UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

PRODUÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO DE UM PEPTÍDEO ANTIMICROBIANO PRODUZIDO POR UMA LINHAGEM DE *Bacillus* sp. P34

AMANDA DE SOUZA DA MOTTA

PORTO ALEGRE-RS
2006

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Autor: AMANDA DE SOUZA DA MOTTA

Tese submetida ao Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do grau de Doutor em Ciências Veterinárias.

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PORTO ALEGRE-RS
2006

Faculdade de Veterinária
Programa de Pós-Graduação em Ciências Veterinárias

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PRODUÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO DE UM PEPTÍDEO ANTIMICROBIANO PRODUZIDO POR UMA LINHAGEM DE *Bacillus* sp. P34

Autor: Amanda de Souza da Motta
Orientador: Prof. Adriano Brandelli

RESUMO

Uma bactéria identificada como *Bacillus* sp. P34 isolada de intestino de peixe (*Leporinus* sp.) da Bacia Amazônica foi estudada quanto a sua capacidade de produzir substâncias do tipo-bacteriocina. As condições ótimas para produção da substância antimicrobiana foram determinadas. A produção da atividade antimicrobiana foi observada começando na fase exponencial de crescimento, sendo a atividade máxima observada no início da fase estacionária. Os resultados da Análise de Superfície de Resposta mostraram que a máxima produção da atividade antimicrobiana ocorreu a pH inicial entre 6.0 e 8.0 e temperaturas entre 25 e 37°C. A substância inibiu bactérias patogênicas e deteriorantes importantes em alimentos como *Listeria monocytogenes*, *Bacillus cereus, Aeromonas hydrophila*, *Erwinia carotovora* e *Pasteurella haemolytica*. O teste de termoestabilidade mostrou a perda de atividade quando a temperatura alcançou 100°C por 15 minutos. Foi sensível à ação das enzimas proteolíticas tripsina, papaína e pronase E. A substância antimicrobiana apresentou efeito bactericida e bacteriolítico sobre *L. monocytogenes* e *B. cereus* a 160 UA ml^{-1}. O crescimento de *Escherichia coli* and *Salmonella* Enteritidis foi inibido somente quando o agente quelante EDTA foi adicionado juntamente. A atividade esporocida não foi observada. A análise da cultura de *L. monocytogenes* depois do tratamento com o composto antimicrobiano, usando espectroscopia de infravermelho com transformada de Fourier mostrou alterações no perfil de ácidos graxos e fosfolipídios da membrana celular bacteriana. Há evidências de que seu modo de ação interfira na membrana e na parede celular. A substância foi purificada pelo seguinte protocolo: precipitação com sulfato de amônio, cromatografias de gel filtração e de troca iônica. O peso molecular da substância foi determinado por espectroscopia de massas sendo 1498.68 Da. A substância antimicrobiana purificada apresentou sensibilidade ao tratamento com proteases e manutenção da atividade foi observada após congelamento e à incubação de 70°C por 30 minutos.

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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THE ANTIBACTERIAL PEPTIDE PRODUCED BY A STRAIN OF Bacillus sp. P34

Author: Amanda de Souza da Motta
Advisor: Prof. Adriano Brandelli

ABSTRACT

A bacterium identified as Bacillus sp. strain P34 isolated from fish intestine (Leporinus sp.) from the Amazon basin was studied in its capacity to produce bacteriocin-like substances. The optimal conditions for producing the antimicrobial activity have been established. The antimicrobial activity was produced starting at the exponencial growth phase, and maximum activity was observed at early stationary phase. Response-surface data showed that maximum antimicrobial activity production was at initial pH between 6.0 and 8.0 and temperature between 25 and 37°C. The antimicrobial substance inhibited pathogenic and spoilage food bacteria such as Listeria monocytogenes, Bacillus cereus, Aeromonas hydrophila, Erwinia carotovora and Pasteurella haemolytica. The thermoestability test showed the loss of activity when the temperature reached 100°C for 15 min. It was sensitive to the proteolytic action of trypsin, papain and pronase E. The antimicrobial substance was bactericidal and bacteriolytic to L. monocytogenes and B. cereus at 160 AU ml^{-1}. Growth of Escherichia coli and Salmonella Enteritidis was inhibited, but only when the chelating agent EDTA was co-added. Sporocidal activity was not observed. The analysis of the culture of L. monocytogenes after being treated with antimicrobial compound, using Fourier transform infrared spectroscopy, established a change in the profile that corresponding assignments of fatty acid and phospholipids. There was evidence that its mode of action to interfere with cell membrane and the cell wall. The substance was purified by the following protocol: precipitation with ammonium sulphate, gel filtration and ion exchange chromatography. The molecular weight was determined by mass spectroscopy as 1498.68 Da. Purified antimicrobial substance has shown sensitivity to protease treatment and maintained activity after freezing and incubation at 70°C for 30 min.

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1 INTRODUÇÃO

O impacto da crescente resistência de microrganismos a medicamentos e substâncias específicas tem movimentado vários grupos de pesquisa. A indústria farmacêutica, tem-se voltado para o desenvolvimento de novas substâncias que sejam capazes de lidar efetivamente com as estratégias de adaptação que esses microrganismos elaboram, em face ao surgimento de situações adversas.

O surgimento da resistência a antimicrobianos é um exemplo clássico da evolução, em resposta a uma forte pressão de seleção. O uso indiscriminado de drogas é o principal fator de indução à resistência. Entretanto, a prevenção desse abuso não é garantia que a susceptibilidade seja um fator garantido, uma vez que os efeitos provocados pelos antimicrobianos na fisiologia e ecologia dos microrganismos, impossibilitam, cada vez mais, o retorno ao estado de susceptibilidade anterior.

Paralelo a este fato**,** um grande interesse por novos métodos de biopreservação de alimentos tem aumentado nos últimos anos, com o objetivo de reduzir o uso de aditivos nestes produtos. Pesquisas têm indicado o potencial de antagonismo de alguns microrganismos, assim como de seus metabólitos antimicrobianos.

Em relação à preocupação com a preservação dos alimentos, tem-se focado nas bactérias lácticas, as quais em seu processo de fermentação normal produzem metabólitos como ácidos orgânicos, peróxido de hidrogênio e bacteriocinas, protegendo o sistema alimentar de microrganismos patogênicos e deteriorantes. As bacteriocinas já possuem uma potencial aplicação prática e um grande número destas já tem sido identificadas e caracterizadas.

Dessa forma, o uso de novas tecnologias para o desenvolvimento de substâncias antimicrobianas mais eficazes, constitui uma estratégia promissora no campo da biotecnologia. Isto pois, possibilitará a prospecção de novas classes de moléculas naturais e/ou sintéticas, capazes de neutralizar ou de eliminar o patógeno alvo (ao invés de inviabilizá-lo geneticamente) inibindo, assim, o desenvolvimento da resistência.

Atualmente peptídeos antimicrobianos estão sendo utilizados como modelos para novas drogas com aplicação na área agrícola e na saúde. Para tanto, seqüências desses

peptídeos vêm sendo desenhadas, sintetizadas e testadas *in vitro*, a fim de permitir a obtenção de genes com potencial de resistência a fitopatógenos, bem como compostos ativos para a fabricação de substâncias antimicrobianas de amplo espectro e de múltipla aplicabilidade.

Muito interesse tem sido focado sobre um grupo de peptídeos antimicrobianos conhecido como bacteriocinas. São substâncias antimicrobianas de natureza protéica, sintetizadas por várias linhagens de bactérias Gram-positivas e Gram-negativas, predominantemente sendo pesquisadas em bactérias lácticas. Esta classe de substâncias apresenta características de estabilidade ao calor, ao baixo pH, refrigeração e congelamento, surgindo como um agente potencial a ser aplicado em sistemas de conservação de alimentos de modo a promover alimentos mais estáveis do ponto de vista microbiológico.

Mais de 700 peptídeos antimicrobianos já foram identificados em todas as espécies vivas, não só em bactérias, mas também em fungos, insetos, moluscos, crustáceos, aracnídeos, plantas, pássaros, anfíbios, peixes e mamíferos. Em geral, são moléculas pequenas de até 5 kDa que exibem um alto teor de aminoácidos básicos e pelo menos 50% de aminoácidos hidrofóbicos, sendo compostos promissores para o desenvolvimento de pesquisas por novas substâncias antimicrobianas, buscando sua aplicação adequada.

Neste contexto, a pesquisa, purificação e caracterização química, biológica e estrutural de novas substâncias antimicrobianas provenientes da fauna e flora brasileira são valiosas, uma vez que a própria evolução tratou de selecionar um vastíssimo espectro de substâncias eficientes que atuem contra infecções.

É importante que se busque a identificação destas novas substâncias assim como se explore o potencial de aplicação farmacológico para o controle de infecções ou como biopreservativo de alimentos, onde temos que considerar todos os fatores intrínsecos e extrínsecos envolvidos no processo de conservação do produto.

Este trabalho propõe a produção, purificação e caracterização de um peptídeo antimicrobiano produzido por uma espécie de *Bacillus* sp. isolada do ambiente aquático da Amazônia.

Mais especificamente, os objetivos deste trabalho são:

1. Identificação do microrganismo produtor da substância antimicrobiana;
2. Avaliação do espectro de ação desta substância antimicrobiana frente a microrganismos patogênicos e deteriorantes;
3. Avaliação da sua estabilidade sob diferentes condições;
4. Determinação das melhores condições de produção;
5. Determinação do seu efeito e modo de ação;
6. Purificação e caracterização química da substância antimicrobiana.

2 REVISÃO BIBLIOGRÁFICA

2.1 Aspectos históricos

No final do século XIX, Pasteur e Joubert reportaram a ocorrência de uma interação antagonista entre bactérias. Estes pesquisadores observaram que uma bactéria isolada de uma cultura de urina, havia sido capaz de interferir no crescimento de *Bacillus anthracis.* Da mesma forma, foi observada uma ação inibitória de um isolado de *Staphylococcus* spp. sobre uma cultura de *Corynebacterium diphyteriae*, descoberta esta que conduziu a aplicação de isolados de estafilococos no tratamento da difteria (JACK, TAGG, RAY, 1995).

Os pesquisadores encontravam-se mais voltados para as implicações biológicas das interações microbianas antagonistas, do que com a caracterização química das substâncias inibitórias. Muitos dos estudos passaram a explorar a possibilidade de controlar doenças como o carbúnculo hemático e a difteria, pelo uso de microrganismos antagonistas não patogênicos. Embora a natureza das substâncias inibitórias e seu mecanismo de ação não tenham sido pesquisados, parece provável que muitas das interações observadas tenham sido causadas por substâncias agora classificadas como bacteriocinas (TAGG, DAJANI, WANNAMAKER, 1976)

Em 1925, Gratia estudou os mecanismos de inibição observados nos processos de interação microbiana. Foi observado que células de *Escherichia coli* produziam uma substância estável às oscilações de temperatura, a qual também apresentava atividade inibitória sobre o crescimento de outros microrganismos similares taxonomicamente. Inicialmente estas substâncias foram denominadas de colicinas, pois nas pesquisas realizadas, havia sido sintetizadas por células de *Escherichia coli*. Pesquisas posteriores mostraram que a síntese destas moléculas era comum em outras bactérias e escolheu-se o nome de bacteriocinas para designá-las. Este foi o passo inicial para os estudos envolvendo essas substâncias (JACOB *et al*., 1953; JACK, TAGG, RAY, 1995).

As colicinas foram extensivamente estudadas e utilizadas como sistemas modelo na investigação dos mecanismos de evolução e diversificação das bacteriocinas. A

comparação de seqüências de proteínas entre colicinas revelou que existiam duas famílias: as colicinas formadoras de poros e as colicinas nucleases, o que as classificava em relação ao seu modo de ação (RILEY, 1998).

Os primeiros relatos de produção de substâncias antimicrobianas por bactérias lácticas, foi feito em 1928 por Rogers. Ele observou a atividade antimicrobiana de *Lactococcus lactis* subsp. *lactis* contra *Lactobacillus delbrueckii* subsp. *bulgaricus*. A substância foi determinada como um polipeptídeo e subseqüentemente denominada de nisina (MATTICK, HIRSH, 1947).

A nisina, produzida por *Lactococcus lactis*, é uma bacteriocina aprovada para uso em, pelo menos 40 países, e já vem sendo empregada por cerca de 50 anos. Ela é a única bacteriocina considerada como substância GRAS (*Generally Regarded as Safe*) e sua utilização está aprovada pelo *Food and Drug Administration* (FDA) (APHA, 1992; CODEX ALIMENTARIUS, 1995).

No Brasil, o uso do NISAPLIN está regulamentado como conservante e biopreservativo em alimentos. É um produto comercialmente liberado pelo Ministério da Saúde através do Regulamento de Inspeção Industrial e Sanitária de Produtos de Origem Animal da Vigilância Sanitária e aprovado pela Portaria N° 29, de 22 de janeiro de 1996, (BRASIL, 2000a, 2000b).

2.2 Definição

Bacteriocinas são peptídeos biologicamente ativos, com atividade antimicrobiana contra bactérias, usualmente, estreitamente relacionadas à bactéria produtora (TAGG, DAJANI, WANNAMAKER, 1976). Estes pesquisadores as caracterizaram como substâncias de estreito espectro de atividade, possuidoras de uma fração protéica ativa, com atividade bactericida, com mecanismo de ação dando-se através da ligação a receptores específicos na parede celular das células sensíveis, com informação genética plasmidial e que sua produção fosse por biossíntese letal.

Entretanto, existe uma diversidade de outras substâncias com atividade antimicrobiana que não, necessariamente, apresentam todas estas características. O termo substância tipo-bacteriocina (*bacteriocin-like*) engloba os compostos antimicrobianos de

natureza protéica que ainda não estão completamente definidos ou não cumprem com todas as características de bacteriocinas. Estas substâncias, geralmente, possuem um espectro de ação maior, atuando contra uma variedade de bactérias Gram-positivas, Gram-negativas e contra alguns fungos (DE VUYST, VANDAMME, 1994).

Substâncias com atividade antimicrobiana têm sido pesquisadas em uma diversidade de microrganismos. Acredita-se que 99% de todas as bactérias possam produzir pelo menos uma bacteriocina, campo este que merece mais pesquisas para a descoberta de substâncias com potencial de aplicação como antimicrobianos (KLAENHAMMER, 1988).

2.3 Classificação

Em 1993, Klaenhammer distinguiu diferentes classes de bacteriocinas.

A Classe I de bacteriocinas, os lantibióticos, são peptídeos pequenos (<5kDa), que possuem de 19 a 50 aminoácidos e são caracterizados pela presença de aminoácidos não usuais como lantionina, β-metil-lantionina, dehidrobutirina e dehidroalanina. Esta classe está subdividida em Classe Ia e Classe Ib. A Classe Ia, que inclui a nisina, consiste de peptídeos hidrofóbicos e catiônicos que formam poros na membrana da célula alvo e possuem uma estrutura flexível, quando comparados com uma estrutura mais rígida dos peptídeos da Classe Ib. Esta possui peptídeos globulares que possuem carga negativa ou não possuem carga (ALTENA *et al.*, 2000).

A Classe II contém peptídeos pequenos, não modificados e estáveis ao calor e que também podem ser subdivididos. A Classe IIa inclui os peptídeos como a pediocina, ativos contra *Listeria*, com a sequência N-terminal conservada Tyr-Gly-Asn-Gly-Val e dois resíduos de cisteína formando a ponte S-S, na metade N-terminal do peptídeo. Já as bacteriocinas compostas de dois peptídeos diferentes pertencem a Classe IIb, necessitando de ambos para ser totalmente ativa. As sequências do aminoácido primário do peptídeo são diferentes. Sabe-se, ainda, que cada um deles é codificado pelo seu próprio gene, havendo um gene comum para a imunidade. A Classe IIc foi proposta para separar as bacteriocinas secretadas pelo sistema *sec*-dependente (NES *et al*., 1996).

Na Classe III temos as bacteriocinas maiores (30kDa) e lábeis ao calor, mas há poucas informações disponíveis; um exemplo é a helveticina J.

Uma quarta Classe tem sido proposta a qual consiste de bacteriocinas que formam complexos com outras macromoléculas (como lipídeos e carboidratos), não sendo uma classe muito bem estudada à nível bioquímico (CLEVELAND *et al*., 2001; MCAULIFFE, ROSS, HILL, 2001). A existência desta classe foi considerada, a partir do momento em que foi observado que algumas atividades de bacteriocinas obtidas de sobrenandantes livres de células, foram perdidas, por tratamento não só com proteases, mas com enzimas lipolíticas e glicolíticas. A plantaricina S é um exemplo de bacteriocina pertencente a esta classe.

2.4 Biossíntese e regulação da produção

2.4.1 Biossíntese

A produção de bacteriocinas parece estar relacionada com a presença de plasmídeos bacteriocinogênicos, embora genes codificando várias bacteriocinas da classe IIa tenham sido localizados sobre fragmentos de cromossomos (AYMERICH *et al.,* 1996; HÜHNE *et al.,* 1996).

A produção, geralmente ocorre na forma de um pré-peptídeo e os genes para a produção da substância ativa estão localizados sobre um operon. Os genes envolvidos são: o gene estrutural que codifica para a produção da pré-bacteriocina; o gene da imunidade, localizado ao lado do estrutural; o gene codificando um transportador ABC, responsável pela externalização da bacteriocina e um gene codificando uma proteína acessória (NES *et al.,* 1996).

As bacteriocinas da classe IIa, assim como as outras bacteriocinas de baixo peso molecular são inicialmente formadas como precursores sintetizados ribossomalmente, na forma de um pré-peptídeo que contém uma extensão N-terminal ou seqüência líder. Posteriormente, ocorre uma clivagem e separação desta seqüência tornando a molécula biologicamente ativa (HAVARSTEIN, DIEP, NES, 1995). A secreção da bacteriocina através da membrana é mediada por um transportador ABC e uma proteína acessória, os quais são duas proteínas de membrana que integram o sistema de transporte da substância ativa (NES *et al.*, 1996; AYMERICH *et al.*, 1996).

O domínio proteolítico do transportador ABC pode ligar-se a seqüência líder do pré-peptídeo, provocando alterações conformacionais no transportador, resultando na clivagem e remoção desta seqüência e externalização da bacteriocina ativa através da membrana citoplasmática (HAVARSTEIN, DIEP, NES, 1995). As proteínas acessórias também parecem estar envolvidas no processo de externalização da substância ativa, porém sua função específica ainda não está bem entendida (VENEMA *et al*., 1995).

As proteínas de imunidade parecem estar envolvidas com a proteção total da cultura produtora, contra os efeitos da sua própria bacteriocina, sendo sugerida a existência de uma proteção parcial contra outras bacteriocinas da mesma classe (EIJSINK *et al.*, 1998).

Quanto a sua produção, estas substâncias podem ser consideradas metabólitos primários, quando sua síntese ocorre na fase exponencial de crescimento, sendo de extrema importância para o desenvolvimento do microrganismo produtor. Quando produzidas após a fase de crescimento ativo, elas passam a ser consideradas metabólitos secundários, não sendo vital para o crescimento do microrganismo produtor, embora estejam envolvidas em processos de manutenção deste, em determinado ambiente (DE VUYST, CALLEWAERT, CRABBÉ, 1996).

2.4.2 Regulação da produção

Sistemas de regulação da produção das bacteriocinas da classe IIa consistem em um sistema com três componentes: uma histidina proteína quinase (HPK), um regulador de resposta (RR) e um fator de indução (IF), o qual é requerido como um sinal para indução da transcrição de genes alvo (Figura 1) (KUIPERS *et al.*, 1998).

Acredita-se que ocorra um aumento gradual do IF, como conseqüência do crescimento celular (NES *et al*., 1996). Com o aumento da biomassa microbiana, o IF passa então a atuar como iniciador do processo. Alguns autores acreditam que este sistema regulatório da produção das bacteriocinas, possa mediar uma resposta para um sinal no meio onde a substância está sendo produzida, sugerindo que fatores ambientais possam afetar a ligação do IF à HPK, influenciando na fosforilação requerida para a ativação do RR (NILSEN, NES, HOLO, 1998).

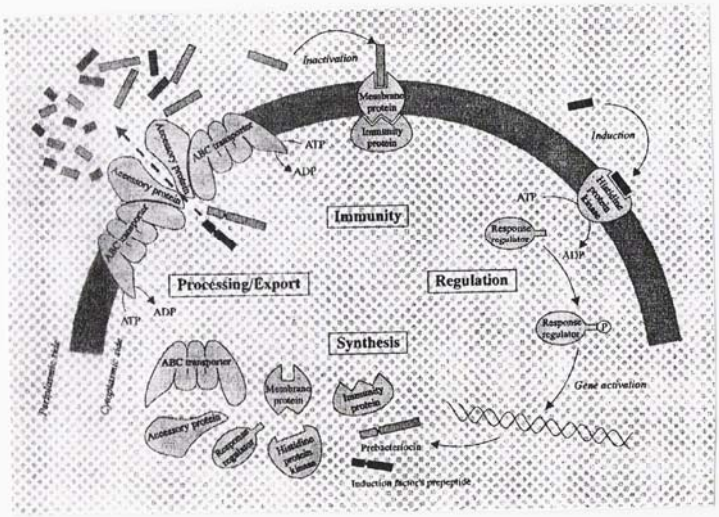
Inactivation
Membrane protein
Immunity protein
ATP
ADP
Induction
Immunity
Histidine protein kinase
ATP
Response regulator
ADP
Processing/Export
Regulation
ADP
ATP
Response regulator
P
Synthesis
ABC transporter
Membrane protein
Immunity protein
Accessory protein
Response regulator
Histidine protein kinase
Gene activation
Prebacteriocin
Induction factor's propeptide

Figura 1. Regulação da produção de bacteriocinas da Classe IIa: sistema regulatório, síntese, processamento, excreção da substância e imunidade. (Fonte: ENNAHAR *et al*., 2000).

2.5 Mecanismo de Ação

O potencial de aplicação das bacteriocinas como biopreservativo de alimentos requer um profundo conhecimento de como elas exercem o seu efeito bactericida. Para muitas bacteriocinas o modo de ação parece se dar inicialmente à nível da membrana citoplasmática. Tem sido proposto que estes peptídeos formem poros transversalmente à bicamada fosfolipídica, causando a permeabilizacão da membrana e perda da força proto-motora das células sensíveis (KLAENHAMMER *et al*., 1993; DRIESSEN *et al*., 1995; CHEN, LUDESCHER, MONTVILLE, 1997).

Considerando a estrutura anfifílica das bacteriocinas da classe IIa, esta forma hélices transmembrana, que possuem solubilidade em água e habilidade de ligar-se à membrana, sugerindo que possa formar poros complexos seguindo o modelo "barrel-stave". O passo inicial para a ligação com a superfície da membrana parece ser por interações eletrostáticas, através de uma molécula receptora ligada à membrana (ABEE, 1995; VENEMA *et al.*, 1995).

Outros estudos realizados com sistemas de vesículas de lipídio, indicam que não seja necessário um receptor protéico à nível da membrana para que ocorra a formação de poros (CHEN, LUDESCHER, MONTVILLE, 1997).

Devido às características das bacteriocinas com seus resíduos carregados positivamente, acredita-se que, inicialmente, ocorra a ligação destas substâncias aos fosfolipídios aniônicos na membrana celular (KAISER, MONTVILLE, 1996). Interações hidrofóbicas poderão ocorrer entre os domínios hidrofóbicos/anfifílicos, dentro da porção C-terminal das bacteriocinas e as cadeias acil dos lipídios, o que tem sido mostrado ser essencial para o processo de formação de poros (MOLL *et al.,* 1996; CHEN, LUDESCHER, MONTVILLE, 1997).

Kaiser e Montville (1996) sugerem que seguindo as interações hidrofóbicas, a bacteriocina pode ser reorientada em uma condição mais favorável de modo que sua inserção e agregação na membrana ocorram de forma a promover a formação de poros como mostra a Figura 2.

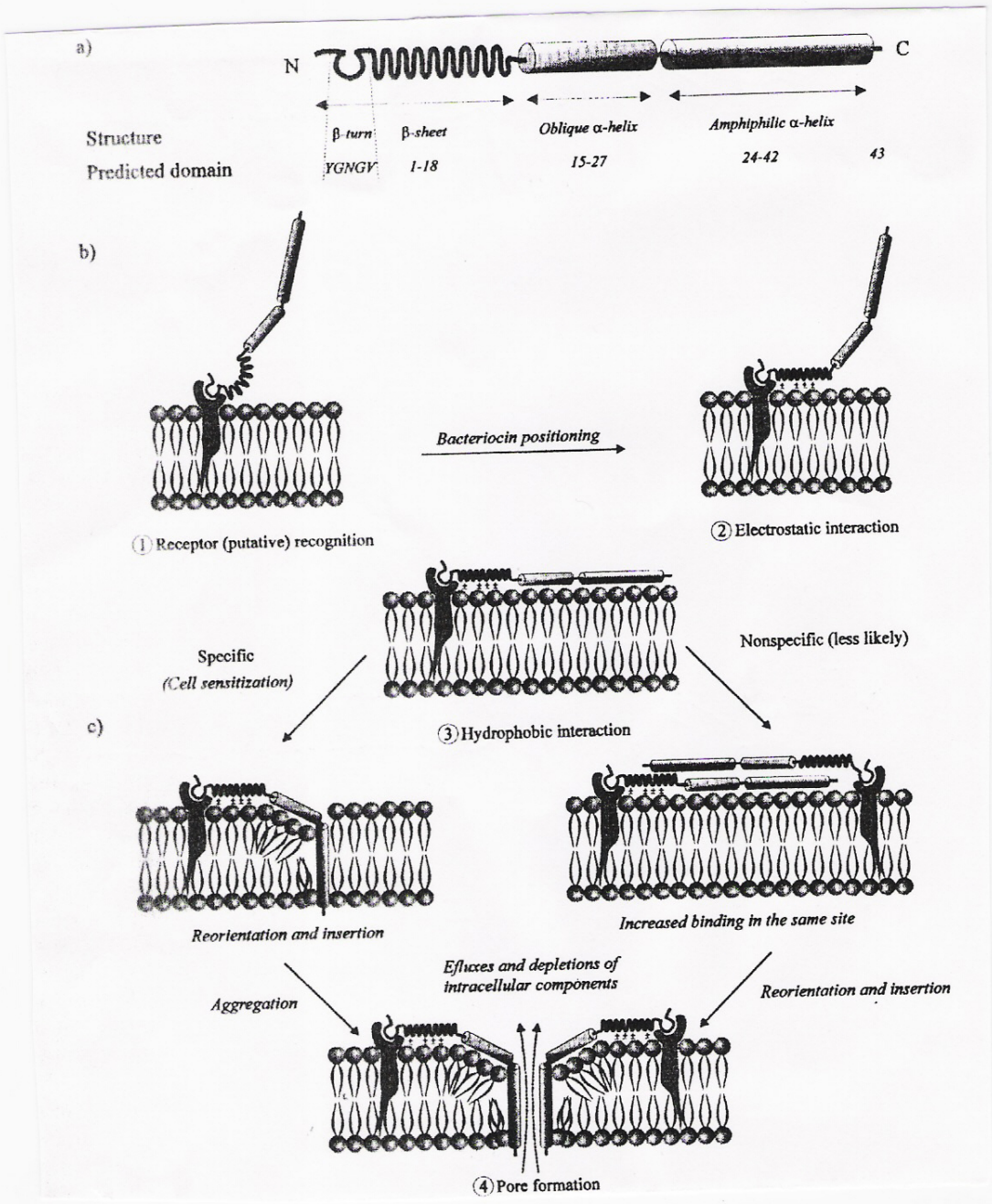
a)
N
C
Structure
Predicted domain
β-turn
β-sheet
Oblique α-helix
Amphiphilic α-helix
YGNGV
1-18
15-27
24-42
43
b)
Bacteriocin positioning
① Receptor (putative) recognition
② Electrostatic interaction
③ Hydrophobic interaction
Specific
(Cell sensitization)
Nonspecific (less likely)
c)
Reorientation and insertion
Increased binding in the same site
Efluxes and depletions of intracellular components
Aggregation
Reorientation and insertion
④ Pore formation

Figura 2. Representação de um modelo de mecanismo de ação proposto para a Classe IIa das bacteriocinas: (a) domínios estruturais da bacteriocina, (b) possíveis interações dos domínios com a superfície da membrana, (c) inserção da bacteriocina e formação de poros hidrofílicos (Fonte: ENNAHAR *et al*., 2000).

A interação destas substâncias com os componentes da membrana, na célula alvo, parece ser determinante para que a ação ocorra. Para isso modelos de membranas têm sido extensivamente estudados para avaliar a dependência da presença de lipídios, nesta interação (DRIESSEN *et al*., 1995).

A nisina é um dos membros da família dos lantibióticos (antibióticos contendo lantionina) e é produzida por *Lactococcus lactis*. Por ser uma das bacteriocinas mais estudadas e caracterizadas, possuindo alta atividade bactericida e ausência de toxicidade para humanos, é empregada como biopreservativo de alimentos. Através dos estudos sobre o modo de ação desta bacteriocina, verificou-se que este se dá por uma interação, de alta afinidade, da bacteriocina com o lipídio II, inibindo a síntese do peptideoglicano e formando poros altamente específicos à nível da membrana citoplasmática. Isto causa a permeabilização desta membrana, resultando em morte celular. De acordo com estudos já realizados, o lipídio II também mostrou-se alvo da vancomicina, um antibiótico amplamente utilizado, onde verificou-se, também, o aparecimento de resistência a vancomicina por alteração da estrutura do lipídio II.

Com isto, os autores evidenciaram que a nisina assim como a vancomicina utilizam o mesmo alvo na membrana celular do microrganismo indicador (*Micrococcus flavus*), ligando-se com alta afinidade ao lipídio II e promovendo a formação de poros na membrana da célula indicadora (BREUKINK *et al*., 1999; BROTZ *et al*., 1998).

O modo de ação da nisina também foi estudado por outros autores, os quais buscaram, através de variantes de nisina, modificadas geneticamente, identificar os requerimentos estruturais para a interação do peptídeo com o lipídio II na parede celular. Observaram que mutações afetando a conformação da porção N-terminal da nisina, conduziram a uma menor especificidade de ligação desta ao lipídio II, necessitando-se de uma maior concentração de peptídeo para a formação de poros (WIEDEMANN *et al*., 2001).

Os achados do envolvimento do lipídio II na sensibilidade às bacteriocinas também sugerem um possível mecanismo de desenvolvimento de resistência contra peptídeos antimicrobianos como a nisina.

O mecanismo pelo qual estas substâncias exercem seu efeito tem sido observado por outros autores e observa-se que a entrada das bacteriocinas, nas células alvo, pode ser

requerida para que atuem à nível de DNA, RNA, enzimas e em outros locais específicos. Há evidências de que a Classe II atue através da inibição da formação do septo, mecanismo este que tem sido proposto para a lactococina 972. Foi observado que esta bacteriocina atua na inibição da biossíntese do septo em células de lactococos suscetíveis. Isto resultou em deformação das células, quando visualizadas em microscópio ótico e alterações estruturais que conduziram a morte celular (MARTINEZ, RODRIGUEZ, SUAREZ, 2000).

Embora a formação de canais iônicos, de poros transmembrana e rupturas à nível da membrana citoplasmática conduzam a lise da célula microbiana, há estudos de que peptídeos antimicrobianos possuam alvos intracelulares. As autolisinas e as fosfolipases também são ativadas por estas substâncias (GENNARO, ZANETTI, 2000)

2.6 Função das bacteriocinas

A produção de substâncias antimicrobianas apresenta-se difundida em uma diversidade de espécies de microrganismos. Com isso surgem questões como: porque ocorre a biossíntese destas substâncias e qual a função destas além da pura e simples atividade inibitória ?

Importante considerar que para que ocorra a produção destas substâncias, existe um gasto energético por parte do microrganismo produtor. As situações em que a biossíntese é requerida, são muitas vezes reguladas por situações de limitação de nutrientes no meio de crescimento tal como na esporulação, desenvolvimento de competência genética e produção de enzimas de degradação extracelulares. Logo, as seqüências de reações envolvidas neste processo serão, de certa forma, em benefício do microrganismo produtor (MARAHIEL, NAKANO, ZUBER, 1993).

Rizobactérias estão presentes no solo em uma média de 10^8 células por grama e, do solo, passam a ser transferidas para vários ambientes associados incluindo plantas, animais, habitats de água doce e marinhos. Um dos principais representantes é *Bacillus subtilis*, produtor de uma diversidade de peptídeos antibióticos: 4 a 5 % do seu genoma é dedicado para a produção destes compostos (PRIEST, 1993).

Os lipopeptídeos antibióticos estão entre os compostos produzidos e são substâncias capazes de alterar as propriedades físico-químicas das interfaces. Acredita-se que eles

teriam a função de aumentar a área de superfície para disponibilizar os substratos para o crescimento do microrganismo produtor, aumentar a biodisponibilidade dos substratos hidrofóbicos, pelo aumento da sua solubilidade aparente e influenciar no condicionamento e fixação dos microrganismos na superfície (ROSENBERG, RON, 1999).

