MAURILIO LOPES MARTINS

CARACTERIZAÇÃO DE PROTEASE E LIPASE DE *Pseudomonas fluorescens* E *QUORUM SENSING* EM BACTÉRIAS PSICROTRÓFICAS ISOLADAS DE LEITE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 23 de março de 2007.

Prof[a]. Elza Fernandes Araújo (Co-orientadora)	Prof. Hilário Cuquetto Mantovani (Co-orientador)
Prof[a]. Andréa de Oliveira Barros Ribon	Dr[a]. Anna Katharina Maria Riedel

Prof[a]. Maria Cristina Dantas Vanetti
(Orientadora)

À minha querida esposa Eliane e

aos meus queridos pais Geralda e Bolivar,

Dedico.

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BIOGRAFIA

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Em setembro de 1996, graduou-se como Tecnólogo em Laticínios pela Universidade Federal de Viçosa (UFV), em Viçosa-MG. No período de 1996 a 2000, foi funcionário do Departamento de Garantia de Qualidade da Cooperativa Central de Laticínios do Estado de São Paulo (Leite Paulista).

Em outubro de 2000, retornou para a UFV onde iniciou o curso de graduação em Ciência e Tecnologia de Laticínios, concluindo-o em 2001. Nesse mesmo ano, iniciou o curso de Mestrado em Microbiologia Agrícola na UFV, concentrando seus estudos na área de Microbiologia de Alimentos, defendendo tese em 26 de fevereiro de 2003.

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LISTA DE ABREVIATURAS

A136 – Estirpe de *Agrobacterium tumefaciens* biosensora de AHL

AB *Agrobacterium* – Meio mínimo para crescimento de estirpes de *Agrobacterium*

ABC – Meio mínimo suplementado com citrato

ABG – Meio mínimo suplementado com glicose

AB *Vibrio* – Meio mínimo para crescimento de estirpes de *Vibrio*

ACN – Acetonitrila

ACP – Proteína carreadora de acil

AHL – Homoserina lactona acilada

AI-2 – autoindutor dois

AI-3 – autoindutor três

AiiA – Enzima lactonase, cliva o anel lactona da molécula de AHL

AprX – Metalloprotease alcalina de *Pseudomonas fluorescens*

BHL – N-butanoil-DL-homoserina lactona

C4-HSL – N-butanoil-DL-homoserina lactona

C5-HSL – N-pentanoil-DL-homoserina lactona

C6-HSL – N-hexanoil-DL-homoserina lactona

C8-HSL – N-octanoil-DL-homoserina lactona

C10-HSL – N-decanoil-DL-homoserina lactona

CFU/ml – Unidade formadora de colônia por mililitro

CV026 – Estirpe mutante de *Chromobacterium violaceum* biosensora de AHL

DHL – N-dodecanoil-DL-homoserina lactona (C12HSL)

DKP – Dicetopiperazina

DNA – Ácido desoxirribonucléico

DPD – 4,5-dihidroxi-2,3-pentanodieno

DTT – Ditiotreitol

dYT – Meio triptona, extrato de levedura, NaCl e glicose

EDTA – ácido etilenodiaminotetracético

ESI-MS – Ionização por *electrospray* – espectrometria de massa

FUR – Regulador de absorção de ferro

GC – Cromatografia gasosa

GC-MS – Cromatografia gasosa acoplada a espectrometria de massa

GMP – Boas práticas de fabricação

HHL – N-hexanoil-DL-homoserina lactona (C6-HSL)

HHQ – 2-heptil-4-quinolona

HPLC – Cromatografia líquida de alta performance

HSL – Homoserina lactona

HTST – Alta temperatura e curto tempo ou pasteurização rápida

IPTG – Isopropil-β-D-tiogalactopiranosideo

KYC55 – Estirpe de *Agrobacterium tumefaciens* biosensora de AHL

LB – Meio Luria Bertani

LC-MS – Cromatografia líquida acoplada a espectrometria de massa

LipM – Lipase de *Pseudomonas fluorescens*

LTLT – Baixa temperatura e longo tempo ou pasteurização lenta

MALDI-TOF – *Matrix assisted laser desorption ionization – time of flight*

MCS – Sítio de clonagem múltipla

MMS – Meio mínimo de sais

MS - Espectrometria de massa

NGMI e NGMII – Meio para crescimento de nematóide

NTL4 – Estirpe de *Agrobacterium tumefaciens* biosensora de AHL

OHHL – N-3-oxohexanoil-L-homoserina lactona (3-oxo-C6-HSL)

OHL - N-octanoil-DL-homoserina lactona

ORF – Quadro de leitura aberto

PCR – Reação de polimerização em cadeia

pMLM – plasmídeo nativo de *Hafnia alvei* 068 e 071

PMSF – Fenilmetilsulfonil fluoride

PQS – 2-heptil-3-hidroxi-4-quinolona ou *quorum sensing* em *Pseudomonas*

QS – *quorum sensing*

RPM – Rotação por minuto

SAH – S-adenosil homosisteína

SAM – S-adenosil metionina

SDS - Dodecil sulfato de sódio

SDS-PAGE – Gel de poliacrilamida-dodecil sulfato de sódio

SRH – S-ribosil homosisteína

TCA – Ácido tricloroacético

TLC – Cromatografia em camada fina

TYEP – Meio triptona, extrato de levedura e fosfato

UHT – Ultra-alta temperatura

X-gal – 5-bromo-4-cloro-3-indolil-β-D-galactopiranosídeo

3-hidroxi-C4-HSL – N-3-hidroxibutanoil-DL-homoserina lactona

3-hidroxi-C12-HSL – N-3-hidroxidodecanoil-DL-homoserina lactona

3-oxo-C6-HSL – 3-oxohexanoil-DL-homoserina lactona

3-oxo-C8-HSL – N-3-oxooctanoil-DL-homoserina lactona

3-oxo-C10-HSL – N-3-oxodecanoil-DL-homoserina lactona

3-oxo-C12-HSL – 3-oxododecanoil-DL-homoserina lactona

RESUMO

MARTINS, Maurilio Lopes, D.Sc., Universidade Federal de Viçosa, março de 2007. **Caracterização de protease e lipase de *Pseudomonas fluorescens* e *quorum sensing* em bactérias psicrotróficas isoladas de leite.** Orientadora: Maria Cristina Dantas Vanetti. Co-orientadores: Elza Fernandes de Araújo, Hilário Cuquetto Mantovani e Célia Alencar de Moraes.

Protease e lipase produzidas por *Pseudomonas fluorescens* foram purificadas e caracterizadas. Os genes *aprX* e *lipM* foram clonados, seqüenciados, e expressos em *Escherichia coli* e apresentaram alta identidade com as seqüências disponíveis no banco de dados. A massa molecular deduzida de ambas as enzimas foi de 50 kDa. Foi verificado que cálcio é essencial para as atividades enzimáticas, uma vez que quando este íon não foi adicionado à solução de diálise nenhuma atividade foi encontrada. A protease foi ativa em ampla faixa de pH, apresentou temperatura ótima de 37 °C, e maior atividade foi verificada sobre caseína e gelatina. Maior estabilidade térmica da enzima foi a 75 °C por 20 s. A lipase foi mais ativa a 25 °C e em pH próximo de 7,5 e p-nitrofenil-palmitato foi o substrato preferencial. Tratamentos térmicos de 65 °C por 30 min e de 75 °C por 1 min reduziram sua atividade para 13,2% e 25,4%, respectivamente. O mecanismo de *quorum sensing* (QS) em *P. fluorescens* foi estudado e verificou-se que as estirpes avaliadas, apesar de induzirem *Agrobacterium tumefaciens* NTL4 e A136, não produziram acil homoserinas lactonas (AHLs). Entretanto, a produção de auto-indutor dois (AI-2) foi detectada e a

presença de dicetopiperazinas (DKPs) nos extratos químicos obtidos a partir de meio TYEP inoculado com *P. fluorescens* foi constatada. O rDNA 16S de seis bactérias gram-negativas isoladas de leite cru foi seqüenciado e o isolado 039 foi identificado como *Pantoea* sp., 059, 068 e 071 foram identificados como *Hafnia alvei*, 067 como *Enterobacter* sp. e 099 como *Aeromonas hydrophila*. Verificou-se que esses isolados apresentam diferenças no potencial deteriorador, na resistência a antibióticos e, após ensaios de estria cruzada em superfície de meio sólido para detecção de AHLs, constatou-se que somente *Pantoea* sp. não foi capaz de induzir nenhuma das estirpes monitoras utilizadas. Os ensaios de cromatografia em camada fina e a caracterização química dos extratos por espectrometria de massa confirmaram que essas bactérias produzem diferentes AHLs. O mecanismo de inibição de quorum foi utilizado e a enzima lactonase foi expressa em *Enterobacter* sp. transconjugante a qual foi incapaz de acumular AHLs em sobrenadante de caldo LB. O transconjugante se mostrou mais proteolítico do que a estirpe selvagem, indicando que o mecanismo de QS regula negativamente a atividade proteolítica neste isolado. Das 32 enzimas de restrição utilizadas para restringir o plasmídeo nativo (pMLM) presente nos isolados de *H. alvei* 068 e 071, apenas *Dde*I, *Hinf*I, *Msp*I, e *Rsa*I foram efetivas. Além disso, não foi possível expressar a enzima lactonase em *H. alvei* 068 e 071 transconjugantes o que impossibilitou a avaliação da influência do mecanismo de QS sobre a atividade proteolítica desses isolados. O gene *halI*, que codifica a sintase de AHLs produzidas por estirpes de *H. alvei*, foi identificado nos isolados 059, 067, 068 e 071. Esse gene foi clonado, seqüenciado e expresso em *E. coli* e verificou-se que codifica uma sintase responsável pela produção de N-hexanoil-DL-homoserina lactona (C6-HSL) e N-3-oxohexanoil-L-homoserina lactona (3-oxo-C6-HSL). Das seis estirpes psicrotróficas proteolíticas avaliadas, apenas *A. hydrophila* foi capaz de produzir quitinase, AI-2 e de ser patogênica contra *Caenorhabditis elegans*.

ABSTRACT

MARTINS, Maurilio Lopes, D.Sc., Universidade Federal de Viçosa, March, 2007. **Characterization of protease and lipase from *Pseudomonas fluorescens* and quorum sensing in psychrotrophic bacteria isolated from milk.** Adviser: Maria Cristina Dantas Vanetti. Co-Advisers: Elza Fernandes de Araújo, Hilário Cuquetto Mantovani and Célia Alencar de Moraes.

A protease and a lipase produced by *Pseudomonas fluorescens* were purified and characterized. The *aprX* and *lipM* genes were cloned, sequenced, and expressed in *Escherichia coli*. These genes presented high identity with the sequences available in the GenBank. The molecular mass of both enzymes were 50 kDa. It was verified that calcium is essential to the enzymatic activities since when this ion was not added into the dialysis solution no activity was found. The protease was active in a large range of pH, had highest activity against casein and gelatin, and its temperature optimum was at 37 °C. Besides, this enzyme showed the highest thermal stability at 75 °C for 20 s. In contrast to the protease, the temperature optimum for the lipase was 25 °C and the pH optimum was close to 7.5. This enzyme showed the highest activity against p-nitrophenyl-palmitate, and the thermal treatments of 65 °C for 30 min and 75 °C for 1 min reduced its activity to 13.2% and 25.4%, respectively. The mechanism of quorum sensing (QS) was studied in *P. fluorescens* and it was verified that although the strains evaluated induced *Agrobacterium tumefaciens* NTL4 and A136, they do not produce acyl-homoserine lactones (AHLs). However, it

was detected production of auto-inducer two (AI-2) and presence of diketopiperizines (DKPs) into the chemical extract obtained from TYEP medium inoculated with *P. fluorescens*. The 16S rDNAs of strains 039, 059, 067, 068, 071, and 099 isolated from raw milk were sequenced and they were identified as *Pantoea* sp., *Hafnia alvei*, *Enterobacter* sp., *Hafnia alvei*, *Hafnia alvei*, and *Aeromonas hydrophila*, respectively. It was verified that these strains presented different spoilage potentials and resistance against different antibiotics. After cross-streak assays in order to detect AHLs, only *Pantoea* sp. was not able to induce the monitor strains. The thin layer chromatography and the chemical characterization of the extracts by mass spectrometry confirmed that these strains produce different AHLs. The quorum quenching mechanism was used and the lactonase enzyme was expressed in the *Enterobacter* sp. transconjugant, which was unable to secrete AHLs into LB medium. This strain was more proteolytic than the wild type, indicating that the QS negatively regulates the proteolytic activity. Of the 32 restriction enzymes used to digest the native plasmid from *H. alvei* 068 and 071, only *Dde*I, *Hinf*I, *Msp*I, and *Rsa*I were effective. Moreover, it was not possible to express lactonase in *H. alvei* 068 and 071 transconjugants which compromised the evaluation of the influence of the QS mechanism on spoilage activity. The *halI* gene, which encodes the AHL synthase in *H. alvei*, was identified in the strains 059, 067, 068, and 071. This gene was cloned, sequenced, and expressed in *E. coli* and it was verified that it encodes a synthase responsible for the production of N-hexanoyl-DL-homoserine lactone (C6-HSL) and N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL). Besides producing AHLs, *A. hydrophila* produced chitinase, AI-2 and was pathogenic against *Caenorhabditis elegans*.

INTRODUÇÃO GERAL

Em produtos lácteos refrigerados, a microbiota Gram-negativa é a mais comumente encontrada, sendo o gênero *Pseudomonas* predominante no leite cru refrigerado. Concentrações significativas de protease são encontradas no leite quando a população de bactérias psicrotróficas está acima de 10^6 UFC/ml. Algumas dessas bactérias secretam lipases que também estão envolvidas na deterioração dos produtos lácteos. As enzimas proteolíticas e lipolíticas secretadas por *Pseudomonas fluorescens* são codificadas pelos genes *aprX* e *lipA*, localizados em um mesmo operon e cuja expressão é regulada por diferentes fatores.

A expressão gênica em muitas bactérias ocorre em resposta à densidade populacional por um mecanismo chamado de *quorum sensing*. Este mecanismo permite que as células controlem muitas de suas funções tais como, colonização de superfície, motilidade, produção de exopolímeros, produção de antibióticos, esporulação, formação de biofilme, bioluminescência, diferenciação celular, competência para absorção de DNA, produção de pigmentos, transferência de plasmídeos, esporulação, produção de toxinas, expressão de genes de virulência e produção de enzimas hidrolíticas.

Quorum sensing é um mecanismo de comunicação entre células e é mediado por sinais químicos extracelulares, denominados de moléculas auto-indutoras ou sinalizadoras, produzidas pelas bactérias e liberadas no ambiente. Quando a concentração do sinal é suficientemente alta, os genes alvo são ativados ou reprimidos. A comunicação entre células pode ser bloqueada por meio da inativação

das moléculas sinalizadoras, pela interrupção dos genes que codificam enzimas que sintetizam essas moléculas, ou pelo uso de aditivos que interfiram na ligação dessas moléculas com a proteína receptora.

A utilização de culturas monitoras e de métodos de cromatografia líquida acoplada a espectrometria de massa é fundamental para o conhecimento da estrutura química das moléculas sinalizadoras e identificação de possíveis fenótipos regulados pelo mecanismo de *quorum sensing*.

