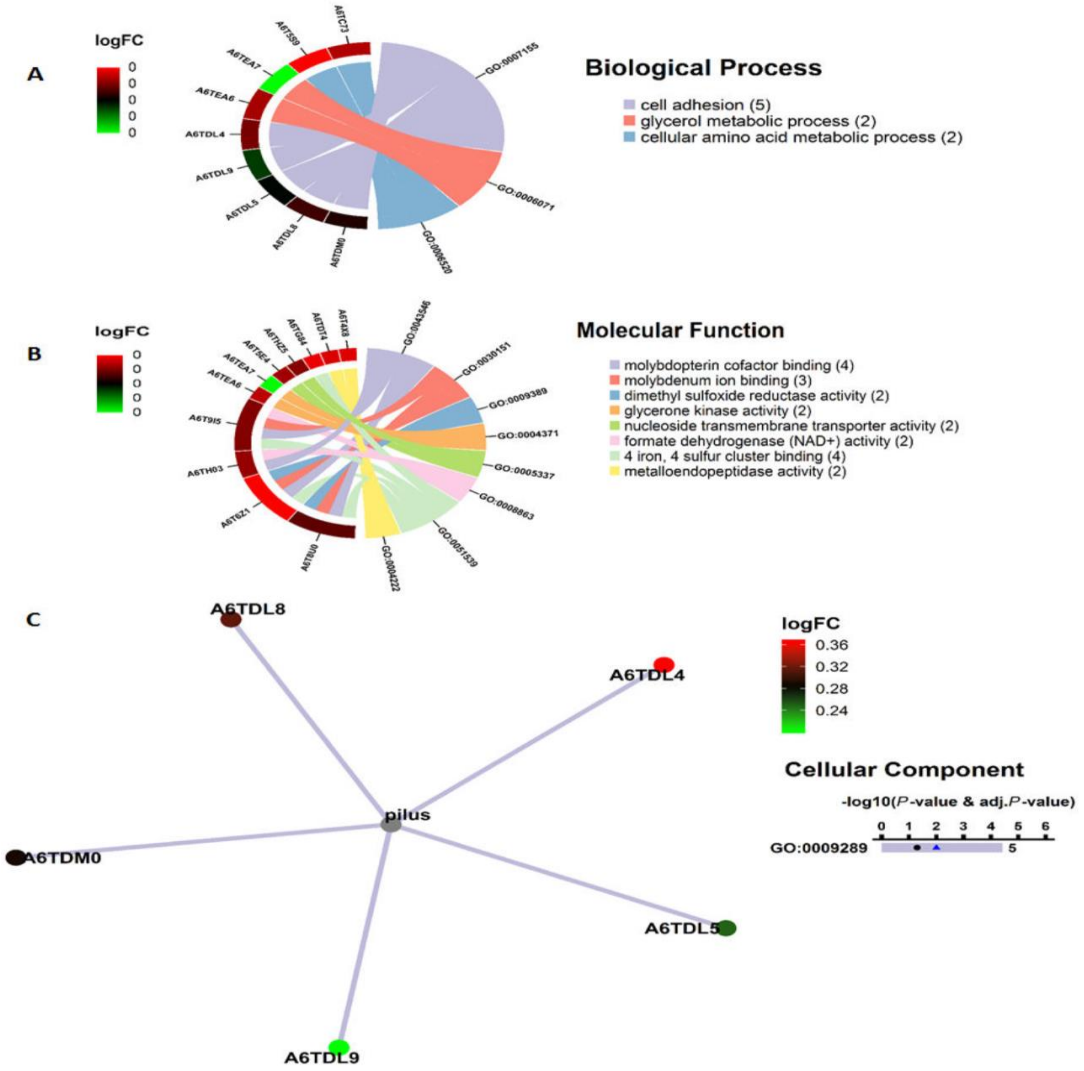


Figure 4. Enriched Gene Ontology terms detected by Nevomics for down-regulated differentially abundant proteins. A) Biological process. B) Molecular function. C) Cellular component. The enriched GO terms associated with the biological process (A) and molecular function (B) categories are represented in chord plots clustered by colors. Each enriched term and its associated proteins are linked by a specific color. Each protein identifier is associated with a colored segment, whose color is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein. The numbers in parenthesis specify the number of proteins detected by the Nevomics tool as associated with each term. The enriched GO terms associated with the cellular component category (C) are displayed as a network clustered by colors. The bar plot contains colored bars that correspond to an enriched GO term with its respective –log10 (p-value). The reference p-value (0.05) is represented as the –log10 (0.05) by the black line in the bar plot, whereas the blue

line represents the –log10 (adjusted p-value) for each enriched term. At the end of each bar in the bar plot, it is specified the number of proteins detected as associated with the respective GO term by Nevomics tool. The color of each node in the network is based on a heat map scale (log FC) that is in correspondence with the log2 (fold-change) of each protein.

Discussion

The increment of resistance by pathogens to antimicrobial compounds has made the search and design of new antimicrobial drugs a priority. Several novel therapeutic strategies are under deep research including the AMPs (de Castro and Franco, 2015; Baptista et al., 2018; Domalaon et al., 2018; Anand et al., 2019; Martínez et al., 2019). These biomolecules enclose characteristics that make them promising antimicrobial candidates since in addition to the microbicide activity; the AMPs could also act as modulators of the immune system (Hancock et al., 2016; Haney et al., 2019). Moreover, they are less prone to the development of resistance than conventional antibiotics (Yu et al., 2018; Spohn et al., 2019). Nevertheless, the AMPs are not exempt from the development of resistance by pathogens, being that multiples AMP-resistance mechanisms have been reported (Maria-Neto et al., 2015; Andersson et al., 2016; Joo et al., 2016a). Therefore, it is required a deep understanding of the mechanisms used by pathogens to circumvent the AMPs activity, if we want to boost the usefulness of AMPs as a therapeutic option against pathogens. Consequently, in the present study, we try to explore from a proteomics perspective the landscape of mechanisms used by *K. pneumoniae* to resist the microbicide activity of the peptide PaDBS1R1, which was recently reported as a powerful AMP (Irazazabal et al., 2019).

Our results suggested that to face the PaDBS1R1-induced stress the resistant *K. pneumoniae* evoke several protective systems (Figure 2A). The activation of multiple defense mechanisms appears to be a signature of the bacterial responses against AMPs as several OMICs-based studies suggest (Maria-Neto et al., 2012; Cardoso et al., 2017; Cardoso et al., 2018b; Fleitas et al., Submitted). Specifically, structural components of the regulatory two-component system PhoPQ and some components of the molecular networks controlled by it were up-regulated (Figure 2A and 3A, Supplemental Tables 2 and 4). The regulatory PhoPQ system plays an active role in the protective response against AMPs (Gunn and Miller, 1996; Shi et al., 2004; Bishop and Finlay, 2006; Tierney and Rather, 2019). This system mediates the sensing of diverse AMPs, which subsequently activates molecular networks involved in bacterial envelope modifications (Bader et al., 2005; Richards et al., 2012; Shprung et al., 2012). Particularly for *K. pneumoniae*, the PhoPQ system seems to exert a significant role *in vivo* since it allows the overcoming of the bactericidal action of the components of the host innate immune

defense system (e.g. host defense peptides) (Llobet et al., 2011; Insua et al., 2013; Llobet et al., 2015; Kidd et al., 2017).

In *K. pneumoniae* the PhoPQ-guided lipid A modifications consist principally of the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (pETN), palmitate and the hydroxylation of myristate (C_{14}) (Llobet et al., 2011; Llobet et al., 2015; Kidd et al., 2017; Leung et al., 2017). Particularly, we observed in the PaDBS1R1-resistant *K. pneumoniae* a significant increased abundance of proteins associated with the molecular machinery involved in lipid A modification with L-Ara4N and hydroxylation of myristate (Figure 3A, Supplemental Tables 1 and 4). In reference to lipid A modification with L-Ara4N, the proteins ArnA, ArnB, ArnC, ArnD and ArnT, which are encoded by the *arnBCADTEF* operon and mediate the addition of L-Ara4N to the lipid A, were detected among the up-regulated DAPs (Supplemental Table 4) (Figure 5) (Raetz et al., 2007; Wang and Quinn, 2010).

Interestingly, we observed changes in the abundance of proteins belonging to pathways linked to glutamate metabolism. In this regard, it was detected an enrichment for histidine catabolic process to glutamate and formate (Figures 2A and 3A, Supplemental Tables 2 and 4), as well as a significantly up-regulated abundance of the protein HisJ that is a component of the histidine ABC transporter (Supplemental Table 1). This suggests that resistant bacteria could prioritize the uptake of extracellular histidine to direct it toward the catabolic pathway to obtain glutamate. Furthermore, it was also observed a significantly up-regulated abundance of the proteins PutP, PutA, GltI and GdhA (Supplemental Table 1). PutP is a proline/sodium symporter that mediates the uptake of proline, whereas the protein PutA is involved in the proline degradation yielding glutamate (Ganea and Fendler, 2009; Liu et al., 2017a). Therefore, as observed for histidine, the resistant bacteria appear to incorporate proline for subsequent degradation yielding glutamate. Moreover, the protein GltI is a component of the glutamate/aspartate ABC transporter, which incorporate extracellular glutamate, whereas the enzyme GdhA could catalyze the production of glutamate from α-ketoglutarate (Barash and Halpern, 1975; Linton and Higgins, 1998; van Heeswijk et al., 2013). Then, these proteome changes suggest that glutamate could play an important role in the anti-PaDBS1R1 response developed by the challenged resistant *K. pneumoniae*.

The amino acid glutamate is a key metabolite that is involved in several metabolic processes, but specifically, it is linked to lipid A modification with L-Ara4N. During the

process of lipid A modification with L-Ara4N, the enzyme ArnB uses glutamate as a donor of amine groups to produce UDP-L-Ara4N, which subsequently is incorporated in the lipid A as L-Ara4N by other enzymes (Breazeale et al., 2003). The incorporated amine group to UDP-L-Ara4N confers cationic properties to the L-Ara4N moiety that is added to lipid A, consequently influencing the electrostatic properties of the LPS molecules, which is key for overcoming the AMPs activity (Raetz et al., 2007; Needham and Trent, 2013; Cole and Nizet, 2016).

The L-Ara4N moiety is added to *de novo* biosynthesized LPS molecules, therefore it is expected that in concordance with the increased abundance of components of the *arnBCADTEF* operon there is also an increased abundance of proteins involved in the LPS biosynthesis. In this regard, the GO enrichment analysis of up-regulated DAPs showed enrichment for lipopolysaccharide biosynthetic process and lipid A biosynthetic process (Figure 3A, Supplemental Table 4). Then, in response to PaDBS1R1 challenge, the PaDBS1R1-resistant *K. pneumoniae* appears to synchronize the production of LPS molecules with the molecular machinery involved in their modifications with L-Ara4N. This harmonization between LPS production and modification machinery could be important for the effectivity of the anti-PaDBS1R1 response; because previously we observed that probably in PaDBS1R1-sensitive *K. pneumoniae* an imbalance between the LPS production and modification machinery took placed when it was challenged with PaDBS1R1 (Fleitas et al., Submitted).

Among the up-regulated DAPs, the enzyme lipid A hydroxylase (LpxO) was also detected (Supplemental Tables 1 and 6). In *K. pneumoniae*, this enzyme is part of the PhoPQ-controlled regulon and catalyzes the hydroxylation of secondary acyl chains in lipid A (Insua et al., 2013; Llobet et al., 2015; Kidd et al., 2017). Specifically, it has been demonstrated that lipid A modified with 2-hydroxymyristate ($C_{14:\,OH}$) is a stamp of the LPS expressed by *K. pneumoniae* in certain tissues *in vivo* (Llobet et al., 2015). The LpxO-mediated lipid A modification was pivotal for *K. pneumoniae* virulence in a murine model and it restricted the inflammatory response activation (Llobet et al., 2015). Besides, the LpxO-mediated lipid A modification seemed to be essential to attenuate the production of AMPs by *Galleria mellonella* during infection with *K. pneumoniae* (Kidd et al., 2017). Furthermore, *K. pneumoniae* strains with lipid A modified with 2-hydroxymyristate showed resistance toward the last resorts antimicrobials as polymyxin B and colistin (Llobet et al., 2015; Kidd et al., 2017). Recently, it has been shown that

LpxO-mediated lipid A modification is a protective strategy used by the pathogen *Acinetobacter baumannii* to overcome the antimicrobials (Bartholomew et al., 2019). Therefore, probably the increased abundance of LpxO in the challenged PaDBS1R1-resistant *K. pneumoniae* constitute an anti-PaDBS1R1 defense mechanism based on lipid A hydroxylation (Figure 5).

The proteome changes in the PaDBS1R1-resistant *K. pneumoniae* suggests that PhoPQ-guided LPS modifications, specifically, the addition of L-Ara4N to lipid A and hydroxylation of lipid A could be essential for the protective response against PaDBS1R1. In fact, the PhoPQ-guided LPS modifications constitute a fundamental defense strategy used by *K. pneumoniae* and other pathogens to circumvent the activity of several antimicrobial compounds including host defense peptides (HDPs) (Olaitan et al., 2014; Llobet et al., 2015; Kidd et al., 2017; Bartholomew et al., 2019). Then, the PaDBS1R1 resistant *K. pneumoniae* potentially might cross-resist the activity of HDPs.