Outro aspecto importante está relacionado com o modo de vida dos microrganismos. Muitas bactérias exibem um estilo de vida livre ou plantônico, através do qual se distribuem rapidamente nos demais micro-ambientes, assim como apresenta outra forma de comportamento, chamado séssil, onde as bactérias ficam condicionadas a uma superfície caracterizando o chamado biofilme (STANLEY, LAZAZZERA, 2004).

A produção de substâncias antimicrobianas no biofilme tem sido reportada com a colonização de raízes de plantas por *B. subtilis*, associada à produção de surfactina, mostrando a proteção da planta contra a infecção por *Pseudomonas syringae* (BAIS, FALL, VIVANCO, 2004).

A ação antibacteriana e antifúngica dos lipopeptídeos, em adição a surfactina, parece ser vantajosa para células de *B. subtilis* em eliminar competidores no mesmo habitat. Parece que a produção destes lipopeptídeos se dá na fase estacionária de crescimento, ao passo que a surfactina é produzida na transição da fase exponencial para a estacionária (ROSENBERG, RON, 1999).

Um grande número de bactérias Gram-positivas que habitam comunidades ecológicas complexas, como as do solo e as de ambientes aquáticos, produzem uma grande quantidade de compostos antimicrobianos, que acredita-se que aumentem a capacidade de sobrevivência desses microrganismos em diferentes condições (KATZ, DEMAIN, 1977; NAKANO, ZUBER, 1990).

Estas substâncias parecem ser produzidas em condições de estresse nutricional, sendo observado o acúmulo destas, na fase estacionária do crescimento microbiano. Isto coloca sua função como estando relacionada com a manutenção ou auxílio ao microrganismo produtor, sob condições de limitação dos recursos disponíveis no meio. Acredita-se que, nestas situações, ocorra o desenvolvimento de rotas alternativas que façam com que o microrganismo se diferencie e se torne resistente às condições inadequadas do meio (VINING, 1990; WILLEY *et al*., 1991).

Avaliando sobre as funções destes compostos antimicrobianos sob ponto de vista ecológico, as bacteriocinas possuem um papel muito importante nas comunidades microbianas mediando interações entre estas. Parecem atuar como anti-competidores, capacitando o estabelecimento de uma linhagem em uma comunidade microbiana pré-existente. Apresentam também uma função de defesa, em impedir a invasão de outras espécies em um nicho já ocupado e formado por microrganismos já estabelecidos (MILLER, BASSLER, 2001).

Tem-se observado uma vantagem competitiva dos microrganismos produtores de bacteriocinas, quando estes disputam nichos ecológicos em ambientes onde eles desenvolvem suas atividades metabólicas (STOCKEWELL, MOORE, LOOPER, 1993). Considerando o estreito espectro de atividade antimicrobiana, a função primária das bacteriocinas é como um mediador intra-específico ou como promotores de interações entre populações microbianas (CLEVELAND *et al*., 2001).

A produção destes compostos desempenha uma função importante no desenvolvimento das comunidades microbianas e contribuem para a sobrevivência das espécies produtoras em seus habitats naturais.

2.7 Aplicações

2.7.1 Alimentos

Na produção de alimentos tem-se despertado o interesse para a obtenção de produtos seguros, com alta qualidade, sem adição de preservativos químicos e que possuam uma maior vida de prateleira. A legislação para alimentos tem buscado restringir o uso de alguns aditivos em diferentes produtos (BRULL, COOTE, 1999). Paralelo a isto, pesquisas sobre novos agentes antimicrobianos têm sido realizadas de modo que possam ser aplicados nos sistemas de conservação de alimentos. Esses compostos podem ser usados em combinação com outros tratamentos, promovendo uma proteção contra microrganismos patogênicos e deteriorantes (PECK, 1997).

Essa nova perspectiva de conservação de alimentos busca a aplicação de sistemas antimicrobianos naturais onde se enfatiza a ação sinérgica de vários elementos, tais como:

produtos com atividade antimicrobiana oriundos de animais, plantas e microrganismos, juntamente com procedimentos de natureza física, procedimentos de embalagem e estocagem dos produtos. Estas ações empregadas de forma associada, produzem um efeito conjunto capaz de criar condições desfavoráveis à sobrevivência dos microrganismos indesejáveis (GOULD, 1995).

Pesquisando novas tecnologias a serem aplicadas na conservação dos alimentos, o uso de substâncias produzidas por microrganismos tem despertado o interesse e o desenvolvimento de pesquisas. Entre estas substâncias, as bacteriocinas têm recebido atenção especial (DAW, FALKINER, 1996), e possuem diferentes características que devem se estudadas antes de sua aplicação como biopreservativo (CLEVELAND *et al*., 2001).

Alguns princípios gerais devem ser observados para o uso destes compostos como aditivos em alimentos: 1. avaliação das suas propriedades toxicológicas, incluindo aspectos relacionados aos potenciais efeitos cumulativos e sinergismos; 2. somente os aditivos considerados seguros podem ser liberados para uso em alimentos; 3. os aditivos devem ser novamente avaliados caso surjam novas informações sobre sua segurança e uso; 4. essas substâncias devem manter-se em conformidade com as especificações do *Codex Alimentarius* Commission; 5. a justificativa do uso dos aditivos deve estar baseada nos requisitos da segurança alimentar dos diferentes tipos de consumidores e deve estar dentro de alternativas técnicas e economicamente praticáveis; 6. a aprovação temporária ou permanente do uso dos aditivos alimentares deve considerar a limitação para alimentos específicos, seu objetivo, condições de uso, descrição das quantidades necessárias para que se alcance os efeitos desejáveis e o nível de tolerância aceitável para humanos e, considerar a provável ingestão por consumidores especiais (COCON, 1988).

Algumas bacteriocinas já possuem seu potencial de ação bem determinado como antimicrobiano, assim como sua possível aplicação como biopreservativo de alimentos. As únicas bacteriocinas com potencial aplicação prática em alimentos são as produzidas por bactérias lácticas, as quais são consideradas GRAS (generally regarded as safe) pelo Food and Drug Administration (FDA) (MONTVILLE, WINKWOSKI, 1997).

A nisina foi a primeira bacteriocina a ser isolada e aprovada para uso em alimentos, especificamente para prevenir a germinação de esporos de *Clostridium botulinum* em

queijos. Em 1988, teve seu uso aprovado para outros alimentos, sendo hoje um produto comercial amplamente aplicado (CHUNG, DICKSON, CROUSE, 1989).

O surgimento de surtos de listeriose, aliados ao fato de seu agente causador, *Listeria monocytogenes* ser capaz de crescer em uma ampla faixa de condições, despertou o interesse para o estudo de antimicrobianos capazes de inibir seu crescimento. *Listeria monocytogenes* é um microrganismo psicrotrófico, capaz de multiplicar-se em temperaturas de 1 a 45°C, sendo capaz de sobreviver no leite por mais de um ano, quando estocados a 5°C. O número de células necessárias para induzir a doença não está bem definido, porém alguns autores acreditam que entre 10^2 e 10^3 células por grama de alimento sejam suficientes para produzir listeriose em pessoas do grupo de risco (gestantes, imunodeprimidos e faixas etárias extremas). A presença de *Listeria monocytogenes* em produtos processados termicamente indica tratamento inadequado ou contaminação pós-processamento (BRASIL, 1991/1992; ENNAHAR *et al.*, 2000).

Esta preocupação tem resultado no isolamento, purificação e caracterização de um grande número de bacteriocinas da Classe IIa, as quais são ativas contra *Listeria monocytogenes* (ENNAHAR *et al.,* 2000).

2.7.2 Outras aplicações

O rápido desenvolvimento de patógenos bacterianos multi-resistentes tem despertado o interesse para novos métodos de terapia antimicrobiana. Uma das limitações do uso de antibióticos de amplo espectro é que eles têm efeito bactericida sobre qualquer espécie bacteriana, o que resulta em uma forte pressão de seleção para a evolução da resistência aos antibióticos (RILEY, WERTZ, 2002).

Como alternativa, tem-se a aplicação das bacteriocinas, as quais possuem um estreito espectro de atividade antimicrobiana podendo ser consideradas como "*designer drugs*". Tendo em vista a diversidade de bacteriocinas produzidas na natureza, a exploração do potencial de aplicação destas substâncias se faz necessário, para que se possa sugerir e aprovar o uso de novas substâncias contra patógenos humanos.

Sob uma perspectiva evolucionária e ecológica, o uso de antimicrobianos com estreito espectro de atividade, direcionará sua ação sobre os microrganismos patogênicos de interesse, reduzindo seus efeitos sobre espécies bacterianas comensais. Isto também faz

com que se reduza a pressão de seleção para o desenvolvimento da resistência (RILEY, WERTZ, 2002).

O uso de bacteriocinas tem sido pesquisado como uma forma segura para controlar doenças de plantas de origem microbiana. Entre as bactérias fitopatogênicas produtoras de bacteriocinas encontramos membros das corinebactérias, erwinias, pseudomonas, xantomonas e agrobaterium. Estes compostos são altamente específicos, efetivos e seguros para os usuários e para o ambiente, e parecem ser uma excelente alternativa para uso na agricultura, prevenindo a formação de tumores na coroa de plantas infectadas (JABBEN *et al*., 2004).

2.8 Bacteriocinas produzidas por espécies de *Bacillus*

O gênero *Bacillus* inclui uma variedade de espécies importantes industrialmente e tem um histórico de uso seguro tanto em alimentos quanto na indústria. Os produtos que existem no comércio hoje, e que são produzidos por *Bacillus,* incluem enzimas, antibióticos, aminoácidos e inseticidas. A produção de bacteriocinas também tem sido descrita para o gênero *Bacillus,* sendo extensivamente pesquisada a subtilina, produzida por *Bacillus subtilis* (KLEIN, KALETTA, ENTIAN, 1993).

Segundo Manual Bergey's de Bacteriologia Sistemática este gênero compreende bactérias Gram-positivas (ou Gram-variáveis), esporogênicas, aeróbias ou anaeróbias facultativas, sendo a maioria catalase positiva. Apresentam uma diversidade de habilidades fisiológicas com espécies psicrotróficas ou termofílicas, acidófilas ou alcalófilas (CLAUS, BERKELEY, 1986).

As espécies de *Bacillus* têm como habitat natural o solo, mas apresentam-se amplamente distribuídas no ambiente. Podem ser isolados de vários habitats como plantas, mananciais de água, águas poluídas, sedimentos marinhos, alimentos e leite. Estes habitats promovem as condições ideais para o crescimento e proliferação das espécies assim como para a manutenção da sua forma de resistência. A capacidade de distribuição e persistência dos esporos faz com que este gênero se mantenha latente nos mais diversos ambientes.

Este gênero constitui um grupo interessante para o estudo de novas substâncias com atividade antimicrobiana, e a produção de peptídeos antimicrobianos com estruturas químicas diferentes tem sido observada (GEBHARDT *et al.,* 2002). Em comparação com os peptídeos antimicrobianos produzidos por bactérias lácticas, pouco é conhecido sobre as bacteriocinas do gênero *Bacillus*, com exceção dos lantibióticos sublancina e subtilina de *B. subtilis* 168 e ATCC 6633, respectivamente (BANERJEE, HANSEN, 1988; PAIK, CHAKICHERLA, HANSEN, 1998). *B. subtilis* é um microrganismo considerado GRAS (generally regarded as safe), mas linhagens bacteriocinogênicas também têm sido encontradas em *Bacillus* spp. patogênicas como *B. cereus* e *B. thuringiensis* (RISOEN *et al*., 2004).

As bacteriocinas têm-se mostrado com espectro maior frente às bactérias Gram-positivas, embora que compostos como polimixina e circulina apresentam atividade contra Gram-negativas (KATZ, DEMAIN, 1977). Alguns dos peptídeos produzidos por espécies de *Bacillus*, especialmente os lipopeptídeos têm apresentado propriedades antifúngicas, como a ituricina e micosubtilina (GALVEZ *et al.,* 1993). Outros peptídeos têm apresentado propriedades de biosurfactante, além da sua atividade antimicrobiana, sendo a surfactina um lipopeptídeo antibiótico já identificado (JENNY, KAPPELI, FIECHTER, 1991).

Em *B. subtillis* 168 foi observada a produção de subtilosina A e estudos de correlação entre a produção do composto e a formação de esporos mostraram que não há nenhuma relação entre os dois fenômenos. Dentre os peptídeos produzidos por outras espécies de *Bacillus* verificou-se que a subtilina produzida por *B. subtilis* ATCC 6633 possui tamanho e mecanismos de síntese semelhantes a subtilosina A, o que sugere que a produção destes compostos é dispensável para o processo de formação de esporos (BABASAKI *et al*., 1985).

Bechard *et al.* (1998) isolaram uma espécie de *B. subtilis* da maçã e observaram a produção de um lipopeptideo com propriedades antimicrobianas interessantes. Esse apresentou um amplo espectro de atividade contra bactérias Gram-negativas e estreito espectro de atividade contra Gram-positivas, sendo ativa contra um dos dois fungos testados. Seu efeito sobre *Agrobacterium vitis* mostrou-se bactericida, não sendo associado com lise celular. O lipopeptídeo apresentou um peso molecular de 1,5 kDa, mas quando na

forma de agregado, 20 kDa foram observados. Suas características evidenciaram sua similaridade com peptídeos antibióticos chamados de surfactantes.

Bactérias produtoras de substâncias antimicrobianas têm sido isoladas de uma diversidade de ambientes. Sugita *et al.* (1998), pesquisaram bactérias de origem intestinal, isoladas de espécies de peixes da costa do Japão. O isolado *Bacillus* sp. NM 12 apresentou um amplo espectro de atividade, sugerindo-se a participação desta espécie no controle biológico de populações bacterianas no intestino. Outros trabalhos também observaram o controle da microflora no intestino de peixes, por bactérias produtoras de bacteriocinas (SMITH, DAVEY, 1993; AUSTIN *et al.,* 1995).

A surfactina produzida por *B. subtilis* é um lipopeptídeo antimicrobiano cíclico, também classificado como biosurfactante. Suas propriedades antivirais e antibacterianas são conhecidas. Seu modo de ação foi estudado e observou-se a desintegração da membrana citoplasmática de micoplasmas assim como, sua baixa citotoxicidade para células de mamíferos. Isto permite a inativação específica dos micoplasmas, sem efeito deletério para o metabolismo celular e para as células do hospedeiro (VOLLENBROICH *et al.*, 1997).

Outra substância também produzida por *B. subtilis* é a subtilina. É um peptídeo antimicrobiano sintetizado ribossomalmente e que contêm aminoácidos não usuais como resultado das modificações pós-traducionais. Para avaliação do mecanismo de ação desta substância, uma variante da subtilina foi feita, com mutação induzida na posição do resíduo dehidroalanina (Dha) substituído para alanina. Observou-se a perda da capacidade de inibição da germinação de esporos de *B. cereus*, porém manteve-se ativa contra as células vegetativas, o que sugere que o mecanismo pelo qual ocorre a inibição da germinação de esporos, não seja o mesmo pelo qual as células vegetativas são inibidas (LIU, HANSEN, 1993).

Uma bacteriocina com atividade antilisteria produzida por *B. coagulans* I_4 tem sido isolada e caracterizada. Análises demonstram que a coagulina I_4 é um novo membro das bacteriocinas pertencentes à família das pediocinas (MARREC *et al*., 2000).

Bacteriocinas produzidas por espécies de *B. cereus* também têm sido descritas. Uma cereína foi produzida por uma espécie de *B. cereus* isolado de alimento. Sua atividade antimicrobiana foi observada apenas contra espécies estreitamente relacionadas à cultura

produtora. Sua caracterização parcial foi feita e seu peso molecular foi estimado por eletroforese em gel de poliacrilamida, sendo de 9 kDa. As características de estabilidade e seu efeito bactericida sugerem seu potencial uso como aditivo em alimentos (NACLERIO et al., 1993).

Cereína 7 foi produzida por uma bactéria isolada de amostras do solo. O peptídeo foi purificado e sua massa determinada, por espectrometria de massa, sendo de 3940 Da. O composto foi ativo contra bactérias Gram-positivas e Gram-negativas. Seu efeito foi estudado usando *Listeria innocua* como indicador e foi observada atividade bactericida, estando à lise celular relacionada com a concentração da substância inibitória. Observou-se que seu modo de ação se dá através da ligação em alvos à nível da membrana citoplasmática (OSCÁRIZ, PISABARRO, 2000).

Bizani e Brandelli (2002) identificaram e caracterizaram a cereína 8A. A espécie produtora foi isolada de amostras do solo e a identificação da sua bacteriocina produzida foi realizada. Apresentou um amplo espectro de atividade inibitória, incluindo *Listeria monocytogenes*, *Clostridium perfringens* e várias espécies de *Bacillus.* Espécies importantes do ponto de vista clínico também foram inibidas como *Streptococcus bovis* e *Micrococcus luteus*. Sua atividade esporocida foi observada. A purificação e caracterização química foram realizadas tratando-se de um peptídeo de aproximadamentede 26 kDa (BIZANI, DOMINGUEZ, BRANDELLI, 2005a). Outra substância antimicrobiana produzida por *B. cereus* ATCC 14579 foi caracterizada, apresentando um amplo espectro de atividade antimicrobiana com potencial de aplicação contra bactérias patogênicas (RISOEN *et al*., 2004).

Uma substância do tipo-bacteriocina, chamada de liquenina foi isolada do suco ruminal de búfalos e apresentou atividade antimicrobiana contra vários microrganismos indicadores. Sua produção e atividade foram realizadas em condições de anaerobiose, sendo sensível ao oxigênio. Sua atividade se manteve a 100°C por 10 minutos e foi ativa em uma faixa de pH de 4.0 a 9.0. Esta bacteriocina de aproximandamente 1,5 kDa mostrou-se bactericida e foi sugerida sua aplicação na manutenção das funções ruminais (PATTNAIK *et al.,* 2001).

Produção de um biosurfactante por *B. licheniformes* F2.2 foi observada. O isolado foi capaz de produzir um novo biosurfactante BL1193, assim como outros dois lipopeptídeos

plipastatina e surfactina. Para a produção destes compostos, diferentes requerimentos nutricionais têm sido observados, predispondo a produção de um ou de outro (THANIYAVARN *et al*., 2003).

Bacteriocinas produzidas por *B. thuringiensis* também têm sido descritas. Esta espécie é amplamente empregada na agricultura produzindo uma diversidade de compostos extracelulares como fosfolipases, quitinases, proteases, exotoxinas, proteínas com propriedades inseticidas e compostos antibióticos com propriedades antifúngicas. Quanto a sua importância biotecnológica e sua versatilidade como agente de controle biológico, poucas linhagens bacteriocinogênicas têm sido caracterizadas. Thuricina 7 é uma bacteriocina produzida por *B. thuringiensis* BMG1.7 isolado do solo. Este composto apresentou atividade contra outras espécies do gênero *Bacillus*, *Streptococcus pyogenes* e *Listeria monocytogenes*. O peso molecular da proteína ativa foi estimado em 11.6 kDa e foi secretada na fase exponencial de crescimento. A thuricina reteve 55 % da sua atividade biológica após incubação a 98°C por 30 minutos. Seu modo de ação foi bactericida e bacteriolítico (CHERIF *et al.,* 2001).

Bacthuricina F4 é uma bacteriocina que apresentou estabilidade frente a uma ampla faixa de pH e temperatura. Sua atividade antimicrobiana foi observada contra outras espécies de *B. thruringiensis* e bactérias Gram-positivas, sendo menos ativa frente às Gram-negativas. As análises de sua massa molecular e seqüência do aminoácido terminal demonstraram a identificação de uma nova bacteriocina produzida por *B. thuringiensis*. Suas características de manutenção de atividade sob condições extremas, tornam este composto uma alternativa para aplicação na agro-indústria (KAMOUN *et al*., 2005). Thuricina B439 também foi identificada e caracterizada. Foram observados dois peptídeos com atividade inibitória, porém com massas moleculares diferentes. De acordo com suas características biológicas e químicas, foi identificada uma nova bacteriocina (AHERN, VERSCHUEREN, VAN SINDEREN, 2003).

Embora a produção de peptídeos antimicrobianos apresente-se difundida em várias espécies do gênero *Bacillus*, o potencial de produção destes compostos por *B. subtilis* tem sido reconhecido por mais de 50 anos, e peptídeos antibióticos representam uma classe predominante. Dentre estes compostos temos os que são produzidos por síntese ribossomal e modificados pós-tradução (peptídeos lantibióticos) e, os que não são sintetizados

ribossomalmente. *B. subtilis* produzem os peptídeos sintetizados ribossomalmente como subtilina, sublancina, assim como os lipopeptídeos surfactina, micosubtilina e fengysina (STEIN, 2005).

A subtilosina A, já identificada e caracterizada, teve sua estrutura bem definida através dos estudos em ressonância magnética nuclear (RMN) e espectrometria de massas (EA). Marx *et al*. (2001). relataram tratar-se de um peptídeo macrocíclico composto de 35 aminoácidos e massa molecular de 3399.7 Da. Bacilisocina também foi estudada e identificada como um novo fosfolipídio antibiótico sendo produzida por *B. subtilis* 168. A produção de outros compostos antimicrobianos por esta linhagem, tem sido observada, o que faz com que sejam questionados aspectos relacionados a função destes compostos para o microrganismo produtor (TAMEHIRO *et al.,* 2002).

Mais recentemente foi observada a produção de substâncias antimicrobianas por *B. subtilis*, *B. firmus* e *B. licheniformis* isolados de reservatórios de óleo do Brasil. Estas substâncias foram selecionadas por sua habilidade de inibir mais de 65% das espécies de *Bacillus* indicadoras e pela capacidade de inibir bactérias redutoras de sulfato. Estas bactérias apresentam-se envolvidas em processos de deterioração de metais de encanamentos e de sistemas de recirculação de água de resfriamento e colonizam as superfícies em consórcio com outras bactérias formando biofilmes, iniciando o processo de corrosão. A caracterização preliminar destes compostos apontam o seu potencial uso como biocidas na indústria de petróleo para o controle dos problemas associados com a bactérias redutoras de sulfato (KOREMBLUM *et al*., 2005).

3 RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de artigos publicados ou enviados para publicação em revistas científicas. Cada subtítulo deste capítulo corresponde a um destes artigos, e os já publicados encontram-se em anexo.

3.1 Screening for antimicrobial activity among bacteria isolated from the Amazon basin.

- Publicado no ***Brazilian Journal of Microbiology*** em 20 de dezembro de 2004 (Braz. J. Microbiol., 2004, 35, 307-310) **(Anexo 1)**

3.2 Characterization of a broad range antibacterial substance from a new *Bacillus* species isolated from Amazon basin.

- Submetido ao ***Archives of Microbiology***

3.3 Bacteriocin-like substance production by a novel *Bacillus* sp. strain P34 using response surface methodology.

- Submetido ao ***Food Technology and Biotechnology***

3.4 Antibacterial activity of a bacteriocin-like substance produced by *Bacillus* sp. P34 that targets the bacterial cell envelope.

- A ser submetido a ***Microbiology Research***

3.5 Purification and partial characterization of an antimicrobial peptide produced by a novel *Bacillus* sp. strain isolated from Amazon basin

- A ser submetido a ***Current Microbiology***

Os artigos apresentados neste trabalho fazem parte do processo de identificação, purificação e caracterização de um peptídeo antimicrobiano.

Este trabalho teve seu início com a triagem de 86 isolados do ambiente aquático da Amazônia. Eles foram obtidos através do Prof. Dr. Spartaco Astolfi Filho, da Universidade Federal do Amazonas.

Estes isolados foram pesquisados para a produção de substâncias com atividade antimicrobiana, sendo selecionadas culturas onde foi observado espectro de atividade contra microrganismos patogênicos importantes. Houve interesse por substâncias do tipo-bacteriocina, o que foi avaliado em testes de estabilidade sob diferentes condições.

Um isolado foi selecionado, identificado e a substância por ele produzida foi pesquisada e caracterizada neste trabalho.

Procedeu-se com a identificação da bactéria produtora do composto e estudos sobre a produção da substância por ela produzida. A determinação do espectro de ação da substância antimicrobiana e sua caracterização foram feitas de modo a verificar sua estabilidade em diferentes condições. Também foi observado seu efeito sobre um patógeno importante que é a *Listeria monocytogenes*.

Após a caracterização parcial da substância antimicrobiana buscou-se a otimização da produção do composto, através do Planejamento Experimental e Análise de Superfície de Resposta. Com este modelamento determinaram-se as condições ótimas de produção da atividade antimicrobiana.

O modo de ação foi avaliado, verificando que este se dá por alterações à nível de membrana celular provocando efeitos bactericidas e bacteriolíticos em *Listeria monocytogenes*.

A purificação e a caracterização do peptídeo antimicrobiano foram realizadas, e por espectrometria de massas mostrou tratar-se de um composto com massa de 1498.68 Da. Esta substância foi denominada de bacteriocina P34.

3.1 ARTIGO 1

3.1.1 Screening for antimicrobial activity among bacteria isolated from the Amazon basin

3.1.2 Abstract

Bacteria producing antimicrobial activity were identified among 86 isolates from aquatic environments of Brazilian Amazon basin. Antimicrobial activity against at least one indicator strain was detected for 59 isolates (68.6%). Inhibitory activity was mostly against Gram-positive bacteria, such as *Listeria monocytogenes* and *Bacillus cereus*. The antimicrobial substances produced by 19 strains that showed higher inhibitory activity were partially characterized. These antimicrobial substances showed thermal resistance for up to 100°C and partial resistance to proteolytic treatment. Some antimicrobials were partially resistant to pronase E at 2 mg ml^{-1} or to treatment with trichloroacetic acid. Detection of antimicrobial activity on polyacrylamide gels revealed that the molecular weight of the compounds was lower than 14 kDa. Several strains showed antibacterial activity, which in some cases appear to be related to antimicrobial peptides. The potential of these microorganisms to produce useful antimicrobial compounds is great and must be better explored.

Key words: antimicrobial activity, antibiotic, *Listeria monocytogenes,* bacteriocins, Amazon Basin

3.1.3 Introduction

Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. An admirable array of microbial defense systems are produced, including broad-spectrum classical antibiotics, metabolic by-products such as organic acids, and lytic agents such as lysozyme. In addition, several types of protein exotoxins, and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action, were described (20,26). This biological arsenal is remarkable in its diversity and natural abundance, since some substances are restricted to some bacterial groups while other are widespread produced (20).

The search for new antimicrobial agents is a field of utmost importance. The prevalence of antimicrobial resistance among key microbial pathogens has increasing at an alarming rate worldwide (22). Current solutions involve development of a more rationale approach to antibiotic use and discover of new antimicrobials, but the problem of antibiotic resistance is increasing globally and may render the current antimicrobial agents insufficient to control at least some bacterial infections (3).

Amazon basin is a source of enormous biological diversity, which is scarcelly studied. Particularly, few reports on microbial life are described (5). More recently, the characterization of microorganisms with biotechnological interest has been reported (1,6), however, the production of antimicrobials by bacteria isolated in this region is poorly described (7). The aim of this work was to investigate the production of antimicrobial activity among bacteria isolated from aquatic environments neighboring Manaus, at the Brazilian Amazon basin.

3.1.4 Materials and Methods

3.1.4.1 Bacterial cultures

The strains used in this work included bacteria from aquatic environments of the Amazon region, near Manaus, Brazil (3°06’S, 60°01’W), and they were kindly provided by Dr. Spartaco Astolfi Filho (Universidade Federal do Amazonas, Brazil). Indicator

microorganisms for the characterization of antimicrobial activity were strains from collections and are listed in Table 1. Bacteria were maintained as stock cultures frozen at -21°C in BHI broth (Difco, Detroit, USA) supplemented with 20% glycerol. Strains were propagated twice before used in experiments. Morphological and biochemical characterization was performed as described elsewhere (8,13).

3.1.4.2 Growth and production of antimicrobial activity

Bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of BHI broth incubated for 48 h at 31 ± 1°C in shaker at 125 cycles/min. After growth, culture media were centrifuged at 10,000 g for 15 min, and the supernatants were filtered through 0.22 μm membranes (Millipore, Bedford, USA). The resulting filtrates were used to evaluate antimicrobial activity. The pH of the filtrates was measured showing values always between 7.0 to 8.0.

3.1.4.3 Detection of antimicrobial activity

Antibacterial activity was determined essentially as described elsewhere (14). An aliquot of 20 μl cell-free culture supernatant was applied on cellulose disks (6 mm) on BHI agar plates previously inoculated with each indicator strain suspension, which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated for 24 h at optimal temperature for the test organism (Table 1). The inhibition zones around the disks were measured.

3.1.4.4 Effect of heat and enzymes on antimicrobial activity

Proteolytic enzymes were tested on cell-free supernatant. Samples of 1 ml were treated at 37°C for 1 h with 2 mg ml^{-1} of either trypsin (Sigma, St. Louis, USA) or pronase E (Sigma). Samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples of the substances were exposed to temperatures ranging 40 to 100°C for 15 min and 121°C/105 kPa for 15 min before being tested for antimicrobial activity. Trichloroacetic acid (TCA) was added to the filtrates to reach a working concentration of 100 mg ml^{-1} and the samples were incubated for 2 h at 4°C. After

treatment with TCA, samples were centrifuged at 10,000 x *g* for 5 min and the supernatant was neutralized to pH 7.0 before testing for antimicrobial activity. After the treatments, the samples were tested for antimicrobial activity against *L. monocytogenes* ATCC 7644.

3.1.4.5 Direct detection on gels

Aliquots of 1 ml of culture filtrates were freeze-dried. Samples were suspended in 0.1 ml 125 mM tris pH 6.8 containing 0.1% SDS, 20% glycerol, and then applied to 14% polyacrylamide gels. Electrophoresis was carried out as described elsewhere (11) using a Mighty Small II apparatus (Hoefer Scientific, San Francisco, USA) and 20 mA per gel. After running, the gels were washed with sterile distilled water for removal of SDS and then flooded on plates containing tempered BHI agar with 10^6 cfu/ml *L. monocytogenes*. Detection of antimicrobial activity was carried out as described by Naclerio *et al*. (15).

3.1.5 Results

Screening of the antimicrobial activity of 86 isolates was carried out against 7 indicator strains. Inhibitory activity against at least one indicator strain was detected for 59 isolates (68.6%). The majority of strains (64%) inhibited *B. cereus* growth (Table 1). Forty three isolates (50%) inhibited the growth of *L. monocytogenes* and the strains P2 and P31A presented highest inhibition zones (17 mm). *Staphylococcus aureus* was inhibited by only two isolates, P45B and P31A, while antimicrobial activity against *Escherichia coli* and *Salmonella* Enteritidis was detected only for isolates P10 and P30, respectively.

Those bacteria producing inhibition zones higher than 10 mm against at least two microorganisms were selected for further characterization of their antimicrobial activity. Based on these criteria 19 bacterial isolates were selected. The isolates were straight Gram-positive rods with endospores, and were strongly catalase positive. Additional biochemical tests indicated that these isolates belonged to the genus *Bacillus*. The antimicrobial substances were partially resistant to proteolytic treatment (Table 2), being only the antimicrobial substances produced by strains P8A and P34 sensitive to both trypsin and pronase. The activity produced by 11 strains, including P8A and P34, was completely lost

after treatment with TCA. The antimicrobial activity of all filtrates was heat resistant for up to 100°C for 15 min. After autoclaving, 14 filtrates still presented some residual activity, and 5 filtrates completely lost the activity after this treatment.

Direct detection of antimicrobial activity on polyacrylamide gels was carried out. All the substances showed inhibitory activity against *L. monocytogenes*, and presented a molecular weight lower than 14 kDa.

Table 1. Antimicrobial activity of bacteria isolated from Amazon basin.

Indicator strain[a]	Temperature (°C)	Inhibitory strains, n (%)
Corynebacterium fimi NCTC 7547	37	44 (51.2)
Listeria monocytogenes ATCC 7644	37	43 (50.0)
Bacillus cereus ATCC 9634	37	55 (64.0)
Lactobacillus acidophilus ATCC 4356	30	47 (54.7)
Staphylococcus aureus ATCC 25923	37	2 (2.3)
Escherichia coli ATCC 25922	37	1 (1.2)
Salmonella Enteritidis ATCC 13076	37	1 (1.2)

[a] Indicator strains were grown on BHI agar plates for 24 h.

Table 2. Partial characterization of 19 selected isolates with antimicrobial activity.