Moléculas sinalizadoras são produzidas por bactérias em alimentos e podem estar associadas a processos de biodeterioração. O esclarecimento dos fatores relacionados à regulação da atividade deterioradora de bactérias psicrotróficas torna-se necessário considerando os diversos problemas tecnológicos e econômicos que esse grupo de bactérias ocasiona à indústria de laticínios. Este estudo buscou a purificação e caracterização de protease e lipase produzidas por *P. fluorescens*, bem como, a caracterização das moléculas auto-indutoras do sistema de *quorum sensing* em bactérias psicrotróficas como *P. fluorescens*, *Pantoea* sp., *Hafnia alvei*, *Enterobacter* sp. e *Aeromonas hydrophila* isoladas de leite cru refrigerado e a possível relação desse mecanismo com o processo de deterioração do leite.

Procedeu-se à redação dos capítulos desta tese, em língua inglesa, pelo fato de a co-orientadora Dra. Anna Katharina Maria Riedel não dominar a língua portuguesa.

CHAPTER 1

LITERATURE REVIEW

1.1. Psychrotrophic bacteria in milk

The microflora present in foods consists of microorganisms associated with the raw material, microorganisms acquired during manipulation and processing, and those which survived the stages of processing and storage of the product. Food spoilage is a complex process and excessive amounts of foods are lost due to microbial spoilage even when modern preservation techniques are employed (GRAM et al., 2002). The establishment of contamination sources of foods is important in order to control this process and to maintain the food microbial population the smallest possible (JAY, 1996).

Milk constitutes an ideal medium for growth of deteriorative and pathogenic microorganisms because of its high nutritional value, water content, and almost neutral pH (FRANK, 1997). Bacteria can access milk and dairy products from several sources, such as water, soil, animal feeding, milking equipments, and manipulators (COUSIN, 1982; MUIR, 1996; ENEROTH et al., 1998; MURPHY and BOOR, 2000).

Maintenance and transport of raw milk under refrigeration eliminate its spoilage by mesophilic bacteria, but results in selection of psychrotrophic bacteria, which are capable of multiply at 7 ºC or below, independent of their optimum growth

temperature (FRANK et al., 1992). In milk, psychrotrophs are mainly represented by the gram-negative genus *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Hafnia*, *Enterobacter*, *Serratia*, *Alcaligenes*, *Burkholderia*, *Chromobacterium* and *Flavobacterium* spp., and by the gram-positive genus *Bacillus, Clostridium, Corynebacterium, Streptococcus, Lactobacillus* and *Micrococcus* spp (SØRHAUG and STEPANIAK, 1997; PINTO 2004; MUNSCH-ALATOSSAVA and ALATOSSAVA, 2006).

In refrigerated dairy products, gram-negative microflora is commonly found (ADAMS et al., 1975; COUSIN, 1982; WIEDMANN et al., 2000; DOGAN and BOOR, 2003). Pinto (2004) verified that gram-negative psychrotrophic proteolytic bacteria constituted 84.6% of the isolates obtained from raw milk in Brazil, while the proteolytic gram-positive bacteria constituted only 15.4%.

Psychrotrophic *Pseudomonas* predominate in refrigerated raw milk (WIEDMANN et al., 2000; DOGAN and BOOR, 2003; PINTO, 2004), since they present a well established physiologic mechanism of adaptation and growth at low temperatures (JAY, 1996).

P. fluorescens constitutes the milk deteriorative species of higher importance (WIEDMANN et al., 2000; DOGAN and BOOR, 2003; PINTO, 2004) due to its ability to produce thermostable proteases that hydrolyze casein and decrease yield and sensory quality of dairy products (SØRHAUG and STEPANIAK, 1997). Some of these bacteria also secrete lecithinases and lipases that can play a significant role in deterioration of these products (WIEDMANN et al., 2000; DOGAN and BOOR, 2003).

Among the microorganisms that survive pasteurization, sporeforming psychrotrophs *Bacillus* spp. dominate (COUSIN, 1982). They secrete heat-resistent extracellular proteases, lipases and phospholipase (lecithinase) that are of comparable heat resistance to those of pseudomonads (SØRHAUG and STEPANIAK, 1997).

1.2. Relevance of spoilage enzymes produced by psychrotrophic bacteria in milk

Spoilage of refrigerated raw milk occurs mainly due to protease and lipase activities. Proteolytic enzymes produced by bacteria act on κ-casein which results in

destabilization of casein micelles and gelation of milk (RECIO et al., 2000). Lipolysis occurs due to the action of natural or microbial lipases. These enzymes are able to hydrolise triglycerides, a milk fat constituent, in fatty acids of small chains such as, butyric, caproic, caprylic and capric acid, mainly responsible for off-flavors in milk and for rancidity in cheese (CHEN et al., 2003). Lipases produced by psychrotrophs are more important than proteases in relation to development of defects of flavor in cheese because proteases are soluble in water and lost in the whey, while lipases are adsorbed in the fatty globules and retained in cheese mass (FOX, 1989).

Proteases, lipases and phospholipases from psychrotrophic bacteria, especially pseudomonads, are stable at high temperatures and survive pasteurization and UHT treatment, but are not active above 50 to 60 °C (Sørhaug and Stepaniak, 1997). They show optima temperature at 30 – 45 °C, have low activation energy and are therefore more active at 4 – 7 °C than enzymes from mesophilic microorganisms (SØRHAUG and STEPANIAK, 1997).

The inactivation of 90% of extracellular activity of proteases produced by *Pseudomonas* can be achieved at 72 °C for 4 – 5 h, or at 120 °C for 7 min (ADAMS et al., 1975). However, these treatments are considered highly detrimental to milk characteristics. Thermoresistant proteases produced by psychrotrophic bacteria from *Pseudomonas* genus are alkaline metalloproteases, which need divalent ions as Ca^{+2} and Zn^{+2} for their stability and activity (RAO et al., 1998). Among the features that stabilize thermoenzymes from psychrotrophic microorganisms are salt bridges, additional hydrogen bonds, tighter Ca^{2+}-binding sites, maximized packing, shorter loops, and an expanded hydrophobic core (SØRHAUG and STEPANIAK, 1997).

Psychrotrophic microorganisms or their enzymes in milk used for cheese manufacture can result in cheese with various defects. In addition, in the cheese industry, the producers have been problems of low yield due to bacterial protease activity against casein (COUSIN, 1982). Development of off-flavours, including bitterness and texture problems in cheese caused by proteases from psychrotrophs have been reported, but only when psychrotroph counts in milk were 2×10^6 to 5×10^8 CFU/ml.

Even though in low concentration, proteases and lipases can cause defects in products of long shelf-life such as UHT milk and milk powder (CELESTINO et al., 1997). The fermentation rates of lactic acid bacteria during production of yogurt can

also be affected by growth of psychrotrophs in milk (FAIRBAIRN and LAW, 1986). In some cases, the action of proteases produced by psychrotrophic bacteria results in increased levels of peptides and free amino acids, which stimulate growth of lactic acid bacteria (COUSIN and MARTH, 1977). On the other hand, lipolysis caused by psychrotrophic bacteria increase the concentration of free fatty acids which may inhibit the lactic acid bacteria (STOFER and HICKS, 1983).

1.3. Regulation of spoilage enzymes expression by *P. fluorescens*

Proteolytic and lipolytic enzymes secreted by *P. fluorescens* are codified by *aprX* and *lipA* genes, respectively (BURGER et al., 2000; WOODS et al., 2001; McCARTHY et al., 2004). These genes are located at opposite ends of an operon, which also includes protease inhibitor (*inh* gene), type I secretion functions (*aprDEF* genes) and two autotransporter proteins (*prtA* and *prtB* genes) (WOODS et al., 2001).

Larger quantities of extracellular enzymes are produced at temperatures below the optimum growth temperature. However, little is known about the regulation mechanism involved in protease production at different temperatures.

According to Burger et al. (2000), the optimum growth temperature for *P. fluorescens* LS107d2 is 27 ºC, and the optimum temperature for metalloprotease production is between 22 and 27 ºC. However, above 27 ºC the proteolytic activity decreased significantly. Production of metalloprotease above the optimum growth temperature by *P. fluorescens* LS107d2 occurs in lower intensity and it is necessary the production of PrtI and PrtR that are encoded by a dicistronic operon. Mutation in *prtI* and *prtR* genes of *P. fluorescens* LS107d2 rendered cells unable to synthesize protease above the optimum growth temperature. The PrtI activates transcription of genes related to extracytoplasmatic functions, and protein PrtR, transmembrane, is an activator of protein PrtI. When temperature increases, a conformational change occurs in PrtR and this enzyme does not activate the PrtI. Then, PrtI does not activate the transcription of *aprX-lipA* operon reducing the proteolytic activity. However, at 23 ºC, temperature below the optimum growth temperature of *P. fluorescens* LS107d2, probably, another system acts controlling the production of protease, since mutants as *prtI*$^{-}$ and *prtR*$^{-}$ showed proteolytic activity in this temperature.

In the soil bacterium, *P. fluorescens* M114, an iron-starvation extracytoplasmatic function sigma factor, PbrA, required for transcription of siderophore biosynthetic genes, was also implicated in protease regulation (SEXTON et al., 1995; SEXTON et al., 1996). A serralysin-type metalloprotease gene, *aprA*, was identified by Maunsell et al. (2006) and it was found to encode the major, if not only, extracellular protease produced by this strain. The expression of *aprA* and its protein product were found to be subject to complex regulation (MAUNSELL et al., 2006). According to these authors, transcription analysis confirmed that PbrA was required for full *aprA* transcription under low iron conditions, while the ferric uptake regulator, Fur, was implicated in *aprA* repression under high iron conditions. Iron regulation of AprA was dependent on culture conditions, with PbrA-independent AprA-mediated proteolytic activity observed on skim milk agar supplemented with yeast extract, when supplied with iron or purified pseudobactin M114. These effects were not observed on skim milk agar without yeast extract. PbrA-independent *aprA* expression was also observed from a truncated transcriptional fusion when grown in sucrose asparagines tryptone broth supplied with iron or purified pseudobactin M114. Thus, experimental evidence suggested that iron mediated its effects via transcriptional activation by PbrA under low iron conditions, while an as-yet-unidentified sigma factor(s) may be required for the PbrA-independent *aprA* expression and AprA proteolytic activity induced by siderophore and iron.

Besides iron, the expression of protease and lipase in *P. fluorescens* B52 is regulated by concentration of ions as Na^+ and K^+ and this involves homologue proteins to the EnvZ-OmpR regulator system present in *Escherichia coli* and *Salmonella* Typhimurium (McCARTHY, 2003). McKellar and Cholette (1986) reported that in absence of ionic calcium an inactive precursor of protease was produced by *P. fluorescens*. This precursor could not be activated proving that calcium is required to stabilize the enzyme. Protease AprX production by *P. fluorescens* CY091 was also dependent of $CaCl_2$ (LIAO and McCALLUS, 1998). Rajmohan et al. (2002) verified that maximum production of protease by an isolate of *P. fluorescens* was in minimal medium containing 1 mM/l of $CaCl_2$. Therefore, besides presence of calcium in the growth medium, carbon source causes a complex effect in the synthesis of protease by *Pseudomonas* spp.

Protease production is induced by many products of protein degradation. Asparagin appears to be the most effective inducer amino acid, whereas citric acid is

an inhibitor of the biosynthesis (McKELLAR, 1989). Glucose, galactose, lactate, glutamine, and glutamic acid also delays or inhibits protease production by *P. fluorescens* in raw milk (JASPE et al., 1994).

Moreover, production of protease and lipase by *P. fluorescens* and other psychrotrophic bacteria in raw milk generally occur between the end of logarithmic phase and beginning of stationary phase of growth (GRIFFITHS, 1989; MATSELIS and ROUSSIS, 1998). Significant concentrations of metalloprotease (1.01 ng/ml) were found into milk when population of *P. fluorescens* was above 10^6 CFU/ml (BIRKELAND et al., 1985; MATTA et al., 1997, PINTO, 2004). Production of extracellular enzymes at low cell density may not be advantageous to the cells due to the distribution of enzymes and nutrients delivered into media (McCARTHY, 2003).

The expression of extracellular enzymes and metabolites by *P. fluorescens* is generally regulated by GacS/GacA two-component system that occurs in a wide range of gram-negative bacteria (BLUMER et al., 1999; KAY et al., 2005). The small (119-nt) RNA, RsmX discovered by Kay et al. (2005), together with RsmY and RsmZ, forms a triad of GacA-dependent small RNAs, which sequester the RNA-binding proteins RsmA and RsmE and thereby, antagonize translational repression exerted by these proteins in strain CHA0 (Figure 1) (KAY et al., 2005). This small RNA triad was found to be both necessary and sufficient for posttranscriptional derepression of biocontrol factors and for protection of cucumber from *Pythium ultimum*. According to Kay et al. (2005), the same three small RNAs also positively regulated swarming motility and synthesis of a quorum-sensing signal which is unrelated to N-acylhomoserine lactones and which autoinduces the Gac/Rsm cascade. Expression of RsmX and RsmY increased in parallel throughout cell growth, whereas RsmZ was produced during the late growth phase. This differential expression is assumed to facilitate fine tuning of GacS/A-controlled cell population density-dependent regulation in *P. fluorescens*.

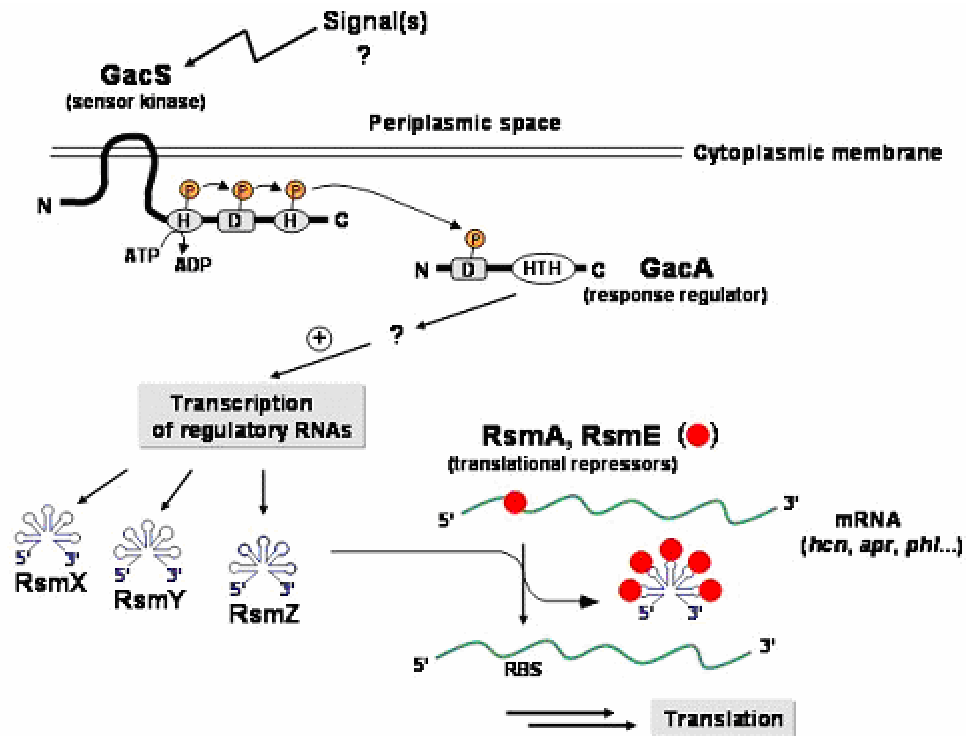

Figure 1 – Post-transcriptional control by Gac/Rsm signal transduction pathway in *P. fluorescens* CHA0. The mRNAs that will be translated in proteins responsible for protease activity (AprX), swarming motility, signal molecules, and antibiotics are repressed by RsmA or RsmE. RsmX together with RsmY and RsmZ forms a triad of GacA-dependent small RNAs, which sequester the RNA-binding proteins RsmA and RsmE and thereby antagonize translational repression exerted by these proteins (source: http://www.unil.ch/dmf/page18042.html. Access on June 4th, 2007).