Also, the C-lysozyme inhibitor and the bifunctional protein Aas were detected among the up-regulated DAPs (Supplemental Table 1). The C-lysozyme inhibitor mediates the resistance to lysozyme, which is a component of the host innate immune system (Callewaert et al., 2008; Liu et al., 2015; Ragland and Criss, 2017). Previously, we observed that PaDBS1R1-sensitive *K. pneumoniae* down-regulated the abundance of C-lysozyme inhibitor when challenged with PaDBS1R1 (Fleitas et al., Submitted). Furthermore, the protein Aas exerts acyltransferase enzymatic activity, making it an essential component of a resistance system (phospholipid-repair system LpLT/Aas) used by bacteria to avoid the antimicrobial effect of mammalian phospholipases A2, which also are components of the innate immune system (Lin et al., 2018b). Gram negative-bacteria tend to be less sensitive than Gram-positive bacteria to phospholipases A2, which could be due to the asymmetry of the Gram-negative bacterial outer membrane (Weiss, 2015; Lin et al., 2018b). Innate immune system components as HDPs and complement membrane-attack complex could compromise the asymmetry of the Gram-negative bacterial outer membrane, which could boost the activity of phospholipases A2 toward the Gram-negative bacteria (Elsbach et al., 1994; Madsen et al., 1996; Weiss, 2015). Interestingly, we observed that in PaDBS1R1-resistant *K. pneumoniae* the abundance of YrbC and YrbB proteins was significantly increased (Supplemental Table 1). Both YrbC and YrbB are constituents of the Mla system that performs the retrograde transport of phospholipids, which guarantees the asymmetry of the outer membrane (Malinverni and

Silhavy, 2009). Besides, the LpLT/Aas system also appears to contribute to bacterial outer membrane asymmetry (Lin et al., 2018b). Therefore, the PaDBS1R1-resistant bacteria potentially could cross-resist several effectors of the innate immune system. The potential cross-resistance toward innate immune system effectors by AMPs-resistant bacteria has been envisioned as one of the negative consequence of inappropriate AMPs-based therapy (Bell and Gouyon, 2003; Fleitas and Franco, 2016a).

In addition to the two-component system PhoPQ, the two-component system CpxRA and the proteins DsbA and PpiA, which are part of the CpxRA-controlled regulon were also associated with the enriched cationic antimicrobial peptide (CAMP) resistance pathway (Figure 2A, Supplemental Tables 1 and 2). The CpxRA system is linked to the network of envelope stress response systems that facilitate bacterial protective response against antimicrobial compounds (Grabowicz and Silhavy, 2017; Mitchell and Silhavy, 2019). Particularly, the CpxRA system has been linked to the protective response against AMPs (Weatherspoon-Griffin et al., 2011; Audrain et al., 2013; Weatherspoon-Griffin et al., 2014). In the context of the anti-AMPs response, the CpxRA system appears to facilitate the alleviating of the envelope stress, the remodeling of the bacterial peptidoglycan and the extrusion of antimicrobial and/or toxic compounds (Weatherspoon-Griffin et al., 2011; Audrain et al., 2013; Weatherspoon-Griffin et al., 2014). Regarding the alleviating of the envelope stress, we observed that in response to PaDBS1R1 challenge the resistant bacteria increased the abundance of several CpxRA-regulated chaperones and proteases including Spy, DegP, DsbA, PpiA and CpxP (Supplemental Tables 1 and 6). Therefore, this suggests that activation of the CpxRA-dependent folding machinery in PaDBS1R1-resistant bacteria is pivotal to overcoming the PaDBS1R-induced envelope stress. Previously, we observed that PaDBS1R1-sensitive *K. pneumoniae* significantly down-regulated the CpxRA-dependent folding machinery when challenged with the peptide (Fleitas et al., Submitted).

Furthermore, as previously mentioned, the CpxRA system facilitates the extrusion of antimicrobial and/or toxic compounds. In this sense, Weatherspoon-Griffin et al. (2014) described the CpxRA-regulated molecular network that operates in the resistance response to the AMP protamine in *Escherichia coli*. The activation of the CpxRA system induced the expression of tripartite multidrug transporters (e.g. AcrAB-TolC) via the *marRAB* operon (Weatherspoon-Griffin et al., 2014). Additionally, it was revealed that CpxRA activation induced the up-regulated expression of the gene *aroK* that encodes the

enzyme shikimate kinase (AroK), which is involved in the chorismate biosynthesis pathway (Weatherspoon-Griffin et al., 2014; Mir et al., 2015). The chorismate is a precursor for the biosynthesis of aromatic amino acids as well as aromatic metabolites like indole, salicylate and 2,3-dihydroxybenzoate (Walsh et al., 1990; Weatherspoon-Griffin et al., 2014; Mir et al., 2015). According to the authors, the produced indole might access to the extracytoplasmic space via the damaged membrane (although indole appears to be able to cross the membrane (Piñero-Fernandez et al., 2011)) and act as signaling molecule that activates the CpxRA system through the CpxA periplasmic domain, thus amplifying the expression of efflux pumps via the *marRAB* operon (Weatherspoon-Griffin et al., 2014). Also, it was suggested that metabolites like salicylate and 2,3-dihydroxybenzoate might promote the expression of the tripartite multidrug transporters via the *marRAB* operon, since they bind to the *marRAB* repressor MarR and modulate its activity (Chubiz and Rao, 2010; Weatherspoon-Griffin et al., 2014).

In agreement with the above described CpxRA-controlled network, we observed that the PaDBS1R-challenged resistant bacteria significantly increased the abundance of TolC and AcrA proteins, which conform the tripartite multidrug transporter TolC-AcrAB (Supplemental Tables 1 and 6). Moreover, an enrichment of the aromatic amino acid family biosynthetic process and chorismate biosynthetic process was observed in addition to the up-regulated abundance of the enzyme AroK (Figure 3A, Supplemental Tables 4 and 6). Therefore, this suggests that in PaDBS1R1-resistant *K. pneumoniae*, the CpxRA-mediated protective response could be operating via a similar molecular network to the described by Weatherspoon-Griffin et al. 2014 (Figure 5).

Interestingly, we observed a significantly down-regulated abundance of the protein AroP (Supplemental Table 1), which mediates the uptake of aromatic amino acids (Cosgriff and Pittard, 1997). However, as it was described above, the aromatic amino acid family biosynthetic process was enriched for the up-regulated DAPs (Figure 3A, Supplemental Table 4). The biosynthesis of aromatic amino acids, specifically, the tryptophan biosynthesis pathway allows the production of indole, whereas, the uptake via AroP and subsequent degradation of tryptophan also favors the production of indole (Dunn et al., 2008; Li and Young, 2013). As indole has been proposed as an activator of the CpxRA system, an overproduction of indole in principle could induce an over activation of the CpxRA system. In this regard, it has been reported that over activation of the CpxRA system could be harmful to the bacteria (Delhaye et al., 2016; Mitchell and

Silhavy, 2019). Therefore, the PaDBS1R1-resistant bacteria might favor the biosynthesis of aromatic amino acids (e.g. tryptophan) but down-regulate the uptake of these (especially tryptophan) as a strategy to control the indole levels during the anti-PaDBS1R1 response and to avoiding over activation of the CpxRA system.

On the other hand, in the PaDBS1R1-resistant *K. pneumoniae* it was observed that proteins associated with bacterial respiration were up-regulated including components of the complexes NADH: quinone oxidoreductase, succinate: quinone oxidoreductase and cytochrome bo_3 ubiquinol oxidase (Figure 3B, Supplemental Tables 1, 4 and 6). This was surprising because it has been reported that activated CpxRA system mediates the down-regulation of proteins associated with respiration, especially the complexes NADH: quinone oxidoreductase and cytochrome bo_3 ubiquinol oxidase (Raivio et al., 2013; Guest et al., 2017). A plausible explanation to the observed up-regulated levels of the respiratory proteins, is that the activity of the AcrAB-TolC efflux pump is dependent of the proton motive force, and the NADH: quinone oxidoreductase and cytochrome bo_3 ubiquinol oxidase complexes are important contributors in the production of the proton motive force (Borisov and Verkhovsky, 2015; Yamaguchi et al., 2015). Therefore, in the context of an anti-AMPs response mediated by the CpxRA system via the AcrAB-TolC efflux pump, the underlying CpxRA-controlled network might operate in a modality that does not interfere with the respiration-associated enzymes, thus ensuring the functionality of AcrAB-TolC efflux pump.

Furthermore, it has been demonstrated that *acrAB, acrB* and *tolC* mutants were sensitive to LL-37, human β-defensin-1, human β-defensin-2, and human neutrophil defensin-1, linking in this way the AcrAB-TolC system with the resistance to these HDPs (Padilla et al., 2010; Warner and Levy, 2010).

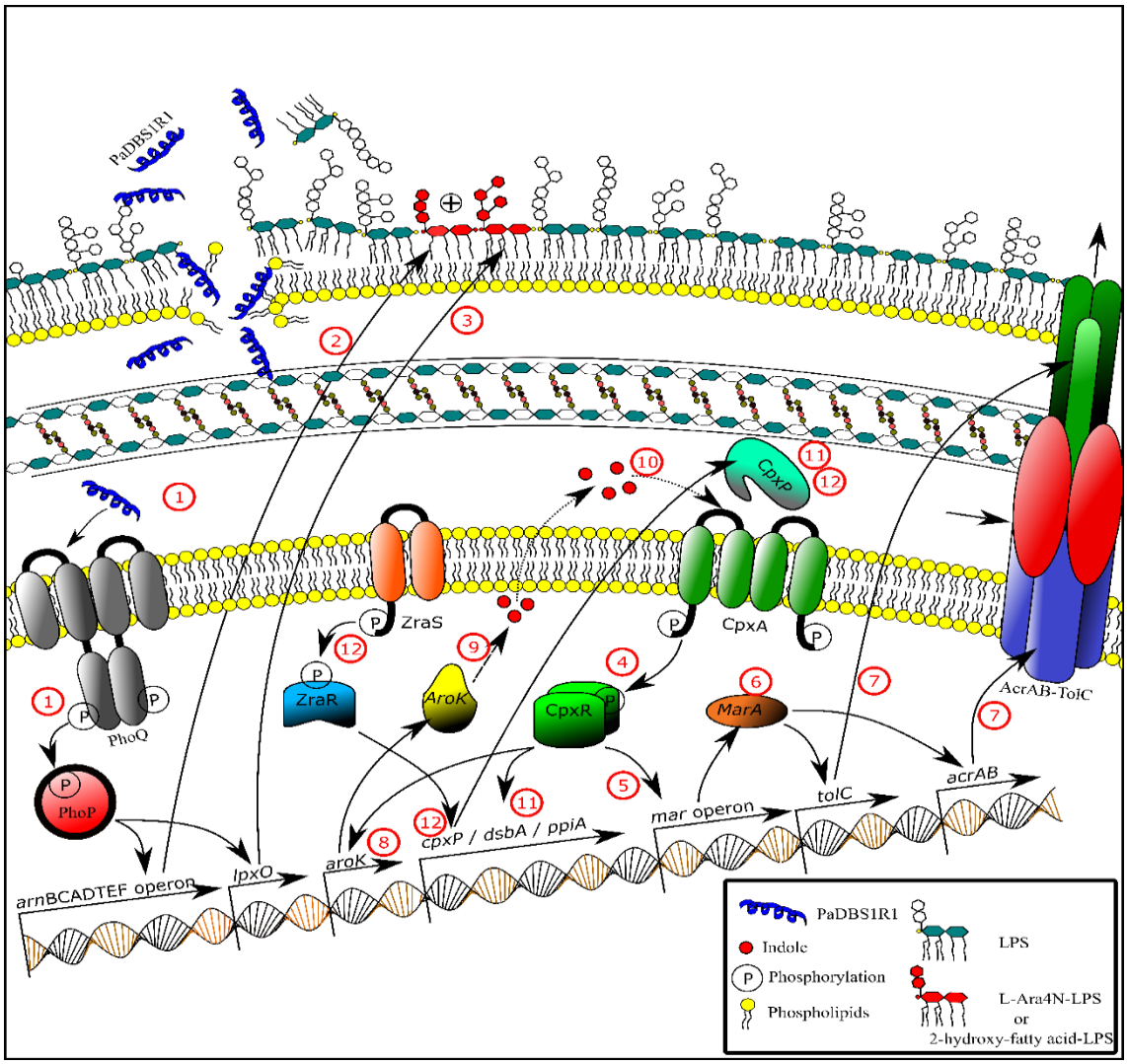


Figure 5. Molecular mechanisms that could be involved in the protective response performed by the PaDBS1R1-resistant *K. pneumoniae* against PaDBS1R1 challenge. 1) The two-component system PhoPQ could sense the peptide and trigger signaling events that activate the *arnBCADTEF* operon and *lpxO* transcription. 2) The proteins encoded by the *arnBCADTEF* operon could mediate the addition of 4-amino-4-deoxy-L-arabinose to the LPS molecules. 3) The produced LpxO enzyme could catalyze the hydroxylation of fatty acids on the LPS molecules. 4) The PaDBS1R1-induced stress could activate the two-component system CpxRA yielding the transcriptional factor CpxR activated (CpxR-P). 5) The activated CpxR could stimulate the transcription of *mar* operon that encodes the transcriptional factor MarA. 6) MarA could stimulate the transcription of *tolC* and *acrAB*, which encode for the components of the AcrAB-TolC efflux pump. 7) The AcrAB-TolC efflux pump could mediate the resistance to PaDBS1R1. 8) The activated CpxR could also stimulate the expression of *aroK*, which encodes the enzyme AroK. 9) The

up-regulated enzyme AroK could stimulate the production of chorismate, which in turn might favor the production of indole. 10) Indole could access to the periplasmic space and activate the CpxRA system. 11) The activated CpxR could also stimulate the transcription of *dsbA*, *ppiA* and *cpxP*, which encode for chaperones that could alleviate the PaDBS1R1-induced envelope stress. Specifically, CpxP could act as a modulator of the CpxRA system activity. 12) The ZraPSR system could also stimulate the production of CpxP.