Isolate	Inhibitory spectra [a]	Properties [b]
P2	Cf, Lm, La, Bc	TH
P5	Cf, Lm, La, Bc	TPH
P7	Cf, Lm, La, Bc	TH
P8A	Lm, La, Bc	H
P10	Cf, Lm, Ec, Bc	TH
P21	Cf, Lm, La, Bc	TAH
P16	Cf, Lm, La, Bc	TAH
P34	Cf, Lm, La, Bc	H
P35	Cf, Lm, La, Bc	TPH
P38	Lm, La, Bc	TPAH
P39A	Lm, La, Bc	TAH
P30	Cf, Se, Lm, La, Bc	TH
P48	Lm, La, Bc	TPAH
P51	Cf, Lm, La, Bc	TPAH
P52	Cf, Lm, La, Bc	TPAH
P71	Cf, Lm, La, Bc	TH
622	Cf, Lm, La, Bc	TH
P45B	Cf, Sa, Lm, La, Bc	TPH
P31A	Cf, Sa, Lm, La, Bc	TH

[a] Cf, *Corynebacterium fimi*; Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Se, *Salmonella* Enteritidis; Lm, *Listeria monocytogenes*; La, *Lactobacillus acidophilus*; Bc, *Bacillus cereus*.

[b] Antimicrobial activity was resistant to the following treatments: (T) trypsin; (P) pronase E; (A) trichloroacetic acid; (H) heating for 15 min at 100°C.

3.1.6 Discussion

Screening for antimicrobial activity of bacteria isolated from Amazon has not been described yet. This study has demonstrated that production of antimicrobial substances is widespread among these bacterial strains. Almost 70% of the isolates exhibited antimicrobial activity against one or more indicator bacteria. This corresponds to a higher proportion than often described in other investigations (9,12,21). Among lactic acid bacteria isolated from raw milk, 82 of 298 strains (27.5%) displayed bacteriocin activity (21), while 8.7% of *S. aureus* strains isolated from cattle produced antimicrobial substances (17). De Vuyst *et al*. (9) found that 122 of 426 *Enterococcus* strains (28.6%) of various origins produced enterocin. Antimicrobial evaluation of cyanobacteria demonstrated that 16.3% was active against Gram-positive and 5.8% against Gram-negative bacteria, while 10.5% possessed antifungal activity (12). Bacilli isolated from the brittlestar *Amphipholis gracillima* exhibited a high range of complete inhibition of several test bacteria (24). The high proportion of antimicrobial producing strains may be associated with an ecological role, playing a defensive action to maintain their niche, or enabling the invasion of a strain into an established microbial community.

The inhibitory effect was mostly against Gram-positive bacteria. Most strains inhibited *B. cereus*. The antimicrobial spectra, essentially restricted to Gram-positive bacteria, may suggest that the produced antimicrobial substances are related to a specific feature of Gram-positive bacteria. It is well known that activity of bacteriocins produced by Gram-positive bacteria is restricted to other Gram-positive bacteria (20).

The pH values of the crude antimicrobial substances indicate that the inhibitory effect was not due to production of organic acids. Most of these substances were partially or completely inactivated by proteases and TCA, suggesting that a protein moiety is involved in the activity. This may indicate that bacteriocin-like substances are implied in antimicrobial activity. These substances showed high thermal resistance and low molecular weight, which are characteristics of small hydrophobic peptides that constitute class II bacteriocins (20).

Although these bacteria were not yet identified to the species level, morphological and biochemical characteristics indicate they belong to the genus *Bacillus*. A variety of antimicrobial compounds are produced by members of the genus *Bacillus*, many of these

identified as peptides, lipopeptides and phenolic derivatives (16). A wide range of antimicrobial substances produced by *Bacillus* spp. isolated from arthropods was recently described, including aromatic acids, acetylamino acids (amino acid analogs), and peptides (10). Bacteriocin-like substances have been related to *Bacillus* spp. isolated from soil (4,18) and vegetal tissues (2,27). Isocoumarin antibiotics are produced by *Bacillus* spp., as demonstrated for *B. subtilis* strains from different habitats and geographic origins (19). A siderophore with wide range of antibacterial spectrum is produced by *Bacillus* sp. NM12 from fish intestine (25) and aminopolyol antibiotic by soil isolates of *B. cereus* (23). Although several antimicrobial substances described in this work appear to be peptides, other substances can not be ruled out since resistance to proteases and even to TCA was observed in some cases.

Results of this study indicate that the potential of these microorganisms to produce antimicrobial compounds that can be useful for many applications is great and must be better explored.

3.1.7 Resumo

Atividade antimicrobiana entre bactérias isoladas da Bacia Amazônica

Bactérias produtoras de atividade antimicrobiana foram identificadas entre 86 isolados de ambientes aquáticos da Bacia Amazônica. Destes, 59 isolados (68.6%) apresentaram atividade antimicrobiana contra pelo menos uma bactéria indicadora. A atividade inibitória foi principalmente observada contra bactérias Gram-positivas, como *Listeria monocytogenes* e *Bacillus cereus*. As substâncias antimicrobianas produzidas por 19 linhagens que demonstraram maior atividade inibitória foram parcialmente caracterizadas, apresentando resistência térmica até 100°C e resistência parcial ao tratamento proteolítico. Algumas substâncias foram parcialmente inativadas somente quando tratadas com ácido tricloroacético ou com pronase E na concentração de 2 mg ml^{-1}. A detecção da atividade antimicrobiana em géis de poliacrilamida mostrou que os compostos apresentaram peso molecular inferior à 14 kDa. Várias linhagens apresentaram atividade antibacteriana, que em alguns casos estaria relacionada com peptídeos

antimicrobianos. O potencial destes microrganismos para produzir substâncias antimicrobianas é grande e merece ser mais explorado.

Palavras-chave: atividade antimicrobiana, *Listeria monocytogenes,* bacteriocinas, Bacia Amazônica

3.1.8 References

1. Bastos, A.E.R.; Moon, D.H.; Rossi, A.; Trevors, J.T.; Tsai, S.M. Salt-tolerant phenol-degrading microorganisms isolated from Amazonian soil samples. *Arch. Microbiol*., 174: 346-352, 2000.
2. Bechard, J.; Eastwell, K.C.; Sholberg, P.L.; Mazza, G.; Skura, B. Isolation and partial chemical characterization of an antimicrobial peptide produced by a strain of *Bacillus subtilis*. *J. Agric. Food Chem*., 46: 5355-5361, 1998.
3. Bhavnani, S.M.; Ballow, C.H. New agents for Gram-positive bacteria. *Curr. Op. Microbiol*., 3: 528-534, 2000.
4. Bizani, D.; Brandelli, A. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. *J. Appl. Microbiol*., 93: 512-519, 2002.
5. Borneman, J.; Triplett, E.W. Molecular microbial diversity in soils from Eastern Amazonia: evidence of unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol*., 63: 2647-2653, 1997.
6. Chies, J.M.; Dias, A.C.O.; Maia, H.M.M.; Astolfi-Filho, S. BanAI a new isoschizomer of the type II restriction endonuclease HaeIII discovered in a *Bacillus anthracis* isolate from Amazon basin. *FEMS Microbiol. Lett*., 215: 97-101, 2002.
7. Cladera-Olivera, F.; Caron, G.R.; Brandelli, A. Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. *Lett. Appl. Microbiol*., 38: 251-256, 2004.
8. Clauss, D.; Berkeley, R.C.W. Genus *Bacillus* Cohn 1872. *In*: Sneath, P.H.A. (ed). *Bergey's Manual of Determinative Bacteriology*, vol 2. Williams & Wilkins: Baltimore: 1986, p. 1105-1141.

9. De Vuyst, L.; Foulquié Moreno, M.R.; Revets, H. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. *Int. J. Food Microbiol*., 84: 299-318, 2003.

10. Gebhardt, K.; Schimana, J.; Müller, J.; Fiedler, H.P.; Kallenborn, H.G.; Holzenkämpfer, M.; Krastel, P.; Zeeck, A.; Vater, J.; Höltzel, A.; Schmid, D.G.; Rheinheimer, J.; Dettner, K. Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS Microbiol. Lett*., 217: 199-205, 2002.

11. Hames, B.D.; Rickwood, D. *Gel electrophoresis of proteins*. IRL Press: Oxford, 1981, 290p.

12. Jaki, B.; Orjala, J.; Burgi, H.R.; Sticher, O. Biological screening of cyanobacteria for antimicrobial and molluscicidal activity, brine shrimp lethality, and cytotoxicity. *Pharm. Biol*., 37: 138-143, 1999.

13. MacFaddin, J.F. *Biochemical test for identification of medical bacteria*. Lippincot, Williams & Wilkins: Baltimore, 2000, 912p.

14. Motta, A.S.; Brandelli, A. Characterization of an antimicrobial peptide produced by *Brevibacterium linens*. *J. Appl. Microbiol*., 92: 63-70, 2002.

15. Naclerio, G.; Ricca, E.; Sacco, M.; De Felice, M. Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. *Appl. Environ. Microbiol*., 59: 4313-4316, 1993.

16. Nakano, M.M.; Zuber, P. Molecular biology of antibiotic production in *Bacillus*. *Crit. Rev. Biotechnol*., 10: 223-240, 1990.

17. Oliveira, S.S.; Póvoa, D.C.; Nascimento, J.S.; Pereira, M.S.V.; Siqueira, J.P.; Bastos, M.C.F. Antimicrobial substances produced by *Staphylococcus aureus* strains isolated from cattle in Brazil. *Lett. Appl. Microbiol*., 27: 229-234, 1998.

18. Oscariz, J.C.; Lasa, I.; Pisabarro, A.G. Detection and characterization of cerein 7, a new bacteriocin produced by Bacillus cereus with a broad spectrum of activity. *FEMS Microbiol. Lett*., 178: 337-341, 1999.

19. Pinchuk, I.V.; Bressollier, P.; Sorokulova, I.B.; Verneuil, B.; Urdaci, M.C. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. *Res. Microbiol*., 153: 269-276, 2002.

20. Riley, M.A.; Wertz, J.E. Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol*., 56: 117-137, 2002.

21. Rodríguez, E.; González, B.; Gaya, P.; Nuñez, M.; Medina, M. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *Int. Dairy J*., 10: 7-15, 2000.

22. Singer, R.S.; Finch, R.; Wegener, H.C.; Bywater, R.; Walters, J.; Lipsitch, M. Antibiotic resistance – the interplay between antibiotic use in animals and human beings. *Lancet Infect. Dis*., 3: 47-51, 2003.

23. Stabb, E.V.; Jacobson, L.M.; Handelsman, J. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. *Appl. Environ. Microbiol*., 60: 4404-4412, 1994.

24. Strahl, E.D.; Dobson, W.E.; Lundie, L.L. Isolation and screening of brittlestar-associated bacteria for antibacterial activity. *Curr. Microbiol*., 44: 450-459, 2002.

25. Sugita, H.; Hirose, Y.; Matsuo, N.; Deguchi, Y. Production of the antibacterial substance by *Bacillus* sp. NM12, an intestinal bacterium of Japanese coastal fish. *Aquaculture,* 165: 269-280, 1998.

26. Yeaman, M.R.; Yount, N.Y. Mechanism of antimicrobial peptide action and resistance. *Pharmacol. Rev*., 55: 27-55, 2003.

27. Yoshida, S.; Hiradate, S.; Tsukamoto, T.; Hatakeda, K.; Shirata, A. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. *Phytopathology*, 91: 181-187, 2001.

3.2 ARTIGO 2

3.2.1 Characterization of a broad range antibacterial substance from a new *Bacillus* species isolated from Amazon basin

3.2.2 Abstract

Bacillus sp. strain producing a bacteriocin-like substance was characterized by biochemical profiling and 16S rDNA sequencing. The phylogenetic analysis indicated that this strain has low sequence similarity with most *Bacillus* spp., suggesting a new specie was isolated. The antimicrobial activity was produced starting at the exponential growth phase, and maximum activity was observed at early stationary phase. The antimicrobial substance was inhibitory to a broad range of indicator strains, incluing pathogenic and food spoilage bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Aeromonas hydrophila*, *Erwinia carotovora*, *Pasteurella haemolytica*, *Salmonella* Gallinarum, among others. The antibacterial substance was stable over a wide pH and temperature range, but the activity was lost when the temperature reached 100°C for 15 min. It was sensitive to the proteolytic action of trypsin, papain and pronase E. The antibacterial substance was bactericidal and bacteriolytic to *L. monocytogenes* and *B. cereus* at 160 AU ml^{-1}. The identification of a broad range bacteriocin-like inhibitory substance active against *L. monocytogenes* addresses an important aspect of food protection against pathogens and spoilage microorganisms.

Key words Amazon, antimicrobial, *Bacillus*, bacteriocin, fish bacteria

3.2.3 Introduction

Antimicrobial substances are widespread produced among bacteria. Bacteriocins and bacteriocin-like substances (BLS) are antimicrobial peptides produced by a number of different bacteria that are often effective against closely related species (Tagg et al. 1976; Riley and Wertz 2002).

Bacteriocins have received increasing attention due to their potential use as natural preservatives in food industry, as probiotics in the human health, and as therapeutic agents against pathogenic microorganisms (Riley and Wertz 2002). Althoug research efforts are mainly focused on bacteriocins produced by lactic acid bacteria, bacteriocins from a variety of Gram-positive and Gram-negative species have been characterized (Gould 1996; McAuliffe et al. 2001). Four distinct classes of bacteriocins have been identified on the basis of biochemical and genetic characterization: (I) lantibiotics; (II) small heat-stable, non-lanthionine peptides including (IIa) *Listeria*-active peptides, (IIb) poration complexes consisting of two peptides for activity, (IIc) thiol-activated peptides; (III) large heat-labile proteins and (IV) complex bacteriocins (Klaenhammer 1993).

The conventional wisdom about the killing range of bacteriocins from Gram-positive bacteria is that they are restricted to killing other Gram-positive bacteria. The range of susceptible strains can vary significantly, from relatively narrow as in the case of lactococcins A, B and M, which have been found to kill only *Lactococcus*, to extraordinarily broad (Ross et al. 1999).

Bacillus species are aerobic spore formers commonly found in soil and ground water and often encountered on plants and animals at the point of harvest or slaughter. *Bacillus* is a genus that have been investigated for producing so-called bacteriocin-like inhibitory substances and strains of *Bacillus thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. cereus* have been reported to produce BLS (Tagg et al. 1976; Stein et al. 2004).

We recently reported antimicrobial activity among several bacteria isolated from aquatic environments of Brazilian Amazon basin (Motta et al. 2004). The bacterium P34 was isolated from the Amazonian fish Piau-com-pinta as a strain producing an antimicrobial substance that inhibits the pathogen *Listeria monocytogenes*. This antimicrobial activity was relatively heat stable and sensitive to proteolytic enzymes,

suggesting a BLS. The objective of this work was the characterization of a new *Bacillus* sp. strain P34 and its antibacterial substance.

3.2.4 Materials and methods

3.2.4.1 Reagents and media

Brain heart infusion (BHI) broth was from Oxoid (Basingstoke, UK). Trypticase soy broth (TSB) was from Acumedia (Baltimore, USA). Trypsin, papain and pronase E were from Sigma (St. Louis, USA). All other media and reagents were from Merck (Darmstadt, Germany).

3.2.4.2 Bacterial strains and culture conditions

The producer strain P34 was given by Universidade Federal do Amazonas (Manaus, Brazil). The organism was isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.) of Amazon basin, at central Amazonia, near Manaus, Brazil (3°06'S, 60°01'W).

The indicator strains used in the study were from ATCC (American Type Culture Collection, Rockville, USA), NCTC (National Collection of Type Culture, Colendale, UK) and our own culture collection (UFRGS, Porto Alegre, Brazil) and were kept frozen at –21°C in BHI containing 20% (v/v) glycerol.

3.2.4.3 Taxonomical studies

Phenotypic characterization of the strain P34 included morphological, cultural, physiological, biochemical and antibiotic susceptibility features, listed in Table 1. All test procedures were carried out as described elsewhere (Claus and Berkeley 1986; MacFaddin 2000). *Bacillus subtilis* ATCC 6633 and *Bacillus licheniformis* ATCC 14580 were used as reference strains. Additionally, an API 50CHB kit was used and the data was submitted to automated interpretation using the APILAB Plus software (BioMérieux, Marcy-l'Etoile, France).

The sequence of 16S rDNA was obtained after genomic DNA extraction, PCR amplification and sequencing based on previous work (Bastos et al. 2000). The DNA was amplified using a Geneamp PCR System 2400 (Perkin Elmer, Norwalk, USA) by denaturation at 96°C (3 min), 30 cycles consisting of 94°C (1 min), 55°C (30 sec) and 72°C (2 min), and a final extension step at 72°C (7min). The PCR-amplified 16S rDNA was sequence by the ABI Prism 377 DNA Sequencer (Perkin Elemer) based on fluorescent-labeled dideoxynucleotide terminators. The 1,522-bp sequence was submitted to Genbank (accession number AY962472). The BLAST algorithm was used to search for homologous sequences in Genbank. The phylogenetic tree was inferred from Jukes-Cantor distances using the neighbor-joining method (software MEGA3, Kumar et al. 2004). The branching pattern was checked by 1,000 bootstrap replicates.

3.2.4.4 Analyses of cellular fatty acids

Whole-cell fatty acids were determined using biomass grown on BHI agar at 37°C for 24 h. Biomass (20 mg) was transferred to screw cap glass vials followed by addition of 0.2 ml of 1.5 mol l^{-1} NaOH in 50% methanol. The released fatty acids were analyzed as fatty acid methyl esters (FAME) essentially as described elsewhere (Buyer 2002).

GC-FID analysis was carried out on a Shimadzu GC-14B chromatograph (Kyoto, Japan). The temperatures of the injector and detector was 250°C. Separation was achieved on a DB-1 (30 m x 0.25 mm I.D.) fused silica capillary column with a 0.25 μm film coating (Chrompack, Walnut Creek, USA), with a maximum operation temperature of 240°C. Hidrogen was used as carrier gas at an initial inlet pressure of 100 kPa. The column temperature was 60°C, 1 min hold, and then programmed to increased at a rate of 20°C/min to 140°C and a further increased at a rate of 3°C/min to 240°C, 5 min hold. Baterial FAME from Supelco (Bellefonte, USA) were used as standards.

3.2.4.5 Transmission electron microscopy

Cells of strain P34 were harvested from BHI agar plates after 24 h of incubation at 37°C. The cells were fixed with 2.5% (v/v) glutaraldehyde, 2% (v/v) formaldehyde in 0.12 mol l^{-1} phosphate buffer for 10 days and then posfixed in 2% (w/v) osmium tetroxide in the same buffer for 45 min before dehydration. Dehydration was done in a graded acetone

series (30-100%) and embeeding in Araldite-Durcupan for 72 h at 60°C. Thin sections were prepared with a Leica Ultracul UCT ultramicrotome (Leica, Benshein, Germany), mounted on grids, covered with collodium film, and poststained with 2% (w/v) uranyl acetate in Reynold's lead citrate. All preparations were observed with a Philips EM 208-5 transmission electron microscope (Philips Electronic Instruments Inc., Mahwah, USA) operating at 100 kV.

3.2.4.6 Production of antimicrobial substance

For the production of antibacterial substance, the strain P34 was grown in 100 ml BHI-medium at 30°C in a rotary shaker at 180 cycles min^{-1} for desired times. Determination of the number of viable cells (CFU ml^{-1}) was carried out as described elsewhere (Motta and Brandelli 2002). The cells were harvested by centrifugation at 10,000 x g for 15 min and the culture supernatant was sterilized by filtration with 0.22 μm membranes (Millipore, Bedford, USA). The filtrate was precipitated with ammonium sulfate at 20% saturation. The precipitate was dissolved in 10 mM phosphate buffer pH 7.0. This solution was further purified by gel filtration chromatography on a Sephadex G-100 column, and active fractions were pooled. The BLS was stored in sterile flasks at 4°C until used.

3.2.4.7 Direct detection on gels

Aliquots of the antimicrobial substance were freeze-dried and analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) using 14% polyacrylamide gels. Electrophoresis and detection of antimicrobial activity were developed as described elsewhere (Bizani et al. 2005), using *Listeria monocytogenes* ATCC 7644 as indicator strain. Molecular weight (MW) standards were from Sigma (St. Louis, USA).

3.2.4.8 Antimicrobial activity assay

Antimicrobial activity was determined essentially as described previously (Motta and Brandelli 2002). An aliquot of 20 μl was applied on discs (6mm) on BHI agar plates previously inoculated with a swab submerged in the indicator strain suspension which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at the

optimal temperature of the test organism. The antimicrobial activity titre was determined by the serial twofold dilution method previously described by Mayr-Harting et al. (1972). Activity was defined as the reciprocal of the dilution after the last serial dilution giving an inhibition zone and expressed as arbitrary unit (AU) per mililitre. The AU ml^{-1} were determined in each experiment against the respective indicator strain.

3.2.4.9 Effect of proteolytic enzymes, heat, pH and chemicals

BLS samples (1 ml) were treated at 37°C for 60 min with 2 mg ml^{-1} (final concentration) of the following enzymes: trypsin, pronase E and papain. Samples were then boiled for 3 min for enzyme inativation. To analyse thermal stability, samples were exposed to 30°C, 40°C, 50°C, 60°C, 70°C, 80°C for 30 min, 100°C for 15 min, 121°C/105 kPa for 15 min, refrigerate at 4°C and frozen at –20°C for up to 13 d and freeze-dried.

The antimicrobial activity at different pH values was estimated by adjusting the pH of samples from pH 3.0 to 10.0. To evaluate pH stability, the antimicrobial substance was incubated at pH 3.0 to 10.0 for 30 min and the pH was neutralized to 7 before testing for antimicrobial activity. Chemicals (working concentration in Table 3) were added to the antimicrobial substance and the samples were incubated for 60 min at 37°C before being tested for antimicrobial activity. After the treatments the samples were tested for antimicrobial activity against *L. monocytogenes* ATCC 7644.

3.2.4.10 Effect on *Listeria monocytogenes* and *Bacillus cereus*

Overnight cultures of *L. monocytogenes* ATCC 7644 and *B. cereus* 8A were obtained by growing in TSB medium at 37°C for 18 h. A sample (1%) of those cultures were inoculated in Erlenmeyer flasks containing 50 ml TSB and incubated at 37°C. The growth was checked at 2-h intervals by O.D. 600 nm and by viable cell counts (CFU ml^{-1}). BLS (final concentration 160 AU ml^{-1}) was added separately to culture of *L. monocytogenes* and *B. cereus* after 6 h of growth and the effect on turbidity and on the number of viable cells was determined at 2-h intervals. The colonies were counted after 24 h of incubation at 37°C.

3.2.5 Results

3.2.5.1 Characterization of strain P34

The morphological and physiological characteristics of the isolate are summarized in Table 1. Microscopic observation of the isolate showed a straight rod with endospores. The spores were eliptical, located at subterminal position. Morphological features were detailed by transmission electron microscopy, revealing a typical Gram-positive cell envelope profile (Fig. 1). The cytoplasmic membrane was surrounde by a thin peptidoglycan layer; an overlaid surface layer was separated from peptidoglycan by a zone of low contrast. The bacterium grew aerobically, was strongly catalase positive, presented variable Gram-stain, and was Gram-positive in the KOH test. Together with additional biochemical tests (Table 1) and the use of an API 50CHB kit, these characteristics indicated that the isolate belongs to the genus *Bacillus* (Claus and Berkeley 1986). The analysis with the APILAB Plus software indicated a very good identity to the genus *Bacillus*.

The phylogenetic analysis confirmed that the isolate was a *Bacillus* sp. and revealed that strain P34 was closest to *Bacillus infernus* (Fig. 2). The P34 sequence shared low similarity with most *Bacillus* species and showed 91% similarity with *B. infernus*. The cluster formed by P34 and *B. infernus* was supported by high bootstrap values (Fig. 2).

Fatty acids were extracted from whole bacterial cells and identified as FAMEs. The cellular fatty acid profiles for *B. cereus* and *B. subtilis* strains used as controls show that there are two major fatty acids consistent with 13-methyltetradecanoate and 15-methylhexadecanoate. These were not observed fatty acids for strain P34, which had a quite distinct fatty acid profile than the other *Bacillus* (not shown).

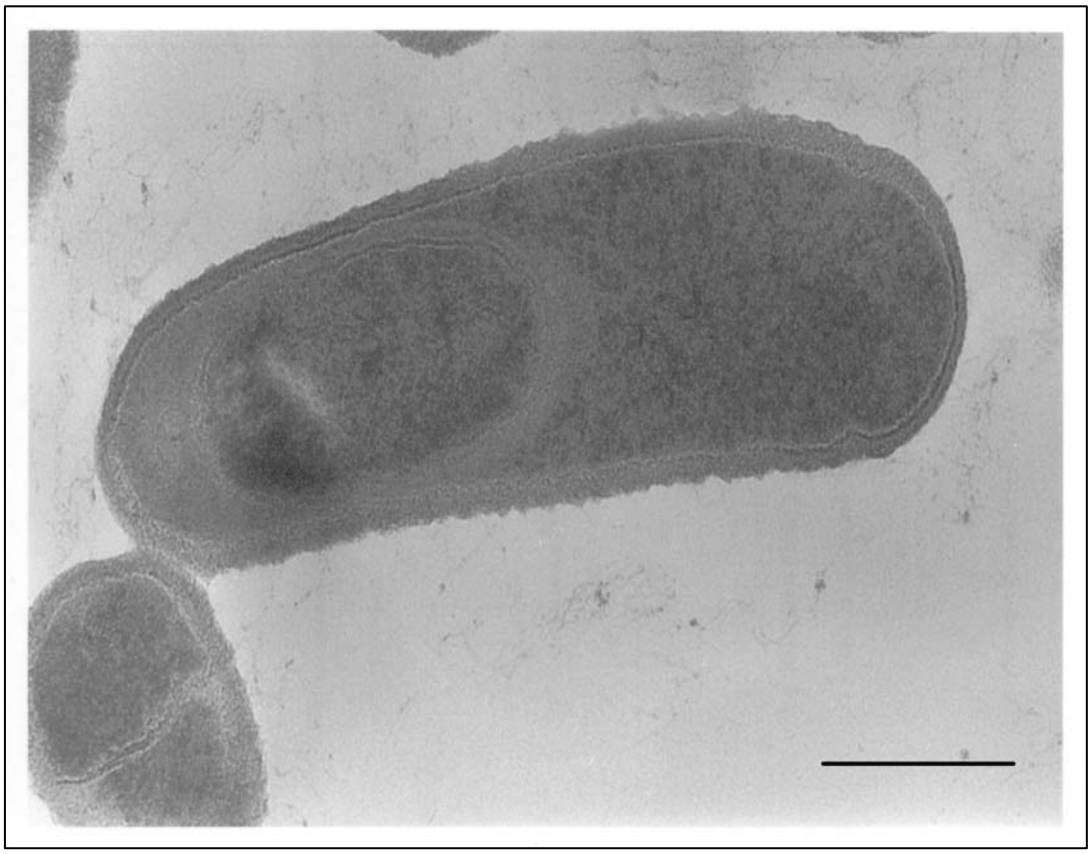

Fig. 1. Thin sections of cells of strain P34, showing round spore located subterminally within sporangium. The cell envelope shows multiple layers, where the outermost layer exhibits the regularly arranged structure. Bar, 300 nm.

Table 1. Phenotypic characteristics of strain P34.

Character	Result
Cells size	2.9 x 0.8 μm
Spores	Elliptical at subterminal position
Gram-stain	Positive
Motility	Positive
Esculin	Positive
Casein hydrolysis	Positive
Starch hydrolysis	Positive
Tyrosine	Negative
Catalase	Positive
Lecithinase	Negative
Acetoin	Positive
Indole	Negative
Citrate	Positive
Gelatin liquefaction	Positive
Nitrate reduction	Positive
Anaerobic growth	Negative
Growth in NaCl at (%, w/v)	
5	Positive
7	Positive
10	Positive
Growth at 50^{o}C	Positive
Growth at pH 5.7	Positive
Fermentation of:	
Glucose	Positive
Arabinose	Positive
Xylose	Negative
Manitol	Positive
Gas production from glucose	Negative
Susceptibility to:	
Penicillin	Sensitive
Streptomycin	Sensitive
Vancomicin	Sensitive
Ceftibuten	Resistant
Novobiocin	Resistant
Ciprofloxacin	Resistant

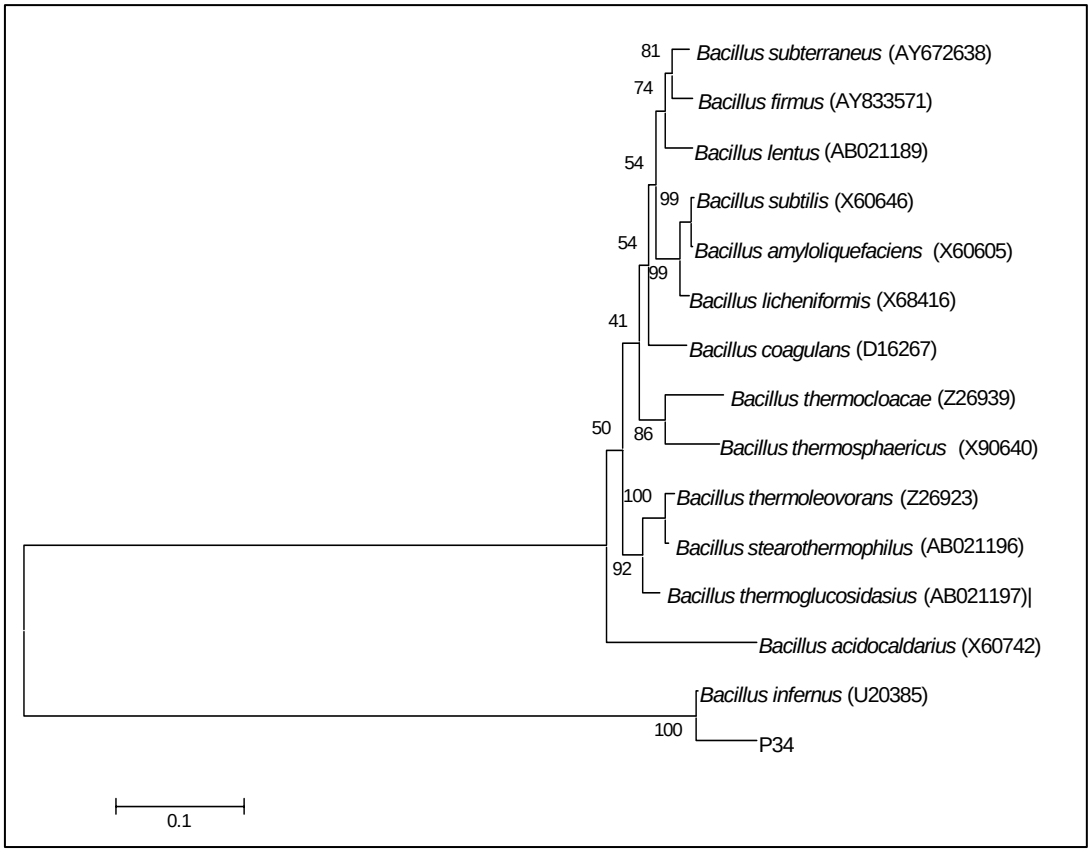

Fig. 2. Phylogenetic position of strain P34 within the genus *Bacillus*. The branching pattern was generated by the neighbour-joining method. The number of each branch indicate the bootstrap values. Bar, 0.1 Jukes-Cantor distance.

3.2.5.2 Production of antimicrobial activity

Bacillus sp. P34 was aerobically incubated in BHI medium at 30°C. Cell growth reached the stationary phase after 12 h of cultivation. Kinetics of antimicrobial substance production showed that synthesis started at the exponencial phase at approximately 6 h, with maximum activity observed at the early stationary phase between 24 h and 30 h of cultivation (Fig. 3). It was observed that pH values were nearly constant (pH = 7.0-7.5) during cultivation.

The antimicrobial substance was then partially purified from the culture supernatant by ammonium sulfate precipitation and gel filtration chromatography. When the fraction containing antimicrobial activity was analyzed by SDS-PAGE, a peptide band with an apparent MW of about 6 kDa was observed (Fig. 4). The peptide band was coinciding with antimicrobial activity observed in gels (Fig. 4).

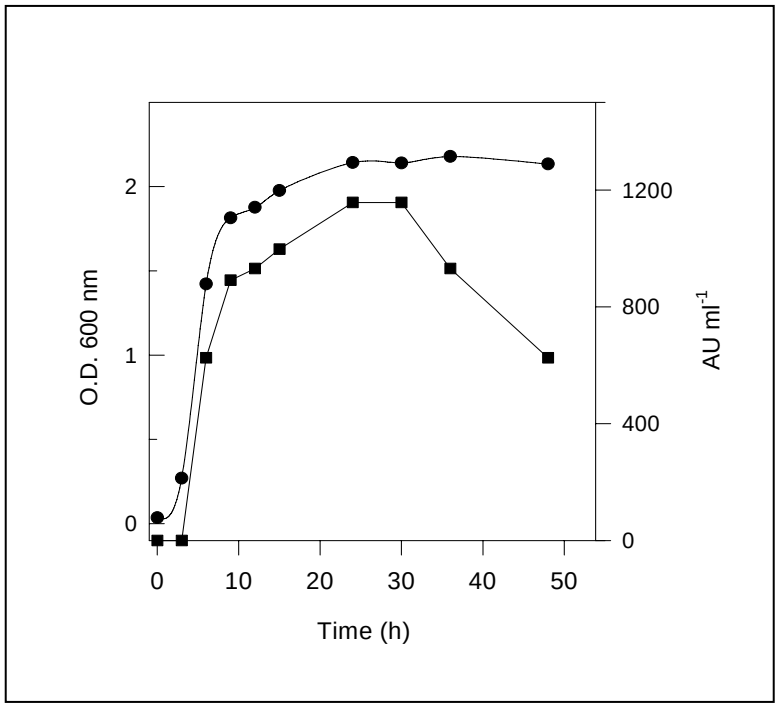

Fig. 3. Production of antimicrobial activity by strain P34. Bacterial growth (circles) and antibacterial activity (squares) were monitored during growth in BHI at 30°C. Each point represents the mean of three independent experiments.