1.4. The mechanism of quorum sensing

Many bacteria regulate the expression of some genes in response to population density in a mechanism known as quorum sensing (QS) (FUQUA et al., 1994; WHITEHEAD et al., 2001). This mechanism allows cells to control many of their functions such as surface colonization and motility, production of exopolymers, production of antibiotics, biofilm development, bioluminescence, cell differentiation, competence for DNA uptake, growth, pigment production, conjugal plasmid transfer, sporulation, toxin production, virulence gene expression, and production of a range of hydrolytic enzymes (SMITH et al., 2004).

Quorum sensing is a mechanism of cell-to-cell communication and it is mediated by extracellular chemical signals denominated signal molecules or autoinducers generated by bacteria when specific cell densities are reached. When concentration of signal is sufficiently high, the target genes are either activated or repressed. Bacteria have been reported to produce many different types of quorum sensing signals. They can be grouped into amino acids and small peptides, commonly used by gram-positive bacteria (MILLER and BASSLER, 2001; WHITEHEAD et al., 2001) and fatty acid derivatives (N-acylhomoserine lactones) or AHLs (autoinducer-1) frequently produced by gram-negative bacteria (FUQUA et al., 1996; EBERL, 1999; WHITEHEAD et al., 2001). Other quorum sensing signaling molecules have been identified in gram-negative bacteria: 2-heptyl-3-hidroxy-4-quinolone (PQS) in *Pseudomonas aeruginosa* (PESCI et al., 1999; EBERL, 2006) and diketopiperazines (DKPs) in *P. aeruginosa* and other bacteria (HOLDEN et al., 1999; DEGRASSI et al., 2002). Recently, the autoinducer-2 (AI-2), first discovered in *Vibrio harveyi* (BASSLER et al., 1993), has been described as a new quorum sensing signal used by both gram-negative and gram-positive bacteria (SCHAUDER and BASSLER, 2001; CHEN et al., 2002). Besides these signal molecules, autoinducer-3 (AI-3) has been involved in QS mechanism, but it has unknown chemical structure (SPERANDIO et al., 2003).

Other different chemical communication systems can also occur such as, in *Vibrio cholerae* that posses, at least, three different QS systems that work in parallel and regulate expression of virulence genes (MILLER et al., 2002). However, among signaling molecules, AHLs, oligopeptides, and AI-2 are more known and used as paradigma of QS (KELLER and SURETTE, 2006).

1.4.1. Biosynthesis and characterization of the signal molecules

1.4.1.1. Acyl-homoserine lactones (AHLs)

Frequently in gram-negative bacteria, AHLs are the signal molecules that are diffused in the medium (EBERL, 1999). Chemical structure of AHLs indicates that the acyl chain is derived from the metabolism of fatty acid, while molecules of homoserine lactones originate from amino acids metabolism. Although all AHL

structures have the homoserine lactone ring moiety in common, the acyl side chain of different AHLs can vary in length, degree of substitution, and saturation. The AHLs currently identified have side chains that range from 4 to 18 carbons in length, usually in increments of to 2-carbon units (ZHU at al., 2003). The overall length of side chain and chemical modification at the *β* position provides specificity to QS systems (FUQUA et al., 2001; ZHU et al., 2003).

Earliest described example of QS system using AHL is found in bioluminescent marine symbiotic bacterium *Vibrio fischeri*. A protein known as LuxI is responsible for production of AHL, and a protein called LuxR is an AHL receptor as well as an AHL-dependent transcriptional activator of the luciferase operon (FUQUA et al., 2001). Concentration of external AHL increases as a function of population density of *V. fischeri*. When the AHL concentration reaches micromolar range, the AHL can bind LuxR, and the resulting complex binds the promoter of *lux* operon and activates its transcription.

LuxI-type proteins must carry out two reactions: formation of a homoserine lactone ring from S-adenosylmethionine (SAM), and acylation of the amine at the expense of acyl-ACP (HANZELKA and GREENBERG, 1995; JIANG et al., 1998; SCHAEFER et al., 1996; VAL and CRONAN, 1998). This explains the variability of these acyl groups, since ACPs can contain over 30 different acyl chains. The homoserine lactone ring of AHL is unstable to hydrolysis, especially at alkaline pH. AHLs are generally thought to diffuse readily across the bacterial envelope. Three families of AHL synthases have been described. While most synthases resemble LuxI of *V. fischeri*, two of these enzymes (AinS of *V. fischeri* and LuxM of *V. harveyi*) resemble each other but do not resemble LuxI (GILSON et al., 1995). However, both families of AHL synthases use the same substrates and may use similar reaction mechanisms. A third AHL synthase, unrelated to either family, was reported in *P. fluorescens* (LAUE et al., 2000).

1.4.1.2. Quinolones

Pesci et al. (1999) discovered that an additional signal, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, affects virulence gene expression in *P. aeruginosa* and more detailed analyses revealed that this *Pseudomonas* quinolone signal (PQS) functions

as an integral component of QS network (DIGGLE et al., 2006). LasR regulates PQS production, which, in turn, is necessary for transcription of the *rhlR* and *rhlI* genes, thereby creating a regulatory link between *las* and *rhl* QS systems (PESCI et al., 1999; McNIGHT et al., 2000). PQS is synthesized from anthranilate by head-to-head condensation reaction between anthranilic acid and various β-keto fatty acids by the products of the *pqsABCDE* operon (BREDENBRUCH et al., 2005). Although additional work will be required to elucidate the complex interrelationships between the two signaling pathways (EBERL, 2006), it is clear that PQS production is modulated by both AHL-dependent QS systems and that exogenous addition of PQS upregulates *rhl*-controlled QS phenotypes in *P. aeruginosa* (DIGGLE et al., 2003).

The *pqsABCDE* operon contains five genes that encode for a putative coenzyme A ligase (*pqsA*), two β-keto-acyl carrier protein synthases (*pqsB*, *pqsC*), and a FabH1 homologous transacetylase (*pqsD*). The *pqsE* gene appears to encode a response effector protein which itself is not involved in biosynthesis of PQS. Although the exact functions of the enzymes remain to be elucidated, it is clear that *pqsABCDE* gene products direct the synthesis of 2-heptyl-4-quinolone (HHQ), the immediate precursor of PQS (GALLAGHER et al., 2002). HHQ is thought to be an extracellular messenger that is released from and taken up by *P. aeruginosa* cells (EBERL, 2006). Once taken up, HHQ is converted into PQS by action of the putative FAD-dependent non-oxygenase PqsH. Expression of the *pqsH* gene, which is not physically linked to the *pqsABCD* operon, is partially controlled by the *las* system, connecting AHL-dependent QS with PQS signaling (GALLAGHER et al., 2002).

Quinolone signaling was thought to be unique to *P. aeruginosa*, since PQS molecules could not be detected in the culture supernatants of several other species of *Pseudomonas*. However, Diggle et al. (2006) presented convincing evidence that quinolone-dependent signaling is more widespread than so far anticipated. Diggle et al. (2006) developed a simple and rapid method for screening bacterial culture supernatants for AHQ production. To this end, a *P. aeruginosa* bioreporter was constructed, which cannot synthesize AHQs due to the inactivation of the *pqs*A gene, but which responds to exogenously supplied AHQs with the emission of light. This biosensor strain can be incorporated within agar and used as an overlay following thin layer chromatography (TLC) of the solvent-extracted culture supernatants.

According to Eberl (2006), using this approach as a fast initial screen and liquid chromatography-mass spectrometry (LC-MS/MS) for confirmation of the

molecules identity, it was shown that several *Burkholderia pseudomallei* strains, *Burkholderia cenocepacia, Burkholderia thailandensis*, and *Pseudomonas putida* produce HHQ, but not PQS.

While in *P. aeruginosa* HHQ has to be converted to PQS before act as a signaling molecule, in *B. pseudomallei*, HHQ serves as a signaling molecule *per se* (DIGGLE et al., 2006). Considering that AHQs are synthesized from key cellular metabolites, it appears likely that this class of signaling molecules is widely used by bacteria (EBERL, 2006). With the AHQ biosensor development, an ideal tool is now available to explore the full extent and diversity of AHQ-mediated signaling between bacteria.

1.4.1.3. Diketopiperazines (DKP)

Although there is abundant literature data about dipeptides isolated from microbial sources (HOLDEN et al., 1999; HOLDEN et al., 2000; BRELLES-MARINÕ and BEDMAR, 2001; DEGRASSI et al., 2002; TAYLOR et al., 2004), their true origin remains controversial. A variety of diketopiperazines (DKPs) were shown to exist in protein hydrolysates as well as fermentation broths and cultures of yeast, lichen, fungi, and bacteria (PRASAD, 1995). Microbial DKPs appear to be both compounds synthesised *de novo* and catabolic products of peptone or other components found in nutrient rich media (PRASAD, 1995; MITOVA et al., 2005).

Holden et al. (1999) found AHL-like molecules, DKPs, in supernatant of many gram-negative bacteria, including *P. fluorescens* and *P. aeruginosa* as a consequence of their ability to activate biosensors previously considered specific for AHLs. Although DKPs are structurally quite distinct from AHLs, at high concentrations they are able to cross-activate AHL-dependent reporter constructs based on several different LuxR homologues (HOLDEN et al., 1999). Detection of these DKPs appears to be an example of fortuitous chemical crosstalk and raises the obvious question as to their origin and biological function(s) (HOLDEN et al., 2000).

Besides *Pseudomonas*, *V. vulnificus* also produces DKP, which was detected by a QS bioindicator and it affected the expression of ToxR-dependent genes *ompU* and *ctxAB* in *Vibrio* strains (PARK et al., 2006). Initially, it was assumed that this pathogen would produce an AHL as do *V. fisheri* and *V. harveyi*, and various

bioindicators sensitive to AHL molecules were employed to detect QS signal affecting expression of *vvp* and *vvh* in *V. vulnificus*. However, the active compound from *V. vulnificus* which activated the QS bioindicator was a DKP molecule.

1.4.1.4. Furanosyl borate diester: auto-inducer two (AI-2)

While AHLs, PQS, and modified peptides (DKPs) described above are confined to a reasonably narrow range of bacteria, recent evidence has suggested the existence of a universal QS language (CÁMARA et al., 2002). A family of molecules, termed AI-2, common to many gram-negative and gram-positive bacteria has been described (FEDERLE and BASSLER, 2003). However, there is no direct evidence for the involvement of AI-2 in regulation of pathogenic traits. Furthermore, whether AI-2 has a true role in QS signaling in general has recently been questioned, with suggestions that in most bacteria AI-2 is simply a metabolic side product, which casts doubts on its suitability as a target in the context of QS inhibition.

Evidence for existence of AI-2 emerged with studies developed with *V. harveyi*. Genetic evaluation of QS circuit in this bacterium showed that it produces two auto-inducer signals in order to regulate bioluminescence. It seems that one signal is used to intra-species communication and another to interspecies communication (FEDERLE and BASSLER, 2003). In *V. harveyi*, the LuxI/R system is absent and light production is controlled by two parallel, not homologue pathways (BASSLER et al., 1993). In one of then, the auto-inducer molecule is not an AHL, but a furanosyl borate diester (CHEN et al., 2002) which synthesis is controlled by *luxS*, and the receptor protein is LuxQ, a kinase sensor of membrane (SURETTE et al., 1999).

The occurrence of *luxS*-dependent AI-2 signaling is widespread among both gram-negative and gram-positive bacteria. Moreover, 130 of 136 bacterial species contain a highly conserved *luxS* homologue and a role for AI-2 in interspecies communication has been proposed (SURETTE et al., 1999; BEESTON and SURETTE, 2002).

The biosynthetic pathway for AI-2 synthesis was elucidated (SCHAUDER et al., 2001; WINZER et al., 2002). In the cell, *S*-adenosylmethionine (SAM) is consumed to form *S*-adenosylhomocysteine (SAH), which in turn, is hydrolysed by

the nucleosidase, Pfs, yielding adenine and *S*-ribosylhomocysteine (SRH). Subsequently, the LuxS protein, a zinc metalloenzyme, converts *S*-ribosylhomocysteine (SRH) to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor probably requiring further rearrangement for AI-2 signal activity (SCHAUDER et al., 2001; WINZER et al., 2002). Both *luxS* and *pfs* are required for AI-2 activity. However, expression of *luxS* is constitutive while the transcription of *pfs* is tightly correlated to AI-2 production and neither is regulated directly by AI-2 (BEESTON and SURETTE, 2002).

Although the role of AI-2 in *V. harveyi* as a density-dependent signal for regulating bioluminescence has been well established, the function of AI-2 in other bacteria has yet to be clarified. It is clear that AI-2 signaling regulates expression of numerous genes and is involved in determining phenotypes, but exactly what AI-2 is signaling is a murky subject. Emerging evidence indicates that AI-2 may not be a density-dependent signal, but rather, a metabolic gauge or waste product (WINANS, 2002).

1.4.1.5. Oligopeptides

Opposite to gram-negative, gram-positive bacteria typically communicate by using oligopeptide signals, which are synthesized as precursors on ribosomes and proteolytically processed and released either by the general secretory pathway or by a dedicated secretory apparatus (DUNNY and LEONARD, 1997). These signals are generally detected using a two-component phosphorelay mechanism. In some cases, the peptide receptor is a histidine kinase, while in other examples, the peptide is imported by an ABC-type permease and the receptor is a cytoplasmic phosphor-aspartate phosphatase (PEREGO, 1998). These signaling systems regulate processes as pathogenesis, endospore formation, genetic competence, conjugation, and production of microcin antimicrobials. Some gram-positive bacteria and at least, one proteobacterium (*Xanthomonas campestris*) communicate via amphipathic compounds called γ-butirolactones rather than peptides (BARBER et al., 1997).

1.4.2. Detection of signal molecules

AHL detection is based on different bacterial bio-assays. Steindler and Venturi (2007) reviewed and discussed the currently available bacterial biosensors which can be used in order to detect and study different AHLs molecules. All bacteria reporter used have the genes that encode the AHL synthase inactivated by mutation, but they have an AHL-responsive reporter gene. Expression of the reporter gene is possible only in presence of exogenous AHLs. Reporter strains display specificity towards different AHL molecules and the use of multiple reporters allow detection of a wide range of AHLs and differentiation among many AHL production patterns (Van HOUDT et al., 2004).

There are limitations when biosensors are used to detect AHL, and one must be cautious in the interpretation of data obtained with AHL biosensors (STEINDLER and VENTURI, 2007). Supernatant medium extracts might contain non-AHL compounds that could potentially interfere or activate the biosensors response (HOLDEN et al., 1999). In addition, from the negative response of AHL biosensors one cannot rigorously conclude that the screened bacterial strain does not produce AHLs. AHLs are usually active at very low concentrations and biosensor technology allows for the quantification of AHLs. As different LuxR homologues have diverse affinities with different AHLs, it is not accurate to compare the intensity of a response of one AHL with the response obtained with a different AHL (STEINDLER and VENTURI, 2007).