Among the up-regulated DAPs, the zinc-binding periplasmic protein ZraP, which is a component of the regulatory system ZraPSR was detected (Supplemental Table 1). The two-component system ZraPSR has been recently reported as a member of the envelope stress response systems since it contributes to the envelope homeostasis under stress conditions (including antimicrobial-induced stress) (Appia-Ayme et al., 2012; Petit-Härtlein et al., 2015; Rome et al., 2018). In line with this, it has been shown that Δ*ZraP* mutants were more susceptible than wild type counterparts to polymyxin B (Appia-Ayme et al., 2012; Rome et al., 2018). The foremost role of ZraP during the bacterial response against antimicrobial compounds appears to be as a modulator of the ZraPSR system activity (Rome et al., 2018). In addition to *zraP* gene (encodes to ZraP), the ZarPSR-controlled regulon comprises genes associated to diverse aspects of the bacterial physiology like motility, metabolism, replication, stress response and transport (Rome et al., 2018). In this regard, under PaDBS1R1-induced stress conditions, we detected several proteins encoded by genes that belong to the ZraPSR-controlled regulon as DAPs in the PaDBS1R-resistant *K. pneumoniae*. These proteins were ZraP, CpxP, GlnH and MtlD (Supplemental Table 1).

The up-regulated abundance of GlnH (a component of the glutamine ABC transporter) could be associated with increased uptake of glutamine. The glutamine is linked to different metabolic pathways, but particularly in the context of the anti-PaDBS1R1 response, the glutamine addressing toward the UDP-N-acetyl-D-glucosamine biosynthetic pathway could be favored. The UDP-N-acetyl-D-glucosamine is a vital precursor for the LPS and peptidoglycan biosynthesis (Raetz et al., 2007; Barreteau et al., 2008). Moreover, we observed an increased abundance of the bifunctional N-acetylglucosamine-1-phosphate-uridyltransferase/glucosamine-1-phosphate acetyltransferase (GlmU) and glutamine-fructose-6-phosphate transaminase (GlmS) enzymes (Supplemental Table 6), which are involved in the UDP-N-acetyl-D-

glucosamine biosynthetic pathway (Mengin-Lecreulx and Van Heijenoort, 1994; Mouilleron et al., 2011). Besides, the LPS biosynthesis was one of the enriched biological processes for the up-regulated DAPs (Figure 3A, Supplemental Table 4). Interestingly, the ZraPSR system could promote the LPS biosynthesis via the protein RfaD, although we did not detect this protein as DAPs in the PaDBS1R1-resistant *K. pneumoniae* (Rome et al., 2018). Then, the activation of the ZraPSR system during the PaDBS1R1 challenge could aid in the LPS biosynthesis process, which ensures the supplying of LPS for the outer membrane reinforcement. Furthermore, it could also ensure the supply of LPS for the PhoPQ-controlled LPS modification machinery, which appears to be pivotal for the anti-PaDBS1R1 resistance response.

Interestingly, protein CpxP is a member of the ZraPSR regulon (Rome et al., 2018). As previously described, CpxP is also part of the CpxRA-controlled regulon and acts as a modulator of the CpxRA system activity (Raivio et al., 1999; Raivio, 2014). This modulatory activity over CpxRA is important because allows the activation of CpxRA system when is required and avoids over activation of the system, which could have dangerous consequences for the bacteria (Delhaye et al., 2016; Mitchell and Silhavy, 2019). Therefore, the fact that CpxP is a member of the ZraPSR regulon, suggests that bacteria use different regulatory systems to guarantee the production of CpxP (Figure 5). Moreover, this also suggests that CpxRA and ZraPSR systems could be functionally associated with each other. In this regard, it is interesting the fact that the proteins tryptophanase (TnaA) and tryptophan: H+ symporter (TnaB) are part of the ZraPSR-controlled regulon (Rome et al., 2018). These proteins are involved in the uptake and degradation of tryptophan yielding indole and pyruvate (Li and Young, 2013). The activated ZraPSR system induces down-regulation of the *tnaA* and *tnaB* expression (Rome et al., 2018). Therefore, the ZraPSR system could influence the indole levels and consequently the CpxRA system activity.

So far, our proteomics data suggest that the regulatory system ZraPSR could be involved in the resistance response against PaDBS1R1. Reinforcing this, we previously observed that when PaDBS1R1-sensitive *K. pneumoniae* is challenged with the peptide, it significantly downregulated the abundances of ZraP as well as of CpxP and GlnH (Fleitas et al., Submitted). Moreover, Cardoso et al. 2017 detected up-regulated abundance of ZraP in magainin I-resistant *E. coli* (Cardoso et al., 2017). Furthermore, transcriptomic analysis of magainin I-sensitive *E. coli* showed significantly down-

regulation of *zraP* and *zraS* expression when the bacteria were challenged with magainin I (Cardoso et al., 2018b).

The alterations in the PaDBS1R1-resistant *K. pneumoniae* proteome suggest that two-component regulatory systems PhoPQ, CpxRA and ZraPSR seem to be essential to develop a successful response against the PaDBS1R1-induced stress. The combination of such regulatory systems might allow *K. pneumoniae* to sense the peptide and/or the peptide-induced stress and consequently assemble a protective anti-PaDBS1R1 response. Therefore, disrupting these regulatory systems could be essential to jeopardize the anti-PaDBS1R1 response effectivity performed by the resistant *K. pneumoniae*. In this regard, it has started to emerge evidence that in bacteria, the two-component regulatory systems are organized in functional membrane microdomains, making them an attractive target to simultaneously impair the several regulatory systems functionality (Bramkamp and Lopez, 2015a; Lopez and Koch, 2017). Then, our future aims are, first confirming the distribution of the regulatory systems PhoPQ, CpxRA and ZraPSR in functional microdomains, and second, testing the impairment of such regulatory systems and consequently the protective response against PaDBS1R1 of resistant-*K. pneumoniae*, by perturbing these microdomains.

Conclusion

In this study a label-free quantitative shotgun proteomic approach was applied to gain understanding about the resistance response developed by *K. pneumoniae* against the AMP PaDBS1R1. Our results show that PaDBS1R1-resistant *K. pneumoniae* assembled a complex anti-PaDBS1R1 response, which appears to be orchestrated by the activity of several regulatory systems as the PhoPQ-, CpxRA- and ZraPSR- two-component systems. The PhoPQ system appears to activate the molecular machinery associated with LPS modification with L-Ara4N and 2-hydroxymyristate since several components of the *arnBCADTEF* operon and the enzyme LpxO were upregulated. On the other hand, the CpxRA system appears to aid the anti-PaDBS1R1 response via the production of chaperones and proteases as well as tripartite multidrug transporters. Furthermore, the ZraPSR system seems to support and modulate the activity of the PhoPQ and CpxRA systems respectively. Besides, the PaDBS1R1-resistant bacteria up-regulated proteins associated with the resistance to effectors of the innate immune system including lysozyme and phospholipases A2. Therefore, future studies focused on the development and evaluation of PaDBS1R1 as a putative therapeutic agent should take into account the possible cross-resistance to innate immune system components.

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Conflict of interest Statement

The authors declare that the research was conducted in the absence of any potential conflict of interest.

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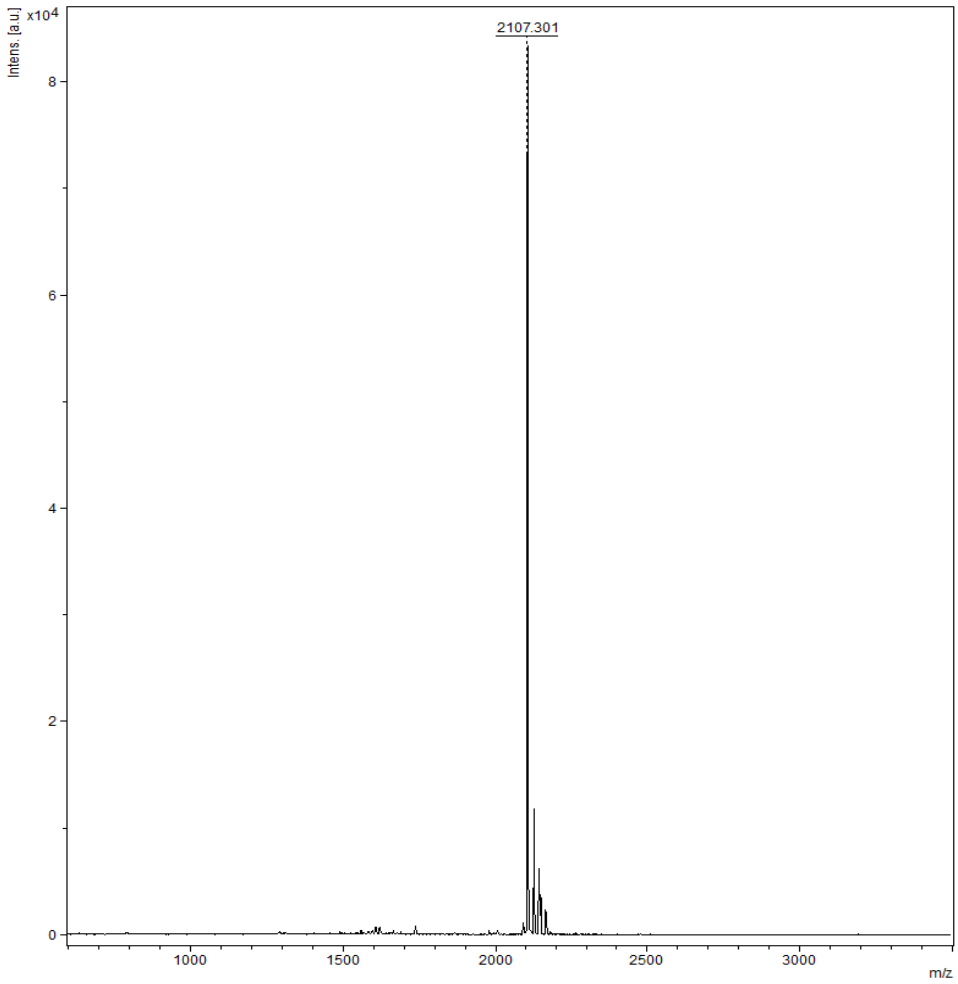
Supplemental Figures



Supplemental Figure 1. MALDI spectrum of the peptide PaDBS1R1

Supplemental Tables

Supplemental Table 1. Proteins considered as differentially abundant in the PaDBS1R1-resistant *Klebsiella pneumoniae*.

Supplemental Table 2. Enriched KEGG pathways for up-regulated differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*.

Supplemental Table 3. Enriched KEGG pathways for down-regulated differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*.

Supplemental Table 4. Enriched GO terms for up-regulated differentially abundant proteins in PaDBS1R1-resistant *Klebsiella pneumoniae*.

Supplemental Table 5. Enriched GO terms for down-regulated differentially abundant proteins in the PaDBS1R1-resistant *Klebsiella pneumoniae*.

Supplemental Table 6. Quantified proteins with significantly normalized abundance differences ($q < 0.05$).

Figures

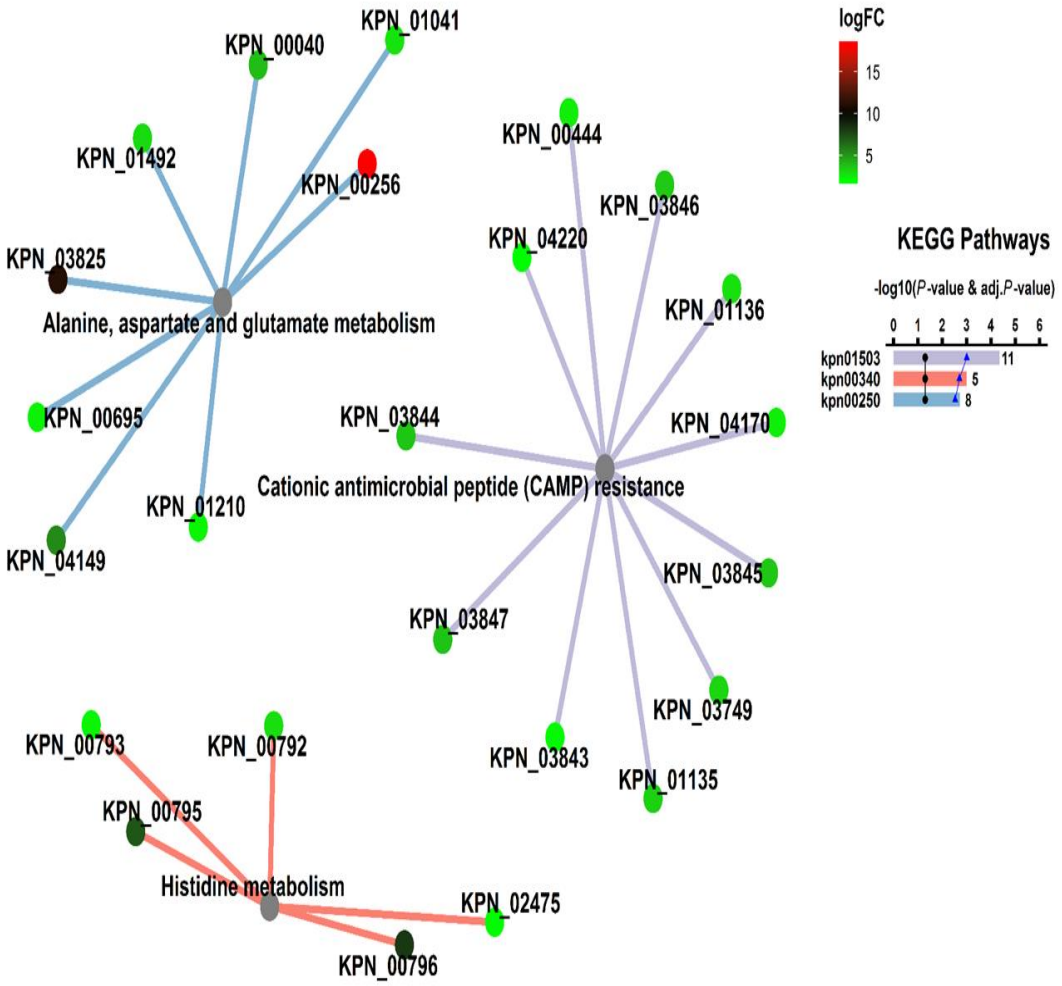


Figure 2A. Enriched KEGG pathways for up-regulated differentially abundant proteins.