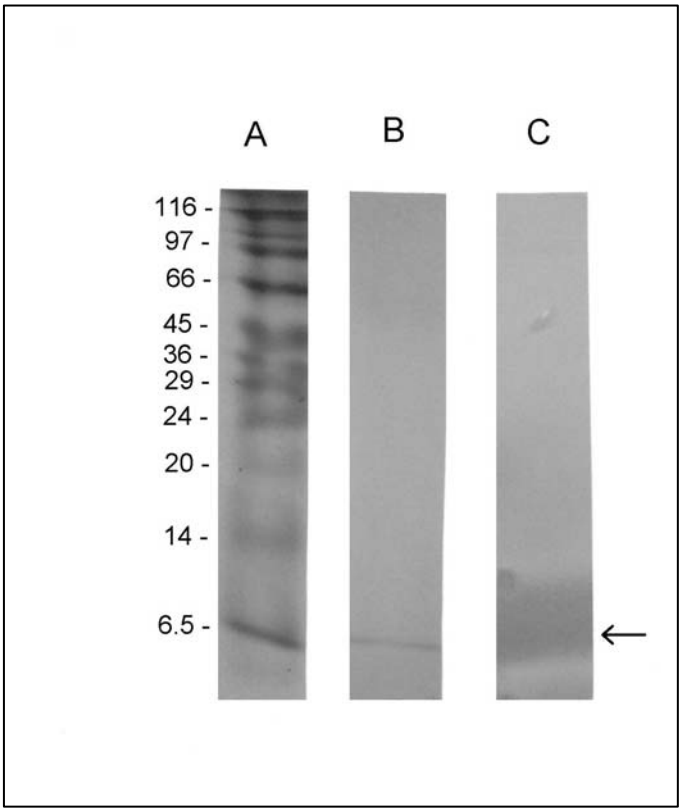

Fig. 4. Gel electrophoresis analysis of BLS P34. Samples of BLS P34 (lanes B and C) were submitted to SDS-PAGE and stained for proteins with Coomassie Brilliant blue (lane B) or tested for antimicrobial activity (lane C). Lane A, molecular weight standards.

3.2.5.3 Inhibitory spectrum

The antimicrobial substance was active against Gram-positive and Gram-negative bacteria, including important pathogenic and spoilage microorganisms. The results are shown in Table 2. The inhibitory activity was observed on *Listeria monocytogenes*, *Listeria innocua*, *Corynebacterium fimi*, *Bacillus cereus*, *Bacillus subtilis*, *Erwinia carotovora, Aeromonas hydrophila, Pasteurella haemolytica*, among other. *Listeria monocytogenes* ATCC 7644 was used as indicator strain in subsequent experiments.

Table 2. Antimicrobial activity spectrum of bacteriocin P34.

Indicator organism	Temperature (°C)	Inhibition zone (mm)*
Gram-positive bacteria		
Bacillus cereus (food isolate)	37	9.8
Bacillus cereus 8A (soil isolate)	37	10.0
Bacillus subtilis (ICBS-1)	37	9.6
Lactobacillus acidophilus ATCC 4356	37	9.8
Brevibacterium linens ATCC 9172	25	9.2
Brevibacterium linens ATCC 9174	25	9.0
Brevibacterium linens ATCC 9175	25	9.2
Brevibacterium linens ATCC 19391	25	9.8
Corynebacterium fimi NCTC 7547	37	9.7
Listeria monocytogenes ATCC 7644	37	12.3
Listeria innocua (food isolate)	37	11.3
Listeria spp. (clinical isolate)	37	11.0
Listeria monocytogenes (clinical isolate)	37	13.0
Listeria monocytogenes 4C	37	13.0
Listeria monocytogenes 1 7D78/03	37	13.0
Listeria innocua ATCC 33090	37	11.5
Listeria innocua 1572	37	12.5
Listeria ivanovii 5 NCTC 11007	37	11.0
Listeria seeligeri AC 82/4	37	9.0
Listeria welshimeri AC 73/2	37	13.0
Brochothrix thermosphacta ATCC	37	0
Staphylococcus aureus ATCC 25923	37	0
Staphylococcus aureus (food isolate)	37	0
Staphylococcus aureus 16070 (clinical isolate)	37	0
Staphylococcus epidermidis (clinical isolate)	37	0
Staphylococcus intermedius (clinical isolate)	37	0
Streptococcus sp. (clinical isolate)	37	0
Rhodococcus sp.	37	13.0

Table 1. cont.

Enterococcus faecalis	37	0
Gram-negative bacteria		
Salmonella Enteritidis ATCC 13076	37	0
Salmonella Enteritidis (food isolate)	37	0
Salmonella Gallinarum (clinical isolate)	37	8.5
Escherichia coli ATCC 25922	37	0
Escherichia coli (food isolate)	37	0
Enterobacter aerogenes (food isolate)	37	0
Enterobacter aerogenes (clinical isolate)	37	0
Erwinia carotovora 413	25	8.2
Erwinia carotovora 416	25	8.2
Erwinia carotovora 325	25	8.7
Erwinia carotovora 365	25	8.2
Aeromonas hydrophila (clinical isolate)	28	8.5
Pasteurella haemolytica (clinical isolate)	37	9.0
Pasteurella haemolytica (clinical isolate)	37	9.0
Pseudomonas aeruginosa (clinical isolate)	37	0
Pseudomonas sp. (clinical isolate)	37	0
Yeast		
Candida sp. (food isolate)	37	0
Candida kefir (food isolate)	37	0
Candida utilis CCT 3469	37	0
Kluyveromyces marxianus CBS 6556	37	0
Malassezia sp. (clinical isolate)	37	0

* Diameter of the inhibition zone in mm around the disk

3.2.5.4 Effect of enzymes, temperature, pH and chemicals on antimicrobial activity

The antimicrobial substance was incubated for 30 min at different temperatures and the residual activity was measured. It was stable up to 80°C, but lost its activity when incubated at 100°C for 15 min, although the crude substance showed resistance remaining 70% its initial activity at 100°C (Table 3). The substance lost the antimicrobial activity after autoclaving at 121°C / 105 kPa for 15 min. Antimicrobial activity was partially lost after 13 d at 4^{o}C (residual 30%) or freezing storage (residual 57%), but not when freeze-dried (residual 100%).

In order to test the effect of proteolytic enzymes, the antimicrobial substance was tested for sensitivity to papain, trypsin and pronase E and residual activity was measured by agar disc diffusion assay against *Listeria monocytogenes* ATCC 7644. The substance was

sensitive to pronase E and partially inactivated by trypsin and papain at the concentration of 2 mg ml^{-1} (Table 3).

The effect of several chemicals on the antimicrobial activity was evaluated. The substance lost its activity after treatment with trichloroacetic acid (Table 3). When treated with organic solvents and other chemicals, antimicrobial activity were only affected by butanol, and in lesser extent by acetone and methanol (Table 3).

The antimicrobial substance was stable in all pH tested (3.0 to 10.0), remaining 100% its initial activity. When the activity was tested within this pH range, at least 70% of maximum activity, observed at pH 6.0 to 8.0, was observed.

Considering the properties of the inhibitory substance produced by *Bacillus* sp. strain P34, it was characterized as a bacteriocin-like compound.

Table 3. Effect of proteases and chemical substances on antimicrobial activity

Treatment	Concentration	Residual activity (%)
Untreated bacteriocin	-	100
Boiled 3 min*	-	100
Trypsin	2 mg ml^{-1}	40
Papain	2 mg ml^{-1}	30
Pronase E	2 mg ml^{-1}	0
Acetone	50% (v/v)	80
Chloroform	50% (v/v)	100
Dimethyl sulfoxide	50% (v/v)	100
Ethanol	50% (v/v)	100
Methanol	50% (v/v)	80
Butanol	50% (v/v)	40
Xylol	50% (v/v)	100
Toluene	50% (v/v)	100
EDTA	10 mmol l^{-1}	100
Trichloroacetic acid	100 mg ml^{-1}	0
Tween 20	10% (v/v)	100
Tween 80	10% (v/v)	100
Triton X-100	1% (v/v)	100

* Control, after proteolytic treatment the bacteriocin was boiled for 3 min at 100°C for protease inactivation.

3.2.5.5 Effect of BLS P34 on *Listeria monocytogenes* and *Bacillus cereus*

The effects of the BLS on the growth of *L. monocytogenes* and *B. cereus* are shown in Fig. 5. The addition of BLS (160 AU ml^{-1}) to cells suspensions of *L. monocytogenes* or *B. cereus* at the exponential growth phase results in a difference in viable counts related to the controls. The addition of the antimicrobial substance inhibited the growth of the indicator strains resulting in a decrease in the number of viable cells and in optical density over a period of 24 h (Fig. 5). This indicated that BLS has a bactericidal and bacteriolytic effect.

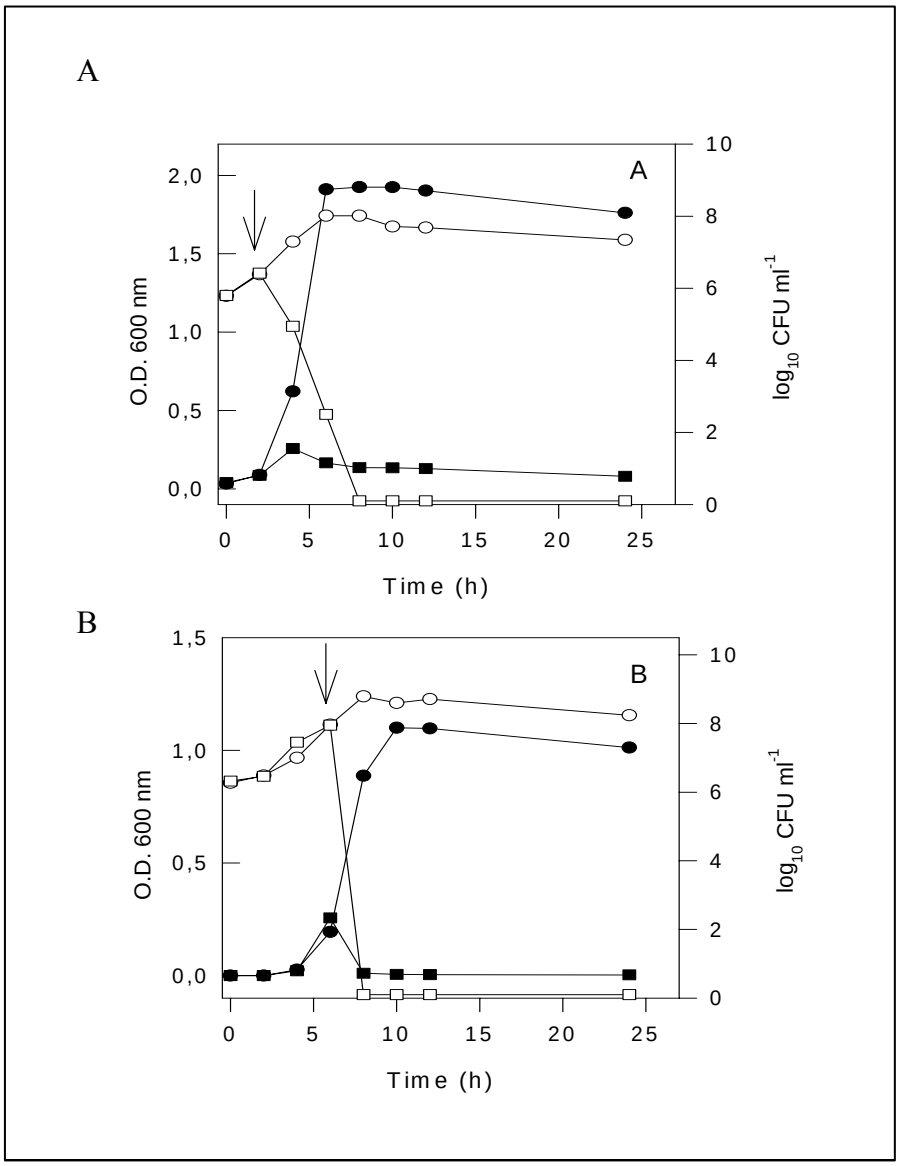

Fig. 5. Effect of BLS P34 on growth of *Bacillus cereus* (A) and *Listeria monocytogenes* (B). Turbidity (black symbols) and viability (open symbols) were monitored in control (circles) and treated (squares) cells. Each point represents the mean of three independent experiments.

3.2.6 Discussion

A bacterium producing a BLS was isolated from the intestinal contents of *Leporinus* sp., a teleost fish of Amazon basin, and was identified as *Bacillus* sp. by biochemical profiling and 16S rDNA sequencing. From the sequence analysis of the 16S rDNA gene, strain P34 was found to be clustered with the *Bacillus infernus*. Although this suggests both bacteria have a common ancestral, an important difference in sequence was observed. In addition, *B. infernus* thrives in heat and anaerobiosis (Boone et al. 1995). Because these characteristics are not in agreement with those of strain P34, this species can be assigned to the genus level as *Bacillus* sp. These data suggest that our isolate is a new *Bacillus* species (Stackebrandt and Goebel 1994; Palys et al. 1997; Goto et al. 2000). Considering that it was isolated from a fish of Brazilian Amazon basin, we propose this bacterium be classified in the genus *Bacillus* as "*Bacillus amazonensis*".

FAME analysis provide additional insight that a new specie was isolated. Comparative analysis of the fatty acid composition of the isolate P34 and stablished *Bacillus* species showed a quite distinct profile (Ivanova et al. 1999). This may suggest that *Bacillus* sp. P34 is a new specie with a cellular fatty acid composition unrelated with most *Bacillus* spp.

The antimicrobial substance showed a broad inhibitory spectrum, including several spoilage and pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, *Pasteurella haemolytica*, *Salmonella* Gallinarum among others. *Corynebacterium fimi* NCTC 7547, described as being susceptible to all bacteriocins tested (Oliveira et al. 1998), was also sensitive to the BLS produced by *Bacillus* sp. P34. The antimicrobial substance was able to inhibit the growth of *Listeria monocytogenes*, a very important property in food safety. *Aeromonas hydrophila*, an important pathogen linked to seafood and water outbreaks (Tsai and Chen 1996) was also inhibited. Therefore, this novel BLS may represent a relevant alternative against several food pathogenic and spoilage microorganisms.

The antimicrobial substance was produced during the exponential growth phase, suggesting a primary metabolite behavior. Some antimicrobial activity was lost at the late stationary phase, which could suggest that the substance was partially sensitive to proteases secreted by the producer strain. Indeed, the production of extracellular proteases by

Bacillus spp. is common and proteolytic activity may be harmful to antimicrobial peptides (Bizani and Brandelli 2002).

Bacteria can produce a variety of inhibitory substances. In this case, organic acids can be ruled out, since the pH in the growth medium was always in the range of 7.0-7.5 during cultivation in BHI. The antimicrobial activity was sensitive to the enzymes tested, with complete inactivation by the broad range protease pronase E, indicating its proteinaceous nature. A bacteriocin produced by *Bacillus licheniformis*, Lichenin A, was completely inactivated by proteinase K treatment but was resistant to trypsin (Pattnaik et al. 2001). Enzymes such as proteinase K and pronase E were shown to eliminate thuricin 439 activity, indicating a bacteriocin-like inhibitory compound (Ahern et al. 2003). The fact that the antimicrobial activity was completely lost by only few enzymes, and exhibited a rather wide antimicrobial spectrum, suggest that the BLS could be a cyclic peptide. This agrees with the fact that the inhibitory compound was relatively heat stable (up to 100°C for 15 min) and shows stability within the pH range of 3.0 to 10.0.

The decline in the number of living cells of *L. monocytogenes* and *B. cereus* after the addition of BLS suggest that the antimicrobial effect was bactericidal. The decrease in O.D. readings indicated that cells of indicator strains were lysed. Exponentially growing cells of *B. cereus* 6A1 were subjected to different amounts of thuricin 439 and assessed for growth and viability. Based on the results, thuricin 439 has a bactericidal and bacteriolytic effect on the indicator strain (Ahern et al. 2003). It has been suggested that the antimicrobial effect can be dependent on the assay conditions, such as the amount and purity of bacteriocin, culture media, indicator strain and its cellular concentration (de Vuyst and Vandamme 1994). The BLS identified in this work showed bactericidal and bacteriolytic effect on *L. monocytogenes* and *B. cereus* at 160 AU ml^{-1}.

Bacteriocins may play a defensive role to hinder the invasion of other strains or species into an occupied niche (Riley and Wertz 2002). Antibacterial substances produced by different bacteria seem to play an important role in the bacterial antagonism in aquatic ecossystems (Dopazo et al. 1988). In addition, it has been found that intestinal bacteria from both freshwater and marine fishes show an inhibitory effect on fish pathogenic bacteria (Olsson et al. 1992; Sugita et al. 1996). A similar role could be assigned to the strain P34, where its BLS may help to avoid pathogen colonization of fish intestines. The

broad inhibitory spectrum of strain P34 may indicate an ecological advantage, since it would be capable to inhibit several competing bacteria.

While many studies on bacteriocins have shown their importance as food preservatives, few atention have been addressed to their application as antimicrobials in clinical studies. Because bacteriocin treatment is effective and non-toxic to human and animals, it has been already proposed as an alternative for disease control (Oliveira et al. 1998; Twomey et al. 2000). The BLS produced by strain P34 may represent an antimicrobial substance with potential application in the prevention and treatment of *Salmonella* Gallinarum, which causes severe systemic disease in domestic poultry (Johnson et al. 1977). In this regard, the lantibiotic mersacidin produced by *B. subtilis* has shown promising applications particularly against methicillin-resistant staphylococci (Bierbaum et al. 1995). The rapid rise and spread of multi-resistant bacterial pathogens have forced the consideration of alternative methods of combating infections. For example, several strains of *Listeria monocytogenes* have acquired resistance to conventional bacteriocins (Rasch and Knochel 1998; van Schaik et al. 1999). Thus, there is a need for new substances that demonstrate efficient antimicrobial activity against such strains. Given the diversity of bacteriocins produced in nature, they can be considered as an alternative to combat infections against specific pathogens (Neu 1992; Riley and Wertz 2002). Therefore, research for new products with antimicrobial activity is a very important field. The identification and chemical characterization of bacteriocins produced by *Bacillus* spp., and exploration for their potential use in the control of pathogenic and spoilage microorganisms adresses this subject.

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3.2.8 References

Ahern M, Verschueren S, van Sindersen D (2003) Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiol Lett 220:127-131

Bastos AER, Moon DH, Rossi A, Trevors JT, Tsai SM (2000) Salt-tolerant phenol-degrading microorganisms isolated from Amazonian soil samples. Arch Microbiol 174:346-352

Bierbaum G, Brotz H, Koller KP, Sahl HG (1995) Cloning, sequencing and production of the lantibiotic mersacidin. FEMS Microbiol Lett 127:121-126

Bizani D, Brandelli A (2002) Characterisation of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. J Appl Microbiol 93:512-519

Bizani D, Dominguez APM, Brandelli A (2005) Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. Lett Appl Microbiol 41:269-273

Boone DR, Liu Y, Zhao ZJ, Balkwill DL, Drake GR, Stevens TO, Aldrich HC (1995) *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. Int J Syst Bacteriol 45: 441-448

Buyer JS (2002) Rapid sample processing and fast gas chromatography for identification of bacteria by fatty acid analysis. J Microbiol Meth 51:209-215

Claus D, Berkeley RCW (1986) Genus *Bacillus* Cohn 1872. In Sneath PHA (ed) Bergey's Manual of Determinative Bacteriology, vol 2, Williams and Wilkins, Baltimore, pp 1105-1141

De Vuyst L, Vandamme EJ (1994) Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic and Professional, London, 540 p.

Dopazo CP, Lemos ML, Lodeiros C, Bolinches J, Barja JL, Toranzo AE (1988) Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J Appl Bacteriol 65 (1): 97-101

Goto K, Omura T, Hara Y, Sadaie Y (2000) Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J Gen Appl Microbiol 46:1-8

Gould G (1996) Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J Food Prot 59:S82-S86

Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova NM, Mikhailov VV, Yumoto N, Shigeri Y, Taguchi T, Yoshikawa S (1999) Characterization of *Bacillus* strains of marine origin. Int Microbiol 2:267-271

Johnson DC, Keenum KG, Perry HP (1977) A fowl typhoid outbreak in a chicken breeder flock. Avian Dis 21:716-719

Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12:39-86

Kumar S, Tamura K, Nei M (2004) Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinf 5:150-163

MacFaddin F (2000) Biochemical test for identification of medical bacteria, 3rd ed. Lippincot, Williams and Wilkins, Baltimore

Mayr-Harting A, Hedjes AJ, Berkeley CW (1972) Methods for studying bacteriocins. In Methods in Microbiology, Vol 7. ed. Norris JB, Ribbons D. New York: Academic Press, pp 315-412

McAuliffe O, Ross RP, Hill C (2001) Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 25:285-308

Motta AS, Brandelli A (2002) Characterization of an antibacterial peptide produced by *Brevibacterium linens*. J Appl Microbiol 92:63-71

Motta AS, Cladera-Olivera F, Brandelli A (2004) Screening for antimicrobial activity among bacteria isolated from Amazon basin. Braz J Microbiol 35:307-310

Neu HC (1992) The crisis in antibiotic resistance. Science 257:1064-1073

Oliveira SS, Abrantes J, Cardoso M, Sordelli D, Bastos MCF (1998) Staphylococcal strains involved in mastitis are inhibited by *Staphylococcus aureus* antimicrobial peptides. Lett Appl Microbiol 27:287-291

Olsson JC, Westerdahl A, Conway PL, Kjelleberg S (1992) Intestinal colonization potential of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl Environ Microbiol 58:551-556

Palys T, Nakamura LK, Cohen FM (1997) Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int J Syst Bacteriol 47:1145-1156

Pattnaik P, Kaushik JK, Grover S, Batish VK (2001) Purification and characterization of a bacteriocin-like compound (Lichenin) produced anaerobically by *Bacillus licheniformes* isolated from water buffalo. J Appl Microbiol 91:636-645

Rasch M, Knochel S (1998) Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA 1 and bavaricin A. Lett Appl Microbiol 27:275-278

Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology and application. Annu Rev Microbiol 56:117-137

Ross RP, Galvin M, McAuliffe O, Morgan SM, Ryan MP (1999) Developing applications for lactococcal bacteriocins. Antonie Van Leeuwenhoek 76:337-346

Stein T, Düsterhus S, Stroh A, Entian KD (2004) Subtilosin production by two *Bacillus subtilis* subspecies and variance of the sob-alb cluster. Appl Environ Microbiol 70 4:2349-2353

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place of DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846-849

Sugita H, Matsuo N, Shibuya K, Deguchi Y (1996) Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. J. Mar Biotechnol 4:220-223

Tagg JR, Dajani AS, Wannamaker LK (1976) Bacteriocins of gram-positive bacteria. Bacteriol Rev 40: 722-756

Tsai GJ, Chen TH (1996) Incidence and toxigenicity of *Aeromonas hydrophila* in seafood. Int J Food Microbiol 31:121-131

Twomey DP, Wheelock AI, FlynnJ, Meaney WJ, Hill C, Ross RP (2000) Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin lacticin 3147. J Dairy Sci 83: 1981-1988

Van Schaik W, Gahan CG, Hill C (1999) Acid adapted *Listeria monocytogenes* displays enhanced tolerance against the lantibiotic nisin and lacticin 3147. J Food Prot 62: 536-539

3.3 ARTIGO 3

3.3.1 Bacteriocin-like substance production by a novel *Bacillus* sp. strain P34 using response surface methodology

3.3.2 Summary

A 2^2 factorial design was performed to optimize the antimicrobial substance production by *Bacillus* sp. P34 using response surface methodology (RSM). The effect of two variables, temperature and initial pH, was tested by RSM. Statistical analysis of results showed that, in the range studied, the two variables have a significant effect on antimicrobial substance production. Response-surface data showed maximum antimicrobial activity production at initial pH between 6.0 and 8.0 and temperature between 25 and 37°C. No relationship between bacterial growth and bacteriocin production was observed. RSM proved to be a powerful tool in optimizing the production of antimicrobial activity by *Bacillus* sp. Optimization by statistical modeling may represent an actual increase in fermentation yields compared with conventional methodology.

Key words: antimicrobial activity; bacteriocin; experimental design; *Listeria monocytogenes*; statistical modeling

3.3.3 Introduction

The bacteriocins produced by lactic acid bacteria (LAB) enjoy the GRAS (generally regarded as safe) status by the U.S. Food and Drug Administration (FDA). This permits their use in fermented foods without additional regulatory approval. However, bacteriocins are found in mostly bacterial species, and within a species several different kinds of bacteriocins may be produced (1). This preservation potential could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food

additive. The latter will necessarily require optimization of their production, which is usually dependent on multiple strain specific factors (2).

Reports on lactic acid bacteria indicate the essential influence of temperature, pH and media composition on bacteriocin production (3,4). Although the production of bacteriocins or bacteriocin-like substances has been already described for many *Bacillus* species, few studies on the influence of growth conditions on bacteriocin production are available (5).

Studies on multiple factors affecting the production of bacteriocins are relatively scarce and it is difficult to optimize them for biotechnological processes. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses (6). RSM has been successfully applied in many areas of biotechnology such as optimization of a culture medium, enzyme synthesis and lactic acid esterification (7-9). Li *et al.* (6) evaluated the effect of medium components on nisin production and cell growth, to search for the optimal medium composition for a higher nisin yield. More recently, Messens *et al.* (10) used RSM to study the growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values for sausage fermentation processes.

We recently reported antimicrobial activity among several bacteria isolated from aquatic environments of Brazilian Amazon basin. A novel *Bacillus* sp. strain P34 was identified, and its antimicrobial activity was characterized as a bacteriocin-like substance (11). In this work, we employed RSM to optimize the production of bacteriocin activity by *Bacillus* sp. strain P34. The purpose of this study was to evaluate the effect of two variables, temperature and initial pH, on production of antimicrobial activity.

3.3.4 Materials and methods

3.3.4.1 Bacterial strains and media

The producer strain was kindly given to us by the Universidade Federal of Amazonas (Manaus, Brazil). The *Bacillus* sp. strain P34 was deposited in our culture collection under the identification P34. The indicator strain was *Listeria monocytogenes* ATCC 7644. Brain heart infusion (BHI) medium (Oxoid) was used for maintenance of strains with 20% (v/v) glycerol at –20 °C. The cultivation of strains was performed aerobically.

3.3.4.2 Production of antimicrobial substance

The inoculum was obtained at 37 °C under shaking (180 rev. min^{-1}), the absorbance at 600 nm was adjusted to obtain approximately 10^7 c.f.u. $ml^{-1,}$ and 1.0 ml was transferred to 250 ml Erlenmeyer flasks containing 100 ml of either BHI, Trypticase soy broth (TSB, Accumedia), or buffered peptone (Difco) at initial pH 7.0. Cultures were incubated in a rotary shaker at 180 rev. min^{-1} for up to 24 h at 37 °C. Aliquots of 1 ml of culture were centrifuged at 10,000 x g for 15 min. Culture supernatants were boiled at 100 °C for 5 min, placed in an ice bath and the pH was measured and adjusted to pH 7.0 with either 5 mol l^{-1} NaOH or 5 mol l^{-1} HCl before being used in the antimicrobial assay. All experiments were done in duplicate.

3.3.4.3 Antimicrobial activity assay

The antimicrobial activity was detected by agar disk diffusion assay (12). Aliquots (20 μl) of culture supernatants were applied to disks on agar plates previously inoculated with a cell suspension of *L. monocytogenes*, which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37 °C. The antimicrobial activity titre was determined by serial twofold dilution method previously described (13). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units per milliliter (AU ml^{-1}). To evaluate whether the antimicrobial activity was due to peptides, culture supernatants were treated with 2 mg ml^{-1} pronase E for 30 min at 37 °C before being tested for antimicrobial activity.

3.3.4.4 Experimental design of RSM

After selection of the best culture medium, the next step was to determine the optimal levels of two variables, initial pH and temperature, on antimicrobial substance activity. To describe the response surface in the optimum region, a central composite design with five coded levels and two variables was used to study the combined influence of pH and temperature. For the two factors, this design was made up of a full 2^2 factorial design with its four points augmented with three replications of the center points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the center of $\pm\alpha$, whereas the other two factors are at level 0. The axial distance α was chosen to be 1.41 to make this design orthogonal. A set of 11 experiments was carried out. The central values (zero level) chosen for experimental design were: temperature, 30 °C and initial pH 7.0. For two factors the equation model is:

$$Y = b_o + b_1x_1 + b_2x_2 + b_{12}\,x_1x_2 + b_{11}\,x_1^2 + b_{22}x_2^2$$

where Y, predicted response, b_o, intercept; b_1, b_2, linear coefficients; b_{11}, b_{22}, squared coefficients and b_{12} interaction coefficients.

The results were analyzed by the Experimental Design Module of the Statistica 5.0 software (Statsoft, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on antimicrobial substance production.

The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and its statistical significance was checked by an *F* test. The significance of the regression coefficient was tested by a *t* test. The level of significance was given as $P< 0.05$.

The optimal values of pH and temperature were obtained by solving the regression equation and also by analyzing the response surface contour plots (14).

The production medium, brain heart infusion broth, (100 ml in 250 ml Erlenmeyer flasks) was inoculated with a 1% inoculum and incubated under different temperatures and

pH, for each set of experiment in a rotary shaker at 180 rev. min^{-1} for 24 h. After incubation, antimicrobial activity, bacterial growth and final pH were determined.

3.3.5 Results

3.3.5.1 Production of antimicrobial activity in different media

Among the cultivation media studied, *Bacillus* sp. P34 produced maximum antimicrobial activity in BHI, followed by TSB and peptone (Fig. 1). Maximum activities in BHI and TSB were reached at the stationary growth phase. However, during cultivation in peptone maximum activity was coinciding with the exponential growth phase (Fig. 1). In all cases the antimicrobial activity was lost when treated with pronase E. BHI was the selected medium for design of RSM.

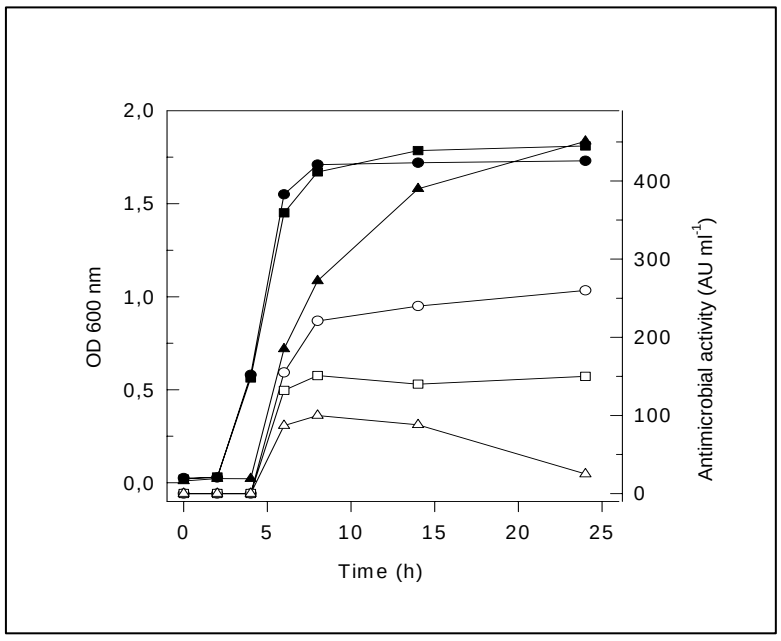


Fig. 1. Growth (black symbols) and bacteriocin production (open symbols) during cultivation of *Bacillus* sp. in BHI (circles), TSB (squares) or buffered peptone (triangles) at 37 °C. Data points are the mean of two independent experiments**.**

3.3.5.2 Response surface methodology

The results of central composite design experiments for studying the effects of two independent variables, initial pH and temperature on antimicrobial substance production and bacterial growth are presented in Table 1 along with the mean observed response.

Statistical analysis of results showed that in the range studied, the two variables had a significant effect on antimicrobial substance production (Fig. 2). Interaction between temperature and initial pH was not significant. Quadratic effect of pH was the most significant effect followed by quadratic effect of temperature (Fig. 2).

The values of coefficients of the regression equation were calculated and the following regression equation was obtained:

$$Y = 313{,}949 - 150{,}532\, X_1^2 - 138{,}407\, X_2^2$$

With Y antimicrobial activity production (response), x_1: initial pH and x_2: temperature.