Methods involving liquid chromatography of reverse phase coupled with mass spectrometry have been used to identify and to quantify AHLs that present different chemistry structures (MIDDELETON et al., 2002).

Quorum-sensing molecule structures can be unequivocally assigned on the basis of spectroscopic properties. Mass spectrometry (MS) detects even picomoles of samples and can be coupled to gas chromatography (GC). Many types of ionization are available, including electron impact (EI-MS), fast atom bombardment (FAB-MS) and chemical ionization, and positive-ion atmospheric pressure chemical ionization (APCI-MS). Analytical HPLC-mass spectrometry (LC-MS) is a very useful technique that couples the resolving power of C18 reverse-phase HPLC directly with mass spectrometry, such that the mass of the molecular ion $(M+H)^+$ and its major component fragments can be determined for a compound with a given retention time.

This technique was used to determine the structure of different AHLs produced by many bacteria.

1.4.3. Cross-communication

Signals released by one species might, under certain circumstances, be detected by another species, either as an agonist or perhaps as antagonists. Synthesis of proteases by *Burkholderia cepacia* was stimulated when cultured in spent medium of *P. aeruginosa*; besides *B. cepacia* was shown to detect AHL released by *P. aeruginosa* (McKENNY et al., 1995; RIEDEL et al., 2001). In some cases this could be accidental rather than intentional. However, *Salmonella* and *E. coli* share a LuxR-type protein called SdiA (MICHAEL et al., 2001), but do not encode a cognate AHL synthase and do not release AHL. SdiA activates several genes in absence of AHL, but only when overexpressed. Expression of SdiA at native levels induces, at least, one gene in the presence of exogenous 3-oxo-octanoyl-HSL (MICHAEL et al., 2001). Apparently, these bacteria, and possible others, have acquired an orphan receptor to detect AHL produced by other bacteria species in mixed microbial communities.

Bruhn et al. (2004) demonstrated that *H. alvei* may induce food quality-relevant phenotypes in other bacterial species in the same environment when *H. alvei* 718 and the *S. proteamaculans* B5a *sprI* mutant were coinoculated into milk. Milk spoilage was observed when *S. proteamaculans* reach 10^9 CFU/ml and *H. alvei* 10^8 CFU/ml. No signs of spoilage were found at comparable cell densities when coinoculating the *S. proteamaculans* B5a *sprI* mutant and *H. alvei* 718 *halI* mutant into milk. Therefore, cross-communication is common among bacteria and it is important to know which microorganisms produce these compounds in the environment in order to control the QS mechanism.

1.4.4. Control of the quorum sensing system

The use of QS as a target to antimicrobial therapy can be done of different ways. One of them consist in blocking microbial communication using auto-inducers in order to prevent that bacteria express its virulence factors becoming, consequently,

harmless (KIEVIT and IGLEWSKI, 2000). This can be obtained by: (1) using specific auto-inducers that bind to proteins like-LuxR but do not promote its activation, and therefore, compromise these proteins to bind to the cognate auto-inducer molecules. (2) Interrupting the biological reactions of syntheses of auto-inducers using analogs of these compounds.

Intervention in bacterial mechanisms of QS can lead to discover new drugs able to combat microorganisms resistant against known antibiotics (GIACOMETTI et al., 2003). In addition to clinical relevance, interventions in QS system to control microorganisms have applicability in agriculture. Auto-inducers produced by plants can activate QS systems of pathogenic bacteria and stimulate early production of virulence factors by bacteria, allowing the defense system of plants to recognize and easily eliminates the infection (DONG et al., 2000). Another possibility to use QS in agriculture consists in introduction of genes encoding enzymes evolved in degradation of auto-inducers in plants, to protect them from infections caused by pathogens as *Erwinia caratovora* (DONG et al., 2001).

Dong et al. (2000) discovered an enzyme in *Bacillus* sp. 240B1, known as AiiA, which is able to degrade AHL. The *aiiA* gene encoding the lactonase from *Bacillus* was cloned, sequenced, expressed in *E. caratovora*, and reduced production of OHHL significantly. Also, it was observed a reduction of concentration of pectinolitic enzymes in 10 folds, and decreased pathogenicity of this microorganism against many plants (DONG et al., 2000). Lactonase enzyme hydrolyses lactone ring of AHL molecules and it is present in many of *Bacillus* strains (LEE et al., 2002). AiiA lactonase expression in *B. thailandensis* reduced accumulation of the signaling molecules such as C6-HSL, C8-HSL, and C10-HSL and bacterial motility; increased cellular generation time; and caused fluctuations in carbon metabolism (ULRICH, 2004). Heterologous expression system of *Bacillus* sp. lactonase in *B. cepacia* species confirms AHL regulation in extracellular proteases production, swarming motility, biofilm formation, and nematode pathogenicity (WOPPERER et al., 2006). Another class of enzymes able to degrade AHL, known as acylase, was cloned, expressed, and inhibited cell-to-cell communication in P. *aeruginosa* (LIN et al., 2003).

Another way to inhibit the gene expression mediated by AHL by interfering with the ligation of the auto-inducer to LuxR (MANEFIELD et al., 2002) is employing brominated furanones produced by *Delisea pulchra* (a microalga). The interference of furanones with QS in bacteria was already showed (MANEFIELD et

al., 1999; RICE et al., 1999; REN et al., 2002). Besides furanones, Tateda et al. (2001) verified that 2 μg/ml of azitromycin inhibited QS mechanism in *P. aeruginosa* PAO1. They proposed that this antibiotic interferes with synthesis of signaling molecules which leaded reduction of virulence factors production.

The screen for other compounds that inhibit QS is currently developed, and Rasmussen et al. (2005) found that garlic extract was one of the most effective to reduce *P. aeruginosa* biofilm tolerance to tobramycin treatment as well as virulence in a *Caenorhabditis elegans* pathogenesis model.

1.4.5. Relevance of quorum sensing mechanism to the food industry

Although studies indicate that signaling compounds are produced by bacteria in foods (GRAM et al., 1999; GRAM et al., 2002; CLOAK et al., 2002; CHRISTENSEN et al., 2003; JAY et al., 2003; BRUHN et al., 2004; KASTBJERG et al., 2007), the role of quorum sensing systems in foods is currently unknown.

Pinto et al. (2007) demonstrated that AHL-production is common among proteolytic psychrotrophic bacteria isolated from raw milk. Once these organisms were isolated from a common source, the possibility of cross-communication between them is relevant and raises the question of what kind of phenotypes might be regulated when they are growing together, and also, the relation of these phenotypes with milk deterioration. The understanding of the role of QS mechanism in regulation of spoilage phenotypes in bacteria from food origin is relevant and may be used to create new ways to preserve food products (PINTO et al., 2007).

According to Pillai and Jesudhasan (2006), to understand the relationships that exist between the food ingredients, food processing, handling, and food consumption methods, and the mechanism of cell-cell communication based microbial activity of pathogens and spoilage bacteria is very important.

Besides, the study of QS and its relationship with spoilage bacteria isolated from foods is necessary in order to obtain a deep comprehension of the role of this mechanism in food ecology. This knowledge may contribute for generation of new strategies to control the growth of undesirable bacteria and the production of detrimental metabolites, presenting a high potential for improving preservation and safety of foods.

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CHAPTER 2

OVEREXPRESSION, PURIFICATION AND CHARACTERIZATION OF MILK-DETERIORATING HYDROLYTIC EXOENZYMES PRODUCED BY *Pseudomonas fluorescens*

2.1. INTRODUCTION

The refrigeration of raw milk in the course of production has generally improved its quality and shelf-life. However, this practice is responsible for new quality problems due to the selection of psychrotrophic bacteria. Refrigeration does not prevent the development of this microflora, which is able to produce heat-stable extracellular enzymes such as proteases and lipases that subsequently degrade milk components such as proteins and fat thereby reducing the shelf-life of milk and dairy products (COUSIN, 1982; DECHEMI et al., 2005).

Many of these enzymes are produced by *Pseudomonas fluorescens* (WIEDMANN et al., 2000; DOGAN and BOOR, 2003; McCARTHY et al., 2004). As hydrolytic enzymes from *P. fluorescens* are not inactivated by pasteurization at 72 ºC for 15 s or by Ultra-High Temperature (UHT) treatment (GRIFFITHS et al., 1981) they cause severe problems in the dairy industry such as, milk protein hydrolysis, development of off-flavors, shelf-life reduction of dairy products,

decrease of yield during the cheese production, milk heat-stability loss, and gelation of UHT milk (FAIRBAIRN and LAW, 1986; DATTA and DEETH, 2001; CHEN et al., 2003).

Sørhaug and Stepaniak (1997) pointed out some important characteristics of the metalloprotease secreted by *P. fluorescens* such as, optimum temperature between 30 to 45 ºC, a significant residual activity at 4 ºC, and a pH optimum in a neutral pH-range. The enzyme was described as metalloprotease, which contains one zinc atom and up to eight calcium atoms conferring the thermostability to the protein.

Besides proteins, fats are important constituents of milk. The nutritional and sensory value and the physical properties of a triglyceride are greatly influenced by factors such as the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation (SHARMA et al., 2001). Microorganisms that produce lipolytic enzymes are important in the dairy industry because they can produce rancid flavors and odors in milk and dairy products that make these foods unacceptable to consumers (COUSIN, 1982). Lipase production by *P. fluorescens* is influenced by the type and concentration of carbon and nitrogen sources, iron, pH, dissolved oxygen concentration, and growth temperature (COUSIN, 1982; BURGER et al., 2000; WOODS et al., 2001; RAJMOHAN et al., 2002). Furthermore, McCarthy et al. (2004) demonstrated that lipase activity in the culture supernatant of *P. fluorescens* B52 is regulated by the homologue of the *Escherichia coli* EnvZ-OmpR two-component regulatory system.

The present work aimed to perform the molecular and biochemical characterization of extracellular protease and lipase produced by the *P. fluorescens* 041. This strain was isolated from refrigerated raw milk, which was kept for 48 h at 4 °C in cooled tanks in a Brazilian farm. Both enzymes were overexpressed in *Escherichia coli*, purified to heterogeneity by affinity chromatography and biochemically characterized in order to evaluate their role in the degradation of milk components.

2.2. MATERIAL AND METHODS

2.2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in table 1.

Table 1 - Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
Strains		
E. coli XL1-Blue	Cloning and subcloning host *sup*E44, *hsd*R17, *end*A1, *rec*A1, g*yr*A96, *thi*1, *rel*A1, *lac*- F´[*pro*AB+, *lacIq*, *lac*ZΔM15, Tn10 (*tet*F)]	Bullock et al., 1987
P. fluorescens 07A and 041	Proteolytic psychrotrophic strains	Martins et al., 2005
Plasmids		
pCR2.1-TOPO	Cloning vector, *lacZα* fragment containing MCS, f1 origin, ColE1, Km^r Ap^r	Invitrogen
pQE30-Xa	Vector for the insertion of a Factor Xa Protease recognition site C-terminal of the 6xHis tag, T5 promoter, *lac* operator, ribosome binding site, ATG start codon, His tag sequence, multiple cloning sites, stop codons in all three reading frames, Col E1 origin of replication, Ap^r	Qiagen
pQE30-Xa-aprX041	1.43 kb fragment containing *aprX* from *P. fluorescens* 041 in pQE30-Xa, Ap^r	This study
pQE30-Xa-lipM041	1.42 kb fragment containing *lipM* from *P. fluorescens* 041 in pQE30-Xa, Ap^r	This study

2.2.2. Growth conditions

P. fluorescens 07A and 041 were cultured in TYEP (tryptone 1%, yeast extract 0.25%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, and $CaCl_2$ 0.25%) broth at 25 °C with aeration. Besides, these strains were inoculated into 12% (w/v) reconstituted skim milk powder in order to verify their capacity to hydrolyze samples of milk after 18 h at 25 °C. *E. coli* XL1-Blue was cultured in Luria-Bertani (LB) broth or on LB agar plates at 37 °C, as required.

2.2.3. Protein quantifiction and enzyme assays

2.2.3.1. Protein quantification

The method of Bradford (BRADFORD, 1976) using bovine serum albumin as a standard was used to quantify protein concentrations.

2.2.3.2. Protease assay

Proteolytic activity was determined as described before (CHRISTENSEN et al., 2003). Briefly, this activity was investigated on azocasein by incubating 250 µl of 2% azocasein (w/v) with 150 µl sterile filtered culture supernatant in TYEP or with 75 µl of the purified AprX protease. The mixture was incubated at 30 °C for 12 h. Subsequently, the mixture was incubated at room temperature for 15 min with 1.2 ml of 10% (w/v) trichloroacetic acid (TCA), and centrifuged for 10 min at 15,000 *g*. Prior to spectroscopic measurement, 600 µl supernatant were rescued and mixed with 750 µl 1M NaOH. The proteolytic activity was quantified by the determination of the OD_{440} against a blank reaction mixture with 150 µl culture media or 75 µl Tris-HCl 20 mM, pH 8.0, $CaCl_2$ 5 mM instead of the enzyme solution. Specific activity was the unit of enzyme activity per hour per µg of protein.

2.2.3.3. Lipase assay

Lipolytic activity was determined as described before (CHRISTENSEN et al., 2003). Briefly, this activity on p-nitrophenylpalmitate was investigated by incubating 1 ml of substrate (one volume 0.3% (w/v) p-nitrophenylpalmitate in isopropanol and nine volumes 0.2% (w/v) sodium desoxycholate and 0.1% (w/v) gummi arabicum in 50 mM sodium phosphate buffer, pH 8.0) with 100 µl culture supernatant from overnight cultures or with 50 µl of the purified lipase LipM for 20 min at room temperature. Prior to spectroscopic measurement, 0.5 ml of 1 M Na_2CO_3 was added. The lipolytic activity was quantified by the determination of the OD_{410} against a blank reaction mixture with 100 µl culture media or 50 µl Tris-HCl 20 mM, pH 8.0, $CaCl_2$ 5 mM instead of the enzyme solution. Specific activity was the unit of enzyme activity per hour per µg of protein.

2.2.4. SDS-PAGE and zymograms

Proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; LAEMMLI, 1970). After electrophoresis the gels were stained with Coomassie brilliant blue.

Exoprotease activities of *P. fluorescens* culture supernatants, resolved proteases after precipitation with ammonium sulfate, and recombinant expressed AprX protease were visualized in SDS-PAGE-gels supplemented with 0.2% (w/v) azocasein as described before (CHRISTENSEN et al., 2003). After electrophoresis, proteins were renaturated by washing them twice in 50 mM Tris-HCl, pH 7.5, 25% (v/v) isopropanol for 15 min at room temperature and once at 50 mM Tris-HCl, pH 7.5. After renaturation overnight at 4 °C in 50 mM Tris-HCl, pH 7.5, the zymogram was incubated for 4 h in 5 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.0 at 40 °C. Prior to detection, the gel was washed in 1 M NaOH for 5 min. Protease activity could be detected as colourless zones in an orange background (CHRISTENSEN et al., 2003).

For the analysis of the lipase pattern after SDS-PAGE, proteins were renaturated as described above. Finally, the gels were overlaid with the fluorescent substrate methylumbelliferyl-butyrate (0.01 M in dimethylformamide) in order to

detect lipolytic activity using UV-light (360 nm) to visualize blue fluorescent bands (RIEDEL et al., 2003).