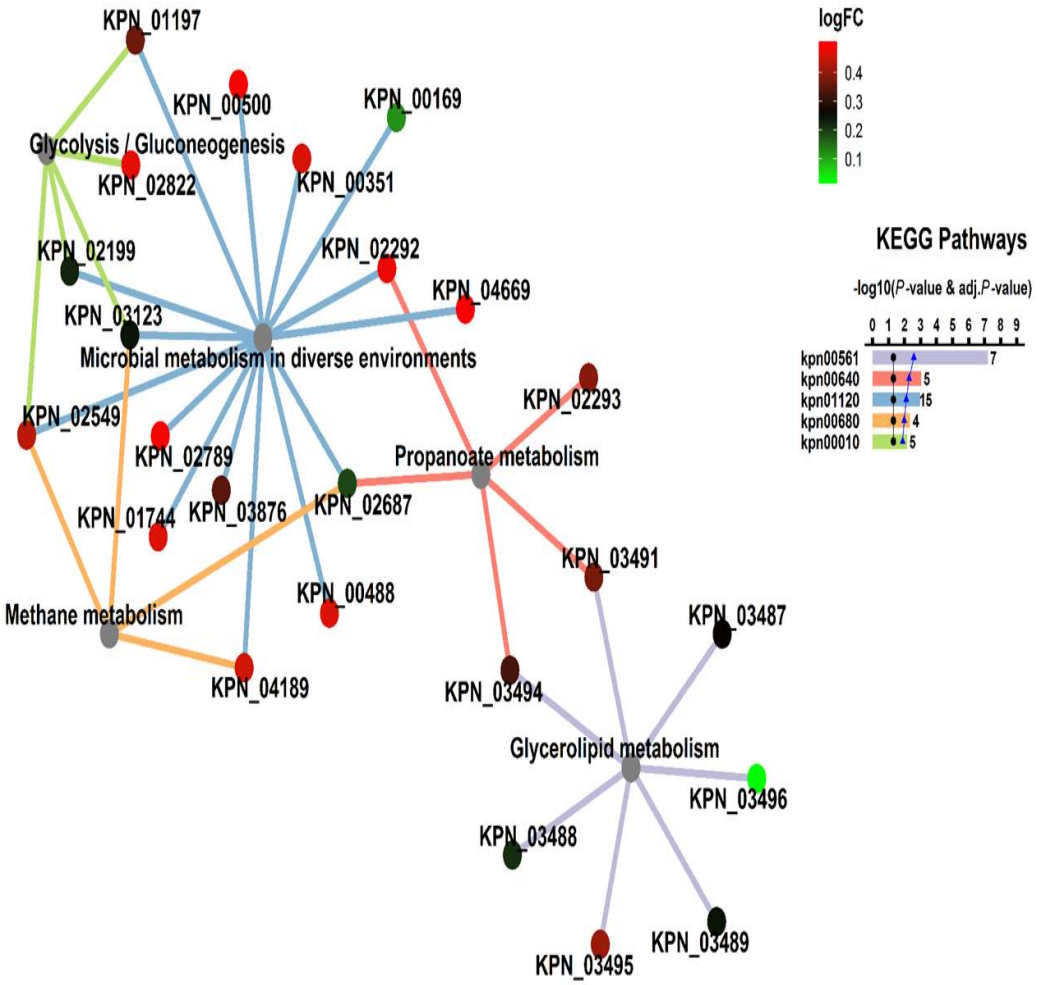


Figure 2B. Enriched KEGG pathways for down-regulated differentially abundant proteins.

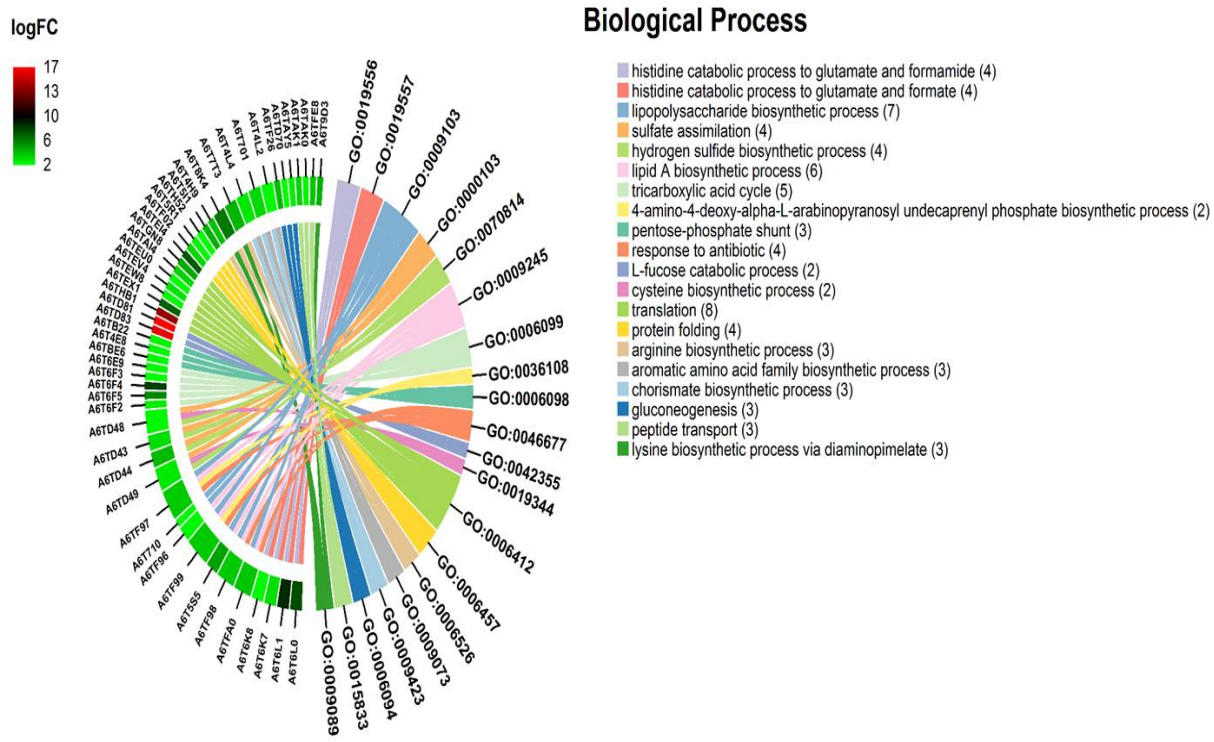


Figure 3. Enriched Gene Ontology terms detected by Nevomics for up-regulated differentially abundant proteins. A) Biological process.

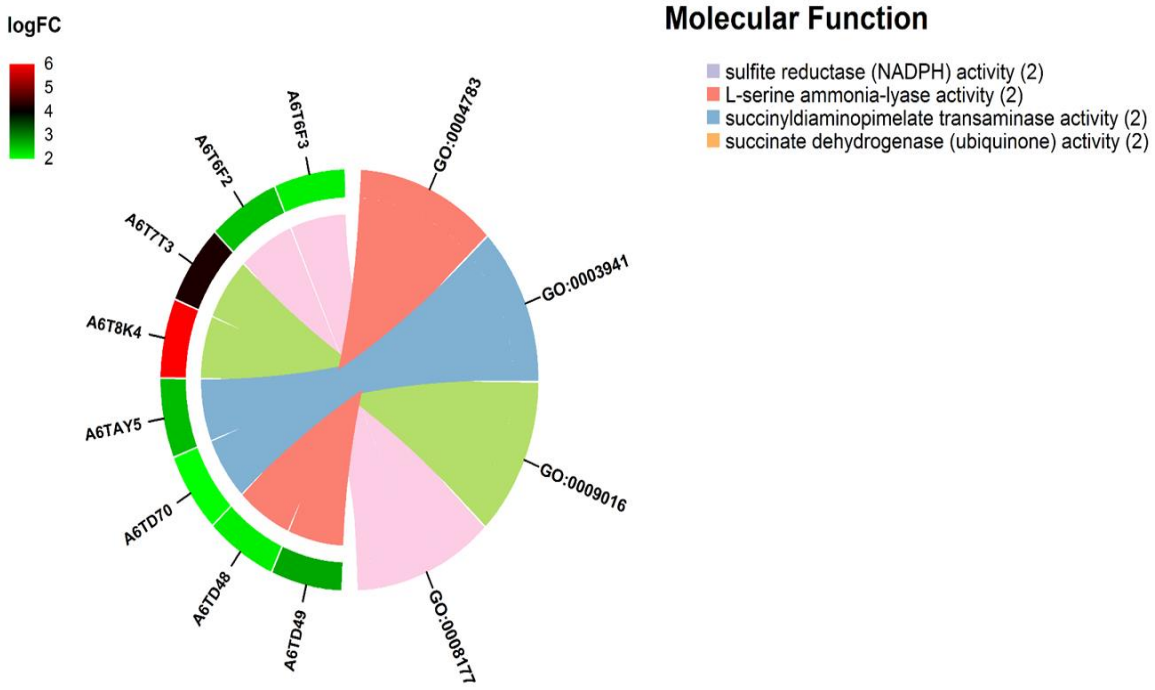


Figure 3. Enriched Gene Ontology terms detected by Nevomics for up-regulated differentially abundant proteins. B) Molecular function.

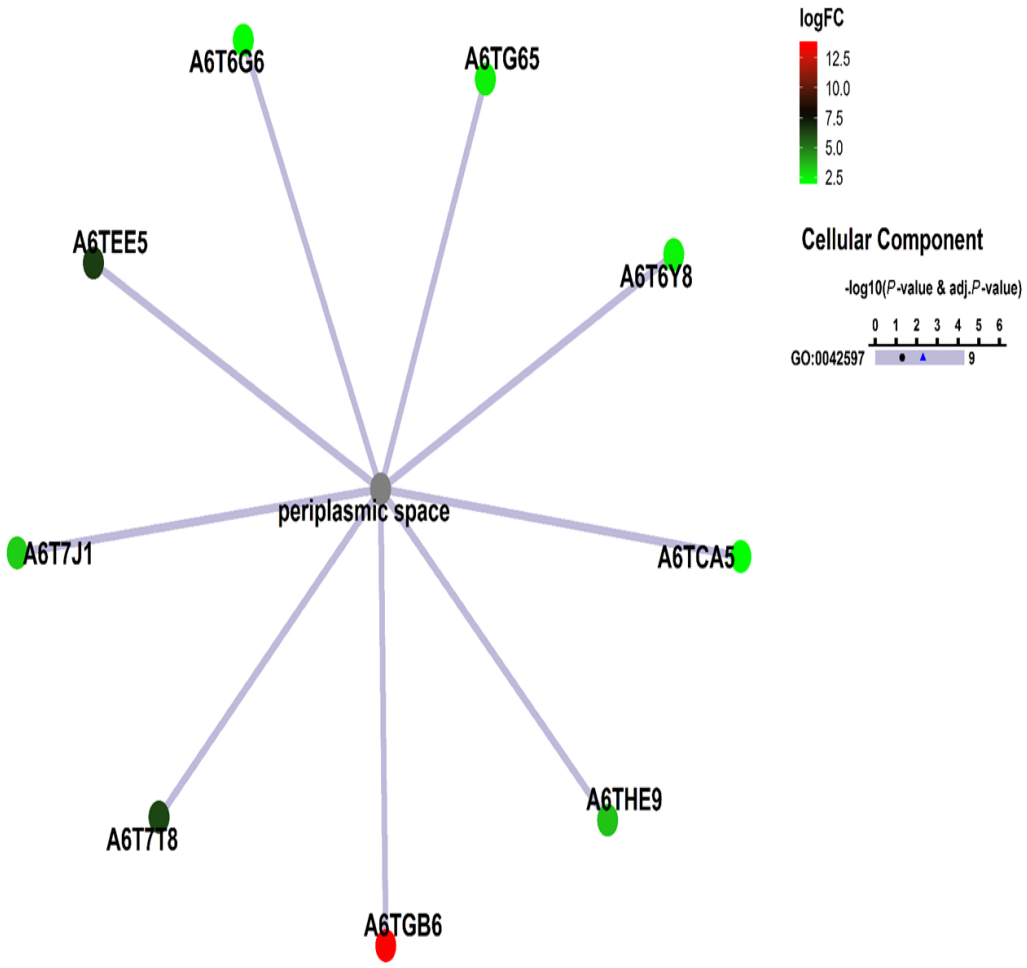


Figure 3. Enriched Gene Ontology terms detected by Nevomics for up-regulated differentially abundant proteins. C) Cellular component.