The regression equation obtained after the analysis of variance (ANOVA) indicated the R^2 value of 0.8716 (a value of $R^2 > 0.75$ indicates the aptness of the model). These values ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that the model could explain 87.16% of the variability in the response. The statistical significance of the model equation was also confirmed by Fischer's *F*-test, and showed value $F = 27.16$, six times bigger than the *F* tabulated ($F = 4.46$) that demonstrates significance for the regression model.

The three-dimensional response surface curve was then plotted to graphically show the interaction of the factors (Fig. 3a). Maximum antimicrobial substance production was achieved at pH between 6.0 and 8.0 and temperature between 25 and 37 °C (Fig. 3b).

With respect to bacterial growth, it was not possible to determine a model because the *F*-test did not showed significance of the regression model ($R^2 = 0.616$). The viable cell counts and final pH values are indicated in the Table 1.

3.3.5.3 Growth and antimicrobial substance production at optimal conditions of temperature and pH

Maximum production was observed when the growth was developed at the central point. *Bacillus* sp. was aerobically incubated at 30 °C, initial pH 7.0 in a rotary shaker. Cell growth reached the stationary phase after 12 h of cultivation and maximum antimicrobial activity was observed from 15 h.

Table 1. Experimental design and results of the 2^2 factorial design

Run	Variables		Antimicrobial activity (AU ml^{-1})	Log_{10} CFU ml^{-1}	Final pH
	Initial pH	Temperature (°C)			
1	5	23	0	7.68	7
2	9	23	0	8.27	8
3	5	37	159	8.33	7
4	9	37	0	8.08	8
5	7	30	317	8.30	7
6	7	30	264	7.69	7
7	7	30	357	8.11	7
8	4	30	0	3.02	4
9	7	20	0	6.96	7
10	10	30	0	3.63	10
11	7	40	149	7.58	7

Data are the mean of two independent experiments.

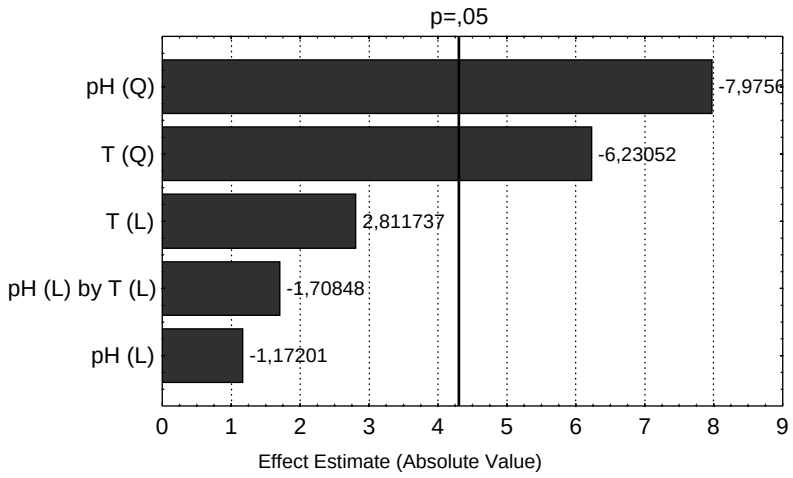
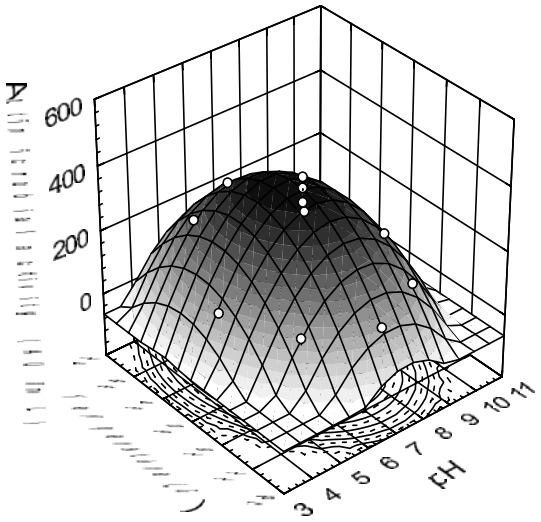


Fig. 2. Bar graph of standardized estimated effects of the different variables tested in experiments on antimicrobial substance production by *Bacillus* sp. The variables tested were pH and temperature (T). The point at which the effect estimates were statistically significant (at $P < 0.05$) is indicated by the vertical line. Q, quadratic; L, linear.



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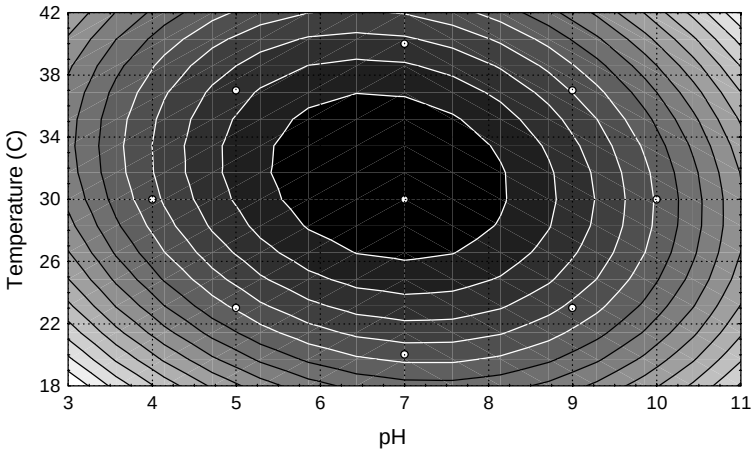

B

Fig. 3. (A) Response surface of antimicrobial substance production by *Bacillus* sp. P34, estimated by AU ml^{-1}, as a function of temperature and pH. (B) Contour plot showing the optimal region of antimicrobial substance production by *Bacillus* sp. P34 estimated by AU ml^{-1}, as a function of temperature and pH.

3.3.6 Discussion

We have shown that a heat-stable antimicrobial substance is produced by the strain P34, which is inhibitory to the foodborne pathogen *Listeria monocytogenes* (11). This activity was always destroyed by proteolytic action of pronase E, indicating that a peptide moiety is essential for its antibacterial activity, a characteristic of bacteriocin-like substances (BLS). In this work, the effect of different media, temperature and initial pH in BLS production by the strain P34 was evaluated. The effect of growth conditions have been studied for the production of bacteriocins and maximum production usually require complex medium and well-controlled physical factors such as temperature and pH (15,16). In this study we demonstrated that these parameters are also important for optimal BLS production by *Bacillus* sp. P34.

The cultivation media influenced the production of BLS. Although prominent growth was observed in BHI and TSB, there was no relationship between BLS activity and biomass since cultivation in TSB produced lower activity. The correlation between biomass production and antimicrobial activity was observed by *Lactococcus lactis* (3) and *Brevibacterium linens* (17). Nevertheless, optimal cell growth does not always result in the highest antimicrobial production levels (18). Production of BLS by *Bacillus* sp. P34 started during the late exponential growth phase, but maximum activity was achieved during the early stationary phase, suggesting a secondary metabolite behavior. However, this was not observed in peptone, which is a meager medium than BHI or TSB.

The traditional “one-factor at time” approach used for optimizing a multivariable system is not only time-consuming, but also may result in wrong conclusions by ignoring the interaction of various parameters (14). RSM used for optimization of BLS production by *Bacillus* sp. P34 indicated that the two variables have significant effects on antimicrobial substance production. The quadratic effect of pH was the more significant effect followed by the quadratic effect of temperature. When production of antimicrobial activity was inspected as a response of temperature and initial pH as variables, it was observed that there was an enhancement in production at the center point.

Krier *et al.* (19) showed that temperature and pH had a strong influence on the production of two bacteriocins by *Leuconostoc mesenteroides*. The production of

antimicrobial activity by *Lactobacillus curvatus* was affected by the manipulation of pH and temperature (20). In those cases, the conditions that caused relatively slow growth rates stimulated the production of antimicrobial substances. The low pH functioned as inducible factor and the optimum value for growth resulted in lower bacteriocin activity (21). The dependence of bacteriocin production on pH suggest that the expression of the biosynthetic genes may be regulated by pH, as has been observed previously for several classes of genes (22). Production of bacteriocin by *Bacillus licheniformis* was studied and response surface data showed maximum bacteriocin production at pH between 6.5 and 7.5 and temperature between 26 and 37 °C, but it was not possible to determine a relation between bacteriocin production and optimum bacterial growth (23). In agreement, Messens *et al*. (10) did not observe a relationship between optimum growth and bacteriocin production by *Lactobacillus curvatus*.

Response surface methodology proved to be a powerful tool in optimizing conditions for BLS production. Maximum antimicrobial activity was achieved at initial pH 7.0 and 30 °C, using BHI broth as cultivation medium. Like other bacteriocins, the antimicrobial peptide produced by *Bacillus* sp. P34 may also have a broad industrial application. Our results show that the production of this substance may be affected by environmental factors. Changes in environmental conditions are relevant when considering the potential biotechnological applications of these substances in food and when testing new isolates for bacteriocin production.

3.3.7 Acknowledgements

We thank Dr. S. Astolfi-Filho from Universidade Federal do Amazonas for isolate and Dr. S.H. Flôres from Universidade Federal do Rio Grande do Sul for helpful in RSM analysis. This work was supported by CAPES and CNPq, Brazil.

3.3.8 References

1. J. Cleveland, T.J. Montville, I.F. Nes, M.L. Chikindas, Bacteriocins: safe, natural antimicrobials for food preservation. *Int.J. Food Microbiol*. 71 (2001) 1-20.
2. M.V. Leal-Sánchez, R. Jiménez-Díaz, A. Maldonado-Barragán, A. Garrido-Fernández, J.L. Ruiz-Barba, Optimization of bacteriocin production by batch fermentation of *Lactobacillus plantarum* LPCO10. *Appl. Environ. Microbiol*. 68 (2002) 4465-4471.
3. L. De Vuyst, Nutritional factors affecting nisin production by *Lactococcus lactis* subsp.*lactis* NIZO 22186 in a synthetic medium. *J. Appl.Bacteriol*. 78 (1995) 28-33.
4. Z. Zalán, E. Németh, A. Baráth, A. Halasz, Influence of growth medium in hydrogen peroxide and bacteriocin production of *Lactobacillus* strains. *Food Technol*. *Biotechnol.* 43 (2005) 219-225.
5. D. Bizani, A. Brandelli, Influence of temperature and pH on bacteriocin production by *Bacillus cereus* 8A during batch cultivation. *Appl. Microbiol. Biotechnol*. 65 (2004) 158-162.
6. C. Li, J. Bai, Z. Cai, F. Ouyang, Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. *J. Biotechnol*. 93 (2002) 27-34.
7. A. Ismail, S. Soultani, M. Ghoul, Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology. *Biotechnol. Prog*. 14 (1998) 874-878.
8. L.P. Ooijkaas, E.C. Wilkinson, J. Tramper, R.M. Buitelaar, Medium optimization for spore production of *Coniothyrium minitans* using statistically-based experimental designs. *Biotechnol. Bioeng*. 64 (1999) 92-100.
9. K.R. Kiran, N.G. Karanth, S. Divakar, Preparation of stearoyl lactic acid ester catalyzed by lipases from *Rhizomucor miehei* and porcine pancreas optimization using response surface methodology. *Appl. Microbiol. Biotechnol*. 52 (1999) 579-584.
10. W. Messens, J. Verluyten, F. Leroy, L. De Vuyst, Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. *Int. J. Food Microbiol*. 81 (2003) 41-52.

11. A.S. Motta, F. Cladera-Olivera, A. Brandelli, Screening for antimicrobial activity among bacteria isolated from Amazon basin. *Braz. J. Microbiol*. 35 (2004) 307-310.
12. A.S. Motta, A. Brandelli, Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J. Appl. Microbiol*. 92 (2002) 63-71.
13. A. Mayr-Harting, A.J. Hedjes, C.W. Berkeley: Methods for studying bacteriocins. In: Methods in Microbiology, vol 7, J.B. Norris, D. Ribbons (Eds.), Academic Press, New York (1972) pp. 315-412.
14. R. Myers, R.C. Montgomery, Response surface methodology: process and product optimization using designed experiments. Wiley, New York (2002) pp.1-154.
15. E. Parente, A. Ricciardi, G. Addario, Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. *Appl. Microbiol. Biotechnol*. 41 (1994) 388-394.
16. L. De Vuyst, R. Callewaert, K. Crabbé, Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable conditions. *Microbiology* 142 (1996) 817-827.
17. A.S. Motta, A. Brandelli, Influence of growth conditions on bacteriocin production by *Brevibacterium linens*. *Appl. Microbiol. Biotechnol*. 62 (2003) 163-167.
18. W.S. Kim, R.J. Hal, N.W. Dunn, The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. *Appl. Microbiol. Biotechnol*. 50 (1997) 429-433.
19. F. Krier, A.M. Revol-Junelles, P. Germain, Influence of temperature and pH on production of two bacteriocins by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 during batch fermentation. *Appl. Microbiol. Biotechnol.* 50 (1998) 359-363.
20. M. Mataragas, J. Metaxopoulos, M. Galiotou, E.H. Drosinos, Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Meat Sci.* 64 (2003) 265-271.
21. F. Leroy, L. De Vuyst, Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. *Int. J. Food Microbiol*. 72 (2002) 155-164.
22. E.R. Olson, Influence of pH on bacterial gene expression. *Mol. Microbiol*. 8 (1993) 5-14.

23. F. Cladera-Oliveira, G.R. Caron, A. Brandelli, Bacteriocin production by *Bacillus licheniformis* P40 in cheese whey using response surface methodology. *Biochem. Eng. J*. 21 (2004) 53-58.

3.4 ARTIGO 4

3.4.1 Antibacterial activity of a bacteriocin-like substance produced by *Bacillus* sp. P34 that targets the bacterial cell envelope

3.4.2 Summary

BLS P34 is an antimicrobial substance produced by *Bacillus* sp. P34 strain isolated from Amazon basin. The mode of action of the BLS was investigated. The effect of the substance was tested against *Listeria monocytogenes*, showing a bactericidal effect at 200 AU ml^{-1}, while slow decrease of spore outgrowth of *Bacillus cereus* was observed with a dose of 1600 AU ml^{-1}. Growth of *Escherichia coli* and *Salmonella* Enteritidis was inhibited, but only when the chelating agent EDTA was co-added with the BLS. The effect of BLS P34 on *L. monocytogenes* was also investigated by Fourier transform infrared spectroscopy (FTIR). Treated cells showed an important frequency increase in 1452 and 1397 cm^{-1} and decrease in 1217 and 1058 cm^{-1}, corresponding assignments of fatty acids and phospholipids. Transmission electron microscopy showed damaged cell envelope and loss of protoplasmic material. Bacteriocin P34 was bactericidal to Gram-positive, and also showed inhibitory effect against Gram-negative bacteria. There is evidence that its mode of action is to interfere at cell membranes and the cell wall.

KEYWORS *Listeria monocytogenes*; bacteriocin; mode of action; infrared spectroscopy, electron microscopy

3.4.3 Introduction

Antibacterial peptides ribossomaly synthesized by bacteria are commonly referred as bacteriocins or bacteriocin-like substances (BLS). Most of those produced by Gram-positive bacteria are synthesized by lactic acid bacteria and have become a focus of interest as an alternative to classical antibiotics (Klaenhammer, 1993; Nes et al., 1996). Antimicrobial peptides which gained increasing interest, exerted in some cases a narrow, or in other cases, a relatively broad spectrum of antimicrobial activity (Atrih et al., 2001). Their proteinaceous nature implies a putative degradation in the gastro-intestinal tract of man and animals, suggesting that some bacteriocin-producing lactic acid bacteria or purified bacteriocins could be used as natural preservatives in food (Daeschel, 1989; Eckner, 1992). Various factors influence the bacteriocin activity on the target bacterial cell. They include the structure and amount of substance, the composition of the cytoplasmic membrane, the structure and the expression level of a protein with an immunity function and the chemical composition of the environment. Thus, the effective use of bacteriocins in food preservation requires the understanding of their mode of action and inhibitory action under different biochemical conditions naturally occurring in food (De Vuyst and Vandamme, 1994).

The large structural variability of the membrane-active peptides argues in favor of a variety of different mechanisms, also including unspecific modes of action. Most authors share the opinion that antibiotic peptides kill a target cell by permeabilization its cytoplasmic membrane (Heerklotz et al., 2004). One of the most important recent findings is that the bacteriocins need a target molecule at the surface of sensitive cells to be active (Héchard and Sahl, 2002). The lantibiotics nisin and epidermin do not merely interact with the phospholipids but target a specific integral component of the cytoplasmic membrane. The lipid-bound-peptidoglycan precursor, Lipid II, significantly facilitates pore formation. It may serve as a docking molecule for the peptides to bind to the membrane surface and to adopt the correct position for pore opening (Brötz and Sahl, 2000). The interaction of mersacidin with Lipid II seems to involve substantial portions of both molecules (Brötz et al., 1998).

Furthermore, the inhibition of the DNA biosynthesis resulting in cell death has also been proposed as a secondary mechanism of action for a number of antimicrobial peptides (Blondelle et al., 1999).

Different species of *Bacillus* produce bacteriocins or BLS with different modes of action. The tochicin (Paik et al., 1997), lichenin (Pattnaik et al., 2001) and thuricin 439 (Ahern et al., 2003) have a demonstrated bactericidal effect, and the inhibition by cerein 8A is most likely due to vesiculization of the protoplasm, pore formation and complete desintegration of the cells (Bizani et al., 2005). Liu and Hansen (1993) observed that molecular mechanism by which subtilin inhibits spore outgrowth is not the same as the mechanism by which it inhibits vegetative cells.

The present report describes the study of the effect and the mode of action of a BLS P34, produced by a new isolated *Bacillus* sp. P34. This work is a step towards exploring and investigate the mode of action of BLS P34 and to evaluate its potential use as antimicrobial to prevent the proliferation of *L. monocytogenes* and other microorganisms.

3.4.4 Materials and methods

3.4.4.1 Bacterial strains and media

The producer strain was kindly given by Universidade Federal of Amazonas (Manaus, Brazil). The organism was isolated from the intestinal contents of Piau-com-pinta (*Leporinus* sp.) of Amazon basin, near Manaus, Brazil. The isolate was identified as *Bacillus* sp. P34 and characterized as described elsewhere (Motta et al., 2004). The indicator strains were *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922 and *Salmonella* Enteritidis ATCC 13076. Brain heart infusion (BHI) medium (Oxoid, Basingstoke) was used for maintenance of strains with 20% (v/v) glycerol at –20°C. The cultivation of strains was performed aerobically.

3.4.4.2 Antimicrobial peptide

For the production of antimicrobial peptide, *Bacillus* sp. P34 was grown in 200 ml BHI-medium at 30°C in a rotary shaker at 180 cycles min^{-1} for 24 h. The cells were

harvested by centrifugation at 10.000 x g for 15 min and the culture supernatant fluid was sterilized by filtration with membranes 0.22 μm (Millipore). Antimicrobial activity was further purified by precipitation with ammonium sulfate at 20% saturation. The pellet was ressuspended in sodium phosphate buffer 10 mM pH 7.0 and submitted to gel filtration chromatography using a Sephadex G-100 column. The active fractions were pooled and stored in sterile flasks at 4°C until used for antimicrobial assay.

3.4.4.3 Antimicrobial activity assay

Antimicrobial activity was determined essentially as described elsewhere (Motta and Brandelli, 2002). An aliquot of 20 μl partially purified antimicrobial substance was applied on discs (6mm) on BHI agar plates previously inoculated with a *swab* submerged in indicator strain suspension which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37°C for 24 h. The antimicrobial activity titre was determined by the serial twofold dilution method previously described by Mayr-Harting et al. (1972). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and was expressed as arbitrary unit (AU) per mililitre.

3.4.4.4 Effect of BLS on *Listeria monocytogenes*

A culture of *L. monocytogenes* with 10^6cfu ml^{-1} was obtained by growing in BHI medium at 37°C. The indicator strain was inoculated with different concentrations of partially purified antimicrobial substance (1 to 1600 AU ml^{-1}). Its effect on growth was monitored after 2 h by viable cells counts (cfu ml^{-1}). The experiment was realized in triplicate.

3.4.4.5 Kinetic of action of antimicrobial substance

L. monocytogenes in BHI broth at 10^6cfu ml^{-1} was inoculated with 800 AU ml^{-1} of partially purified antimicrobial substance. Growth was monitored at different intervals by determining of cfu ml^{-1} in 2, 4, 10, 20, 30, 40, 50, 60, 75, 90 e 105 min of incubation at 37°C. The control was taken with addition of sodium phosphate buffer 10 mM pH 7,0. The experiment was realized in triplicate.

3.4.4.6 Effect against Gram-negative bacteria

The effect of antimicrobial substance on gram-negative bacteria (*E. coli* and *S.* Enteritidis) was evaluated at a concentration of 800 AU ml^{-1} for 2 h. To evaluate the effect of chelator on bacteriocin activity, the addition of 20 mM disodium EDTA (Riedel, Hannover) was added. A control with EDTA alone was done.

3.4.4.7 Effect on *Bacillus cereus* spores

Spore production by *B. cereus* was carried out on TSB agar for 7 days. After incubation at 37°C for 1 week samples were harvested, ressuspended in sterile MilliQ water and was treated at 80°C for 10 min to kill vegetative cells. Aliquots were diluted with sterile water to the initial concentration of 10^4 at 10^6 spores ml^{-1}. After centrifugation (8000 g for 10 min) the pellet was ressuspended with antimicrobial substance at concentration of 1600 AU ml^{-1} and was incubated for 2 h and the cfu ml^{-1} was determined.

3.4.4.8 Release of UV-absorbing material

Leakage of ultraviolet (UV) light-absorbing material was used as indicator of the loss of cell membrane integrity. *L. monocytogenes* cell suspension, which corresponded to a 0.5 McFarland turbidity standard solution in phosphate buffer (10 mM pH 7.0), was mixed with bacteriocin (800 AU ml^{-1}) and incubated at 37°C. Sample was removed after 3 h and filtered through 0.22 μm pore size sterile membrane (Millipore)

The absorbance of the filtrates was measured at 260 and 280 nm using a Ultrospec 3100 spectrophotometer (Amersham Pharmacia).

3.4.4.9 Fourier Transform Infrared (FTIR) spectroscopy

Antimicrobial substance (400 AU ml^{-1}) was added to cell suspension of about 10^6cfu ml^{-1} of *L. monocytogenes*. After incubation for 60 min, both treated and control cells were washed three times with MilliQ water. Ten microliter of each bacterial sample was evenly applied onto a ZnSe optical plate, dried for approximately 5 min under vacuum, and then analyzed by FTIR spectroscopy. The curves represent the average of three individual measurements of the same experiments. All IR spectra (4000-650 cm^{-1}) were obtained by

the attenuated total reflection (ATR) technique, using a Perkin-Elmer Spectrum One spectrometer (Perkin Elmer, Uberlingen, Germany) with a horizontal ATR device (Se, 45°). The resolution was 2 cm^{-1} and at least 4 scans were co-added for each sample. Reproducibility of the normalized spectra was ± 2%.

3.4.4.10 Transmission electron microscopy

Samples were taken from exponentially growing cultures (10^6 cfu ml^{-1}) of *L. monocytogenes* treated and non-treated with antimicrobial substance (800 AU ml^{-1}). Cells were harvested by centrifugation and washed twice and 0,1 M phosphate buffered (pH 7.3). The cells were fixed with 2.5% (v/v) glutaraldehyde for 10 d; (2.5% glutaraldehyde + 2% paraformaldehyde + 0.12 M phosphate buffer) and then posfixed in 2% (w/v) osmium tetroxido in the same buffer for 45 min before dehydration. Dehydration was done in a graded acetone series (30-100%) and embedding in Araldite-Durcupan for 72 h at 60°C. Thin sections (Ultramicrotomo UPC 2.0, Leica) were mounted on grids, covered with collodion film, and poststained with 2% uranyl acetate and Reynold's lead citrate. All preparations were observed with a JEOL JEM 1200ExII transmission electron microscope (JEOL, Tokyo) operating at 120 kV.

3.4.5 Results

3.4.5.1 Effect on *Listeria monocytogenes*

The effect of bacteriocin P34 concentration on the survival of *L. monocytogenes* was observed. The number of viable cells was reduced as increased the concentration of the antimicrobial substance. Complete inhibition of growth was observed from 200 AU ml^{-1} to 1600 AU ml^{-1} in 2 h of incubation with antimicrobial substance (Fig.1).

The kinetics of the bacteriocin effect on growth of *L. monocytogenes* is shown in Fig. 2. When the concentration of 800 AU ml^{-1} was tested, the number of viable cells of *L. monocytogenes* decreased in 10 min with complete inhibition of growth until 105 min of incubation. The inhibition of *L. monocytogenes* growth resulted in decreased OD_{600} during incubation time.

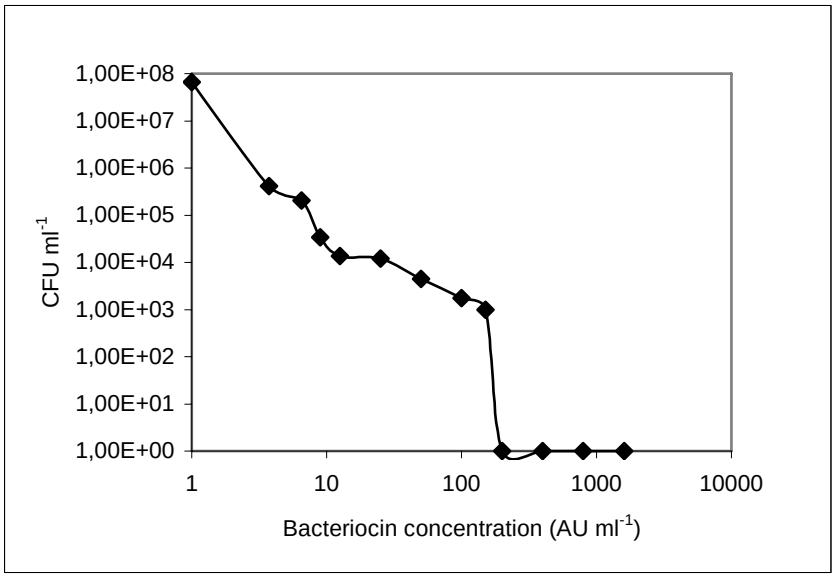

Fig. 1. Effect of BLS P34 concentration on the survival of *Listeria monocytogenes*. Bacterial cells were incubated for 60 min in the presence of increasing concentrations of BLS and then viable cells were counted. Each point represents the mean of three independent experiments.

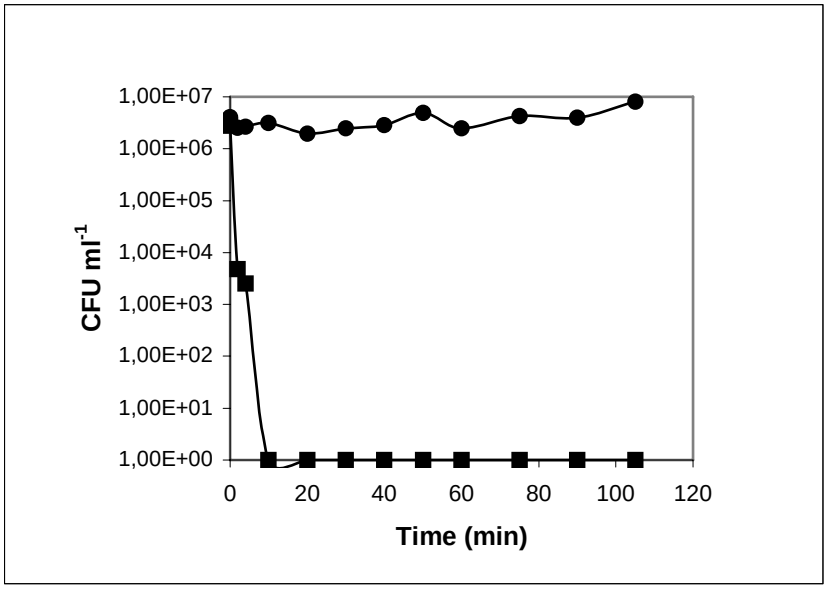

Fig. 2. Effect of BLS P34 on the growth of *Listeria monocytogenes* incubated for 105 min. Control (circles) and treated (squares) cells using a final concentration of 800 AU ml^{-1}. Each point represents the mean of three independent experiments.

3.4.5.2 Effect on Gram-negative bacteria

The addition of BLS plus EDTA to cell suspensions of either *Salmonella* Enteritidis or *Escherichia coli* was tested. When the BLS P34 was co-added with EDTA the viable cell counts were lower than those of the controls without BLS or EDTA (Fig. 3). This inhibitory effect was observed for both strains. The BLS or EDTA alone had no inhibitory effect on the Gram-negative bacteria tested (Fig. 3).

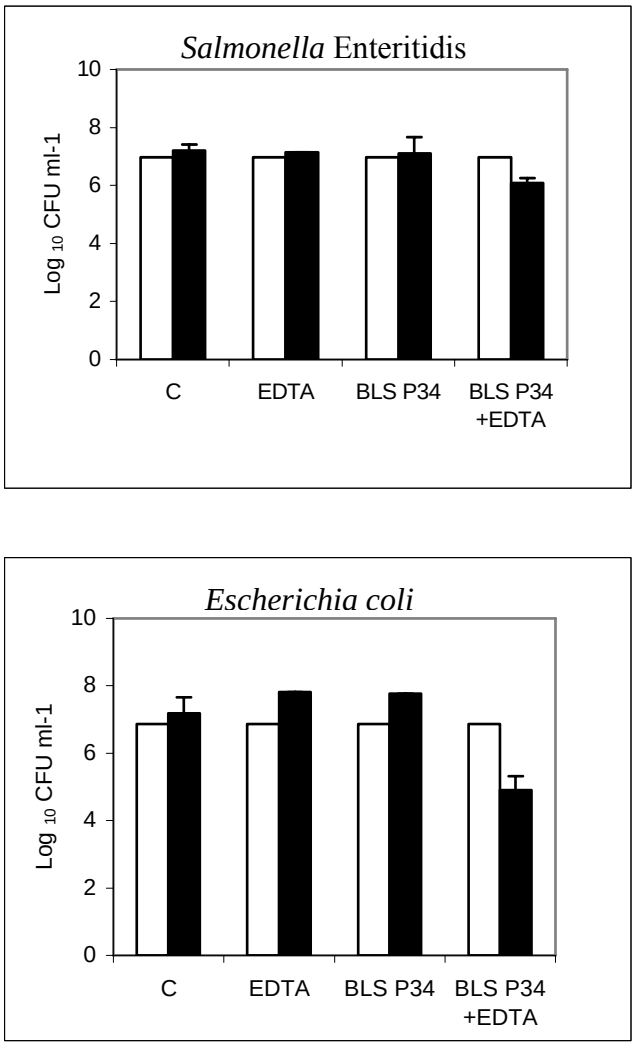

Fig. 3. Effect of BLS P34 on Gram-negative bacteria. The viability of *Salmonella* Enteritidis and *Escherichia coli* was monitored after treatment with 800 AU ml^{-1} BLS, 20 mM EDTA or BLS plus EDTA. Each point represents the mean of three independent experiments.

3.4.5.3 Effect on spore outgrowth

The effect of BLS P34 on spore outgrowth of *Bacillus cereus* was investigated. No inhibition of spore outgrowth was observed with the BLS concentration tested.

3.4.5.4 Release of cellular materials

The effect of 800 AU ml^{-1} of BLS P34 on the integrity of cell membranes of *L. monocytogenes* was measured using different parameters. The release of UV-absorbing molecules by treatment with the antimicrobial substance was monitored at 260 and 280 nm. Treatment of *L. monocytogenes* cells with BLS caused a leakage of UV-absorbing materials measured at both 260 and 280 nm (Table 1).

Table 1. Release of intracellular UV-absorbing materials of *Listeria monocytogenes*

	Control	Bacteriocin-treated
Nucleic acids ($A_{260\ nm}$)	0.004 ± 0,003	0.188 ± 0,043
Proteins ($A_{280\ nm}$)	0.021 ± 0,019	0.340 ± 0,043

3.4.5.5 Infrared spectroscopy

The effect of BLS P34 on structural macromolecules of *L. monocytogenes* was analyzed by FTIR spectroscopy. The possible assignments of the individual bands are listed in Table 2.

Treated cells of *L. monocytogenes* showed an important frequency increase at 1452 and 1397 cm^{-1} and a decrease at 1217 and 1058 cm^{-1} (Fig. 4). Smaller differences were observed in the range 1300-1350 cm^{-1}. The carbon-hydrogen stretching vibrations of the lipid acyl chains gave rise to bands in the spectral region 3100-2800 cm^{-1} (Fig. 5). The strongest bands corresponded to the CH_2 antisymmetric and symmetric stretching modes at around 2960-2920 and 2850 cm^{-1}. The addition of BLS shifted both the antisymmetric and symmetric bands maxima to lower values, and displaced the maximum from 2870 to 2840 cm^{-1}.