2.2.5. Identification of proteins by mass spectrometry

P. fluorescens 041 and *P. fluorescens* 07A were grown in 1000 ml of TYEP medium at 25 °C for 48 h. The cells were removed from the medium by centrifugation at 10,000 *g* for 30 min, the supernatant was sterile filtered, and the proteins were precipitated with ammonium sulfate (85% saturation). The samples were centrifuged 20 min at 10,000 *g* and the supernatant was discarded. Then, the pellets were washed twice with a 85% (w/v) ammonium sulfate and centrifuged again. The pellets were dissolved in 50 mM Tris-HCl, pH 8.0 and dialyzed overnight at 4 °C against 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$. Aliquots of 15 µl of the dialysed samples were loaded on the SDS-PAGE (12%) and Coomassie-stained protein bands were excised, digested with trypsin and analysed by mass spectrometry as described before (RIEDEL et al., 2005). Briefly, gel pieces stained were treated with 50 µl of destaining solution (50% methanol in 100 mM NH_4HCO_3, pH 8.0) for 30 min at 37 °C. The gel pieces were washed twice with 100 µl of water and once with 100 µl of 100 mM NH_4HCO_3 buffer. This buffer was removed and the gel pieces were dehydrated in 100 µl of 80% (v/v) acetonitrile in 20% water for 10 min. After solvent removal, the residual solvent was evaporated for 30 min at 50 °C. Aliquots of 10 µl of trypsin solution (20 ng of trypsin in 2 ml of 5 mM Tris buffer, pH 8.4) were incubated 15 min with the gel pieces at room temperature. Finally, 10 µl of 5 mM Tris buffer, pH 8.4 were added and the samples were incubated for at least 3 h at 37 °C. The samples were kept at -20 °C until they were analysed by mass spectrometry.

The samples were analyzed on a 4700 Proteomics Analyzer MALDI TOF/TOF system (Applied Biosystems, Framingham, MA). The instrument was equipped with an Nd:YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode, and were generated by accumulating data from 5,000 laser pulses. First, MS spectra were recorded from the standard peptides on each of the six calibration spots, and the default calibration parameters of the instrument were updated. Subsequently, MS spectra were recorded for all sample spots on the plate

and internally calibrated using signals from autoproteolytic fragments of trypsin. Up to five spectral peaks per spot that met the threshold criteria were included in the acquisition list for the MS/MS spectra. Peptide fragmentation was performed at collision energy of 1 kV and a collision gas pressure of approximately 2.561027 Torr. During MS/MS data acquisition, a method with a stop condition was used. In this method, a minimum of 3000 laser pulses and a maximum of 6000 laser pulses were allowed for each spectrum.

For the protein identification, MS data were searched using MASCOT version 1.9.05 (Matrix Science, London, UK) as the search engine. All searches were performed against a database comprising annotated proteins. GPS (Global Proteomics Server) Explorer Software (Applied Biosystems) was used for submitting data acquired with the MALDI-TOF/TOF mass spectrometer for database searching. The following search settings were used: maximum number of missed cleavages: 1; peptide tolerance: 25 ppm; MS/MS tolerance: 0.2 kDa. Carboxyamidomethylation of cysteine was set as fixed modification, and oxidation of methionine was selected as variable modification.

2.2.6. DNA manipulation, PCR reaction and sequencing

2.2.6.1. DNA manipulations

Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (SAMBROOK et al., 1989). PCR was performed with TaKaRa Ex Taq polymerase (TaKaRa Shuzo, Shiga, Japan). Plasmid DNA was isolated using the QIAprep Spin Miniprep kit, and chromosomal DNA was purified with the DNeasy tissue kit. DNA fragments were purified from agarose gels by using the QIAquick gel extraction kit (all kits from Qiagen, Hilden, Germany).

2.2.6.2. Amplification of the protease and lipase genes by PCR

The PCR reaction consisted of 2.0 mM $MgCl_2$, 5.0 µl of 10X buffer Ex *Taq*, 2.5 mM deoxynucleotide triphosphates (dNTPs), 25 pmol of each primer, 1 U Ex *Taq* DNA polymerase, and 40 ng of DNA in a final volume of 50 µl. Primers based

on the sequences of the *aprX* (GenBank accession numbers DQ146945, AY298902, AF216700) and *lip* gene (GenBank accession numbers AF216702, AY694785, M86350, S77830, D11455, AB063391, AY304500, AY673674) of other *P. fluorescens* strains were designed (Table 2), and synthesized by Microsynth (Zürich, Switzerland). PCR-reactions were carried out in a T3 thermocycler (Biometra®, Biolabo Scientific Instruments, Zürich, Switzerland).

Table 2 - Primers used to amplify the *aprX* and *lipM* genes by PCR

Primer	Sequence (5'-3')	Aplification
Apr-F	TTATGTCAAAAGTAAAAGAC	Amplification of the *aprX* gene
Apr-R	TCAGGCTACGATGTCACTG	Amplification of the *aprX* gene
APRX-F	ATT<u>GGATCC</u>AAAGCTATTGTATCTGCCGCG	Amplification of the *aprX* gene and preparation for cloning in pQE-30Xa
APRX-R	ATT<u>GAGCTC</u>TCAGGCTACGATGTCACTGGC	Amplification of the *aprX* gene and preparation for cloning in pQE-30Xa
Lip-F	ATGGGTRTSTTYGACTATAAAAACC	Amplification of the *lipM* gene
Lip-R	TTAACCGATCACAATCCCCTCC	Amplification of the *lipM* gene
LIPM-F	ATT<u>GGATCC</u>AACCTCGGTACCGAGGACTC	Amplification of the *lipM* gene and preparation for cloning in pQE-30Xa
LIPM-R	ATT<u>GAGCTC</u>TTAACCGATCACAATCCCCTCCC	Amplification of the *lipM* gene and preparation for cloning in pQE-30Xa

The introduced restriction sites *Bam*HI and *Sac*I are underlined.

2.2.6.3. Sequencing of the protease and lipase genes

The M13 Forward and Reverse primers were used to sequence the *aprX* and *lipM* genes of *P. fluorescens* 07A and 041 cloned into pCR2.1-TOPO.

2.2.7. Cloning, heterologous expression and purification of *P. fluorescens* 041 protease and lipase

Once the complete sequences of the *aprX* and *lipM* genes were obtained, primers (Table 2) were designed to amplify the open reading frame (ORF) by PCR using the bacterial genomic DNA as a template and TaKaRa Ex Taq as DNA-polymerase. The primers generated *Bam*HI and *Sac*I sites at the 5' and 3' ends of the amplificates, respectively.

The DNA amplificates, 1434 bp and 1422 bp, containing the *aprX* and *lipM* structural genes respectively were digested with *Bam*HI and *Sac*I and ligated into the vector pQE-30Xa (Qiagen), which contains 6xHis-tag coding sequence either 5' or 3' to the cloning region, cut with the same restriction enzymes. Plasmids harbouring the ORF of *aprX* or *lipM* inserted downstream of the T5 promoter and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter were selected and named pQE-30Xa-aprX041 or pQE-30Xa-lipM041, respectively. The plasmids were subsequently transformed into expression strain *E. coli* XL1-Blue.

For overproduction of AprX and LipM, *E. coli* XL1-Blue cells carrying pQE-30Xa-aprX041 or pQE-30Xa-lipM041 were grown in dYT medium (tryptone 1.6%, yeast extract 1.0%, NaCl 0.5%, and glucose 0.2%) containing ampicillin (100 µg ml^{-1}) at 37 °C under vigorous shaking. At an optical density of 0.5 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) which binds to the *lac* repressor protein and inactivates it was added to the culture to a final concentration of 1 mM in order to induce the expression of *aprX* and *lipM*.

After 5 h incubation at 37 °C, the cells were collected by centrifugation at 10,000 *g* for 30 min, resuspended in 50 mM Tris-HCl (pH 8.0) and centrifuged at 10,000 *g* for 30 min followed by two washing steps with 50 mM Tris-HCl pH 8.0, NaCl 150 mM. The resulting cell pellets were finally resuspended in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0) and the recombinant enzymes were purified under denaturing conditions using the Ni-NTA Spin Columns (Qiagen) according to the suppliers' instructions. After purification, the enzymes were subjected to dialysis (20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$) overnight at 4 °C to allow renaturation of the enzymes AprX and LipM.

2.2.8. Biochemical characterization of purified enzymes

2.2.8.1. Temperature optimum

Proteolytic and lipolytic activities of purified AprX and LipM were determined as described above on azocasein and p-nitrophenyl palmitate, respectively, at various incubation temperatures (4, 25, 30, 37, 40, 45, 50, and 60 ºC).

2.2.8.2. pH optimum

Proteolytic and lipolytic activities of purified AprX and LipM were determined as described above on azocasein and p-nitrophenyl palmitate, respectively, at various pH. The following buffer systems in a final concentration of 50 mM were used: sodium succinate (pH 4.0, 5.0, 6.0), Tris-HCl (pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0), and glycine-NaOH (pH 9.0, 10.0, 11.0, 12.0, 13.0).

2.2.8.3. Heat stability

Purified enzymes AprX and LipM were incubated for 5, 10, 15, 20, 30 and 60 min. at 50, 60, 70, 80, 90, and 100 °C to determine their thermostability. Moreover, they were incubated at 65 °C for 30 min. and 72 °C for 20 s to simulate the milk pasteurization treatments. The final activity was determined as described above.

2.2.8.4. Metal ions

To investigate the effect of metal ions on purified AprX and LipM, the reaction mixture was supplemented with 1 mM of each compound ($MnS0_4$, $CoCl_2$, $ZnSO_4$, $FeSO_4$, $MgSO_4$, or $FeCl_3$) as described by Setyorini et al. (2006). Proteolytic and lipolytic activities were determined on azocasein or p-nitrophenyl palmitate as described above.

2.2.8.5. Protease inhibitors

The effect of potential protease inhibitor on the proteolytic activity of purified AprX was determined as described by Setyorini et al. (2006) by supplementing the reaction mixture with 1 mM PMSF, 1 mM EDTA, 1 mM Pefabloc SC, 2% (w/v) SDS, 4 M urea, 0.1% (w/v) DTT, and 0.1% (v/v) *β*-mercaptoethanol and subsequent measurement of residual activities on azocasein as described above.

2.2.9. Substrate specificity

2.2.9.1. Protease

The substrate specificity of purified AprX was determined on casein, elastin, collagen, bovine serum albumin, and gelatine. The reaction mixture consisted of 0.4% (w/v) of each protein in 400 µl of 50 mM Tris-HCl, pH 6.5 and 150 µl of enzyme solution. After incubation at 37 °C for 1 h, the mixture was withdrawn and the increase in the amount of free amino groups was determined by the ninhydrin method according to Setyorini et al. (2006).

2.2.9.2. Lipase

Activities of purified LipM on different p-nitrophenyl fatty acid esters (p-nitrophenyl acetate, p-nitrophenyl butyrate, p-nitrophenyl palmitate, and p-nitrophenyl phosphorylcholine) were measured according to the assay for lipolytic activity as described above.

2.3. RESULTS AND DISCUSSION

2.3.1. Milk-deteriorating hydrolytic activities of *P. fluorescens*

P. fluorescens 041 showed higher proteolytic (Figure 1A) and lipolytic (Figure 1B) activities in the supernatant of TYEP medium than the strain 07A. Moreover, strain 041 exhibited a higher capacity to hydrolyse milk than *P. fluorescens* 07A when both strains were inoculated into 12% (w/v) reconstituted skim milk powder (Figure 1C). SDS-PAGE analysis of ammonium sulfate precipitated protein from supernatants of TYEP cultures of *P. fluorescens* 07A and 041 demonstrated the presence of multiple protein bands (Figure 2, lines 1 and 3). Proteolytic activity of the dominant 50 kDa band was demonstrated by a zymogram incorporating azocasein (Figure 2, lines 2 and 4). Mass spectrometry analysis of the major proteolytic protein band identified this protein as metalloprotease, which was designated as AprX. Previously, numerous *Pseudomonas* spp. have been shown to produce and secrete hydrolytic enzymes (LIAO and MCCALLUS, 1998; BURGER et al., 2000; WOODS et al., 2001; McCARTHY et al., 2004; MAUNSELL et al., 2006). Rajmohan et al. (2002) verified the production of five proteases by a single strain of *P. fluorescens*. However, in this work, mass spectrometry analysis of low molecular bands that showed also proteolytic activity (Figure 2) revealed that these bands were degradation products of AprX.

Surprisingly, no lipolytic activity could be detected when the renaturated SDS-PAGE was overlayed with the lipase substrate methylumbeliferyl-butyrate.

Probably it occurred due to the degradation of lipase by protease or because Ca^{+2} was not added into the renaturation buffer and LipM needed this ion for correct folding.

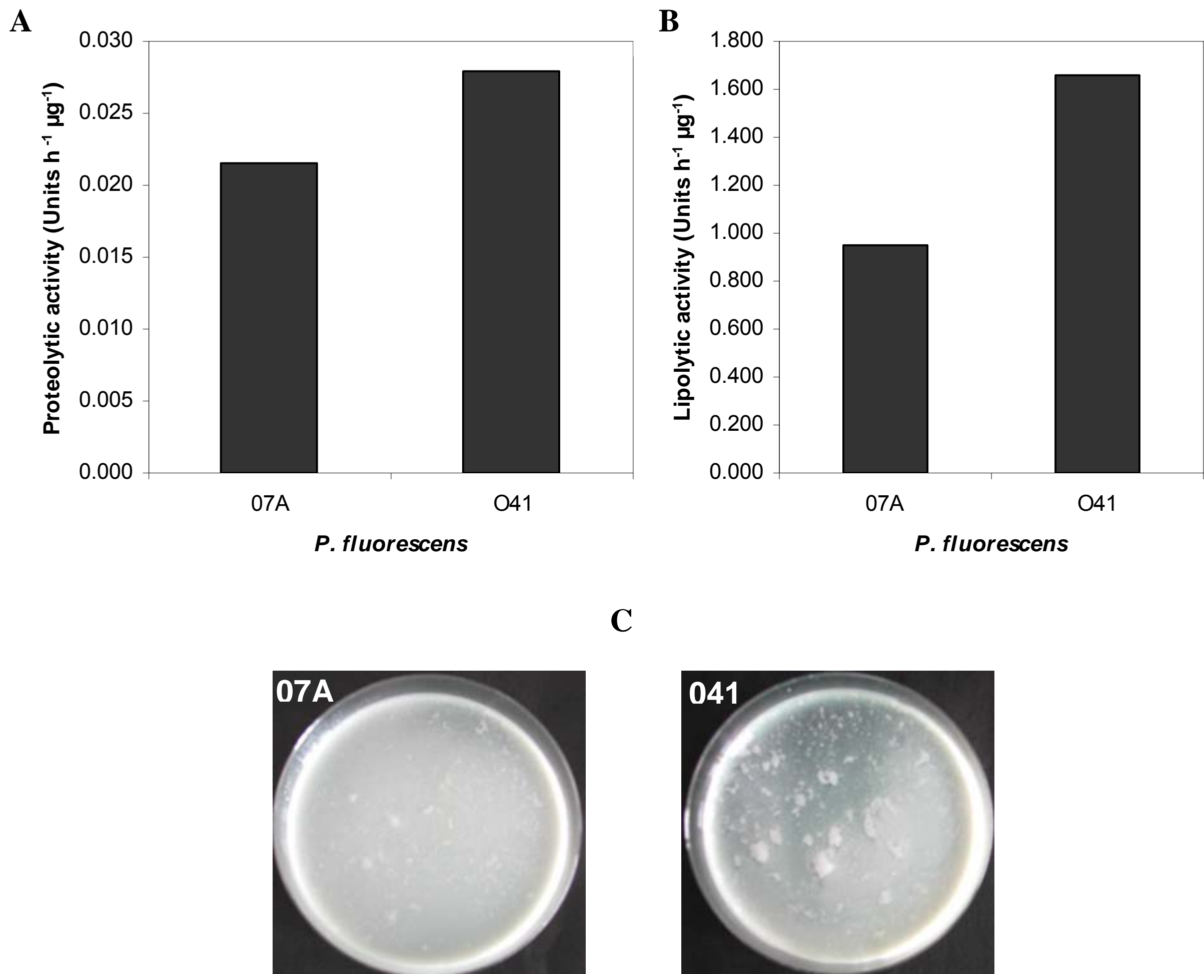

Figure 1 – Production of extracellular hydrolytic enzymes by *P. fluorescens*. A: Proteolytic activity in the supernatant of TYEP medium; B: Lipolytic activity in the supernatant of TYEP medium; C: Samples of reconstituted skin milk powder (12%) inoculated with *P. fluorescens* 07A and 041 after 18 h of incubation at 25 °C. Data represent the average of duplicate experiments.