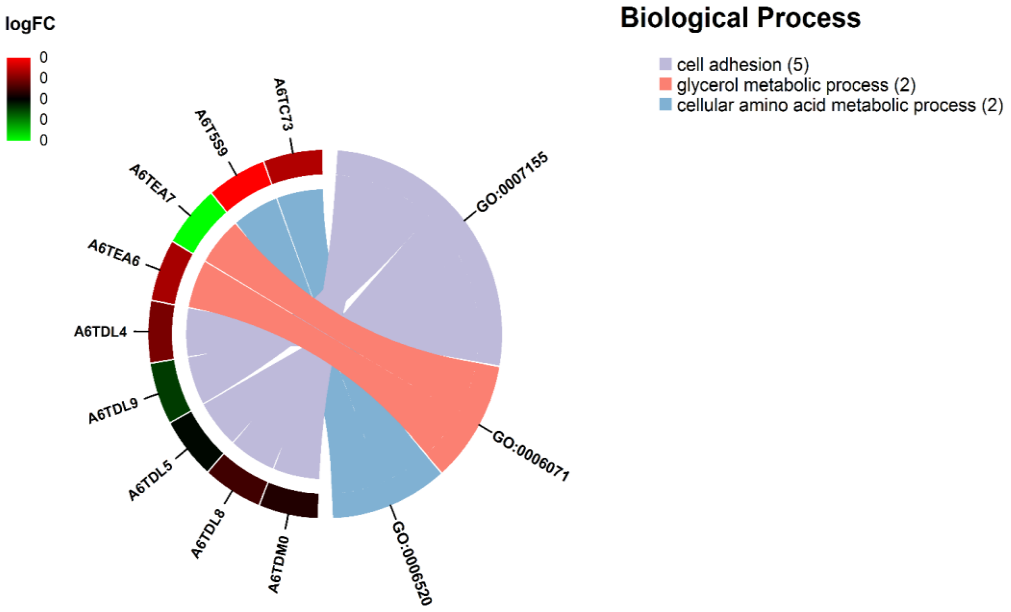


Figure 4. Enriched Gene Ontology terms detected by Nevomics for down-regulated differentially abundant proteins. A) Biological process.

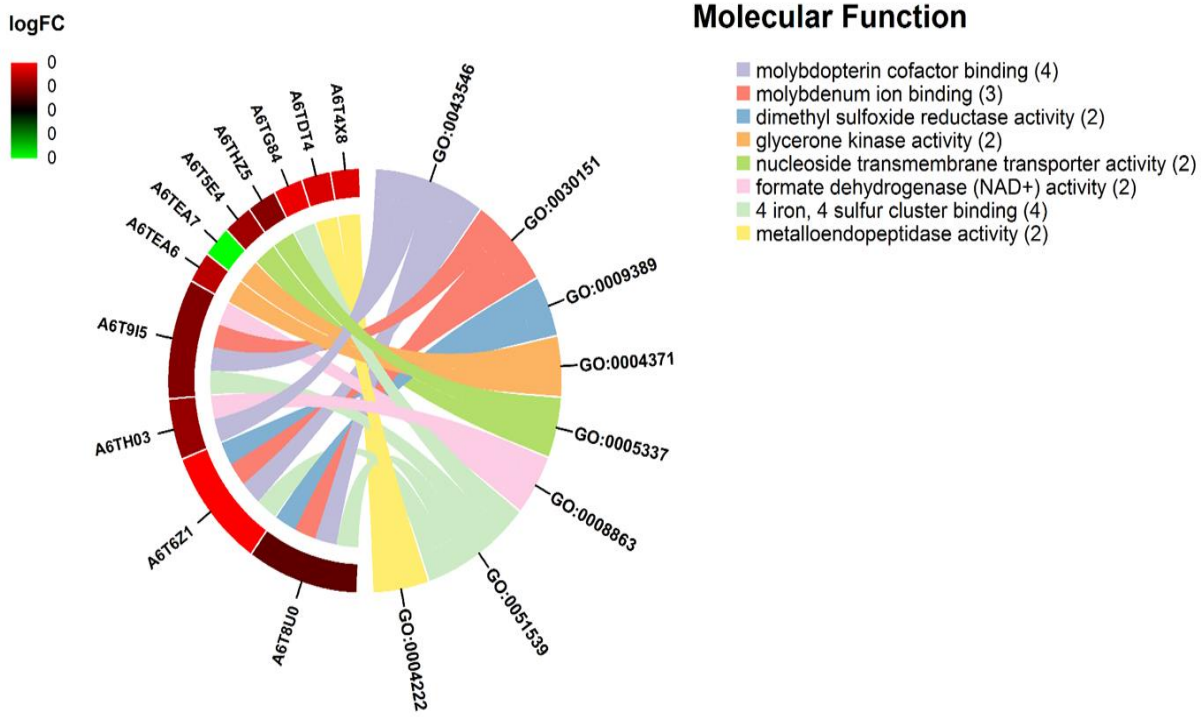


Figure 4. Enriched Gene Ontology terms detected by Nevomics for down-regulated differentially abundant proteins. B) Molecular function.

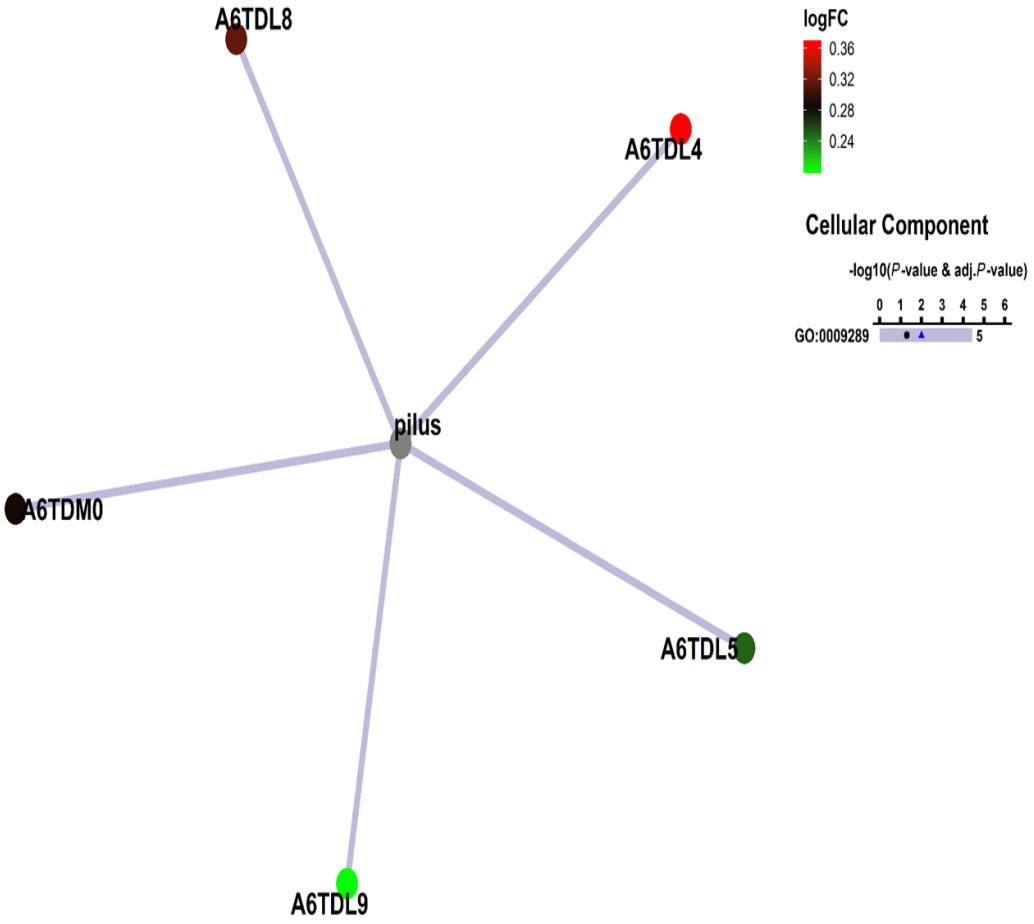


Figure 4. Enriched Gene Ontology terms detected by Nevomics for down-regulated differentially abundant proteins. C) Cellular component.

Discussão Geral

Como foi exposto e discutido no capítulo I, e em concordância com o descrito por outros autores na literatura para vários peptídeos antimicrobianos (PAMs), o peptídeo PaDBS1R1 parece induzir um remodelamento da maquinaria metabólica da bactéria (Maria-Neto et al., 2012; Wenzel et al., 2014a; Wenzel et al., 2016; Cardoso et al., 2017). Esse remodelamento implica mudanças nos níveis de abundância de enzimas diretamente vinculadas à produção de energia metabólica. Especificamente, na *K. pneumoniae* sensível desafiada com PaDBS1R1, observou-se uma diminuição na abundância de enzimas associadas ao ciclo de Krebs; sendo observado, concomitantemente, um significativo incremento na abundância dos componentes do sistema Pta-AckA (Capítulo I, Figura 3A e 4A, Tabelas suplementares 1, 3 e 4), sugerindo que a fosforilação ao nível de substrato mediada pelo sistema Pta-AckA poderia ser chave para a produção de energia na *K. pneumoniae* sensível desafiada. A ação sequencial das enzimas Pta e AckA permite a produção de ATP a partir de acetil-CoA (Wolfe, 2005).

Desta forma, se em *K. pneumoniae* sensível desafiada com PaDBS1R1, o sistema Pta-AckA constitui um fator chave para a produção de energia, a mesma deve ativar rotas metabólicas que garantem o fornecimento de acetil-CoA para o sistema Pta-AckA. Nesse contexto, como exposto no capítulo I, foi observada uma abundância aumentada de enzimas envolvidas na via catabólica do inositol (Capítulo I, Figura 2 e 3, Tabelas suplementares 2 e 3), permitindo o potencial uso de compostos externos contendo inositol como fonte de carbono, cuja degradação poderia produzir acetil-CoA (Berman and Magasanik, 1966a; b; Anderson and Magasanik, 1971). Além disso, observou-se um aumento significativo da abundância da enzima 3-hidroxi-5-fosfonooxipentano-2,4-diona tiolase (LsrF) (Capítulo I, Tabelas suplementares 1 e 3), que está envolvida na etapa terminal da degradação da molécula de sinalização autoindutor-2 (AI-2) (*quorum-sensing*). A reação enzimática catalisada por LsrF libera como produtos acetil-CoA e fosfato de di-hidroxiacetona (FDHA) (Marques et al., 2014). Dessa forma, a degradação do AI-2 poderia constituir uma via adicional para fornecer acetil-CoA para o sistema Pta-AckA. Adicionalmente, as análises de enriquecimento das vias metabólicas do KEGG mostraram um enriquecimento para o metabolismo do propanoato (Capítulo I, Figura 2),

no qual estão envolvidos componentes do operon *tdc*, que mediam a degradação de aminoácidos (Sawers, 1998; Simanshu et al., 2007). Especificamente, observou-se um aumento significativo na abundância das enzimas TdcB e TdcE (Capítulo I, Figura 2, Tabelas suplementares 1 e 2), as quais estão envolvidas na degradação de serina e treonina produzindo os metabólitos de acetil-CoA e propanoil-CoA respectivamente, que poderiam ser direcionados para a produção de ATP pelo sistema Pta-AckA (Shizuta et al., 1969; Hesslinger et al., 1998; Simanshu et al., 2007).Também foi observado um incremento significativo da enzima propionato quinase TdcD (Capítulo I, Tabela suplementar 2), a qual pode assistir na produção de ATP a partir da transformação do propanoil-CoA (produzido na degradação da treonina) em propionato pelo sistema Pta-TdcD (Simanshu et al., 2007).

Como o operon *tdc* media a degradação de aminoácidos, então em consequência ao aumento de sua expressão, deve acontecer também um aumento na abundância de sistemas que permitam a incorporação de peptídeos e/ou aminoácidos para serem utilizados pela maquinaria de degradação codificada pelo operon *tdc*. Neste sentido, foi observada uma abundância significativamente incrementada do simportador treonina/serina: H+ (TdcC) (Capítulo I, Tabela suplementar 1). Esse transportador poderia mediar, portanto, a incorporação desses aminoácidos presentes no meio LB, um meio complexo e enriquecido utilizado em nosso sistema experimental para o crescimento bacteriano (Sezonov et al., 2007), para posteriormente serem degradados e utilizados na obtenção de energia metabólica pelos sistemas Pta-AckA e Pta-TdcD. Além disso, na *K. pneumoniae* sensível desafiada foi observado um aumento na abundância do transportador YjiY (Capítulo I, Tabela suplementar 1), o qual media a incorporação de piruvato (Kristoficova et al., 2018). Esse piruvato incorporado poderia ser direcionado para a formação da cápsula bacteriana como previamente exposto no capítulo I; além de ser substrato da enzima TdcE, a qual media a transformação do piruvato em acetil-CoA (Hesslinger et al., 1998) .

Portanto, todas essas mudanças no proteoma da *K. pneumoniae* sensível desafiada com PaDBS1R1, sugerem que a bactéria ativa diferentes vias metabólicas que garantem o fornecimento de acetil-CoA e propanoil-CoA para os sistemas Pta-AckA e Pta-TdcD, permitindo dessa forma a obtenção de energia metabólica mediante a fosforilação ao nível de substrato.

No entanto, o incremento na abundância dos componentes do operon *tdc* na bactéria sensível desafiada mostrou-se como um resultado surpreendente, dado o fato que esse operon é expresso sob condições de anaerobioses (Sawers, 2001); e em nossas condições experimentais o sistema não se encontrava em condições de anaerobioses. Nesse contexto, a presença de O_2 poderia ser tóxica para os componentes do operon *tdc*, especialmente para a enzima TdcE, uma enzima que opera baseada na formação de um radical-glicilo. Esses tipos de enzimas tendem a ser sensíveis ao O_2 (Zhang et al., 2001; Simanshu et al., 2007; Shisler and Broderick, 2014). Portanto, é de esperar que *K. pneumoniae* utilize estratégias complementares que garantam a funcionalidade dos componentes do operon *tdc* para que este possa ser expresso em condições com presença de O_2. Nesse sentido, foi interessante observar o aumento na abundância das proteínas CydA e CydB (componentes do complexo citocromo bd-I), assim como da proteína GrcA (Capítulo I, Tabelas suplementares 1 e 5), uma vez que essas proteínas podem participar de estratégias de proteção a presença de O_2. Nesse contexto, as proteínas CydA e CydB são componentes do complexo bd-I o qual apresenta uma alta afinidade pelo O_2, o que permite, portanto, atuar como protetor de enzimas sensíveis ao O_2. Já a proteína GrcA, por sua vez, pode restaurar a atividade de enzimas radical-glicilo fragmentadas por O_2, através de sua associação com as mesmas, realizando também, dessa maneira um efeito protetivo ao O_2 (Hill et al., 1990; Alexeeva et al., 2000; Wagner et al., 2001; Zhu et al., 2007; Borisov and Verkhovsky, 2015). Dessa forma, o uso combinado do complexo citocromo bd-I e GrcA poderia ser uma estratégia usada pela *K. pneumoniae* sensível desafiada com PaDBS1R1 para facilitar a produção e funcionalidade dos componentes do operon *tdc*.