Table 2. Fourier transformed infrared (FTIR) absorption bands

Frequency (cm^{-1})	Possible assignment*
3280	H-bonded OH groups, NH_2 stretching
2960-2920	Aliphatic C-H stretching (fatty acids)
1660-1535	NH_2 bending, C=O, C=N stretching (amide I and II)
1450	C-H deformation in aliphatics
1400	C=O stretching (symmetric) of COO^-
1398-1390	C-H bending, $-CH_3$ stretching (fatty acids)
1310-1240	C-N stretching (amide III)
1250-1220	P=O stretching antisymmetric $>PO_2^-$
1200-1000	C-O, PO_2^-, C-C stretching, C-O-C deformation (glycopeptides, phosphodiester, polysaccharides)

* Assignments according Rodriguez (2000); Maquelin et al. (2002).

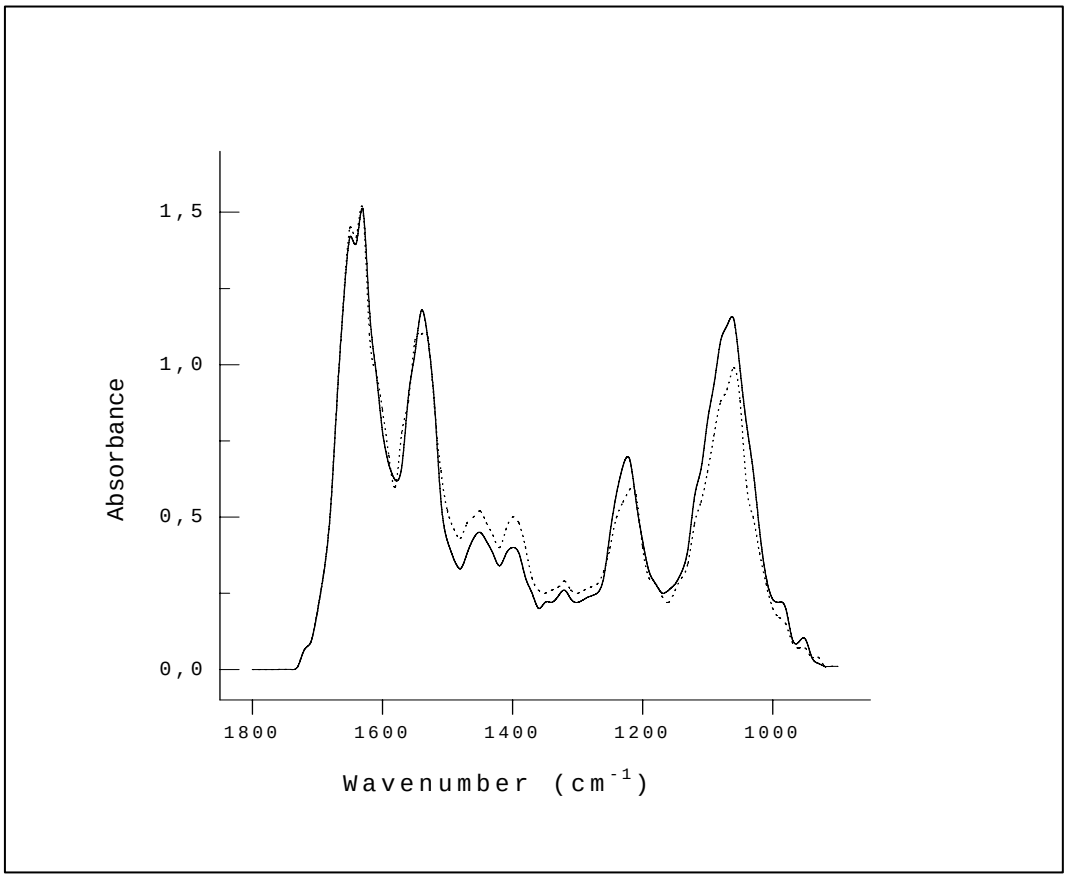

Fig. 4. Fourier transformed infrared (FTIR) spectroscopy spectra (1800-1000 cm^{-1}) of cell biomass of *Listeria monocytogenes*. Cell suspensions of *L. monocytogenes* were treated with 800 AU ml^{-1} of BLS for 60 min, then washed and dried onto a ZnSe optical plate. The infrared spectra of treated (dashed line) or untreated (solid line) biomass were recorded using the ATR method.

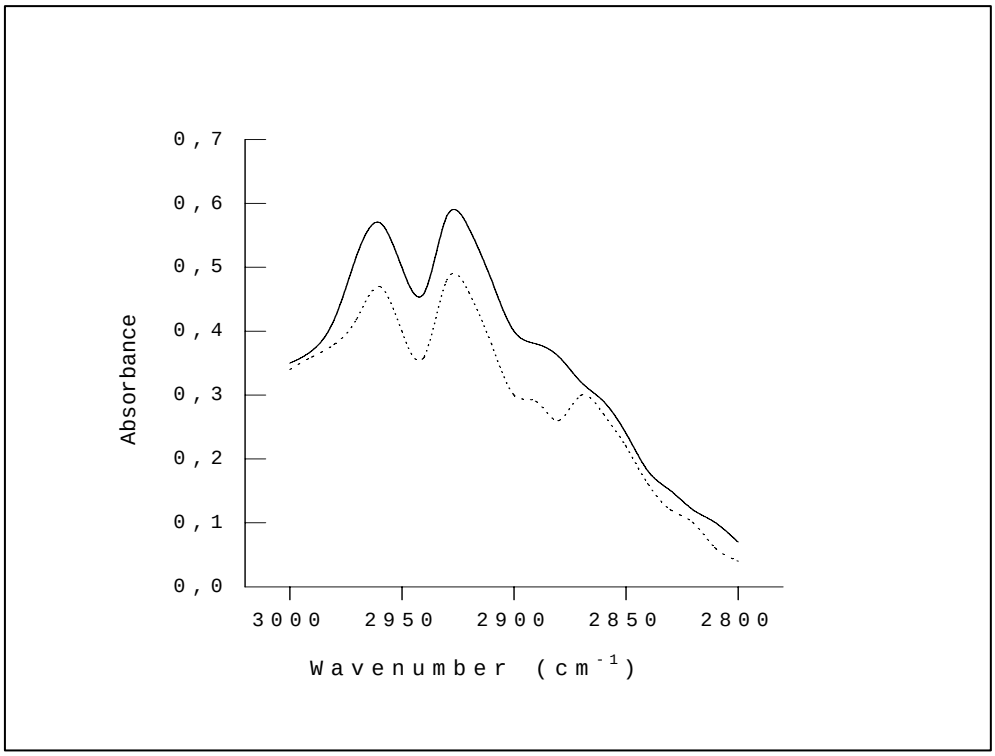

Fig. 5. Fourier transformed infrared (FTIR) spectroscopy spectra (3000-2800 cm^{-1}) of cell biomass of *Listeria monocytogenes*. Cell suspensions of *L. monocytogenes* were treated with 800 AU ml^{-1} of BLS for 60 min, then washed and dried onto a ZnSe optical plate. The infrared spectra of treated (dashed line) or untreated (solid line) biomass were recorded using the ATR method.

3.4.5.6 Transmission electron microscopy

L. monocytogenes harvested from culture an early-stationary-phase culture (10^6cfu ml^{-1}) were incubated with 800 AU ml^{-1} of BLS P34 for 2 h. After incubation, the microorganisms were prepared for transmission electron microscopy. *L. monocytogenes* cells treated with BLS P34 showed vesiculization of the protoplasm, pore formation and desintegration of the cells (Fig. 6).

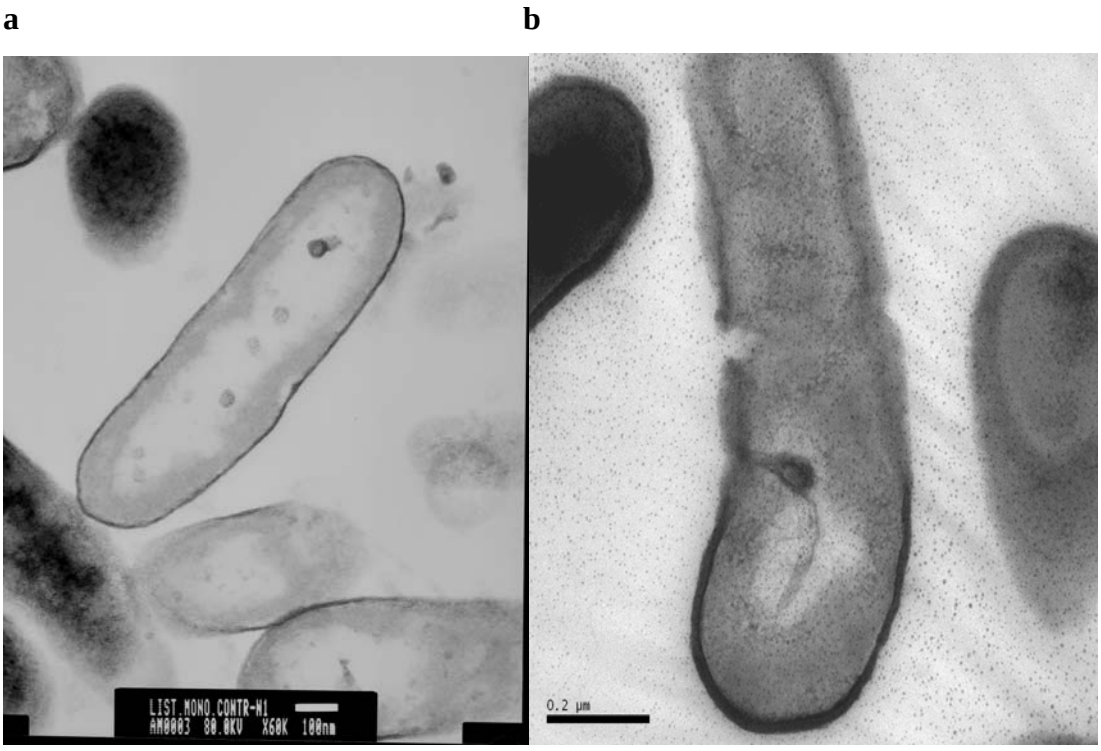

Fig. 6. Transmission electron microscopy of cells of *Listeria monocytogenes:* control cells (a) and after treatment with 800 AU ml^{-1} of BLS P34 for 2 h (b).

3.4.6 Discussion

It is well documented that different *Bacillus* species are able to produce substances with antimicrobial activity against a wide variety of microorganisms. Many of them are indicated as potential biopreservatives in food systems and beverages (Zheng and Slavic, 1999), as biological control agents against phytopathogens, and as antibiotic producers (Zuber et al., 1993). BLS P34 is an antimicrobial peptide produced by a novel species of *Bacillus* P34. In this study, the effect and the mode of action of BLS P34 on indicator bacteria was addressed, particularly using *L. monocytogenes* as the target organism.

The study on the mode of inhibition of the BLS P34 on *L. monocytogenes* revealed that this substance has a bactericidal effect, based on the observed decline in the number of the living cells of indicator strain within 2 h after the addition of antimicrobial substance. The rate of both biomass (measured as OD_{600}) and the viable cell accumulation was immediately affected by the presence of BLS P34 in exponentially growing cultures.

BLS P34 effectively inhibited the growth of *L. monocytogenes,* which have been already detected after 10 min of treatment. This effect is dependent on the antimicrobial substance concentration present in the culture medium. In this context, some authors suggest that bacteriocin-induced lysis could be due to the liberation of autolytic enzymes, that are usually electrostatically bound to anionic polymers (teichoic and lipoteichoic acids) of the cell wall, that are displaced by cationic bacteriocins from their binding sites (Bierbaum and Sahl, 1987, Jack et al., 1995).

Gram-negative bacteria are not generally sensitive to most bacteriocins. However, EDTA is thought to chelate magnesium ions from the lipopolysaccharide (LPS) layer of the outer membrane of Gram-negative bacteria. It has been suggested that alterations in the LPS layer may cause the outer cell surface to become permeable, thereby allowing the bacteriocins access to the cytoplamic membrane where bacteriocin-mediate inactivation occurs (Cutter and Siragusa, 1995). *Escherichia coli* and *Salmonella* Enteritidis were not inhibited by BLS P34 alone. However, the number of viable cells decreased when EDTA was tested in combination with BLS, indicating a synergistic effect. In this case, inhibition is most likely due to weakening of the outer plasma membrane following sequestration of magnesium ions by the chelating agent (Montville and Bruno, 1994). Cutter and Siragusa (1995) observed that nisin is effective for reducing populations of Gram-negative bacteria

when used in combination with chelating agents such as EDTA, citrate and phosphate. Inactivation of *Salmonella* species and other Gram-negative bacteria in applications involving the simultaneous treatment with nisin and chelating agents was also observed by Stevens et al. (1991).

The BLS P34 caused no inhibition of spore outgrowth nor sporecidal activity in the concentrations tested. The ability to make spores more sensitive to antimicrobial agents has been investigated by other researchers. Data suggest that bacteriocins may have synergistic effect when used in association with pressurization, heat treatment and low pH. Thermally injured spores become more sensitive to bacteriocins (Roberts and Hoover, 1996; Faille et al., 2002).

The fact that subtilin inhibits spore outgrowth at a very low concentration (80 nM) by a mechanism that involves a critical (Dha-5) residue argues that subtilin is highly evolved to perform this specific function and hence that this is a role that was optimized by natural selection. In contrast, the amount of subtilin required to lyse vegetative cells is about 30-fold higher than this, is independent of an intact Dha-5 residue, and hence could be a fortuitous consequence of the structural features that enable subtilin to inhibit spore outgrowth. This would help explain why subtilin and other lantibiotics encompass such broad spectra of action (Liu and Hansen, 1993).

The exact mode of action of antimicrobial peptides on Gram-positive bacteria is not completely elucidated. However, it has been proposed and is widely believed that the peptides interact with and disrupt the cytoplasmic membrane, leading to the dissolution of the proton motive force and leakage the essential molecules, resulting in the cell death. The view that BLS P34 was bactericidal and bacteriolytic is consistent with the fact that it damaged the cell membrane of *L. monocytogenes*. Indeed, release of UV-absorbing materials was detected after treatment of cells with the BLS.

FTIR spectroscopy of *L. monocytogenes* treated with BLS P34 revealed major changes in assignments for phospholipids and fatty acids, suggesting that the cell membrane is the target to the antimicrobial substance. FTIR was used to get further insight into the molecular mechanism by which BLS P34 is able to destabilize lipid membranes. This technique allowed us to know in which way the bacteriocin phospholipid interaction may alter membrane integrity, by observing effects both at the level of the polar head

groups (C=O stretching) and in the region of the phospholipid acyl chains (C-H stretching). The pronounced effect caused by BLS addition in the antisymmetric and symmetric stretching bands indicate a strong interaction with the phospholipid acyl chains, resulting a net fluidizing effect of the apolar part of the bilayer (Casal and Mantsch 1984).

FTIR spectroscopy was also useful to determine the degree of hydration of the phospholipid-water interface membrane bilayers in the presence of BLS P34. The antisymmetric PO_2^- double bond stretching band has been used to monitor the hydration state of the polar head group of the phospholipids (Casal et al. 1987; Ortiz et al. 1992). Phosphate bands showing maximum frequencies corresponding to 1220 cm^{-1} and 1200 cm^{-1} characterize mono- and dihydrated PO_2^- groups, respectively, whereas its dehydrated PO_2^- groups appear at a higher wavenumber (approximately 1235–1240 cm^{-1}). The antisymmetric PO_2^- stretching band of *L. monocytogenes* biomass presented a maximum corresponding to a partially hydrated phosphate group, i.e., 1217 cm^{-1} (Fig. 5). The spectra of the antisymmetric PO_2^- stretching of BLS-treated cell biomass presented a very similar profile with a maximum located at 1210 cm^{-1}, indicating a similar proportion of hydrated and dehydrated phosphate groups.

The peptide antibiotic surfactin exerts its strong biological activities mainly by altering membrane integrity, as a consequence of the establishment of the strong interactions with the phospholipid membrane constituents and altering membrane permeability leading to the loss of the internal vesicle contents (Carrilo et al., 2003).

Electron microscopy showed that the effect on *L. monocytogenes* must be regarded as indicative of cytoplasmic membrane alteration induced by BLS P34, since untreated cells were not injured. Furthermore, since the cytoplasmic membrane is cooperative in the cell wall synthesis and turnover, perturbation of this membrane may also affect cell wall integrity and autolysis regulation (Kemper et al., 1993).

All the membrane effects of BLS P34 described in this work suggest it acts through desestabilization of the lipid packing, or pore formation as some authors have proposed. Recently, it has been shown that certain types of cyclic peptide can kill Gram-positive and Gram-negative bacteria by forming self-assembled pores in lipid membranes (Fernandéz-Lopes, 2001). There is good evidence from our study that the membrane barrier properties are likely to be damaged in the domains where BLS P34 interact with phospholipids. This

will cause structural fluctuations that may well be the primary mode of the antibiotic action and the other important biological effects of this peptide. This type of peptides, that act rapidly on membrane integrity rather than on other vital process might perhaps constitute the next generation of antibiotics.

3.4.7 Acknowledgements

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3.4.8 References

Ahern, M., Verschueren, S., van Sinderen, D., 2003. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiol. Lett. 220, 127-131.

Atrih, A., Rekhif, N., Moir, A.J.G., Lebrihi, A., Lefebvre, G., 2001. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. Int. J. Food Microbiol. 68, 93-104.

Bierbaum, G., Sahl, H.G., 1987. Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. J. Bacteriol. 169, 5452-5458.

Bizani, D., Motta, A.S., Morrissy, J.A.C., Terra, M.S.T., Souto, A.A., Brandelli, A., 2005. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. Int. Microbiol. 8, 125-131.

Blondelle, S.E., Lohner, K., Aguilar, M.I., 1999. Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity. Biochim. Biophys. Acta 1462, 89-108.

Brötz, H., Bierbaum, G., Leopold, K., Reynolds, P.E., Sahl, H.G., 1998. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob. Agents Chemother. 42, 154-160.

Brötz, H., Sahl, H.G., 2000. New insights into the mechanism of action of lantibiotics-diverse biological effects by binding to the same molecular target. J. Antimicrob. Chemother. 46, 1-6.

Carrilo, C., Teruel, J.A., Aranda, F.J., Ortiz, A., 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim. Biophys. Acta 1611, 91-97.

Casal, H.L., Mantsch, H.H., 1984. Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy. Biochim. Biophys. Acta 774, 381-401.

Casal, H.L., Mantsch, H.H., Hauser, H., 1987. Infrared studies of fully hydrated saturated phosphatidylserine bilayers. Effect of Li+ and Ca2+. Biochemistry 26, 4408-4416.

Cutter, C.N., Siragusa, G.R., 1995. Population reductions of gram-negative pathogens following treatments with nisin and chelators under various conditions. J. Food Prot. 58, 9, 977-983.

Daeschel, M., 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43, 164-167.

De Vuyst, L., Vandamme, E.J., 1994. Lactic acid bacteria and bacteriocins: their pratical importance. In: De Vuyst, L., Vandamme, E.J., (Eds.), Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications. Blackie Academic and Professional, London, pp. 1-11.

Eckner, F.K., 1992. Bacteriocins and food application. Dairy Food Environ. Sanit. 12, 204-209.

Faille, C., Membre, J.M., Kubaczka, M., Gavini, F., 2002. Altered ability of *Bacillus cereus* spores to grow under unfavorable condition (presence of nisin, low temperature, acidic pH, presence of NaCl) following heat treatment during sporulation. J. Food Prot. 65, 12, 1930-1936.

Fernandéz-Lopes, S., Kim, H.S., Choi, E.C., Delgado, M., Granja, J.R., Khasanov, A., Kraehenbuehl, A., Long, G., Weinberger, D.A., Wilcoxen, K.M., Ghadiri, M.R., 2001. Antibacterial agents based on the cyclic D,L-α-peptide architecture. Nature 412, 452-455.

Héchard, Y., Sahl, H.G., 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84, 545-557.

Heerklotz, H., Wieprecht, T., Seelig, J., 2004. Membrane perturbation by the lipopeptide surfactin and detergents as studied by deuterium NMR. J. Phys. Chem. B. 108, 4909-4915.

Jack, R.W., Tagg, J.R., Ray, B., 1995. Bacteriocins of Gram-positive bacteria. Microbiol. Rev. 59, 171-200.

Kemper, M.A., Urrotia, M.M., Beveridge, T.J., Koch, A.L., Doyle, R.J., 1993. Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. J. Bacteriol. 175, 5690-569.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39-85.

Liu, W., Hansen, J.N., 1993. The antimicrobial effect of a structural variant of subtilin against outgrowing *Bacillus cereus* T spores and vegetative cells occurs by different mechanism. Appl. Environ. Microbiol. 59, 648-651.

Maquelin, K., Kirschner, C., Choo-Smith, L.P., van de Braak, N., Endtz, H.P., Naumann, D., Puppels, G.J. 2002. Identification of medically relevant microorganisms by vibrational spectroscopy. J. Microbiol. Meth. 51, 255-271.

Mayr-Harting, A., Hedjes, A.J., Berkeley, C.W., 1972. Methods for studying bacteriocins. In: Norris, J.R., Ribbons, D., (Eds), Methods in Microbiology, vol.7. Academic Press, New York, pp. 315-412.

Montville, T.J., Bruno, M.E.C., 1994. Evidence that dissipation of proton motive force is a common mechanism of action for bateriocins and other antimicrobial proteins. Int. J. Food Microbiol. 24, 53-74.

Motta, A.S., Brandelli, A., 2002. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. J. Appl. Microbiol. 92,63-71.

Motta, A.S., Cladera-Olivera, F., Brandelli, A., 2004. Screening for antimicrobial activity among bacteria isolated from Amazon basin. Braz. J. Microbiol. 35, 307-310, 2004.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., Holo, H., 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70, 113-128.

Ortiz, A., Aranda, F.J., Villalaín, J., San Martin, C., Micol, V., Gómez-Fernández, J.C., 1992. 1,2-Dioleoylglycerol promotes calcium-induced fusion in phospholipid vesicles. Chem. Phys. Lipids 62, 215-224.

Paik, H.D., Bae, S.S., Park, S.H., Pan, J.G., 1997. Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thurigiensis* subsp. *tochigiensis.* J. Indust. Microbiol. Biotechnol. 19, 294-298.

Pattnaik, P., Kaushik, J.K., Grover, S., Batish, V.K., 2001. Purification and characterization of a bacteriocin-like compound (lichenin) produced anaerobically by *Bacillus licheniformes* isolated from water buffalo. J. Appl. Microbiol. 91, 636-645.

Roberts, C.M., Hoover, D.G., 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J. Appl. Bacteriol. 81, 363-369.

Rodriguez, M.P.Q. 2000. Fourier transform infrared (FTIR) technology for the identification of microorganisms. Clin. Microbiol. New. 22, 57-61.

Stevens, K.A., Sheldon, B.W., Klapes, N.A., Klaenhammer, T.R., 1991. Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. Appl. Environ. Microbiol. 57, 12, 3613-3615.

Zheng, G., Slavic, M.F., 1999. Isolation, purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. Lett. Appl. Microbiol. 28, 363-367.

Zuber, P., Nakano, M.M., Marahiel, M.A., 1993. Peptide antibiotics. In: Sonenshein, A.L., Hoch, J.A., Losick, R., (Eds), *Bacillus subtilis* and other Gram-positive Bacteria. American Society for Microbiology, Washington, pp. 897-916.

3.5 ARTIGO 5

3.5.1 Purification and partial characterization of an antimicrobial peptide produced by a novel *Bacillus* sp. strain isolated from Amazon basin

3.5.2 Abstract

An antimicrobial peptide-producing strain P34 was isolated from Amazon basin and identified as a new *Bacillus* species. The corresponding antimicrobial peptide was purified by ammonium sulfate precipitation, gel filtration and ion exchange chromatography, and after the final purification step, one active fraction was obtained, designated as BLS P34. Direct activity on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was observed. A single band on SDS-PAGE suggested that the peptide was purified at homogeneity and had a molecular mass less than 5 kDa. The native protein eluted at the void volume of Sephadex G-100, but within the included volume when a 1.5 M NaCl buffer was used, indicating that BLS P34 aggregates extracellularly. The molecular weight (MW) was determined by mass spectroscopy as 1498.68 Da. The purified BLS P34 remained active over a wide range temperature and was susceptible to proteases tested.

Key words: antimicrobial, *Bacillus*, bacteriocin, peptide

3.5.3 Introduction

Bacteriocins are ribosomally synthesized antimicrobial polypeptides that are usually inhibitory only to closely related strains to the producing bacteria. These antimicrobial compounds are thought to provide the producer strain with the selective advantage over others strains. Bacteriocins produced by gram-positive bacteria are often membrane permeabilizing cationic peptides with fewer than 60 amino acid residues [9].

In recent decades, the major advances in bacteriocin research have been made in the lactic acid bacterium (LAB) family, due to eminent economic importance of these microorganisms. Many bacteriocin producing LAB are present in a variety of naturally fermented food and feed products has led to a great interest in the potential of these bacteria as biopreservatives [26].

The vast number of bacteriocins described so far allows different classifications of these compounds. The classification of bacteriocins put forward by [13] takes into account the chemical structure, heat stability, molecular mass, enzymatic sensitivity, presence of modified amino acids and mode of action of these chemicals. Four classes of bacteriocins can be distinguished. I) Lanthibiotics containing the modified amino acids lanthionine, and nisin [8], epidermin [1] belong to this group. II) Low-MW bacteriocins (smaller that 10 kDa) formed exclusively by unmodified amino acids. Within this group, specific antilisterial compounds, bacteriocins formed by two peptides acting synergistically and thiol-activated peptides can be found. III) High-MW bacteriocins: heat labile proteins larger than 30 kDa. IV) Bacteriocins carrying lipid or carbohydrate moieties.

The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for food biopreservation but also in other areas, especially for therapeutic applications and petroleum industry (biosurfactants) [3].

The genus *Bacillus* includes a variety of industrially important species and has a history of safe use in both food and industry. The products in commerce today that are produced by *Bacillus* fermentation include enzymes, antibiotics, amino acids and inseticides. Many bacteriocins or bacteriocin-like substance (BLS) in the genus *Bacillus* have been reported; with cerein 8A of *Bacillus cereus* [2], subtilosin A and surfactin of

Bacillus subtilis [25]. The potential of *Bacillus* species to produce antibiotics has been recognized for 50 years, and peptides antibiotics represent the predominat class.

We have screened a number of bacterial cultures obtained from Amazon basin for production of antimicrobial substances [18]. The microorganism *Bacillus* sp. P34 was isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.) of Amazon basin and produces an antimicrobial peptide, which inhibit *Listeria monocytogenes*, an important food-borne pathogen. This paper describes a purification procedure and some properties of BLS P34.

3.5.4 Materials and Methods

3.5.4.1 Bacterial strains and media

The producer strain was kindly given by Universidade Federal of Amazonas (Manaus, Brazil). The isolate was identified as *Bacillus* sp. P34 and characterized as described elsewhere [18]. The indicator strain was *Listeria monocytogenes* ATCC 7644. Brain heart infusion (BHI) medium (Oxoid, Basingstoke) was used for maintenance of strains with 20% (v/v) glycerol at –20°C. The cultivation of strains was performed aerobically.

3.5.4.2 Detection of antimicrobial activity

Antimicrobial activity was determined essentially as described elsewhere [17]. An aliquot of 20 µl antimicrobial substance was applied on discs (6mm) on TSB agar plates previously inoculated with a *swab* submerged in indicator strain suspension which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37°C for 24 h. The antimicrobial activity titre was determined by the serial twofold dilution method previously described by [16]. Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as arbitrary units per milliliter (AU ml^{-1}). The haemolytic activity was determined on sheep blood agar plates. A isolate of *Staphylococcus aureus* with known haemolytic activity was used as positive control.

3.5.4.3 Purification protocol

Bacillus sp. was cultivated aerobically in 500 ml Erlenmeyer flasks containing 200 ml of TSB broth at 30°C, 180 cycles min^{-1} for 24 h. Cells were harvested by centrifugation at 10,000 x g for 15 min at 12°C, and the resulting supernatant was filtered through 0.22 μm membranes (Millipore, Bedford, MA, USA). The cell free culture filtrate was submitted to precipitation with ammonium sulfate to 20% saturation. The resulting pellet was ressuspended in 10 mM sodium phosphate buffer pH 7.0 and applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala) and eluted with 10 mM sodium phosphate buffer pH 7.0. Fractions positive for antimicrobial activity were pooled and applied to a column of DEAE-Sepharose (Pharmacia Biotech, Uppsala, Sweden), eluted with this same buffer followed by a gradient from 0 to 1.5 M NaCl. The active peaks were dialyzed and rechromatographed according to the same process. Fractions were monitored for A280 nm using an EM-1 EconoUV monitor (Bio-Rad Laboratories, Hercules, CA, USA).

The determination of soluble protein was realized by the Folin phenol reagent method [14] with bovine serum albumin as standard.

3.5.4.4 Direct detection on gels

Aliquots of purified BLS P34 were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were suspended in 125 mM tris pH 6.8 containing 0.1% SDS, 20% glycerol, and then applied to 14% polyacrylamide gels. Electrophoresis was carried out as described elsewhere [6] using a Mighty Small II apparatus (Hoefer Scientific, San Francisco, CA, USA) and 20 mA per gel. After running the gels were washed with sterile distilled water for removal of SDS and then flooded on plates containing BHI agar with 10^6 CFU ml^{-1} *L. monocytogenes*. Detection of antimicrobial activity was developed as described by [19]. Plates were incubated for 24 h at 37°C and the presence of clearing zones was observed. Other gels were stained with Commassie blue to observe peptide bands [20]. MW standards were from Sigma (St. Louis, MO, USA).

3.5.4.5 Effects of enzymes and heat on bacteriocin activity

Samples of purified bacteriocin were treated at 37°C for 1 h with 2 mg ml^{-1} final concentration of the following enzymes: trypsin, papain, pronase E and proteinase K. Samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples of bacteriocin were exposed to temperatures ranging to 40 to 90°C for 30 min, 100°C for 5, 10, 15, 20, 30, 40, 50 and 60 min and 121°C/ 15 lb in^{-2} for 15 min. Samples also exposed to temperatures of refrigeration for 10 d, freeze for 15 d and freeze-dried. After the treatments, the samples were tested for antimicrobial activity against *L. monocytogenes*.

3.5.4.6 Mass spectroscopy

A sample of purified BLS P34 was dialyzed against MilliQ water and freeze-dried. This material was dissolved in 0.046% trifluoroacetic acid and applied to a C18 chromatographic resin (Vydac, USA). The column was eluted with 80% acetonitrile 0.046% TFA and concentrated in a vacuum centrifuge (SpeedVac SC100, Savant, USA). The sample was analyzed in a MALDI-TOF mass spectrometer (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflecton mode and using a matrix of α-ciano-4-hydroxycinnamic acid.

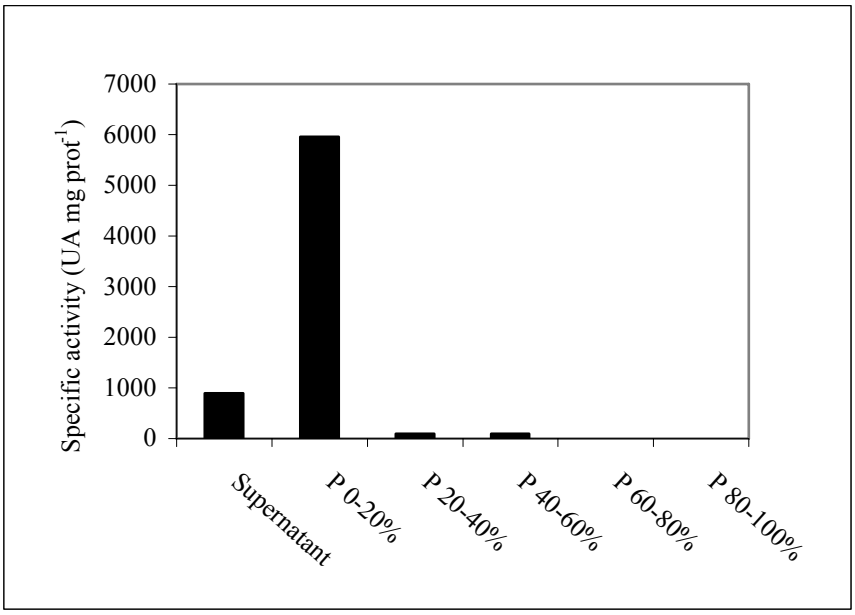
3.5.5 Results

The antimicrobial peptide produced by *Bacillus* sp. P34 was purified from the culture supernatant by combination of ammonium sulfate precipitation, gel filtration and ion exchange chromatography. The purification steps and recoveries of BLS P34 are given in Table 1 and the BLS activity was determined at each step of the purification process.