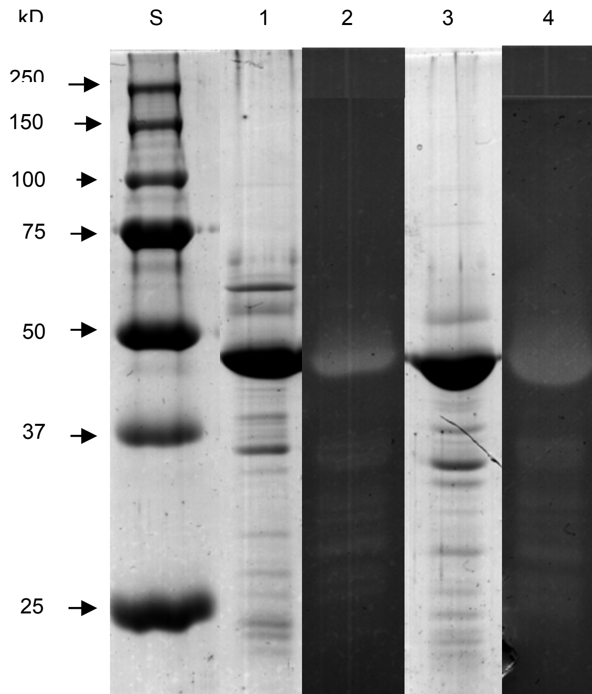

Figure 2 – Coomassie-stained SDS-PAGE and azocasein zymogram on 12% sodium dodecyl sulfate-polyacrylamide gels visualizing protease production by *P. fluorescens* after grown in TYEP medium supplemented with 0.25% $CaCl_2$. Lane S: molar mass standards (BioRad); lane 1: SDS-PAGE of ammonium sulfate precipitated proteins of *P. fluorescens* 07A supernatant; lane 2: azocasein zymogram of ammonium sulfate precipitated proteins of *P. fluorescens* 07A supernatant; lane 3: SDS-PAGE of ammonium sulfate precipitated proteins of *P. fluorescens* 041 supernatant; lane 4: azocasein zymogram of ammonium sulfate precipitated proteins of *P. fluorescens* 041 supernatant.

2.3.2. Cloning and sequencing of protease and lipase genes

Primers based on sequences of protease and lipase from other *P. fluorescens* strains were synthesized and used to amplify a segment encoding these enzymes in *P. fluorescens* 041. Electrophoresis of the PCR products revealed a single product at about 1,500 bp for both enzymes.

Subsequently, the protease and lipase genes were sequenced. The *aprX* and the *lipM* genes of *P. fluorescens* 041 comprised 1,434 and 1,425 bp, respectively, and coded for proteins with 477 and 474 amino acids. Based on amino acid sequence, the the molecular mass of both enzymes was predicted to be 49.365 kDa and 49.811 kDa, which could be confirmed by SDS-PAGE analysis of purified

enzymes (Figure 3, lane 3 and 5). These results were similar to those found for protease and lipase of *Pseudomonas* strains isolated from raw milk (MAKHZOUM et al., 1996; KIM et al., 1997; LIAO and MCCALLUS, 1998; RAJMOHAN et al., 2002; KOJIMA and SHIMIZU, 2003).

The *aprX* gene of *P. fluorescens* 07A and *P. fluorescens* 041 presented 96% identity with each other. When the sequence of *aprX* gene of *P. fluorescens* 041 was compared to the sequences of the GenBank, it was verified 97% identity with both extracellular alkaline metalloprotease (*aprX*) gene of *P. fluorescens* strain A506 (accession number AY298902) and with the protease (*aprX*) gene of *P. fluorescens* strain F (accession number DQ146945). On the other hand, the *lipM* gene of *P. fluorescens* 07A and *P. fluorescens* 041 showed 94% identity with each other, and this gene of *P. fluorescens* 041 showed 93% identity with polyurethanase lipase A (*pul*A) gene of *P. fluorescens* (accession number AF144089) and 86% with the lipase (*lip*A) gene of *P. fluorescens* (accession number AF216702).

Once the AprX and LipM produced by strains of *P. fluorescens* isolated from raw milk showed high identity with the sequences from homologous enzymes in the data base, it will be possible to use the *aprX* and *lipM* genes as markers to detect spoilage psychrotrophic bacteria in milk using the PCR technique as described by Martins et al. (2005). According to these authors, the technique could reduce the time for detection of these bacteria in raw milk allowing processor to decide about the best use of milk during processing. Besides, the characterization of these spoilage enzymes is important to estimate the degradation of milk components, and thus further improve enzymatic methods to access the quality of milk.

2.3.3. Overexpression and purification of AprX and LipM

The expression and purification of recombinant proteins facilitate production and detailed characterization of proteins. After cloning of alkaline metalloprotease AprX and lipase LipM in the vector pQE30Xa and heterologous expression in *E. coli* XL1-Blue (Figure 3, lane 1 and 2), the two proteins were purified under denaturing conditions as the purification of overexpressed AprX and LipM under native conditions was hampered by massive occurrence of inclusion bodies. Purified proteins were renatured by dialysis against 20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$.

The AprX and LipM were purified to homogeneity (Figure 3, lane 3 and 5), showed the expected molecular mass and were active on zymograms after renaturation when 1 mM of $CaCl_2$ was added into the renaturation buffer (Figure 3, lane 4 and 6).

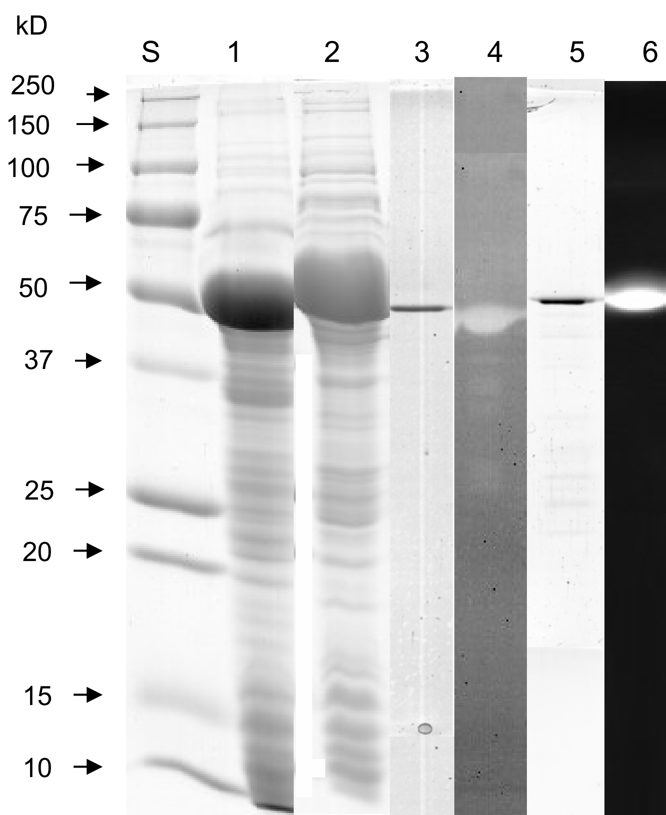

Figure 3 - Coomassie-stained SDS-PAGE and zymogram on 12% sodium dodecyl sulfate-polyacrylamide gels visualizing recombinant AprX and LipM. Lane S: molar mass standard (BioRad); lane 1: SDS-PAGE of crude extract of *E. coli* XL1-Bue carrying pQE30-Xa-aprX-041; lane 2: SDS-PAGE of crude extract of *E. coli* XL1-Bue carrying pQE30-Xa-lipM-041; lane 3: SDS-PAGE of purified AprX; lane 4: azocasein zymogram of purified AprX; lane 5: SDS-PAGE of purified LipM; lane 6: methylumbelliferyl-butyrate zymogram of purified LipM.

2.3.4. Biochemical characterization of AprX and LipM

The temperature optimum of the purified protease of *P. fluorescens* 041 was 37 °C (Figure 4). This is in agreement to the temperature optimum of the most other pseudomonal proteases, which are between 30 and 45 °C (FAIRBAIRN and LAW,

1986; SØRHAUG and STEPANIAK, 1997). Besides, the protease showed considerable activity under conditions of refrigeration from 4 °C to 7 °C, and low activity in temperatures higher than 45 °C (Figure 4). As this enzyme has a low activation energy and is therefore more active at 4 °C to 7 °C than enzymes from mesophilic microorganisms (SØRHAUG e STEPANIAK, 1997), it is readily able to degrade casein and cause many problems to the dairy industry.

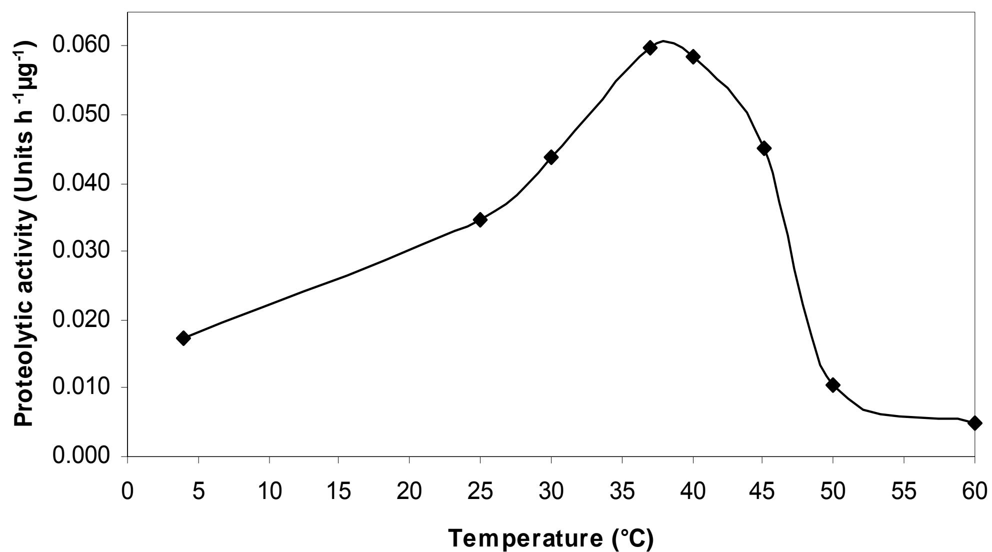

Figure 4 – Effect of temperature on activity of purified AprX of *P. fluorescens* 041 on azocasein**.** Data represent the average of duplicate experiments.

The temperature optimum of the purified lipase was 25 °C (Figure 5). Different from AprX, LipM showed only a residual activity of 3.7% at 4 °C, and exhibited low activities at temperatures higher than 37 °C (Figure 5). These data were different from thouse obtained by Makhzoum et al. (1996) who detected temperature optimum of a purified lipase from *P. fluorescens* 2D as 40 °C. Temperature optimum from 20 until 35 °C has also been reported for lipases from *P. fluorescens* (DRING and FOX, 1983; ROUSSIS et al., 1998).

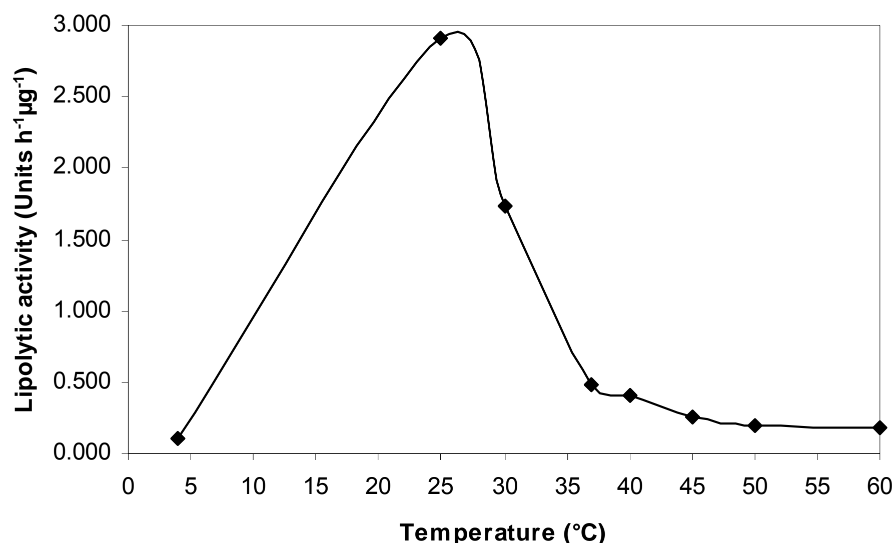

Figure 5 - Effect of temperature on activity of purified LipM of *P. fluorescens* 041 on p-nitrophenyl palmitate. Data represent the average of duplicate experiments.

The effect of pH of the assay conditions on extracellular protease activity is shown in Figure 6. It had a broad pH range of activity towards azocasein as substrate. The pH optimum of AprX is at 6.0 to 6.5 (Figure 6), which is close to the pH of bovine milk (6.66). Sørhaug and Stepaniak (1997) also pointed out the pH optimum of this enzyme close to neutrality. Besides, the protease exhibits still 36% residual activity at pH 4.0 and 62% at pH 9.0.

On the other hand, LipM showed a narrow range of activity with the highest at pH 7.5 (Figure 7). At pH values lower than 6.0 and higher than 11.0 only residual lipase activities could be detected (Figure 7). Range of pH optimum from 7.0 to 9.0 has been reported for lipases from *P. fluorescens* (SZTAJER et al., 1991; MAKHZOUM et al., 1996; ROUSSIS et al., 1998).