Por outro lado, a formação de acetil-CoA e propanoil-CoA mediada pela enzima TdcE implica a formação de formato como produto das reações (Simanshu et al., 2007). Um acúmulo de formato no citoplasma bacteriano poderia acidificar este compartimento e atuar como um desacoplador do gradiente de prótons, consequentemente afetando a força motriz protônica (Sawers, 2005). Por isso, as bactérias devem ativar mecanismos que evitem afetar potencialmente a força motriz protônica pela acumulação de formato. Nesse sentido, foi observada na *K. pneumoniae* sensível desafiada um incremento na abundância das proteínas FocA e FdnG (Capítulo I, Tabela suplementar 5). A proteína FocA atua como um canal que permite o transporte bidirecional de formato o qual dependendo das condições do cultivo bacteriano pode exportá-lo do citoplasma (ex. pelas

reações catalisadas por TdcE) para o periplasma bacteriano (Suppmann and Sawers, 1994; Lü et al., 2013). Adicionalmente, a proteína FdnG constitui uma subunidade do complexo formato dehidrogenase (FDH-N), o qual media a oxidação do formato no periplasma bacteriano (Pinske and Sawers, 2016). Portanto, o aumento na abundância de FocA e FdnG sugere que, na *K. pneumoniae* sensível desafiada com PaDBS1R1, a estratégia utilizada para evitar os potenciais efeitos adversos do formato produzido pela atividade do operon *tdc* (especificamente TdcE), se baseia na exportação e subsequente oxidação do formato no periplasma bacteriano.

As mudanças nos níveis de proteínas na *K. pneumoniae* sensível desafiada com PaDBS1R1 descritas acima, sugerem que a mesma ativa vias metabólicas que garantem o fornecimento de substratos para os sistemas de produção de energia Pta-AckA e Pta-TdcD, assim como ativa sistemas de ”suporte” que sustentam o funcionamento dessas vias de fornecimento. Isto em conjunto com a diminuição na abundância de enzimas associadas ao ciclo de Krebs e a cadeia de transporte eletrônico (Capítulo I, Figuras 4A e B, Tabelas suplementares 4 e 5) indicam que na *K. pneumoniae* sensível desafiada provavelmente a produção de ATP recai principalmente (mas não exclusivamente) na fosforilação ao nível de substrato mediante os sistemas Pta-AckA e Pta-TdcD.

As quantidades de ATP produzidas pelos sistemas de fosforilação ao nível de substrato são menores que as quantidades produzidas pelos sistemas respiratórios aeróbicos (ex. ciclo de Krebs-cadeia de transporte eletrônico) (Nelson et al., 2008). Portanto, se na *K. pneumoniae* sensível desafiada a produção de ATP recai principalmente sobre sistemas de fosforilação ao nível de substrato é de esperar que a disponibilidade de ATP decresça e consequentemente os processos metabólicos que consumem ATP. Em concordância com isto, como previamente exposto, observou-se para as proteínas abundantes diferencialmente (PADs) reguladas negativamente, um enriquecimento de processos biológicos que consumem ATP assim como para proteínas cujas funções moleculares depende do ATP (ex. transportadores ABC) (Capitulo I, Figuras 4A e B, Tabelas suplementares 1 e 4). Então, nossos resultados sugerem que quando a *K. pneumoniae* sensível está submetida ao estresse induzido por PaDBS1R1 adopta um estado de atividade metabólica reduzida (em comparação a quando não está desafiada com PaDBS1R1).

Por outro lado, como exposto acima, o possível redirecionamento da produção de ATP para sistemas de fosforilação ao nível substrato faz com que a *K. pneumoniae*

sensível tenha que produzir proteínas associadas ao metabolismo anaeróbico (ex. componentes do operon *tdc*) num ambiente com presença de O_2, o qual poderia comprometer a atividade catalítica e estabilidade estrutural dessas enzimas, mesmo ativando mecanismos de proteção para as mesmas (Buckel and Golding, 2006; Imlay, 2006; Broderick et al., 2014). Além disso, a produção de energia metabólica baseada em fosforilação ao nível de substrato pode ser menor em comparação com a produção de energia metabólica realizada mediante os sistemas respiratórios aeróbicos bacterianos. Então, por que redirecionar a produção de energia metabólica para sistemas baseado na fosforilação ao nível de substrato? Uma plausível explicação poderia ser que PaDBS1R1 interfere com a via metabólica ciclo de Krebs-cadeia de transporte eletrônico e em reposta a isso *K. pneumoniae* redireciona a produção de ATP para sistemas de fosforilação ao nível de substrato.

Em referência a essa hipótese, estudos reportaram que alguns PAMs podem induzir a produção de espécies reativas do oxigênio (EROs) como parte do mecanismo de ação dos mesmos, provavelmente mediante interferência com a cadeia de transporte eletrônico de forma tal que se favorece a liberação de EROs (Choi et al., 2015; 2017; Rowe-Magnus et al., 2019). Aparentemente, um fluxo ativo de elétrons pela cadeia de transporte eletrônico assim como uma elevada força motriz protônica favorece a formação e liberação das EROs promovida pelos PAMs (Choi et al., 2015; 2017). Interessantemente, alguns desses estudos mostraram que quando as bactérias eram submetidas ao crescimento em condições de fermentação (fosforilação ao nível de substrato constituindo a principal forma de obtenção de ATP) ou respiração anaeróbica, as concentrações inibitórias mínimas (CIMs) dos peptídeos para essas bactérias aumentava, sugerindo um enfraquecimento dos efeitos microbicidas dos PAMs (mediados pelas EROs) (Choi et al., 2015; 2017). Então, baseados nessas descobertas e especificamente em nossos dados proteômicos foi possível sugerir que o redirecionamento da obtenção de ATP para sistemas de fosforilação ao nível de substrato poderia ser uma estratégia usada pela *K. pneumoniae* sensível para superar o estresse induzido por PaDBS1R1.

As mudanças nos níveis de abundâncias de algumas proteínas na *K. pneumoniae* sensível desafiada com PaDBS1R1 sugere que a mesma pode estar submetida a um estresse oxidativo induzido por PaDBS1R1. Na bactéria desafiada observou-se um incremento significativo da abundância de proteínas como CydB, YieF, MacA, e Tpx as

quais estão associadas à resposta ao estresse oxidativo (Capítulo I, Tabela suplementar 1) (Ackerley et al., 2004a; Ackerley et al., 2004b; Cha et al., 2004; Imlay, 2008; Bogomolnaya et al., 2013; Al-Attar et al., 2016). Também foi detectada uma abundância significativamente incrementada da enzima agmatinasa (SpeB) a qual catalisa a formação de putrescina (Capítulo I, Tabela suplementar 1) (Igarashi and Kashiwagi, 2010; Pegg, 2018). A putrescina forma parte da família das poliaminas, as quais estão envolvidas na resposta contra o estresse oxidativo promovido por antimicrobianos (Tkachenko and Fedotova, 2007; Tkachenko et al., 2012; El-Halfawy and Valvano, 2014). Especificamente, a putrescina poderia atuar como sequestrador de EROs assim como unindo-se ao DNA e deslocando os cátions Fe^{2+} associados ao mesmo, o qual evita a formação de radicais hidroxilas sobre a molécula de DNA (Tadolini, 1988; Tkachenko and Fedotova, 2007; El-Halfawy and Valvano, 2014). Interessantemente, as poliaminas também têm sido associadas à defesa contra PAMs (Goytia and Shafer, 2010; Johnson et al., 2012; El-Halfawy and Valvano, 2013). Além disso, também foi detectado um aumento na abundância das proteínas glicerol dehidrogenase (GldA) e YhbL (ElbB) (Capítulo I, Tabela suplementar 1), as quais estão associadas aos sistemas de defesas contra espécies eletrofílicas reativas (EERs) (ex. glioxal e o metilglioxal)(Lee and Park, 2017). Essas EERs podem se formar pela peroxidação lipídica assim como pela oxidação do DNA e de aminoácidos (Lee and Park, 2017). Então, todas essas mudanças no proteoma da bactéria sensível desafiada sugerem que PaDBS1R1 promove a produção de EROs.

Por outro lado, como previamente exposto, estudos sugerem que os PAMs podem induzir a produção de EROs via perturbações na cadeia de transporte eletrônico e que essa produção de EROs poderia se favorecer mediante uma elevada força motriz protônica (Choi et al., 2015; 2017). Nesse sentido, na *K. pneumoniae* sensível desafiada foram observadas mudanças na abundância de proteínas associadas a cadeia de transporte eletrônico, sendo que a enzima NADH dehidrogenase respiratória (Ndh) apresentou um incremento na sua abundância enquanto subunidades da NADH:ubiquinona oxidoreductase mostraram uma redução na abundância (NuoC, NuoE,NuoF,NuoG e NuoK) (Capítulo I, Tabela suplementar 5). Adicionalmente, foi observado um incremento nos níveis das proteínas CydA e CydB (componentes do complexo citocromo bd-I), enquanto as proteínas CyoB e CyoA (componentes do complexo citocromo bo_3 oxidase) mostraram uma abundância diminuída (Capítulo I, Tabelas suplementares 1 e 5). Isto

sugere que na cadeia de transporte eletrônico da bactéria sensível desafiada poderia estar atuando principalmente a Ndh acoplada com o complexo citocromo bd-I.

Interessantemente, o acoplamento de Ndh com o complexo citocromo bd-I (ambas com abundância incrementada) resulta numa proporção (próton /elétron) de $2H^+/2e^-$, enquanto o acoplamento de NADH:ubiquinona oxidoreductase com complexo citocromo *bo₃* oxidase (ambas com abundância diminuída) resulta numa proporção ($8H^+/4e^-$). Portanto, a força motriz protônica gerada pelo acoplamento de Ndh com o complexo citocromo bd-I pode ser menor que a gerada pelo acoplamento de NADH:ubiquinona oxidoreductase com o complexo citocromo *bo₃* oxidase (Unden et al., 2014). Tomando isto em consideração e baseados nas evidências experimentais previamente expostas de que uma elevada força motriz protônica parece favorecer a geração de EROs pelos PAMs, é concebível sugerir que os incrementados níveis de Ndh e do complexo citocromo bd-I, assim como os reduzidos níveis de NADH:ubiquinona oxidoreductase e do complexo citocromo *bo₃* oxidase obedecem a uma reconfiguração da cadeia de transporte eletrônico frente ao desafio com PaDBS1R1, de forma tal que a força motriz protônica gerada seja menor e consequentemente haja uma diminuição da possibilidade de geração de EROs. No entanto, como previamente exposto no capítulo I, a enzima Ndh tem sido associada à geração de EROs (Messner and Imlay, 1999; Seaver and Imlay, 2004). Outra enzima vinculada com o transporte eletrônico e que também tem sido associada à geração de EROs é a fumarato reductase (Imlay, 1995; Korshunov and Imlay, 2010). Nesse sentido, como exposto no capítulo I se observou na *K. pneumoniae* sensível desafiada um incremento significativo na abundância da subunidade FrdA da fumarato reductase, a qual contém o cofator FAD que pode reagir com o O_2 produzindo EROs (Imlay, 1995; Korshunov and Imlay, 2010).

De acordo com as mudanças observadas acima no proteoma da *K.pneumoniae* sensível desafiada com PaDBS1R1, e nas evidências experimentais reportadas na literatura, pode ser admissível sugerir que o PaDBS1R1 poderia induzir a formação de EROS via perturbação da cadeia de transporte de elétrons. Em resposta a esse estresse oxidativo gerado, a *K. pneumoniae* sensível reestrutura a maquinaria metabólica associada a produção de ATP mediante ativação de sistemas de fosforilação ao nível de substrato e reconfigura a cadeia de transporte de elétrons de forma tal que a força motriz protônica gerada por esta seja menor. Ademais, a bactéria também ativa sistemas de defesa ao estresse oxidativo envolvendo provavelmente a produção de moléculas

sequestradoras de EROs (ex. putrescina) assim como bombas de efluxo (ex. MacAB-TolC) e enzimas que degradam as EROs (ex. Tpx).

Em contrapartida, as mudanças no proteoma da *K. pneumoniae* resistente desafiada com PaDBS1R1 também suportam a indução de estresse oxidativo pelo peptídeo. Nesse sentido foram detectadas proteínas com abundância aumentada que são associadas aos sistemas de defesas contra o estresse oxidativo. Essas proteínas incluíram a TrxA, TrxB, tiol peroxidase (Tpx), tioredoxina peroxidase, glutaredoxina, tioredoxina/glutationa peroxidase (BtuE), o regulador OxyR, a proteína YaaA, proteína de proteção ao dano oxidativo, superóxido dismutase SodA e MsrA (Capítulo II, Tabelas suplementares 1 e 6) (Arenas et al., 2010; Liu et al., 2011; Chiang and Schellhorn, 2012; Lu and Holmgren, 2014).

Adicionalmente, as análises de ontologia de genes para as PADs reguladas positivamente na *K. pneumoniae* resistente mostraram os processos biológicos de assimilação de sulfato, biossínteses de sulfureto de hidrogênio e biossínteses de cisteína como enriquecidos assim como o processo de catabolismo da histidina formando glutamato (Capítulo II, Figura 2A e 3A, Tabelas suplementares 2 e 4). Esses processos favorecem a obtenção de cisteína e glutamato os quais são precursores da glutationa (Masip et al., 2006). Também foi incrementada significativamente a abundância da proteína YliB a qual forma parte de um possível transportador ABC que media a incorporação de glutationa (Capítulo II, Tabela suplementar 1) (Suzuki et al., 2005). A glutationa é um componente essencial do sistema antioxidativo glutationa-glutarredoxina (Masip et al., 2006; Lu and Holmgren, 2014). Além disso, houve um enriquecimento da via das pentoses fosfato (o ramo oxidativo) na qual estão associadas as enzimas Gnd e Wzf as quais produzem NADPH que é um fator essencial para os sistemas antioxidativos glutationa-glutarredoxina e tioredoxina (Capítulo II, Figura 3A,Tabela suplementar 4) (Lu and Holmgren, 2014; Stincone et al., 2015).