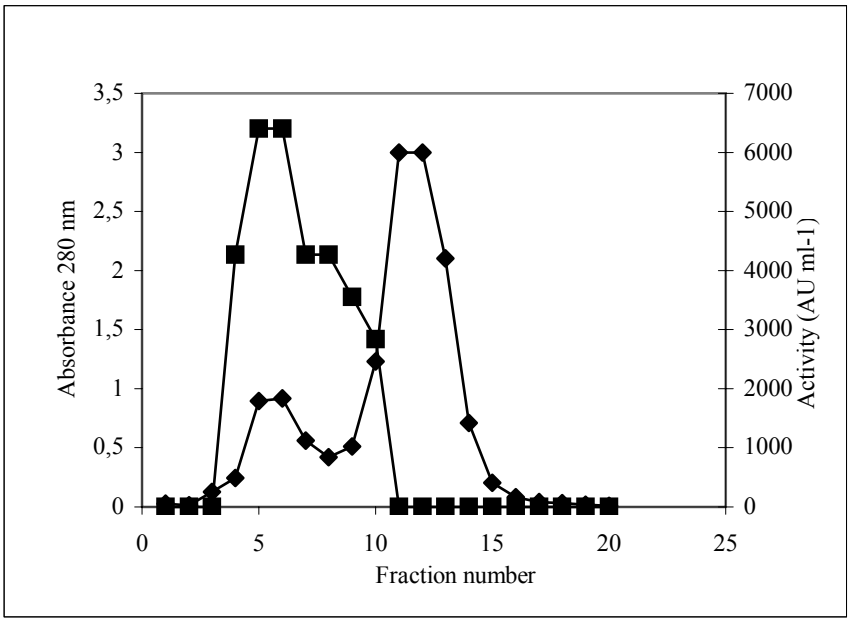
Table 1. Purification of BLS P34 produced by *Bacillus* sp. P34

	Activity (AU ml^{-1})	Volume (ml)	Total Activity (AU)	Prot ml^{-1}	Prot mg^{-1}	Specific Activity (AU mg^{-1})	Purification fold	Yield (%)
Supernatant	800	180	144000	10,591	1906,38	76	1,0	100,00
Precipitation	25600	3,6	92160	5,839	21,02	4384	57,7	64,00
Sephadex G-100	6400	8	51200	1,05	8,4	6095	80,2	35,56
DEAE Sepharose	400	30	12000	0,03	0,9	13333	175,43	8,33
DEAE Sepharose R	200	27	5400	0,01	0,27	20000	263,15	3,75

Fractionated precipitation of bacteriocin P34 showed that the maximum of activity was obtained with 20% ammonium sulfate (Fig 1A). The ammonium sulfate precipitation resulted in important increased in purification fold. Gel filtration chromatography using Sephadex G-100 resulted in fractions exhibiting antibacterial activity that eluted apart of the main protein peak (Fig.1B). When ion exchange chromatography using DEAE-Sepharose was carried out, a protein peak eluted at nearly 1.2 M NaCl contained the antimicrobial activity (Fig. 1C). The active peak was rechromatographed according to the same protocol, resulting in the purified BLS (Fig1D). The final specific activity of the BLS was approximately 263-fold greater than that in the culture supernatant and the final recovery was of 3.75% (Table 1).

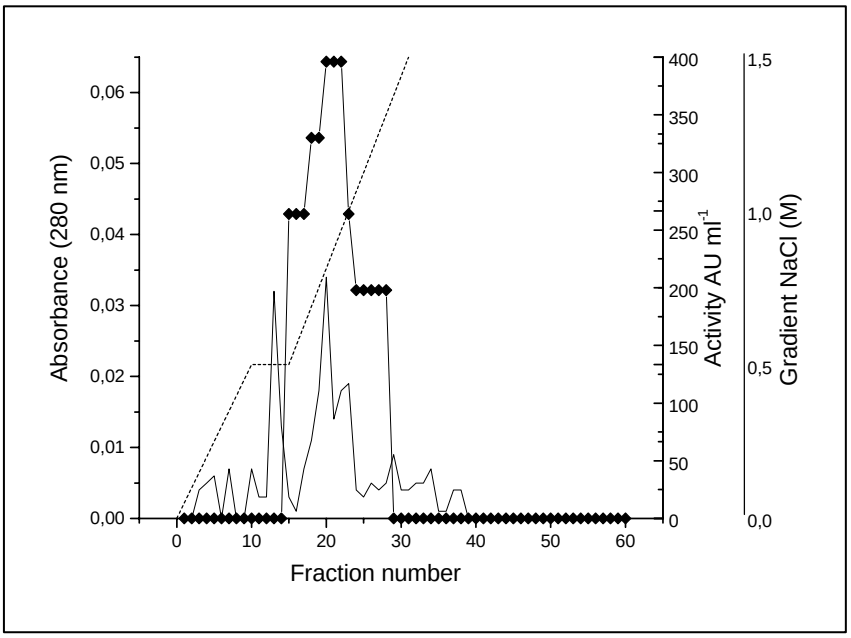


1A



1B

Fig. 1. Purification of BLS P34. (A) Fractionated ammonium sulfate precipitation. (B) Gel filtration chromatography on Sephadex G-100 eluted with 10 mM sodium phosphate buffer pH 7.0. (■) Activity (AU ml^{-1}) and (◆) Absorbance 280 nm.



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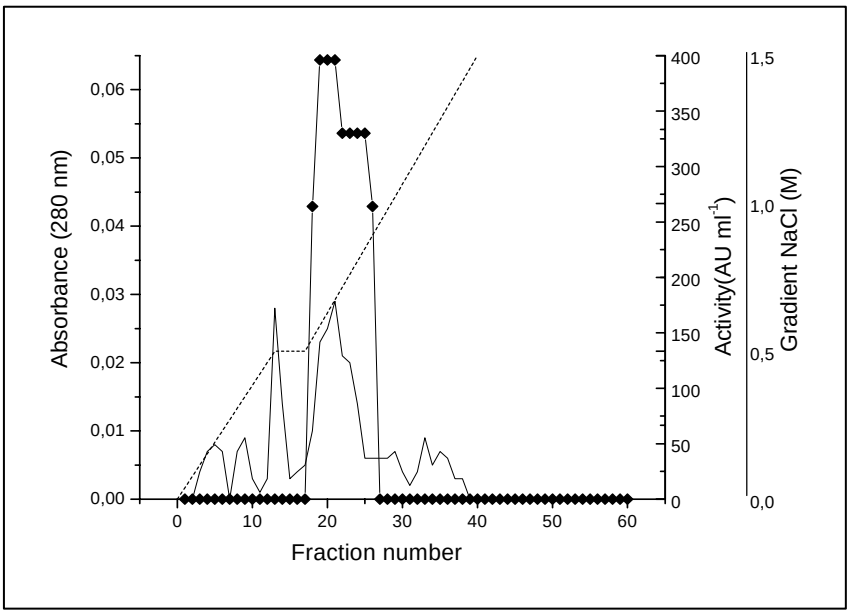


D

Fig. 1. Purification of BLS P34. (C) Ion exchange chromatography on DEAE-Sepharose eluted with 10 mM sodium phosphate buffer pH 7.0, followed by a gradient from 0 to 1,5 M NaCl. (D) Rechromatography on DEAE-Sepharose. Fractions were monitored for absorbance at 280 nm (◆) and assayed for antimicrobial activity against *L. monocytogenes* (■).

The purified BLS P34 was then concentrated by freeze-dried and analyzed by SDS-PAGE (Fig. 2). This analysis of the purified peptide in 14% polyacrylamide gel, revealed a single band of about 5 kDa suggesting that the BLS P34 had been purified to homogeneity. This antibacterial activity of this single peptide could be demonstrated by overlaying the other part of the gel which contains the same purified protein, with the indicator strain *L. monocytogenes* revealing an inhibitory zone at the same R_f than that visualized in the stained gel (Fig. 2).

Studies on native protein revealed that is eluted at the void volume of Sephadex G-100, but enter at the included volume when the elution buffer containing 1.5 M NaCl.

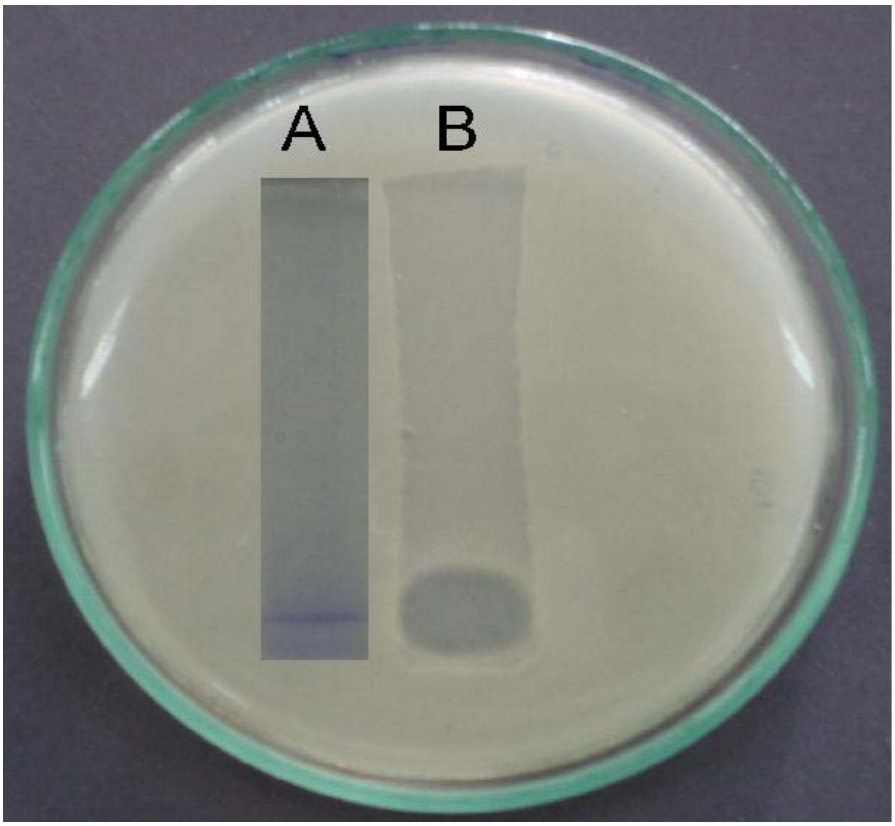


Fig. 2. Gel electrophoresis analysis of BLS P34. Samples of the purified bacteriocin submitted to SDS-PAGE and stained for proteins with Comassie blue (A) and tested for antimicrobial activity (B).

Aliquots of BLS P34 were treated with trypsin, papain, pronase E and proteinase K and it was observed that the inhibitory activity was lost with all the proteolytic enzymes tested (Table 2). When examining the thermal stability, the antimicrobial compound retained activity over a wide range of temperatures even up 100°C. Purified BLS retained biological activity at heat treatment and resulted in residual activity of 59% at 90°C/ 30 min (Table 2). When submitted at 100°C antimicrobial activity was observed at 60 min of treatment (Fig 3), indicating that the active substance is relatively heat stable. There was total loss of antibacterial activity when stored at refrigerated temperature for 10 days and at 121°C for 15 min (autoclaving).

To test whether BLS P34 had haemolytic activity, sheep blood agar plates were treated with the antimicrobial substance and no haemolytic activity was detected in preparations of freeze-dried bacteriocin (data not shown).

Table 2. Properties of the purified BLS P34

Treatment*	**Residual activity (%)**
None	100
Trypsin	0
Papain	0
Pronase E	0
Proteinase K	0
60°C/ 30 min	71
70°C/ 30 min	76
80°C/ 30 min	59
90°C/ 30 min	59
121°C/ 15 min	0
4^{o}C / 10 days	0
-20^{o}C / 15 days	65

* Enzymes were used at 2 mg ml^{-1}.

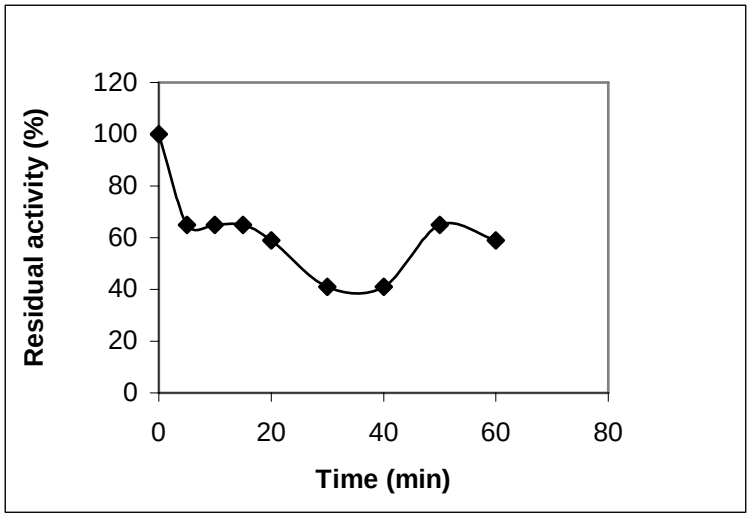


Fig. 3. Thermal stability of purified bacteriocin P34 treated at 100°C for 5, 10, 15, 20 30, 40, 50 and 60 min.

In order to prove the purity and to determine accurately the molecular mass of the BLS P34, mass spectroscopy analysis was carried out revealing a molecular mass of 1498 Da (Fig 4). The mass spectroscopy analysis showed a cluster of 6 peaks that were observed at m/z 1498, 1484, 1470, 1458, 1442 and 1428, differing by 14 Da each other.

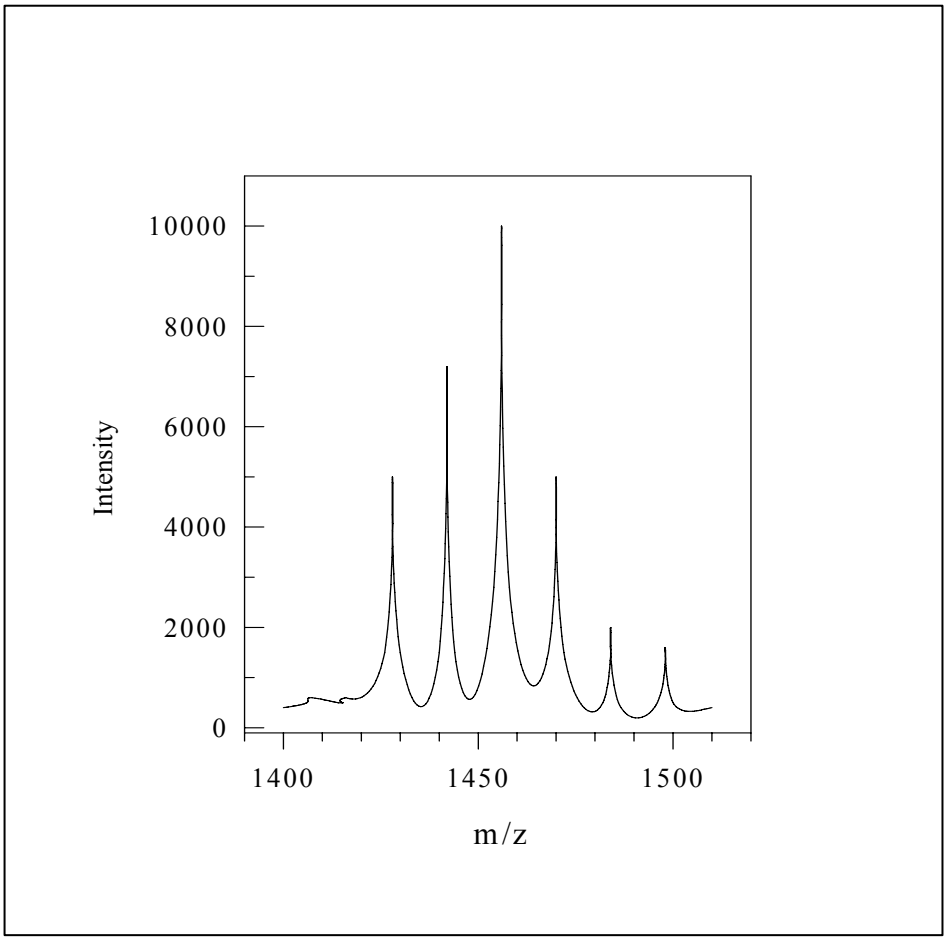


Fig. 4. Mass spectroscopy spectrum of purified bacteriocin P34.

3.5.6 Discussion

A variety of bacteriocins such as those of Gram-positive bacteria have attracted attention for their potential use in many fields. Bacteriocins produced by *Bacillus* spp. demonstrate a distinct diversity in their inhibitory activities and have been studied in different species including *Bacillus subtilis* [12,27,28] *Bacillus licheniformis* [22] and *Bacillus cereus* [2]. In this work, a new bacteriocin-like substance named BLS P34

produced by *Bacillus* sp. P34 strain isolated from Amazon basin, was purified and characterized.

The antimicrobial substance was purified by sequential precipitation, gel filtration and ion-exchange chromatography process. The substance bond to the DEAE-Sepharose matrix, indicating the nature of the peptide is anionic. Upon gel filtration BLS P34 was eluted with a MW higher than 150 kDa. However, lower MW was observed when the elution was carried out with a high ionic strength buffer (containing 1.5 M NaCl), or when estimated by SDS-PAGE. The high apparent MW of BLS P34 may be due to polymer formation and/or association of substance with macromolecules present in the culture medium, and has been associated with the strong hydrophobic nature of the peptides [15, 28]. This discrepancy can be explained on the basis of the abnormal behavior of some highly hydrophobic proteins [11]. These data suggest that high MW aggregates of BLS P34 can be formed in aqueous solution, which could be maintained by hydrophobic interactions. *Bacillus* sp. P34 was isolated from Amazon basin and its natural environment is essentially polar one. Thus, formation of BLS P34 aggregates, can very likely occur in natural conditions in which a large number of bacteria simultaneously produce antibiotics as the nutrients become limited. These aggregates can prevent diffusion and loss of the antibacterial activity maintaining its concentration at high levels in the surrounding of the bacterial population.

In addition, the experiments involving organic solvents revealed a rather hydrophobic (amphipathic) nature for the peptide indicating a potential for interaction between the substance and the cell membrane [23].

The estimation of the apparent molecular mass of the purified substance was made by direct detection of the bactericidal activity on SDS-PAGE, which gives indication of the low MW of the peptide. The molecular mass was accurately determined by mass spectroscopy as 1498 Da. The mass spectroscopy analysis showed a cluster of 6 peaks that were observed at m/z 1498, 1484, 1470, 1458, 1442 and 1428. These peaks differ by 14 Da, suggesting a series of homologous molecules or fragments having different length of fatty acid chain (CH_2 = 14 Da). This substance may be a surfactin-like compound, a family of lipopeptide antibiotics often termed “biosurfactants” [3].

According with its properties of size, protein stability data and the lack enzymatic activity, bacteriocin P34 could be from the group of *Listeria*-active class I bacteriocins of the classification described by [13]. However there are a broader category of antimicrobial peptides. They are common antimicrobial substances found not only among bacteria, but also as part of defense system in higher organisms [7,21]. It has been proposed that antagonism mediated by cationic peptides may represent the conservation through the course of evolution of a general mechanism of antibiosis [24].

BLS P34 is a low MW peptide sensitive to proteases that maintained its activity after freezing and after incubation at 70°C during 30 min. Many of these properties are also common to other bacteriocins, like bacthuricin F4 [10], and have been said to be a value in substances intend to be used as preservatives in food products that undergo pasteurization treatments and/or cold storage [4,5].

Nevertheless, with basis in the characteristics described above, the antimicrobial substance reported in this work could have a potential to be implemented as a substance in controlling pathogenic and food spoilage microorganisms.

The role of antimicrobial production for the producing microorganism is still under speculation. The best-accepted theory is that non-ribosomal antibiotics may play a role in competition with other microorganisms during spore germination. The detection of novel antibiotics produced by *Bacillus* species would therefore be helpful in providing an understanding of the intrinsic (if any) role of antimicrobial activity in the life cycle of those organisms.

3.5.7 Acknowledgements

This work received financial support of CNPq, Brazil.

3.5.8 Literature Cited

1. Allgaier H, Jung G, Werner RG, Schneider U, Zahner H (1986) Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amine antibiotic. Eur J Biochem 160: 9-22
2. Bizani D, Brandelli A (2002) Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. J Appl Microbiol 93: 512-519
3. Fiechter A (1992) Biosurfactants: moving towards industrial application. Trends Food Sci Technol 3: 286-293
4. Gálvez A, Valdivia E, Abriouel H, Camafeita E, Mendez E, Martinez-Bueno M, Maqueda M (1998) Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Arch Microbiol 171: 59-65
5. Giraffa G (1995) Enterococcal bacteriocins: their potential as anti-*Listeria* factor in dairy technology. Food Microbe 12: 291-299
6. Hames BD (1981) An introduction to polyacrylamide gel electrophoresis. In: Hames BD and Rickwood D (eds) Gel Electrophoresis of Proteins. Oxford, UK, IRL Press, pp. 1-92
7. Hancock RE (1997) Peptide antibiotics. Lancet 349: 418-422.
8. Hurst A (1981) Nisin. Adv Appl Microbiol 27: 85-123
9. Jack RW, Tagg JR, Ray B (1995) Bacteriocins of gram-positive bacteria. Microbiol Rev 59: 171-200
10. Kamoun F, Mejdoub H, Aouissaoiu H, Reinbolt J, Hammami A, Jaoua S (2005) Purification, amino acid sequence and characterization of bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. J Appl Microbiol 98: 881-888
11. Kaufmann E, Geisler N, Weber K (1984) SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins. *FEBS* Letters 170: 81-84.
12. Kawulka KE, Sprules T, Diaper CM, Whittal RM, McKay RT, Mercier P, Zuber P, Vederas JC (2004) Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α-carbon cross-links: formation and reduction of α-thio-α-amino acid derivative. Biochemistry 43: 3385-3395

13. Klaenhammer T (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12: 39-86
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 267-275
15. Maisnier-Patin S, Richard J (1995) Activity and purification of linenscin OC2, an antibacterial substance produced by *Brevibacterium linens* OC2, an orange cheese coryneform bacterium. Appl Environ Microbiol 61: 847-1852
16. Mayr-Harting A, Hedjes AJ, Berkeley CW (1972) Methods for studying bacteriocins. In: Norris JB, Ribbons D (eds) Methods in Microbiology, vol. 7. New York, Academic Press, pp 315-412
17. Motta AS, Brandelli A (2002) Characterization of an antimicrobial peptide produced by *Brevibacterium linens*. J Appl Microbiol 92: 63-70
18. Motta AS, Olivera FC, Brandelli A (2004) Screening for antimicrobial activity among bacteria isolated from the amazon basin. Braz J Microbiol 35: 307-310
19. Naclerio G, Ricca, E, Sacco M, de Felice M (1993) Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. Appl Environ Microbiol 59: 4313-4316
20. Neuhoff V, Arold N, Trauber D, Ehrhardt W (1994) Improved staining of proteins in polyacrylamide gels including isoeletric focusing with clear background at nanogram sensitivity using Comassie Brilliant blue G-250 and R-250, Electrophoresis 9: 255-262
21. Niessen-Meyer J, Nes IF (1997) Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis and mechanism of action. Arch Microbiol 167: 67-77
22. Pattnaik P, Kaushik JK, Grover S, Batish VK (2001) Purification and characterization of bacteriocin-like compound (lichenin) produced anaerobically by *Bacillus licheniformes* isolated from water buffalo. J Appl Microbiol 91: 636-645
23. Risoen PA, Ronning P, Hegna IK, Kolsto A (2004) Characterization of a broad range antimicrobial substance from *Bacillus cereus*. J Appl Microbiol 96: 648-655
24. Sahl HG (1995) Bactericidal cationic peptides involved in bacterial antagonism and host defense. Microbiol Sci 2: 212-217

25. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 1-13
26. Stiles ME (1996) Biopreservation by lactic acid bacteria. Antonie Leeuwenhock 70: 331-345
27. Tamehiro N, Okamoto-Hosoya Y, Okamoto S, Ubukata M, Hamada M, Naganawa H, Ochi K (2002) Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Chemother 46: 315-320
28. Valdés-Satuber N, Scherer S (1994) solation and characterization of linocin M18, a bacteriocin produced by *Brevibacterium linens*. Appl Environ Microbiol 60: 3809-3814

4 DISCUSSÃO GERAL

Uma diversidade de compostos resultantes do metabolismo microbiano como antibióticos e bacteriocinas tem sido pesquisados, de forma a explorar o potencial biotecnológico destas substâncias, seja do ponto de vista farmacológico ou como um bioconservante na indústria de alimentos.

As bacteriocinas vêm sendo extensivamente estudadas, sendo as bactérias lácticas as pioneiras para o estudo destes compostos visando à conservação dos alimentos. Bactérias lácticas são encontradas em vários produtos e alimentos fermentados, sendo empregadas como "*culturas starters*", de modo a conferir certas propriedades a estes produtos. Também encontram-se envolvidas na manutenção da qualidade, do ponto de vista microbiológico, a qual ocorre através da produção de substâncias com atividade antimicrobiana. Bacteriocinas devidamente purificadas e caracterizadas podem ser diretamente aplicadas ao produto sendo a nisina, produzida por *Lactococcus lactis,* a única bacteriocina aprovada para uso em alimentos.

Vários fatores devem ser analisados quando da aplicação destes compostos no alimento, de modo a garantir o efeito desejado, promovendo a proteção do consumidor. Estes fatores vão desde os relacionados com as características inerentes ao alimento, chamados de fatores intrínsecos, como sua composição e microflora; assim como os fatores extrínsecos (DEVLIEGHERE, VERMEIREN, DEBEVERE, 2004).

Em relação à questão da bioconservação de alimentos, um conceito importante e que permeia este ponto é o de "barreira". Neste, pode-se considerar a ação sinérgica de vários elementos considerando os compostos com atividade antimicrobiana oriundos de microrganismos, animais e plantas; os procedimentos de natureza física; o emprego de embalagens; os procedimentos de fabricação e a obtenção do produto assim como a estocagem do mesmo. Todos estes influenciarão na qualidade final do produto. Logo, é interessante que haja um controle de todas estas variáveis para que elas atuem conjuntamente na manutenção das qualidades físicas e microbiológicas do alimento em questão (SOUZA, SILVA, SOUSA, 2005).

A bacia Amazônica possui uma biodiversidade de microrganismos ainda não identificados, com um potencial a ser explorado. Alguns microrganismos foram estudados para fins biotecnológicos, mas poucos são os relatos de produção de substâncias por bactérias isoladas da região amazônica (HECK, HERTZ, AYUB, 2002; MOTTA CLADERA-OLIVERA, BRANDELLI, 2004).

A partir de 86 isolados da região Amazônica buscou-se identificar a produção de substâncias do tipo-bacteriocina e o isolado P34 foi selecionado por produzir um composto com amplo espectro de ação e sensível às enzimas proteolíticas testadas.

Neste trabalho, a substância antimicrobiana produzida pelo isolado P34 foi identificada, purificada e caracterizada, e denominada de bacteriocina P34. A bactéria produtora foi identificada como sendo uma espécie do gênero *Bacillus*, porém a análise filogenética desta espécie agrupou com a espécie *Bacillus infernus*. Esses resultados indicam que existe uma relação de ancestralidade comum entre estas duas espécies, embora apresentem características fisiológicas e bioquímicas bastante distintas. Isto indica a possibilidade do isolamento e identificação de uma nova espécie de *Bacillus*, sugerindo-se sua classificação como *Bacillus amazonensis*.

A bacteriocina P34 apresentou um espectro de atividade relativamente amplo. Inibiu bactérias do mesmo gênero como *Bacillus cereus,* assim como várias linhagens de *Listeria*, incluindo *Listeria monocytogenes,* patógenos muito importantes do ponto de vista de segurança alimentar. A atividade antimicrobiana também foi observada sobre *Erwinia carotovora, Pasteurella haemolytica* e *Rhodococcus* sp.

Listeria corresponde a um gênero de microrganismos amplamente distribuído no meio ambiente. É composto por várias espécies, entre as quais apenas *Listeria monocytogenes* é importante em alimentos. Essa espécie causa a listeriose em homens e animais e é largamente distribuída na natureza, podendo ser encontrada no solo, vegetais e na água. Os vegetais se contaminam através da água de irrigação e do adubo usado como fertilizante. Os animais podem conter a bactéria sem apresentar nenhum sintoma de doença e contaminar os alimentos de origem animal, como leite e carne. Esta espécie já foi detectada em uma grande variedade de alimentos crus, como carnes e vegetais não cozidos e também em alimentos processados com contaminação pós-processamento, como queijos. Leite não pasteurizado, bem como laticínios preparados com leite não pasteurizado

também podem conter o microrganismo. *Listeria* é destruída pela pasteurização, assim como pelo calor empregado no processamento de alimentos. Entretanto, se não forem empregadas práticas de higiene adequadas pode ocorrer a contaminação após o processamento. *Listeria monocytogenes* tem a capacidade de crescer a baixas temperaturas, sendo, portanto, um potencial contaminante de produtos refrigerados. Neste sentido, a inibição desta espécie pela bacteriocina P34 representa uma importante aplicação para esta substância.

As características de termoestabilidade da bacteriocina P34 purificada, permitem sugerir sua aplicação em produtos pasteurizados e congelados. Importante considerar o conceito de "barreira", onde há vários fatores atuando sinergicamente na conservação do produto, garantindo a manutenção da sua qualidade microbiológica. Ao mesmo tempo, isto permite que se possa reduzir os limites utilizados para cada sistema de conservação, mantendo as características e propriedades organolépticas do alimento. A natureza do produto no qual a bacteriocina será empregada merece atenção, para que se possa estimar a possibilidade de interação da substância com os componentes do alimento, diminuindo sua disponibilidade para exercer seu efeito bactericida.

Manutenção da atividade antimicrobiana foi observada, quando a bacteriocina P34 foi submetida à liofilização.

A avaliação da estabilidade da bacteriocina P34 quando submetida à diferentes pHs mostrou que esta se mantém numa ampla faixa, sendo ativa no intervalo de pH 3 a 10. As características destas substâncias indicam o seu potencial uso em uma ampla faixa de produtos, seja em produtos liofilizados, congelados ou produtos com pHs diferentes.

As avaliações quanto à estabilidade da bacteriocina P34 parcialmente purificada frente às enzimas proteolíticas mostram que ela perdeu completamente a atividade quando tratada com pronase E. Porém, manteve 40 e 30% de sua atividade residual quando tratada com tripsina e papaína, respectivamente. Quando a bacteriocina P34 foi purificada, os testes com as enzimas proteolíticas foram repetidos e foi observada perda total da sua atividade nas mesmas concentrações empregadas anteriormente (2 mg ml^{-1}). Isto sugere que a substância em uma forma mais bruta, possa estar na forma de agregados, inclusive

com outras proteínas, indisponibilizando sítios de ação específicos para que a substância atue. Estas propriedades deste composto também possibilitam classificar como uma substância do tipo-bacteriocina.

Quanto a inibição das bactérias Gram-negativas, observa-se a inibição de *Salmonella* Gallinarum, *Escherichia coli* e *Salmonella* Enteritidis não apresentaram sensibilidade, sendo observada uma tênue inibição do crescimento quando da adição de 20 mM de EDTA. Neste caso, a atividade inibitória deveu-se a ação do quelante EDTA. A ação desta substância dá-se pela remoção dos íons Mg^{+2} e Ca^{+2} da membrana externa das bactérias Gram-negativas, favorecendo a liberação de lipoproteínas e fosfolipídeos, o que aumenta a permeabilidade da parede celular, expondo a célula à ação da bacteriocina P34 (MONTVILLE , BRUNO, 1994).

Os resultados obtidos em cromatografia de gel filtração indicam que a substância forma agregados extracelulares. Foi observado que quando eluída com tampão fosfato de sódio 10 mM pH 7.0, um pico com atividade foi verificado, sendo eluída no volume morto da coluna. Entretanto, quando se aumentou a força iônica do tampão, a atividade foi observada em outras frações, dentro do limite de resolução da coluna. Por outro lado, em condições desnaturantes observou-se que a substância tem baixo peso molecular. Eletroforese (SDS-PAGE) foi conduzida com a substância purificada, observando-se uma banda de atividade no gel correspondente a um peso molecular inferior a 5 kDa. Espectrometria de massas foi empregada para a determinação exata do peso molecular da bacteriocina P34, confirmando seu baixo peso molecular de 1498.68 Da. Essas diferenças em relação a determinação do peso molecular têm sido observadas para outras bacteriocinas (OSCÁRIZ, PISABARRO, 2000; BIZANI *et al*., 2005b) estando associadas à peptídeos com natureza altamente hidrofóbica.

Este fenômeno tem sido observado por outros pesquisadores, onde verificaram que peptídeos antimicrobianos têm sido eluídos de duas formas diferentes em cromatografia de gel filtração: monômeros e agregados. Foi observado que estes peptídeos formam alguma estrutura em micela, o que é relativamente comum para peptídeos antimicrobianos que apresentam propriedades de superfície ativa tal como a surfactina e outros surfactantes

produzidos por espécies de *Bacillus licheniformes*. Estes substâncias são lipopeptídeos e por suas propriedades formam agregados com alto peso molecular (LIN *et al*., 1994).