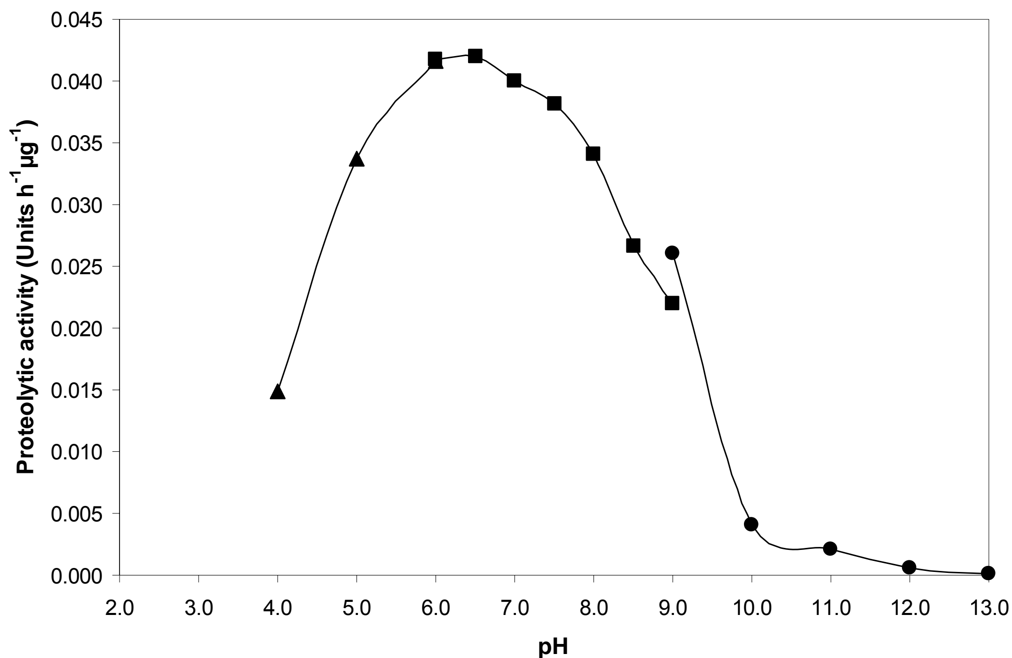

Figure 6 – Effect of pH on proteolytic activity of purified AprX of *P. fluorescens* 041 on azocasein. Data represent the average of duplicate experiments. (▲) Succinate buffer, (■) Tris/HCl buffer, and (●) NaOH-glycine buffer.

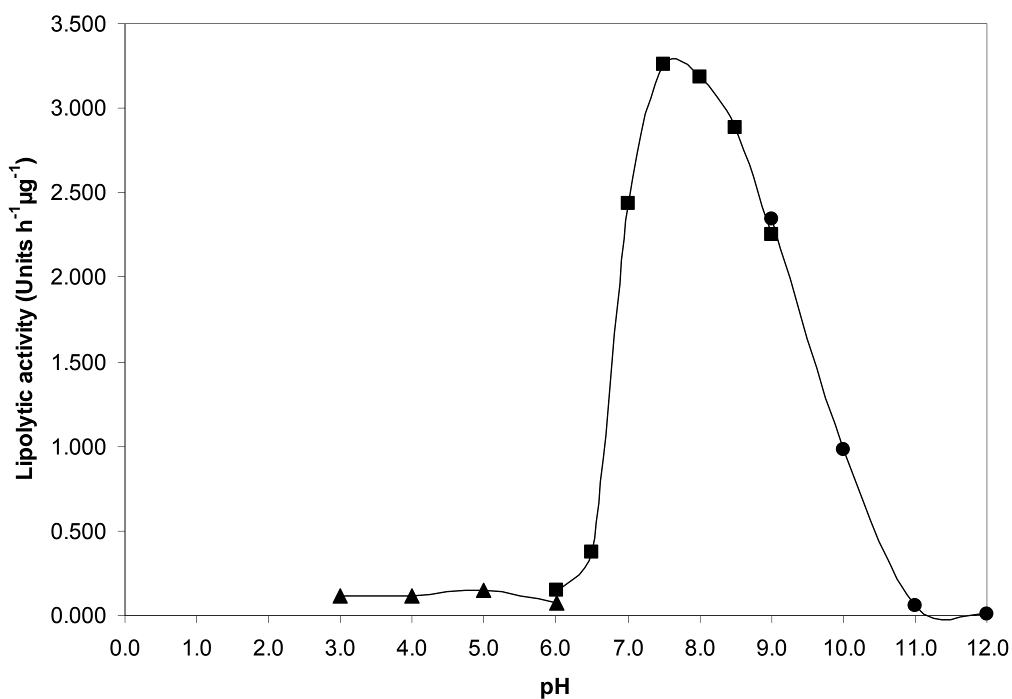

Figure 7 – Effect of pH on lipolytic activity of purified LipM of *P. fluorescens* 041 on p-nitrophenyl palmitate. Data represent the average of duplicate experiments. (▲) Succinate buffer, (■) Tris/HCl buffer, and (●) NaOH-glycine buffer.

The protease activity was strongly decreased by temperatures of 50, 60, 70, 80, 90 and 100 °C maintaining the residual activity between 2 and 4% after 60 min (Figure 8). The inactivation of 90% of activity of extracellular protease produced by *Pseudomonas* could be reached at 72 °C for 4 to 5 h or at 120 °C for 7 min (ADAMS et al., 1975).

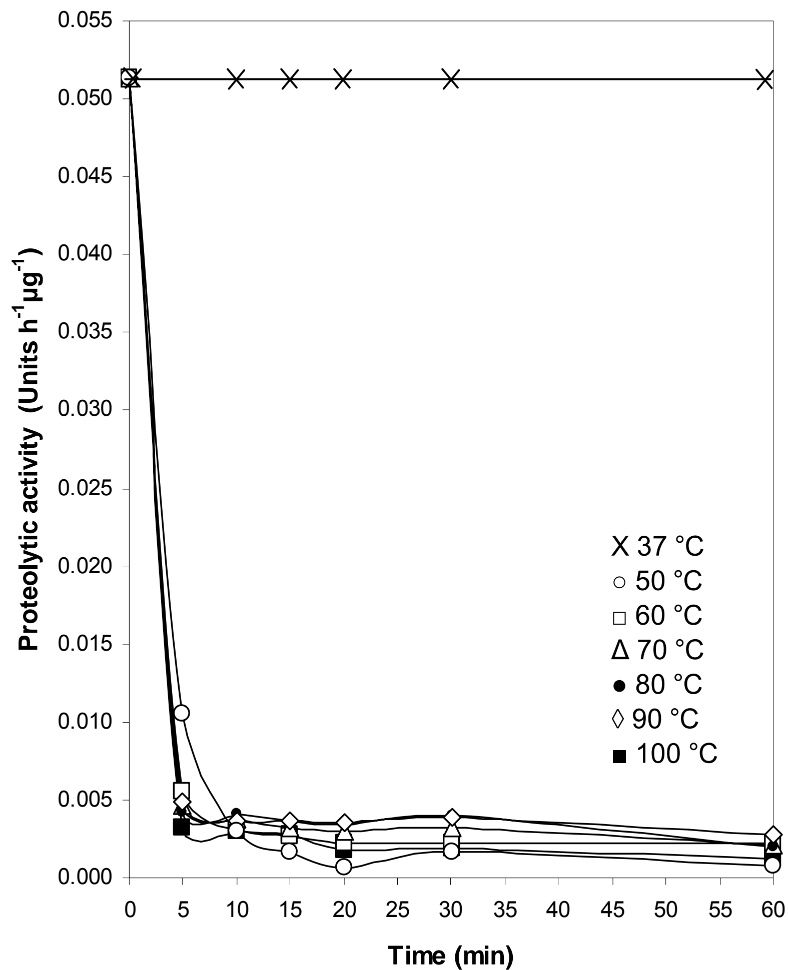

Figure 8 – Effect of heat treatment for 60 min on proteolytic activity of purified AprX of *P. fluorescens* 041**.** Data represent the average of duplicate experiments.

However, these treatments are considered highly prejudicial to the milk characteristics. Inactivation of the metalloprotease at temperature and time conditions used during the pasteurization process was also evaluated. AprX showed 70% residual activity when it was treated at 75 °C for 20 s (HTST treatment - high temperature and short time) and 4% residual activity when it was incubated at 65 °C

for 30 min (LTLT - low temperature and long time). This demonstrates the importance of the combination of time and temperature during the heat treatment once, when the time was increased the activity decreased significantly. As the heat treatment and the refrigeration temperature adopted during the storage of milk neither complet inhibit the activity of this spoilage enzyme nor the growth of psychrotrophs, it is necessary to adopt the good manufactory practices (GMP) to limit the contamination of raw milk.

Besides protease, the inactivation of lipase by heat has been important to dairy processors because enzymes that survive pasteurization can be detrimental to keeping quality of products. The residual activity of the lipase after 60 min pre-incubation at 50, 60, 70, 80, 90 and 100°C was between 0 and 6%. It was observed a little residual activity after all thermal treatment, but the residual activity was abolished after the treatment of the lipase at 100 °C for 30 min (Figure 9).

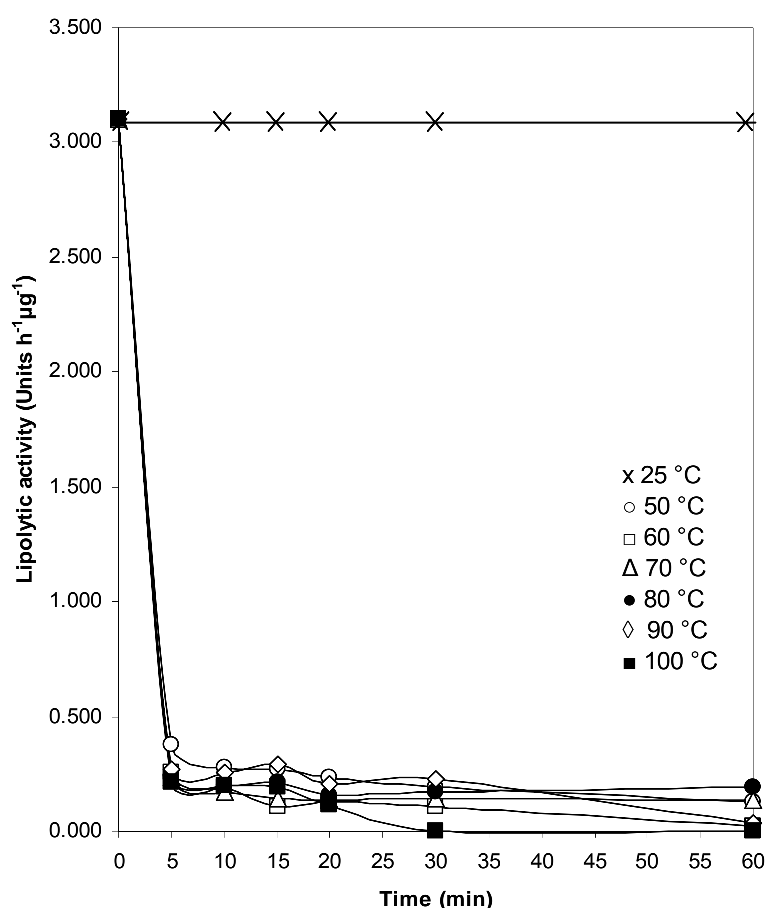

Figure 9 – Effect of heat treatment for 60 min on lipolytic activity of purified LipM of *P. fluorescens* 041. Data represent the average of duplicate experiments.

The treatment of 65 °C for 30 min (LTLT), and 75 °C for 20 s (HTST) similar to pasteurization conditions reduced the lipolytic activity to 13.2% and 25.4%, respectively. According to Cousin (1982), complete inactivation of lipases was obtained by autoclaving milk at 121 °C for 15 min. Knaut (1978) observed that lipases from *P. fluorescens* species were stable above 100 °C, and a heat-treatment of 98 °C for 14 to 25 min was necessary to inactivate the lipases of some *Pseudomonas* species, including *P. fluorescens* and *P. fragi*.

The correct protein folding of AprX seems strongly dependent of Ca^{+2} as no renaturation of AprX could be achieved when the purified enzyme solution was dialyzed against buffer lacking this metal ion. Surprisingly, some metal ions e.g. Co^{+2} even reduced the proteolytic activity significantly (Table 3).

Like protease, the maximum activity of the lipase was observed when Ca^{+2} was added in the dialyse solution. On the other hand, 1 mM of the other ions strongly reduced the lipolytic activity (Table 3). According to Makhzoum et al. (1996), calcium had a strong activating effect on the lipase (125% increase in activity), suggesting the possible effect of this cation on the activity and stability of the enzyme.

Table 3 - Effects of metal ions on the activities of alkaline metalloprotease and lipase

Metal ion	Relative activity (%)	
	Alkaline metalloprotease[a]	Lipase[b]
None	100	100
Mn^{2+}	73	61
Co^{2+}	48	59
Zn^{2+}	86	49
Fe^{2+}	90	48
Fe^{3+}	102	65
Mg^{2+}	100	50

[a]A reaction mixture containing 250 µl of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µl of AprX, and 1 mM of each metal ion was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text.
[b]A reaction mixture containing 1 ml of substrate (one volume of 0.3% (w/v) p-nitrophenyl palmitate in isopropanol and nine volumes 0.2% (w/v) sodium desoxycholate and 0.1% (w/v) gummi arabicum in 50 mM sodium phosphate buffer, pH 8.0), 50 µl of LipM, and 1 mM of each metal ions was incubated at 25 °C for 30 min. The remaining activity was then measured, as described in the text.

The effect of different protease inhibitors on AprX activity is shown in Table 4. The activity was strongly decreased when 1 mM of EDTA, an inhibitor that specifically inhibits metalloproteases was added to the reaction mixture confirming the metalloprotease nature of the enzyme. In addition, AprX was strongly inhibited by denaturing and reducing agents such as SDS, dithiothreitol (DTT), β-mercaptoethanol, and urea (Table 4).

Table 4 - Effects of inhibitors and denaturing and reducing agents on the activity of alkaline metalloprotease

Compound	Relative activity (%)
Inhibitor[a]	
None	100
PMSF	95
EDTA	51
Pefabloc SC	89
Denaturing and reducing agent[b]	
None	100
SDS	6
Urea	38
DTT	24
β-mercaptoethanol	44

[a]A reaction mixture containing 250 µl of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µl of AprX, and 1 mM of each inhibitor was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text.
[b]A reaction mixture containing 250 µl of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µl of AprX, and 2% (w/v) SDS, 4 M urea, 0.1% (w/v) DTT, or 0.1% (v/v) β-mercaptoethanol in 50 mM Tris/HCl (pH 8.0) was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text.

The alkaline metalloprotease was furthermore tested for its capability to hydrolyse different substrates. Highest activity was found on gelatine (100%) and casein (87.6%), followed by collagen (57%), elastin (41.2%), and bovine serum albumin (39.8%).

LipM was also tested for its capability to hydrolyse different substrates. Highest activity was found on p-nitrophenyl palmitate (100%), followed by p-nitrophenyl butyrate (73%), p-nitrophenyl acetate (20%), and p-nitrophenyl phosphorylcholine (11%). These results confirmed that this enzyme has lipolytic activity rather than esterase activity.

2.4. CONCLUSIONS

The *aprX* and the *lipM* genes are highly conserved among *P. fluorescens* strains and encode protease (AprX) and lipase (LipM), which have molecular mass of 50 kDa and are dependent of Ca^{2+}.

Metalloprotease from *P. fluorescens* 041 exhibits temperature optimum at 37 °C, pH optimum of 6.5, and the highest activity on gelatin and casein. On the other hand, lipase from *P. fluorescens* 041 exhibits temperature optimum at 25 °C, pH optimum of 7.5, and the highest activity on p-nitrophenyl palmitate.