Dessa forma, esses dados também mostram que a *K. pneumoniae* resistente ao peptídeo parece acionar uma resposta defensiva robusta contra o estresse oxidativo. Essa resposta parece estar baseada principalmente nos sistemas antioxidativos glutationa-glutarredoxina e tioredoxina. Interessantemente, foi constatado diferenças na resposta contra o estresse oxidativo desenvolvida pela *K. pneumoniae* sensível ao peptídeo e a *K. pneumoniae* resistente. Como foi descrito previamente a bactéria sensível parece basear a sua resposta em reconfiguração da maquinaria metabólica associada a geração de ATP

assim como em moléculas sequestradoras de EROs, bombas de efluxo e enzimas que degradam as EROs. No entanto, a *K. pneumoniae* sensível parece não ativar todo o arsenal enzimático ativado pela *K. pneumoniae* resistente já que os sistemas enzimáticos incrementados em abundância na bactéria resistentes foram regulados negativamente na bactéria sensível quando desafiada com o peptídeo. As proteínas reguladas negativamente na *K. pneumoniae* sensível incluíram a glutationa transferase (GstA), TrxA, TrxB, TrxC, tioredoxina peroxidase, BtuE, glutationa sintase, YliB, glutationa reductase, alkil hidroperoxide reductase, YaaA, peroxidase, superóxido dismutase, peroxiredoxine e MsrA (Capítulo I, Tabelas suplementares 1 e 5). Isto, portanto, sugere que uma resposta robusta contra o estresse oxidativo é essencial para a resistência ao peptídeo PaDBS1R1.

Além de ser essencial para a resistência a PaDBS1R1, a resposta contra o estresse oxidativo poderia ser também importante para a resistência cruzada a outros PAMs. Particularmente, tem sido mostrado que o peptídeo LL-37 entre seus mecanismos de ação contempla o desencadeamento de estresse oxidativo (Choi et al., 2015).

Como foi exposto no capítulo I um estudo prévio desenvolvido por Irazazabal e colaboradores (2019) mostrou que o peptídeo PaDBS1R1 perturba a permeabilidade da membrana citoplasmática bacteriana (Irazazabal et al., 2019). Por outro lado, Rodríguez-Rojas e colaboradores (2015) mostraram que peptídeos que afetam a permeabilidade da membrana podem facilitar a entrada dos íons ferroso (Fe^{2+}) e férrico (Fe^{3+}) no citoplasma bacteriano (Rodríguez-Rojas et al., 2015). Isto pode desencadear e/ou amplificar o estresse oxidativo já que os íons Fe^{2+} em presença de EROs como o H_2O_2 podem formar radicais hidroxilas mediante reação de Fenton (Rodríguez-Rojas et al., 2015). À vista disso, devido ao fato que PaDBS1R1 pode afetar a permeabilidade da membrana e que nossos experimentos foram desenvolvidos em meio LB o qual tem alto conteúdo de ferro é provável que PaDBS1R1 facilite a entrada de íons Fe^{2+} potenciando dessa forma o estresse oxidativo. De fato, como foi descrito no capítulo I, na *K. pneumoniae* sensível desafiada foi detectada uma abundância significativamente incrementada da proteína ferritina (FtnA) (Capítulo I, Tabela suplementar 1), a qual funciona como uma espécie de sequestrador de Fe^{2+} (Bou-Abdallah et al., 2014). Isto sugere que ocorreu um incremento nos níveis intracelulares de Fe^{2+} provavelmente facilitado pela atividade desestabilizadora de membrana do peptídeo PaDBS1R1, já que proteínas associadas ao transporte de ferro como YdcO e SitA foram significativamente reduzidas em abundância (Capítulo I, Tabela suplementar 1).

De forma semelhante, as mudanças no proteoma da *K. pneumoniae* resistente desafiada com o peptídeo também suportam um aumento nos níveis intracelulares de Fe^{2+}. Particularmente, foi observado um aumento significativo da ferritina (FtnA) (Capítulo II, Tabela suplementar 1). No entanto, como previamente exposto também foram detectados incrementados níveis das proteínas YaaA e a proteína de proteção ao dano oxidativo (Capítulo II, Tabela suplementar 6). As proteínas YaaA, e proteína de proteção ao dano oxidativo parecem atenuar a ocorrência da reação de Fenton mediante a diminuição dos níveis intracelulares de ferro, contudo os mecanismos exatos ainda não são conhecidos (Gralnick and Downs, 2003; Liu et al., 2011). Também foi observado, na *K. pneumoniae* resistente desafiada, um incremento significativo da proteína YbbL (Capítulo II, Tabela suplementar 1), a qual forma parte de um possível sistema exportador de ferro que confere resistência ao estresse oxidativo mediante a diminuição intracelular de ferro (Nicolaou et al., 2013).

As mudanças no proteoma associado ao metabolismo do ferro acima descritos mostram que a resposta ao provável incremento intracelular de Fe^{2+} desenvolvida pela *K. pneumonaie* resistente é mais robusta que a desenvolvida pela *K. pneumoniae* sensível. Isto pode ser de uma grande importância para evitar uma potenciação do aparente estresse oxidativo promovido pelo peptídeo. Em seu conjunto, os nossos resultados sugerem que o peptídeo PaDBS1R1 induz estresse oxidativo provavelmente perturbando a cadeia de transporte eletrônico. Esse estresse poderia se amplificar dada a atividade perturbadora de membrana do peptídeo já que facilitaria a entrada não controlada de Fe^{2+} na bactéria. Portanto, uma resposta antioxidante robusta pode ser essencial para resistir a atividade bactericida de PaDBS1R1.

De maneira geral nossos dados das análises proteômicas sugerem que a resposta desenvolvida pela *K. pneumoniae* ao desafio com o peptídeo PaDBS1R1 é uma resposta complexa que compreende um arsenal de mecanismos moleculares. Especificamente, a resposta anti-PaDBS1R1 desenvolvida pela *K. pneumoniae* sensível ao peptídeo parece se basear na modificação da superfície bacteriana mediante a produção de cápsula polissacarídica aniônica, assim como na modificação dos LPS com 4-amino-4-deoxi-L-arabinose. Além disso, o sistema de resposta a estresse baseado no fator sigma (σ^E) também parece estar envolvido na resposta anti-PaDBS1R1, provavelmente aliviando o estresse da superfície bacteriana mediante a maquinaria BAM (*β-barrel assembly machinery*). Adicionalmente, bombas de efluxo (MacAB-TolC e AcrAB-TolC) parecem

ser ativadas durante a resposta protetora. Uma vez que, essas bombas de efluxo têm sido vinculadas à resistência a vários antimicrobianos, uma das consequências da utilização de PaDBS1R1 a concentrações subinibitórias pode ser a indução de resistência cruzada a outros antimicrobianos. Outra das consequências da utilização de concentrações subinibitórias de PaDBS1R1 é a emergência de bactérias resistentes ao peptídeo. Nesse cenário, foi constatado que na *K. pneumoniae* sensível quando desafiada com concentrações subinibitórias do peptídeo incrementou-se os níveis de componentes de sistemas de reparação do DNA sugerindo que o peptídeo promovia danos no DNA. Nesse sentido, nossos dados sugerem que PaDBS1R1 poderia atuar induzindo estresse oxidativo na bactéria provavelmente via perturbação da cadeia de transporte eletrônico, ao mesmo tempo a sua atividade membranolítica pode facilitar a entrada de íons Fe^{2+} na bactéria potenciando dessa forma o estresse oxidativo. Esse estresse oxidativo gera espécies moleculares reativas com potencial mutagênico o que poderia facilitar danos no DNA e a emergência de bactérias mutantes com capacidade de resistência ao peptídeo.

Por outro lado, no caso da *K. pneumoniae* resistente ao peptídeo se observou uma resposta anti-PaDBS1R1 na qual estão envoltos diferentes sistemas regulatórios. Particularmente, os sistemas de dois componentes PhoPQ, CpxRA e ZraPSR parecem orquestrar de maneira coordenada a resposta protetora. Essa reposta protetora parece incluir modificações da envoltura bacteriana mediante a adição de 4-amino-4-deoxi-L-arabinose e hidroxilação do lipídeo A dos LPS via enzimas codificadas pelo operon *arnBCADTEF* e a enzima LpxO, respectivamente. Ambos sistemas estão sob o controle do sistema PhoPQ. Além disso, o sistema CpxRA parece ativar uma rede molecular que garante a produção e funcionamento da bomba de effluxo AcrAB-TolC a qual tem sido vinculada com a resposta de resistência a PAMs e outros antimicrobianos. Tanto a modificação dos LPS via hidroxilação e/ou com 4-amino-4-deoxi-L-arabinose assim como a ativação de AcrAB-TolC têm sido vinculadas com a resistência a peptídeos de defesa do hospedeiro, os quais são efetores do sistema imune do hospedeiro. Nesse sentido, também foram detectados um incremento em proteínas (ex. inibidor de lisozima e aciltransferase) que podem mediar a resistência a outros componentes do sistema imune como a lisozima e fosfolipases. Então, potencialmente a resistência a PaDBS1R1 poderia favorecer a resistência cruzada a uma variedade de efetores do sistema imune. Adicionalmente, foi observado que *K. pneumoniae* resistente a PaDBS1R1 incrementou significativamente os níveis de proteínas associadas a sistemas defensivos contra o

estresse oxidativo, enquanto a *K. pneumoniae* sensível a PaDBS1R1 parece não ativar uma resposta anti-estresse oxidativo potente. Isso sugere que os mecanismos de defesa contra o estresse oxidativo são de grande importância para a resistência ao peptídeo PaDBS1R1.

Conclusões

- PaDBS1R1 poderia induzir estresse oxidativo em *K. pneumoniae*
- O desafio continuo com concentrações sub-inibitórias de PaDBS1R1 pode facilitar a emergência de *K. pneumoniae* com susceptibilidade reduzida ao peptídeo
- *K. pneumoniae* desenvolve uma resposta anti-PaDBS1R1 complexa
- A resposta anti-PaDBS1R1 desenvolvida por *K. pneumoniae* sensível ao peptídeo, assim como *K. pneumoniae* resistente ao peptídeo, poderia envolver uma restruturação do proteoma associado ao metabolismo bacteriano, além da ativação de diferentes mecanismos defensivos.
- Uma resposta robusta contra o estresse oxidativo poderia ser essencial para resistir a atividade microbicida de PaDBS1R1

Perspectivas

O peptídeo antimicrobiano PaDBS1R1 constitui um antimicrobiano com potencialidades para ser explorado no futuro desenvolvimento de estratégias terapêuticas, particularmente devido a sua potente atividade contra bactérias gram-negativas. No entanto, ainda é necessário adquirir uma profunda compreensão sobre as redes moleculares que regem a interação bactéria-peptídeo. Nesse sentido, o presente trabalho aporta informações que podem servir como ponto de partida para o desenvolvimento de futuras pesquisas. Nossos dados sugerem que PaDBS1R1 pode desencadear um estresse oxidativo na bactéria e que uma potente resposta anti-estresse oxidativo poderia ser essencial para resistir a ação do peptídeo. Nesse sentido, futuras pesquisas podem elucidar como PaDBS1R1 ativa o estresse oxidativo, além de comprovar o papel da resposta anti-estresse oxidativo na resistência. Adicionalmente, nossos dados também sugerem que vários sistemas regulatórios de dois componentes orquestram a resposta de resistência ao peptídeo. Portanto, a procura de estratégias que visem interferir com a atividade desses sistemas regulatórios poderia ser de grande utilidade, não só na compreensão da resposta de resistência, mas também na formulação de terapias combinadas com o peptídeo. Outro elemento interessante que sugere os resultados da presente pesquisa é que na resposta anti-PaDBS1R1 desenvolvida pela *K. pneumoniae* resistente poderiam ser ativados mecanismos de resistência a componentes do sistema imune inato do hospedeiro. Consequentemente, isto constitui um aspecto que precisa de uma pesquisa mais detalhada para comprovar se acontece resistência cruzada aos efetores do sistema imune inato.

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Anexos

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Bacterial resistance to antimicrobial peptides: an evolving phenomenon

Osmel Fleitas[1,2], Caleb M. Agbale[2,3], Octavio L. Franco[2,4]

[1]Pos-Graduacao da Faculdade de Medicina, Campus Universitario Darcy Ribeiro, Universidade de Brasilia, Asa Norte, Brasília, DF, Brazil, [2]S-Inova, Programa de Pos-Graduacao em Biotecnologia, Universidade Catolica Dom Bosco, 79117-900 Campo Grande, MS, Brazil, [3]Department of Biochemistry, School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, Cape Coast, Ghana, [4]Centro de Analises Proteomicas e Bioquimicas, Programa de Pos-Graduacao em Ciencias Genomicas e Biotecnologia, Universidade Catolica de Brasilia, 70719-100 Brasília, DF, Brazil

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1. ABSTRACT

Bacterial resistance to conventional antibiotics is currently a real problem all over the world, making novel antimicrobial compounds a real research priority. Some of the most promising compounds found to date are antimicrobial peptides (AMPs). The benefits of these drugs include their broad spectrum of activity that affects several microbial processes, making the emergence of resistance less likely. However, bacterial resistance to AMPs is an evolving phenomenon that compromises the therapeutic potential of these compounds. Therefore, it is mandatory to understand bacterial mechanisms of resistance to AMPs in depth, in order to develop more powerful AMPs that overcome the bacterial resistance response.