Foi estudado o efeito da bacteriocina P34 sobre *L. monocytogenes* e *B. cereus*. Culturas na fase exponencial de crescimento destas linhagens foram tratadas com a substância e foi observado um efeito bactericida e bacteriolítico após 12 horas de experimento. Para verificação e confirmação do efeito e modo de ação da bacteriocina, espectroscopia de infra-vermelho foi empregada, onde verificou-se que células de *L. monocytogenes* tratadas tiveram alteração nos constituintes lipídicos da membrana, sugerindo-se desestruturação e permeabilização da membrana celular, levando a formação de poros; mecanismo este descrito para uma diversidade de peptídeos antimicrobianos (HANCOCK, ROZEK, 2002; CARRILO *et al.,* 2003; BIZANI *et al*., 2005b).

Outros trabalhos têm evidenciado efeitos bacteriostáticos. Alguns fatores devem ser considerados aqui, quando os experimentos são conduzidos *in vitro*, como a relação da concentração de bacteriocina e a quantidade de células a serem inibidas, o grau de pureza da substância, assim como as condições físicas do meio em que o ensaio será realizado.

O modo de ação de algumas bacteriocinas já tem sido descrito e observa-se a interação do composto na superfície da membrana das células sensíveis, quando então começa a exercer seu efeito propriamente dito. Para nisina é bem descrita a participação do lipídio II para que a interação ocorra. A interação nisina-lipídio II é considerada de alta afinidade, inibindo a síntese do peptideoglicano e formando poros altamente específicos à nível da membrana, resultando em morte celular. A vancomicina também exerce seu efeito através de sua ligação ao lipídio II da membrana (BREUKINK *et al.,* 1999).

De acordo com a classificação de Klaenhammer (1993) para as bacteriocinas, pode-se sugerir que a bacteriocina P34 pertença a Classe I. A substância apresenta um baixo peso molecular assim como uma natureza aniônica, o que a coloca como pertecendo a Classe Ib. A mersacidina é um lantibiótico produzido por espécies de *Bacillus* sp. e é ativa contra várias espécies de bactérias Gram-positivas, incluindo *Staphylococcus aureus* meticilina-resistentes. Possui característica que a coloca na Classe Ib, ou seja, possui carga negativa ou não possui carga. Possui um peso molecular de 1825 Da. Um modo de ação

tem sido proposto para esta substância, onde observa-se a inibição da biossíntese do peptideoglicano e uma diminuição da espessura da parede celular; sendo a inibição da biossíntese da parede celular proposta como o modo de ação primário da mersacidina (BROTZ *et al*., 1995). Estudos subseqüentes vieram a identificar que o mecanismso molecular para a inibição é a interação da mersacidina com um intermediário do processo de síntese do peptideoglicano, chamado lipídio II. Através de sua ligação a este precursor, verificou-se a inibição do processo de formação do peptideoglicano levando a lise das células tratadas (BROTZ *et al.,* 1998).

Em relação às aplicações terapêuticas, a mersacidina é considerada um dos mais promissores lantibióticos da Classe Ib devido a sua ação *in vivo* contra *Staphylococus aureus* meticilina-resistentes. A mersacidina, como a vancomicina, atua pela ligação ao lipídio II, embora isto aconteça em sítios alvo diferentes. Isto faz com que esta substância venha a ser uma nova classe de antimicrobianos a ser explorada (CHATTERJEE *et al*., 1992).

Diferente do que foi descrito para a mersacidina, a bacteriocina P34 em seu modo de ação mostrou afetar a permeabilidade da membrana celular o que pode se dar através da formação de poros nesta membrana. O mecanismo molecular exato, pelo qual este processo ocorre merece futuros estudos.

A bacteriocina P34 apresentou um peso molecular de 1498 Da, sendo que várias substâncias de baixo peso molecular têm sido descritas. A liquenisina A produzida por *Bacillus licheniformis* possui 1034 Da e é um biosurfactante com propriedades antimicrobianas (YAMIKOV *et al*., 1995). A surfactina produzida por *B. subtilis*, é um lipopeptídeo cíclico de peso molecular de 1036 Da, com atividade antimicrobiana já descrita (VOLLENBROICH *et al.,* 1997). Outro lipopeptideo antimicrobiano foi descrito sendo produzido por *B. subtilis* e seu peso molecular foi determinado como sendo de 1500 Da (BECHARD *et al.,* 1998). A pesquisa de biosurfactantes em espécies de *Bacillus* foi realizada por outros pesquisadores e *B. licheniformis* F2.2 produziu uma nova substância BL 1193 de 1193 Da, porém o peso molecular destas substâncias difere do obtido para a bacteriocina P34. Quanto ao peso molecular e as características até então estudadas sobre a

bacteriocina P34 sugere-se que ela possa fazer parte da Classe Ib assim como possa ser uma substância com características de biosurfactante, sendo necessários mais estudos para afirmar com segurança estas proposições, assim como sugerir que se trate da identificação de uma nova substância.

Figura 1. Estrutura química de substâncias já identificadas e descritas (YAKIMOV *et al*., 1995; VOLLENBROICH *et al*., 1997; STEIN, 2005).

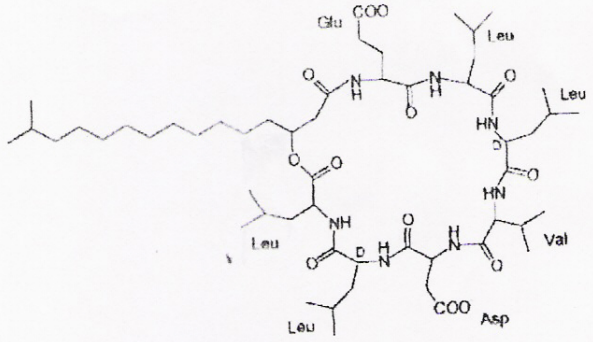
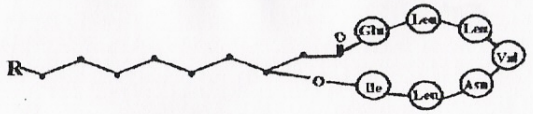
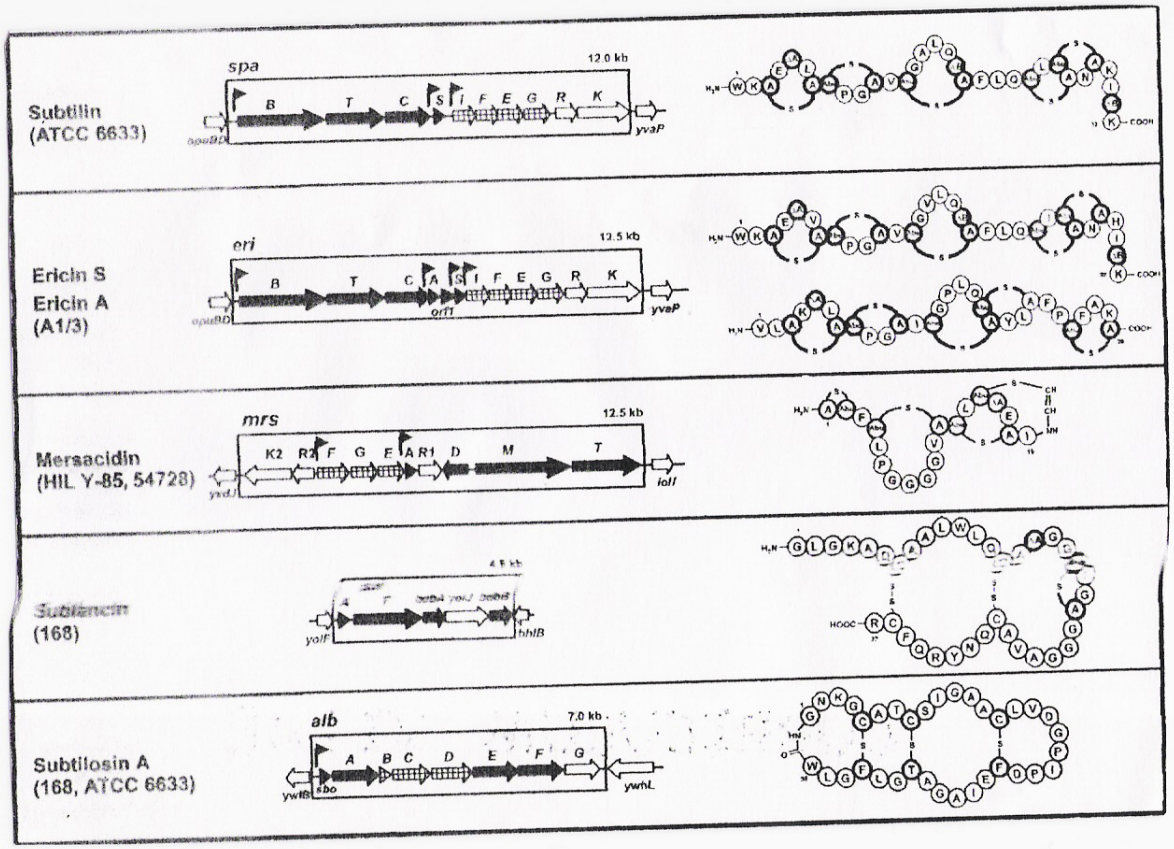


FIG. 1. Structure of surfactin.



Proposed structure of lichenysin A.


Subtilin
(ATCC 6633)
spa
12.0 kb
B T C S I F E G R K
spaBD
yvaP
Ericin S
Ericin A
(A1/3)
eri
12.5 kb
B T C A S I F E G R K
spaBD
orf1
yvaP
Mersacidin
(HIL Y-85, 54728)
mrs
12.5 kb
K2 R2 F G E AR1 D M T
yxdJ
ioll
Subtilomycin
(168)
4.5 kb
yolF
hhlB
Subtilosin A
(168, ATCC 6633)
alb
7.0 kb
A B C D E F G
ywfB
sbo
ywhL

De acordo com as características da bacteriocina P34 e com os resultados até então observados pode-se sugerir um modo de ação para esta substância. O peptídeo apresenta características aniônicas e é altamente hidrofóbico, sendo a maior parte dos aminoácidos que compõem a bacteriocina P34 de natureza apolar. A natureza altamente hidrofóbica desta substância sugere que ocorram interações hidrofóbicas entre seus aminoácidos com os grupamentos hidrofóbicos das proteínas da membrana da célula sensível. Para conclusões mais precisas sobre o exato modo de ação, são necessários mais estudos.

A bacteriocina P34 foi purificada e caracterizada. O conhecimento de suas propriedades faz com que se possa explorar seu potencial de forma a otimizar sua obtenção uma vez que existe interesse para aplicação em alimentos. Para isso vários estudos para avaliar sua toxicidade se fazem necessários justificando seu uso de forma segura e eficaz no alimento. O estudo dos fatores inerentes a cada produto devem ser avaliados, de modo a que se obtenha o retorno desejado, do ponto de vista de manutenção da qualidade microbiológica do alimento.

Estudos sobre peptídeos antimicrobianos são extremamente importantes se considerarmos a diversidade de funções ecológicas que estas substâncias apresentam em comunidades microbianas. Embora saiba-se que o potencial de aplicação destas bacteriocinas vêm sendo estudado de modo a explorar seu uso como bioconservantes em alimentos, outras áreas de aplicação merecem pesquisas para justificar seu emprego.

Por serem substâncias amplamente produzidas, deve-se buscar explorar o potencial de aplicação destas, tendo em vista a diversidade de propriedades por elas apresentadas.

5 CONCLUSÕES

As características de amplo espectro de ação, principalmente considerando sua atividade bactericida sobre *Listeria monocytogenes* e *Bacillus cereus*, assim como suas propriedades de termoestabilidade e manutenção de sua atividade em uma ampla faixa de pH, tornam a bacteriocina P34 bastante atrativa se considerarmos seu potencial de aplicação como biopreservativo.

O uso da Metodologia de Planejamento Experimental e de Análise de Superfície de Resposta foi empregada para a otimização da produção da substância, onde se determinou o melhor meio de cultivo para produção assim como a influência combinada dos fatores pH e temperatura. Determinou-se os intervalos de pH 6.0 a 8.0 e temperaturas de 25 a 37°C, como ótimos, para a produção da bacteriocina P34.

Na biossíntese da bacteriocina P34, sua concentração máxima foi observada na fase estacionária de crescimento. Embora sua produção tenha se iniciado na fase exponencial de crescimento de *Bacillus* sp. P34. O que sugere uma cinética de metabólito secundário.

Nos experimentos de efeito e modo de ação a substância mostrou-se bactericida e bacteriolítica para *Listeria monocytogenes* e *Bacillus cereus*, nas condições testadas.

As características químicas indicam se tratar de uma bacteriocina com massa molecular de aproximadamente 1498.68 Da.

Suas características como sensibilidade às proteases, estabilidade térmica, atividade em um amplo intervalo de pH, aniônica e hidrofóbica, e sendo uma substância com baixo peso molecular, permitem caracterizar a substância P34 como uma substância do tipo-bacteriocina, e sugere-se como pertencente à Classe I, de acordo com a classificação de Klaenhammer (1993).

Este composto possui potencial de aplicação como substância antimicrobiana em alimentos, merecendo estudos e pesquisas *in situ,* para avaliação de sua eficácia.

6 PERSPECTIVAS

Os resultados deste trabalho despertam interesses para novos estudos de modo a explorar o potencial biotecnológico deste peptídeo, com vistas a sua utilização, propriamente dita. Resultados quanto à caracterização do peptídeo são ainda necessários para que se possa afirmar a descoberta de uma nova substância com atividade antimicrobiana.

Para tanto é interessante:

- Finalizar os estudos de caracterização química do composto determinando sua seqüência de aminoácidos.
- Realizar testes para avaliar sua toxicidade e citotoxicidade *in vivo*.
- Pesquisar o modo de ação desta substância e sugerir seu mecanismo, comparando-o com outros antibióticos, através da espectroscopia de infra-vermelho.
- Aplicar a bacteriocina P34 no alimento verificando sua eficácia na manutenção da qualidade microbiológica dos produtos.

7 BIBLIOGRAFIA

ABEE, T. Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanisms of producer organisms. **FEMS Microbiology Letters**, Amsterdam, v.129, n. 1, p. 1-10, June 1995.

AHERN, M.; VERSCHUEREN, S.; VAN SINDEREN, D. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. **FEMS Microbiology Letters**, Amsterdam, v. 220, n. 1, p. 127-131, March 2003.

ALTENA, K. et al. Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. **Applied and Environmental Microbiology**, Washington, v. 66, p. 2565-2571, 2000.

APHA **Compendium of Methods for the Microbiological Examination of Food**. Cap. 24, EUA, Washington, 1992.

AUSTIN, B. et al. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*. **Journal of Fish Diseases**, Oxford, v. 18, n. 1, p. 93-96, 1995.

AYMERICH, T. et al. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. **Applied and Environmental Microbiology**, Washington, v. 62, n. 5, p. 1676-1682, May 1996.

BABASAKI, K. et al. Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: Isolation, structural analysis, and biogenesis. **Journal of Biochemistry**, Tokyo, v. 98, n.3, p. 585-603, 1985.

BAIS, H.P.; FALL, R.; VIVANCO, J.M. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis roots* by *Pseudomonas syrinagae* is facilitated by biofilm formation and surfactin production. **Journal of Plant Physiology**, Stuttgart, v. 134, p. 307-319, 2004.

BANERJEE, S.; HANSEN, J.N. Structure and expression of a gene encoding the precursor of subtilin, a small peptide antibiotic. **Journal of Biological Chemistry**, Bethesda, v. 263, n. 19, p. 9508-9514, 1988.

BECHARD, J. et al. Isolation and partial chemical characterization of an antimicrobial peptide produced by a strain of *Bacillus subtilis*. **Journal of Agricultural and Food Chemistry**, Bethesda, v. 46, p. 5355-5361, 1998.

BIZANI, D.; BRANDELLI, A. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. **Journal of Applied Microbiology**, Oxford, v. 93, n. 3, p. 512-519, September 2002.

BIZANI, D.; DOMINGUEZ, A.P.M.; BRANDELLI, A. Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. **Letters in Applied Microbiology**, London, v. 41, n. 3, p. 269-273, September 2005a.

BIZANI, D. et al. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus.* **International Microbiology**, Barcelona, v. 8, n. 2, p. 125-131, June 2005b.

BRASIL. Ministério da Agricultura, do Abastecimento e da Reforma Agrária. **Métodos de análise microbiológica para alimentos**. [s.l.]. 1991/ 1992. 136p.

BRASIL. Resolução n° 23 de 15 de março de 2000. **Agência Nacional de Vigilância Sanitária**. Ministério da Saúde, Brasília-DF, 2000a.

BRASIL. Resolução n°1001, 1002, 1003 e 1004. **Agência Nacional de Vigilância Sanitária**. Ministério da Saúde, Brasília-DF. 2000b.

BREUKINK, E. et al. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. **Science**, Washington, v. 286, p. 2361-2367, December 1999.

BROTZ, H. et al. Mode of action of the lantibiotic mersacidin: inhibition of peptidoglycan biosynthesis via a novel mechanism? **Antimicrobial Agents and Chemotherapy**, Washington, v. 39, n. 3, p. 714-719, 1995.

BROTZ, H. et al. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. **Antimicrobial Agenst and Chemotherapy,** Washington, v. 42, n.1, p. 154-160, January 1998.

BRULL, S.; COOTE, P. Preservatives agents in food: mode of action and microbial resistance mechanisms. **International Journal of Food Microbiology**, Amsterdam, v. 50, n. 1-2, p. 1-17, September 1999.

CARRILO, C. et al. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. **Biochimica et Biophysica Acta**, Amsterdam, v. 1611, n. 1-2, p. 91-97, April 2003.

CHATTERJEE, S. et al. Mersacidin, a new antibiotic from *Bacillus*: in vitro and in vivo antibacterial activity. **The Journal of Antibiotic**, Tokyo, v. 45, p. 839-845, 1992.

CHEN, Y.; LUDESCHER, R.D.; MONTVILLE, T.J. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments of phospholipid vesicles. **Applied and Environmental Microbiology**, Washington, v. 63, n. 13, p. 4770-4777, December 1997.

CHERIF, A. et al. A. Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. **Letters in Applied Microbiology**, London, v. 32, n. 4, p. 243-247, April 2001.

CHUNG, K.; DICKSON, J.; CROUSE, J. Effects of nisin on growth of bacteria attached to meat. **Applied and Environmental Microbiology**, Washington, v. 55, p. 1329-1333, 1989.

CLAUS, D.; BERKELEY, R.C.W. Genus *Bacillus* Cohn 1872. In: SNEATH P.H.A. (Ed.) **Bergey's manual of determinative bacteriology**. Baltimore: Williams and Wilkins, 1986. v. 2, p. 1105-1141.

CLEVELAND, J. et al. Bacteriocins: safe antimicrobials for food preservation. **International Journal of Food Microbiology**, Amsterdam, v. 71, n. 1, p. 1-20, December 2001.

COCON, J.M. **Food toxicology: contaminants and additives** (part B). New York: Marcel Deker, 1988. 1083p.

CODEX ALIMENTARIUS. **Princípio de transferência dos aditivos alimentares**. 1995. Seção 5.3, Vol 1A.

DAW, M.A.; FALKINER, F. Bacteriocins: nature, functions and structure. **Micron**, New York, v. 27, n. 6, p. 467-479, December 1996.

DEVLIEGHERE, F.; VERMEIREN, L.; DEBEVERE, J. New preservation technologies: possibilities and limitations. **International Dairy Journal**, Barking, v. 14, n. 4, p. 273-285, April 2004.

DE VUYST, L.; CALLEWAERT, R.; CRABBÉ, K. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavorable growth conditions. **Microbiology**, London, v. 142, p. 817-827, 1996.

DE VUYST, L.; VANDAMME, E.J. **Bacteriocins of lactic acid bacteria**: microbiology, genetics and applications. London: Blackie Academic & Professional, 1994. 540p.

DRIESSEN, A.J.M. et al. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. **Biochemistry,** Washington, v. 34, p. 1606-1614, 1995.

EIJSINK, V.G.H. et al. Comparative studies of class IIa bacteriocins of lactic acid bacteria. **Applied and Environmental Microbiology**, Washington, v. 64, n. 9, p. 3275-3281, September 1998.

ENNAHAR, S. et al. Class IIa bacteriocins: biosynthesis, structure and activity. **FEMS Microbiology Reviews**, Amsterdam, v. 24, n. 1, p. 85-106, January 2000.

GALVEZ, A. et al. Isolation and physico-chemical characterization of an antifungal and antibacterial peptide produced by *Bacillus licheniformes* A12. **Applied Microbiology and Biotechnology,** Washington, v. 39, n. 4-5, p. 438-442, July 1993.

GEBHARDT, K. et al. Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. **FEMS Microbiology Letters**, Amsterdam, v. 217, n. 2, p. 199-205, December 2002.

GENNARO, R.; ZANETTI, M. Structural features and biological activies of the cathelicidin-derived antimicrobial peptides. **Biopolymers**, New York, v. 55, p. 31-49, 2000.

GOULD, G.W. Industry perspective on the use of natural antimicrobial and inhibitors for food applications. **Journal of Food Protection**, Ames, v. 35, p. 82-86, 1995.

HANCOCK, R.E.W.; ROZEK, A. Role of membranes in the activities of antimicrobial cationic peptides. **FEMS Microbiology Letters**, Amsterdam, v. 206, n. 2, p. 143-149, January 2002.

HAVARSTEIN, L.S.; DIEP, B.D.; NIES, I.F. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. **Molecular Microbiology**, Salem, v. 16, p. 229-240, 1995.

HECK, J.X.; HERTZ, P.F.; AYUB, A.Z. Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. **Brazilian Journal of Microbiology**, São Paulo, v. 33, n. 3, p. 213-218, September 2002.

HÜHNE, K.; AXELSSON, L.; HOLCK, A.; KRÖCKEL, L. Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *L. sake* strains. **Microbiology**, London, v. 142, p. 1437-1448, June 1996.

JABEEN, N. et al. Isolation, identification and bacteriocin production by indigenous diseased plant and soil associated bacteria. **Pakistan Journal of Biological Science,** Faisalabad, Pakistan, v. 7, n. 11, p. 1893-1897, 2004.

JACK, R.W.; TAGG, J.R.; RAY, E. Bacteriocins of gram-positive bacteria. **Microbiology Reviews,** Washington, v. 59, p. 171-200, 1995.

JACOB, F. et al. Definition of qualques terms relatifs à la lysogenies. **Annales de Institut Pasteur**, Paris, v. 84, p. 222-224, 1953.

JENNY, K.; KAPPELI, O.; FIECHTER, A. Biosurfactanst from *Bacillus licheniformes:* structural analysis and characterization. **Applied Microbiology and Biotechnology**, Washington, v. 36, n. 1, p. 5-13, October 1991.

KAISER, A.L.; MONTVILLE, T.J. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. **Applied and Environmental Microbiology**, Washington, v. 62, n. 12, p. 4529-4535, December 1996.

KAMOUN, F. et al. Purification, amino acid sequence and characterization of bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. **Journal of Applied Microbiology**, London, v. 98, n. 4, p. 881-888, April 2005.

KATZ, E.; DEMAIN, A.L. The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible function. **Bacteriological Reviews**, Washington, v. 41, p. 449-474, 1977.

KLAENHAMMER, T.R. Bacteriocins of lactic acid bacteria. **Biochimie**, Paris, v. 70, n. 3, p. 337-349, March 1988.

KLAENHAMMER, T.R. Genetics of bacteriocins produced by lactic acid bacteria. **FEMS Microbiology Reviews**, Amsterdam, v. 12, n. 1-3, p. 39-45, September 1993.

KLEIN, C.; KALETTA, C.; ENTIAN, K.D. Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. **Applied and Environmental Microbiology**, Washington, v. 59, n. 1, p. 296-303, 1993.

KOREMBLUM, E. et al. Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* H20-1 and *B.licheniformis* T6-5 isolated from an oil reservoir in Brazil. **Journal of Applied Microbiology**, London, v. 98, n. 3, p. 667-675, March 2005.

KUIPERS, O.P. et al. Quorum sensing-controlled gene expression in lactic acid bacteria. **Journal of Biotechnology**, Amsterdam, v. 64, n. 1, p. 15-21, September 1998.

LIN, S.C. et al. Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformes* JF-2. **Applied Microbiology and Biotechnology**, Washington, v. 41, p. 281-285, 1994.

LIU, W.; HANSEN, N. The antimicrobial effect of a structural variant of subtilin against outgrowing *Bacillus cereus* T spores and vegetative cells occurs by different mechanisms. **Applied and Environmental Microbiology**, Washington, v. 59, n. 2, p. 648-651, 1993.

MARAHIEL, M.A.; NAKANO, M.M.; ZUBER, P. Regulation of peptide antibiotic production in *Bacillus*. **Molecular Microbiology**, Salem, v. 7, p. 631-636, 1993.

MARREC, C.L.E. et al. Biochemical and genetic characterization of Coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I_4. **Applied and Environmental Microbiology**, Washington, v. 66, n. 12, p. 5213-5220, December 2000.

MARTINEZ, B.; RODRIGUEZ, A.; SUAREZ, J.E. Lactococcin 972, a bacteriocin that inhibits spetum formatiom in lactococci. **Microbiology**, London, v. 146, n. 4, p. 949-955, April 2000.

MARX, R. et al. Structure of the *Bacillus subtilis* peptide antibiotic subtilosin A determined by ^{1}H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. **Journal of Protein Chemistry**, New York, v. 20, n. 6, p. 501-506, August 2001.

MATTICK, A.T.R.; HIRSH, A. Further observations on an inhibitory substance (nisin) from lactic streptococci. **Lancet**, Minneapolis, v. 2, n. 27, p. 5-7, 1947.

MCAULIFFE, O.; ROSS, R.P.; HILL, C. Lantibiotics: structure, biosynthesis and mode of action. **FEMS Microbiology Reviews**, Amsterdam, v. 25, p. 285-308, 2001.

MILLER, M.; BASSER, B. Quorum sensing in bacteria. **Annual Review Microbiology,** Palo Alto, v. 55, p. 165-199, 2001.

MOLL, G.N. et al. Mechanism of the lantibiotic-induced pore formation. Antonie Van Leeuwenhoek. **International Journal of General Molecular Biology**, Amsterdam, v. 69, p. 185-195, 1996.

MONTVILLE, T.J.; BRUNO, M.C.E. Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and other antimicrobial proteins. International **Journal of Food Microbiology**, Amsterdam, v. 24, n. 1-2, p. 53-74, December 1994.

MONTVILLE, T.J.; WINCOWSKI, K. Biologically based preservation systems and probiotic bacteria. In: DOYLE, M.P.; BEUCHAT, L.R.; MONTVILLE, T.J. **Food Microbiology**: fundamentals and frontiers. Washington, DC: American Society for Microbiology, 1997.

MOTTA, A.S.; CLADERA-OLIVERA, F.; BRANDELLI, A. Screening for antimicrobial activity among bactéria isolated from the Amazon basin**. Brazilian Journal of Microbiology**, São Paulo, v. 35, n. 4, p. 307-310, October/December 2004.

NACLERIO, G. et al. Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. **Applied and Environmental Microbiology**, Washington, v. 59, n. 12, p. 4313-4316, 1993.

NAKANO, M.M.; ZUBER, P. Molecular biology of antibiotic production in *Bacillus*. **Critical Reviews in Biotechnology**, London, v. 10, p. 223-240, 1990.

NES, I.F. et al. Biosynthesis of bacteriocins in lactic acid bacteria. **Antonie van Leeuwenhoek**, Amsterdam, v. 70, n. 2-4, p. 113-128, October 1996.

NILSEN, T.; NES, I.F.; HOLO, H. An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. **Journal of Bacteriology**, Washington, v. 180, n. 7, p. 1848-1854, April 1998.

OSCÁRIZ, J.C.; PISABARRO, A.G. Characterization and mechanism of action of cerein 7, a bacteriocin produced by *Bacillus cereus* Bc7. **Journal of Applied Microbiology**, London, v. 89, n. 2, p. 361-369, 2000.

PAIK, S.H.; CHAKICHERLA, A.; HANSEN, J.N. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. **Journal of Biological Chemistry**, Bethesda, v. 273, n. 36, p. 23134-23142, September 1998.

PATTNAIK, P. et al. Purification and characterization of a bacteriocin-like compound (lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. **Journal of Applied Microbiology**, London, v. 91, n. 4, p. 636-645, October 2001.

PECK, M.W. *Clostridium botulinum* and the safety of refrigerated processed foods of extended durability. **Trends in Food Science and Technology**, Cambridge, v. 8, n. 4, p. 186-192, June 1997.

PRIEST, F.G. Systematics and ecology of *Bacillus*. SONENSHEIN, A.L.; HOCH, J.A.; LOSICK, R. (Eds). In: ***Bacillus subtilis* and other Gram-positiva Bacteria**. Washington, DC: American Society for Microbiology Press, 1993. pp. 3-16

RILEY, M.A. Molecular mechanism of bacteriocin evolutionary. **Annual Reviews of Genetics,** Palo Alto, v. 32, p. 255-278, December 1998.

RILEY, M.A.; WERTZ, J.E. Bacteriocins: evolution, ecology and application. **Annual Reviews of Microbiology**, Palo Alto, v. 56, n. 8, p. 117-137, April 2002.

RISOEN, P.A. et al. Characterization of a broad range antimicrobial substance from *Bacillus cereus*. **Journal of Applied Microbiology**, London, v. 96, n. 4, p. 648-655, April 2004.

ROSENBERG, E.; RON, E.Z. High and low-molecular-mass microbial surfactants. **Applied Microbiology and Biotechnology**, Washington, v. 52, n. 2, p. 154-162, August 1999.

SMITH, P.; DAVEY, S. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. **Journal of Fish Disease**, Oxford, v. 16, p. 521-524, 1993.

SOUZA, E.V.; SILVA, C.A.; SOUSA, C.P. Bacteriocins: molecules of fundamental impact on the microbial ecology and potential food biopreservatives. **Brazilian Archives of Biology and Technology**, Curitiba, v. 48, n. 4, p. 559-566, July 2005.

STANLEY, N.R.; LAZAZZERA, B.A. Environmental signals and regulatory pathways that influence biofilm formation. **Molecular Microbiology**, Salem, v. 52, p. 917-924, 2004.

STEIN, T. *Bacillus subtilis* antibiotics: structure, syntheses and specific functions. **Molecular Microbiology**, Salem, v. 56, n. 4, p. 845-857, May 2005.

STOCKEWELL, V.O.; MOORE, L.W.; LOOPER, J.E. Fat of *Agrobacterium radiobacter* K84 in the environment. **Applied and Environmental Microbiology**, Washington, v. 59, n. 7, p. 2112-2120, 1993.

SUGITA, H. et al. Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. **Aquaculture**, Amsterdam, v. 165, p. 269-280, 1998.

TAGG, J.R.; DAJANI, A.S.; WANNAMAKER, L.W. Bacteriocins of gram-positive bacteria. **Bacteriological Reviews**, Washington, v. 40, n. 3, p. 722-756, 1976.

TAMEHIRO, N. et al. Bacilysocina, a novel phospholipidic antibiotic produced by Bacillus subtilis 168. **Antimicrobials Agents and Chemoterapy**, Washington, v. 46, n. 2, p. 315-320, February 2002.

THANIYAVARN, J. et al. Production and characterization of biosurfactants from *Bacillus licheniformis* F2.2. **Bioscience Biotechnology and Biochemistry**, Tokyo, v. 67, n. 6, p. 1239-1244, 2003

VENEMA, K. et al. Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. **Molecular Microbiology**, Salem, v. 17, p. 515-522, 1995.

VINING, L.C. Functions of secondary metabolites. **Annual Review of Microbiology**, Palo Alto, v. 44, p. 395-427, 1990.

VOLLENBROICH, D. et al. Antimycoplasm properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. **Applied and Environmental Microbiology**, Washington, v. 63, n. 1, p. 44-49, January 1997.

YAKIMOV, M.M. et al. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. **Applied and Environmental Microbiology**, Washington, v. 61, p. 1706-1713, 1995.

WIEDEMANN, I. et al. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of the cell wall biosynthesis for potent antibiotic activity. **Journal of Biological Chemistry**, Bethesda, v. 276, n. 1, p. 1772-1779, January 2001.

WILLEY, J. et al. Extracellular complementation of a developmental mutations implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. **Cell**, Cambridge, v. 65, n. 17, p. 641-650, May 1991.

ANEXO 1

Screening for Antimicrobial Activity Among Bacteria Isolated from the Amazon Basin

- Brazilian Journal of Microbiology (2004), **35**: 307-310

ANEXO 2

Antibacterial Activity of Cerein 8A, a Bacteriocin-like peptide produced by *Bacillus cereus*

- International Microbiology (2005), **8:** 125-131.