2. INTRODUCTION

Since the first reports of bacterial resistance to conventional antibiotics the spread of antimicrobial resistance has been such that at present constitute one of the major health challenge. The threat to human health has reached such magnitude that the World Health Organization Antimicrobial Resistance Global Report on Surveillance (2014) by Dr Keiji Fukuda, Assistant Director-General, Health Security, stated that: "A post-antibiotic era - in which common infections and minor injuries can kill - far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century" (1).

Antimicrobial resistance is a complex and multifactorial phenomenon. A factor that facilitated the emergence of resistance to conventional antibiotics is that most of them target proteins involved in specific vital functions for bacteria, therefore exerting a great selective pressure that expedites the generation of mutations to counteract the antibiotic action (2).

It is currently necessary to search for and design new antimicrobial compounds that can be directed towards different targets and exercise a broad spectrum of activities. Among the compounds that carry these characteristics are the antimicrobial peptides (AMPs). They have become attractive as therapeutic drugs since they are less likely to develop resistance. However, resistance to AMPs has been reported since AMPs were first discovered as natural antibiotics. For example, in 1949 Joseph L. Stone reported resistance to bacitracin in cultures of *Staphylococcus aureus*, only four years after bacitracin had been launched as a new antibiotic by Johnson *et al.*, 1945 (3,4). Over the years, a growing number of AMPs resistance mechanisms have been discovered in bacteria, which include mechanisms at the

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Induced Bacterial Cross-Resistance toward Host Antimicrobial Peptides: A Worrying Phenomenon

Osmel Fleitas[1,2] and Octávio L. Franco[1,2,3]*

[1] Programa de Pós-Graduação em Patologia Molecular, Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil, [2] Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil, [3] Programa de Pós-Graduação em Biotecnologia, S-Inova Biotech, Universidade Católica Dom Bosco, Campo Grande, Brazil

Bacterial resistance to conventional antibiotics has reached alarming levels, threatening to return to the pre-antibiotic era. Therefore, the search for new antimicrobial compounds that overcome the resistance phenomenon has become a priority. Antimicrobial peptides (AMPs) appear as one of the most promising antibiotic medicines. However, in recent years several AMP-resistance mechanisms have been described. Moreover, the AMP-resistance phenomenon has become more complex due to its association with cross-resistance toward AMP effectors of the host innate immune system. In this context, the use of AMPs as a therapeutic option could be potentially hazardous, since bacteria could develop resistance toward our innate immune system. Here, we review the findings of major studies that deal with the AMP cross-resistance phenomenon.

Keywords: antimicrobial peptides, cross-resistance, bacterial infection, antibiotics

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***Correspondence:**
Octávio L. Franco
ocfranco@gmail.com

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INTRODUCTION

With the discovery and introduction in the early 20th century of antimicrobial compounds for the treatment of infections caused by microorganisms it was thought that infections would no longer endanger human health. However, one century later, infectious diseases still constitute a threat (Saga and Yamaguchi, 2009).

Undoubtedly, the capacity of microorganisms to develop resistance to antimicrobial compounds has been one major cause of this situation. Therefore, the scientific community is deeply involved in the search for new and more powerful antimicrobial compounds that overcome pathogen resistance. In this search, among the most promising compounds that have been found are the antimicrobial peptides (AMPs). AMPs are short amphipathic peptides, generally cationic, produced by a wide variety of organisms that range from bacteria to humans. They perform antimicrobial activities by dissimilar mechanisms of action, including cell membrane permeability and inhibition of the synthesis of proteins, nucleic acids and the cell wall, among others (Jenssen et al., 2006; Guilhelmelli et al., 2013).

It is assumed that bacterial resistance to AMPs is unlikely because bacteria have to change conserved targets, such as the cell membrane, and this could be costly (Zasloff, 2002). However, the reality is quite different, since several AMP resistance mechanisms have been described (as reviewed in Maria-Neto et al., 2015). The resistance to AMPs would compromise their use and effectiveness as therapeutic agents. Moreover, within the issue of resistance to AMPs a more

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REVIEW

Deciphering bioactive peptides and their action mechanisms through proteomics

Osmel Fleitas Martinez[a,b], Caleb Mawuli Agbale[c,d], Fernanda Nomiyama[b] and Octávio Luiz Franco[a,b,c]

[a]Pos-Graduação em Patologia olecular, Universidade de Brasilia, Brasilia-DF Brazil; [b]Centro de Analises Proteomicas e Bioquimicas, Programa de Pos-Graduacao em Ciencias Genomicas e Biotecnologia, Universidade Catolica de Brasilia, Brasília, Brazil; [c]S-Inova Biotech, Programa de Pos-Graduacao em Biotecnologia, Universidade Catolica Dom Bosco, Campo Grande, Brazil; [d]Department of Biochemistry, School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, Cape Coast, Ghana

ABSTRACT

Introduction: Bioactive peptides such as antimicrobial peptides (AMPs), ribosomally synthesized and post translationally modified peptides (RiPPs) and the non-ribosomal peptides (NRPs) have emerged with promising applications in medicine, agriculture and industry. However, their development has been limited by several difficulties making it necessary to search for novel discovery methods. In this context, proteomics has been considered a reliable tool.

Areas covered: This review highlights recent developments in proteomic tools that facilitate the discovery of AMPs, RiPPs and NRPs as well as the elucidation of action mechanisms of AMPs and resistance mechanisms of pathogens to them.

Expert commentary: Proteomic approaches have emerged as useful tools for the study of bioactive peptides, especially mass spectrometry-based peptidomics profiling, a promising strategy for AMP discovery. Furthermore, the rapidly expanding fields of genome mining and genome sequencing techniques, as well as mass spectrometry, have revolutionized the discovery of novel RiPPs and NRPs from complex biological samples.

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Antimicrobial peptides; non-ribosomal peptides; ribosomally synthesized and post translationally modified peptides; peptidomics; genome mining tools

1. Introduction

Interest in natural products from all sources has been at an all-time high in the past decade. This has been boosted by the need for more potent drugs to deal with the constantly evolving threat of chemoresistance in microbes, parasites, and cancers as well as the need for novel chemicals for agriculture and industry [1]. Special interest to natural product researchers are antimicrobial peptides (AMPs), ribosomally synthesized and post translationally modified peptides (RiPPs), and non-ribosomal peptides (NRPs) [2–5]. These peptides pose unique action mechanisms quite different from conventional antibiotics and should therefore prevent or delay the emergence of chemoresistance in microbes [6,7]. Research on these peptides has been revolutionized by recent advances in genomics and proteomics, which have enabled a comprehensive study of a potential array of peptides within various eukaryotes and prokaryotes, as well as their biosynthetic pathways and how their effects are neutralized by resistance mechanisms in microbes. For instance, the qualitative and quantitative information obtained by the proteomics approach to the pathogen proteome under conditions of AMP challenge allows molecular networks to be established, revealing possible mechanisms of AMP action or resistance to AMPs [8,9]. Additionally, using a mass spectrometry-based peptidomics approach, it is possible to discover AMPs from natural sources, avoiding some limitations inherent in the conventional methods [10]. Unlike AMPs, identification and characterization of RiPPs and NRPs from bacteria and fungi is a challenging task and requires the use of genome mining tools that have been trained to identify and predict biosynthetic gene clusters and gene products. Fortunately, these biosynthetic genes and their tailoring enzymes are clustered, making their identification easier. Indeed, each new class of RiPPs or NRPs discovered leads to the refinement of existing *in silico* approaches to peptide discovery. Thus, the current state of genome mining tools, gene expression systems, and chromatographic and mass spectrometry (MS) techniques is a reflection of discoveries made so far, although obviously the potential for further refinement exists. Here, we present computational and MS-based strategies to highlight various themes that have emerged recently in this exciting field of genome-guided AMP discovery. AMPs hold immense potential for the development of novel compounds for chemotherapy, agriculture, and industry.

2. Novel platforms for AMP, RiPP, and NRP discovery

2.1. *Mass spectrometry-based peptidomics*

Initially, the isolation and identification of AMPs from natural sources were based on conventional biochemistry methods. This involved complete purification of the extract followed by Edman degradation to identify the peptide sequence. Obviously, this bioassay-guided fractionation was a laborious task fraught with numerous limitations, including the need for large sample volumes, loss of activity and in most cases low

CONTACT Octávio Luiz Franco ocfranco@gmail.com; ocfranco@ucdb.br; ocfranco@pos.ucb.br

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Interference With Quorum-Sensing Signal Biosynthesis as a Promising Therapeutic Strategy Against Multidrug-Resistant Pathogens

Osmel Fleitas Martinez[1,2], *Pietra Orlandi Rigueiras*[2], *Állan da Silva Pires*[2], *William Farias Porto*[2,3,4], *Osmar Nascimento Silva*[3], *Cesar de la Fuente-Nunez*[5,6,7,8,9*] and *Octavio Luiz Franco*[1,2,3*]

[1] Programa de Pós-Graduação em Patologia Molecular, Universidade de Brasília, Brasília, Brazil, [2] Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil, [3] S-Inova Biotech, Programa de Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, Brazil, [4] Porto Reports, Brasília, Brazil, [5] Synthetic Biology Group, MIT Synthetic Biology Center, Massachusetts Institute of Technology, Cambridge, MA, United States, [6] Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, MA, United States, [7] Department of Biological Engineering, Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA, United States, [8] Broad Institute of MIT and Harvard, Cambridge, MA, United States, [9] The Center for Microbiome Informatics and Therapeutics, Cambridge, MA, United States

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Rodolfo García-Contreras, National Autonomous University of Mexico, Mexico

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Hanne Ingmer, University of Copenhagen, Denmark
Yael González Tinoco, Ensenada Center for Scientific Research and Higher Education (CICESE), Mexico

***Correspondence:**
Cesar de la Fuente-Nunez
cfuente@mit.edu
Octavio Luiz Franco
ocfranco@gmail.com

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Faced with the global health threat of increasing resistance to antibiotics, researchers are exploring interventions that target bacterial virulence factors. Quorum sensing is a particularly attractive target because several bacterial virulence factors are controlled by this mechanism. Furthermore, attacking the quorum-sensing signaling network is less likely to select for resistant strains than using conventional antibiotics. Strategies that focus on the inhibition of quorum-sensing signal production are especially attractive because the enzymes involved are expressed in bacterial cells but are not present in their mammalian counterparts. We review here various approaches that are being taken to interfere with quorum-sensing signal production via the inhibition of autoinducer-2 synthesis, PQS synthesis, peptide autoinducer synthesis, and N-acyl-homoserine lactone synthesis. We expect these approaches will lead to the discovery of new quorum-sensing inhibitors that can help to stem the tide of antibiotic resistance.

Keywords: virulence, antibiotic resistance, quorum sensing, quorum-sensing inhibition, anti-virulence therapy

INTRODUCTION

The increase in bacterial resistance to antimicrobial compounds and the spread of drug-resistant pathogens have become serious threats to human health. Currently, most antimicrobial compounds target essential bacterial physiological processes, thereby exerting a strong selective pressure on bacteria and facilitating the emergence and dissemination of resistant strains (Munguia and Nizet, 2017). Therapeutic strategies that circumvent the emergence and spread of multidrug-resistant pathogens are, therefore, urgently needed.

New attractive approaches for generating new therapeutics have focused on interfering with bacterial virulence factors, specifically, interfering with compounds synthesized by pathogens that facilitate colonization of the host and subsequent infection (Kong et al., 2016; Vale et al., 2016; Dickey et al., 2017; Munguia and Nizet, 2017). Because interference with virulence factors does

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Recent Advances in Anti-virulence Therapeutic Strategies With a Focus on Dismantling Bacterial Membrane Microdomains, Toxin Neutralization, Quorum-Sensing Interference and Biofilm Inhibition

Osmel Fleitas Martínez [1,2], Marlon Henrique Cardoso [1,2,3], Suzana Meira Ribeiro [4] and Octavio Luiz Franco [1,2,3]*

[1] Programa de Pós-Graduação em Patologia Molecular, Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil, [2] Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil, [3] S-inova Biotech, Programa de Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, Brazil, [4] Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal da Grande Dourados, Dourados, Brazil

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Edited by:
Natalia V. Kirienko,
Rice University, United States

Reviewed by:
Michael L. Vasil,
School of Medicine, University of Colorado Denver, United States
Michael Otto,
National Institute of Allergy and Infectious Diseases (NIAID), United States

***Correspondence:**
Octavio Luiz Franco
ocfranco@gmail.com

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Antimicrobial resistance constitutes one of the major challenges facing humanity in the Twenty-First century. The spread of resistant pathogens has been such that the possibility of returning to a pre-antibiotic era is real. In this scenario, innovative therapeutic strategies must be employed to restrict resistance. Among the innovative proposed strategies, anti-virulence therapy has been envisioned as a promising alternative for effective control of the emergence and spread of resistant pathogens. This review presents some of the anti-virulence strategies that are currently being developed, it will cover strategies focused on quench pathogen quorum sensing (QS) systems, disassemble of bacterial functional membrane microdomains (FMMs), disruption of biofilm formation and bacterial toxin neutralization.

Keywords: anti-virulence therapy, antibiotic resistance, bacterial membrane microdomains, quorum sensing, biofilms, bacterial toxins

INTRODUCTION

Antimicrobial resistance has turned a serious concern to the human health, because in addition to the death caused by drug-resistant pathogens (~700,000 death annually and it is estimated ~10 million for the year 2050), important medical procedures such as organ transplantation, cancer chemotherapy and surgery are also compromised (O'Neill, 2016). Antimicrobial resistance is a multifactorial phenomenon. Therefore, to circumvent it, a range of actions are needed (WHO, 2018). According that, the innovative antimicrobial compounds development that operate under different principles to those of conventional antibiotics constitutes an important element in the