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CHAPTER 3

INVESTIGATION OF QUORUM SENSING IN STRAINS OF *Pseudomonas fluorescens* ISOLATED FROM REFRIGERATED RAW MILK

3.1. INTRODUCTION

Gram-negative bacteria are the predominant psychrotrophic microflora encountered in raw milk (URAZ and ÇITAK, 1998; DOGAN and BOOR, 2003), with *Pseudomonas* spp. comprising at least, 50% of the total bacteria (CHAMPAGNE et al., 1994). Many gram-negative bacteria, including members of the genus *Pseudomonas* regulate gene expression in response to population density by sensing the level of signal molecules produced and liberated into the environment (SHAW et al., 1997). This phenomenon is termed quorum sensing and appears to be a conserved process amongst prokaryotes since many bacteria have been shown to use cell-cell communication to regulate diverse physiological processes (FUQUA et al., 1996).

Several types of quorum sensing signals have been reported from different *P. fluorescens* strains. Three different acyl-homoserine lactones (AHLs) were detected in *P. fluorescens* 2-79 isolated from wheat by thin layer chromatography (TLC) and mass spectrometry (MS) analysis, which were identified as N-(3-

hydroxyhexanoyl)-HSL, N-(3-hydroxyoctanoyl)-HSL, and N-(3-hydroxydecanoyl)-HSL (SHAW et al., 1997). Later on, N-octanoyl-HSL and N-hexanoyl-HSL were identified in this strain by Cha et al. (1998).

P. fluorescens F113 obtained from rhizosphere of sugarbeets produces N-(3-hydroxy-7-cis-tetradecenoyl)-HSL, N-(decanoyl)-HSL, and N-(hexanoyl)-HSL (LAUE et al., 2000). The gene *hdtS* capable of directing synthesis of all three *P. fluorescens* F113 AHL does not belong to either of the known AHL synthase families (LuxI or LuxM). However, Khan et al. (2005) showed that the *phz* operon of *P. fluorescens* 2-79 is preceded by two genes, *phzR* and *phzI*, that are homologs of quorum sensing gene pairs of the *luxR-luxI* family. Deleting *phzR* and *phzI* from strain 2-79 led to loss of production of the antibiotics, as well as suite of AHLs.

El-Sayed et al. (2001) identified, when they studied the mupirocin antibiotic biosynthetic cluster in a soil-borne bacterium, *P. fluorescens* NCIMB 10586, two putative regulatory genes, *mupR* and *mupI*, whose predicted amino acid sequences showed significant identity to proteins involved in quorum sensing dependent regulatory systems such as LasR-LuxR and LasI-LuxI. This bacterium produced a diffusible substance that overcomes the defect of a *mupI* mutant, and the use of biosensor strains showed that MupI product can activate the *P. aeruginosa lasR-lasI* system and that *P. aeruginosa* produces one or more compounds that can replace the MupI product.

However, no AHL molecules were detected in *P. fluorescens* 1855.344, a plant-growth-promoting rhizosphere bacterium (CHA et al., 1998), and in *P. fluorescens* pf 7-14 isolated from rice rhizosphere (DUMENYO et al., 1998). According to Cui et al. (2005), given the differences in AHL profiles from *P. fluorescens* strains examined, it would clearly be of future interest to determine whether there is any association between AHL profile and strain habitat. These authors reported the identification of an AHL, N-(3-hydroxyoctanoyl)-HSL, in *P. fluorescens* 5064 isolated from infected broccoli and the evidence for regulation of biosurfactant production via this quorum sensing signal. AHL production appears to be more common among plant-associated than among soilborne *Pseudomonas* spp. (ELASRI et al., 2001).

Quorum sensing systems were involved in promoting cell attachment and biofilm formation in *P. fluorescens* B52 isolated from milk, but these did not involve short chain HSLs (ALLISON et al., 1998). Dunstall et al. (2005) evaluated nine AHL

compounds on lag phase duration and exponential growth rate of three strains of *P. fluorescens* isolated from refrigerated raw milk. Two compounds N-(benzoyloxycarbonyl)-L-homoserine lactone and N-(3-oxyhexanoyl)-DL-homoserine lactone were found to significantly ($p<0.001$) reduce the lag phase duration and increase the exponential growth rate of these strains. However, *P. fluoresecens* strains isolated from cooled raw milk induced only the monitor strains of *Agrobacterium tumefaciens* ultrasensitive for AHL (PINTO et al., 2007).

A novel family of signaling compounds identified as diketopiperazines (DKPs) has been discovered in cell-free supernatants of *P. aeruginosa*, *P. fluorescens*, *Pseudomonas alcaligenes*, *Enterobacter agglomerans* and *Citrobacter freundii* (HOLDEN et al., 1999). Although there is abundant literature data about dipeptides isolated from microbial sources their true origin remains controversial (HERNÁNDEZ et al., 2004). However, several dipeptides isolated from microorganisms display relevant biological activities (HOLDEN et al., 1999; HOLDEN et al., 2000; HERNÁNDEZ et al., 2004). Microbial DKPs found to date appear to be catabolic products of peptone or other components of the nutrient rich media (PRASAD, 1995).

While the AHLs are confined to a reasonably narrow range of bacteria, recent evidence has suggested the existence of a universal quorum sensing language (CÁMARA et al., 2002) known as a family of molecules termed AI-2, common to many gram-negative and gram-positive bacteria, including some pathogens (MILLER and BASSLER, 2001). However, there is no direct evidence for the involvement of AI-2 in regulation of pathogenic characteristics. AI-2 has evolved several diverse species specific roles while simultaneously remaining a universal signal recognizable across numerous species of bacteria (DeLISA et al., 2002). This autoinducer and its biosynthetic via are the same among all species that have *luxS* (MOK et al., 2003).

Considering that some strains of *P. fluorescens* use the quorum sensing mechanism to regulate antibiotic production and other phenotypes, and considering that expression of spoilage enzymes occurs in high population density, this research focused on elucidation of quorum sensing in strains of *P. fluorescens* isolated from cooled raw milk.

3.2. MATERIAL AND METHODS

3.2.1. Bacterial strains and growth conditions

The strains used in the present study are listed in Table 1. Unless otherwise stated, *P. fluorescens* was grown at 25 °C and other strains were grown at 30 °C in Luria-Bertani (LB) medium (ANDERSEN et al., 1998) or AB minimal medium (CLARK and MAALOE, 1967) supplemented with 10 mM citrate (ABC). *Vibrio harveyi* strains were grown at 30 °C in AB *Vibrio* medium (BASSLER et al., 1994). Solid media were routinely solidified with 1.4% agar, while growth media for examination of swarming motility contained 0.4% (w/v) agar (EBERL et al., 1996). Antibiotics were added as required at final concentrations of 20 µg/ml for gentamicin and tetracycline, 50 µg/ml for trimethroprim and spectinomycin, 100 µg/ml for ampicillin, and 10 µg/ml for chloramphenicol. Kanamycin was used at 30 µg/ml for *V. harveyi* and at 50 µg/ml for *E. coli* S17-1 and *P. fluorescens*. Besides, tellurite, an inhibitor agent, was used at 100 µg/ml. Growth of liquid cultures was monitored spectrophotometrically with an Ultrospec 3100 Pro spectrophotometer (Biochrom, Ltd., Cambridge, England) by measurement of the optical density at 600 nm.

Table 1 – Bacterial strains and plasmids used in this study

Strain	Plasmid	Description	Reference or source
Agrobacterium tumefaciens A136	pCF373, pCF218, Tc^r, Spc^r	Monitor strain: detects AHL with 3-oxo, 3-hydroxy, and 3-unsubstituted side chain	Fuqua and Winans, 1996; Shaw et al., 1997
A. tumefaciens KYC55	pJZ410, pJZ372, pJZ384, Tc^r, Spc^r, Gm^r	Ultrasensitive strain: detects many diverse AHLs at extremely low concentrations	Zu et al., 2003
A. tumefaciens NTL4	pZLR4, Gm^r	Monitor strain: detects AHL with 3-oxo, 3-hydroxy, and 3-unsubstituted side chain	Cha et al., 1998
Burkholderia cepacia H111		Positive control in the cross-streak to *E. coli* pSB403, and *P. putida* F117 pAS-C8	Riedel et al., 2003
Burkholderia vietinamensis		Positive control in the cross-streak to *P. putida* F117 pKR-C12	Wopperer et al., 2006
Chromobacterium violaceum CV026		Monitor strain: detects AHL compounds with unsubstituted side chains from C4 to C8 in length.	Ravn et al., 2001
Escherichia coli HB101	pRK600, Cm^r	Helper	Laboratory of Microbiology, University of Zürich
E. coli MT102	pSB403, Tc^r	Monitor strain: exhibits the highest sensitivity for 3-oxo-C6-HSL. However, several other AHL molecules are detected by this sensor.	Winson et al., 1998; Gotschlich et al., 2001; Steidle et al., 2001
E. coli XL1-Blue	pMLBAD-aiiA-Gm^r	Donor of pMLBAD-aiiA, Gm^r that codify the lactonase enzyme	This study
E. coli XL1-Blue	pMLBAD-aiiA-Tel^r	Donor of pMLBAD-aiiA, Tel^r that codify the lactonase enzyme	Laboratory of Microbiology, University of Zürich
E. coli XL1-Blue	pMLBAD-aiiA-Trm^r	Donor of pMLBAD-aiiA, Trm^r that codify the lactonase enzyme	Wopperer et al., 2006
Pseudomonas aeruginosa PAO1		Positive control in the cross-streak to *C. violaceum* CV026, *A. tumefaciens* NTL4, and *A. tumefaciens* A136	Laboratory of Microbiology, University of Zürich
Pseudomonas fluorescens 07A		Wild type	Martins et al., 2005
P. fluorescens 07A-2	pMLBAD-aiiA-Trm^r-Gm^r	Transconjugant, express the lactonase enzyme	This study
P. fluorescens 041		Wild type	Martins et al., 2005
P. fluorescens 041-3	pMLBAD-aiiA-Trm^r-Gm^r	Transconjugant, express the lactonase enzyme	This study
P. fluorescens 097		Wild type	Martins et al., 2005
P. fluorescens 0109		Wild type	Pinto, 2004; Pinto et al., 2007
Pseudomonas putida F117	pAS-C8, Gm^r	Monitor strain: exhibits the highest sensitivity for OHL	Wopperer et al., 2006
P. putida F117	pKR-C12, Gm^r	Monitor strain: it detects 3-oxo-C12- and 3-oxo-C10-HSL	Wopperer et al., 2006
Vibrio harveyi BB170		Monitor strain: detects AI2	Schauder et al., 2001
V. harveyi BB120		Positive control: AI2 producer	Schauder et al., 2001

3.2.2. Detection and quantification of signal molecules

AHL production was investigated by cross-streaking *P. fluorescens* strains against *E. coli* MT102 pSB403, *C. violaceum* CV026, *A. tumefaciens* NTL4, and *A. tumefaciens* A136. The cross-streak of *P. fluorescens* against *E. coli* MT102 and *C. violaceum* was done on LB agar plates, whereas the cross-streak of *P. fluorescens* against *A. tumefaciens* strains was done on LB agar plates supplemented with 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Also the *A. tumefaciens* strains were cross-streaked against themselves in AB medium. After incubation up to 2 days at 30 °C, bioluminescence produced by *E. coli* MT102 was detected into a dark box containing a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics Herrsching, Germany) (STEIDLE et al., 2001). Violacein production by *C. violaceum* CV026 was detected as violet pigments in the medium and β-galactosidase activity was identified by forming blue pigments. These results were documented by photographing the plates.

Pseudomonas putida F117 harbour a plasmid pKR-C12, which contains a P*lasB*-*gfp*(ASV) translational fusion, together with the *lasR* gene placed under control of P*lac*. This sensor strain is highly sensitive for 3-oxo-C12- and 3-oxo-C10-HSL. Plasmid pAS-C8 was constructed from components of the *cep* system of *Burkholderia cenocepacia* H111 and contains a P*cepI*-*gfp*(ASV) translational fusion together with the *cepR* gene transcribed from the P*lac* promoter of the broad-host-range plasmid pBBR1MCS-5. This sensor plasmid responds very efficiently to C8-HSL and only with low efficiency to other AHL molecules. After overnight incubation of cross-streaking plates at 30 °C, fluorescence was detected by illumination with blue light by using an HQ 480/40 filter (AHF-Analysentechnik, Tübingen, Germany) in combination with a halogen lamp (Volpi, Schlieren, Switzerland) as a light source in a dark box equipped with a light-sensitive camera (Hamamatsu Photonics, Herrsching, Germany) with a Pentax CCTV camera lens and an HQ 535/20 filter.

Since *E. coli* MT102 pSB403 is able to detect low amount of AHL, it was used for quantification of these molecules. The plasmid pSB403 contains the *Photobacterium fischeri luxR* gene together with *luxI* promoter region as a transcriptional fusion to bioluminescence genes *luxCDABE* of *Photorhabdus luminescens*. The quorum sensing system of *P. fischeri* relies on 3-oxo-C6-HSL, and

the sensor plasmid consequently exhibits the highest sensitivity for this AHL molecule. However, several other AHL molecules are detected by the sensor, albeit with some what reduced sensitivity (WINSON et al. 1998). A volume of 1 ml of overnight culture of *E. coli* MT102 pSB403 was inoculated into 5 ml LB supplemented with tetracycline and incubated at 30 °C for 1 h. Then, 100 µl of filter-sterilized culture supernatants in LB of *P. fluorescens* were added to 100 µl of an exponential culture of the sensor strain in the wells of a microtiter dish. After incubation at 30 °C for 3 h, the expression of the bioluminescence reporter genes was measured using the program KC4 (Bio-Tek Instruments, Highland Park, Box 998, Vermont, USA). AHL concentrations were determined by comparing bioluminescence signal intensities with a defined concentration (0.1 mg/ml) of pure 3-oxo-C6-HSL.

3.2.3. Extraction of quorum sensing signal from supernatants

P. fluorescens 07A and 041 (10^4 CFU/ml) was inoculated in 600 ml of LB, TYEP, King's B, and AB minimal medium supplemented with 10 mM citrate (ABC). The cultures were incubated with aeration at 25 °C for 20 h or until the population reach 10^9 CFU/ml. Then, the cells were harvested by centrifugation at 10,000 *g* for 20 min at 4 °C and 250 ml of the cell free supernatants were mixed with 100 ml of dichloromethane stabilized with ethanol in a 1,000 ml separating funnel. The mixture was shacked for 3 min with aeraton every 20 s. When the two phases were separated, the dichloromethane-phase was collected (lower phase). The upper phase (aqueous phase) was mixed with 100 ml of dichloromethane and shaked again as described above. Lower dichloromethane-phase was collected and mixed with the first one. These steps were repeated until finishing the 600 ml of supernatant. Then, the remaining water was removed with water free $MgSO_4$ and it was filtrated using Whatman paper. The filtered extracts were concentrated in a rotary evaporator at 40 °C, resuspended in 250 µl ethyl acetate, and maintained at -20 °C.

3.2.4. Detection of signal molecules in supernatant of *P. fluorescens*

Thirty milliliter of overnight culture of *E. coli* MT102 pSB403 were inoculated in 150 ml of LB agar. The inoculated LB plates were solidified and 6 µL of AHL extracts obtained from the supernatant of King's B, LB, and TYEP inoculated with *P. fluorescens* 07A were transferred as drops to the plate's surface. Aliquots of 0.6 µL of HHL 1 mg/ml were used as positive controls. The plates were incubated overnight and the activation of the AHL monitor strain *E. coli* MT102 pSB403 was observed into a dark box that contained a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics Herrsching, Germany) as described by Steidle et al. (2001).

3.2.5. Detection of signal molecules using Thin Layer Chromatography (TLC)

A line of 1.5 cm afar from the borders of the TLC plate (C_{18} reversed-phase thin-layer plate, hydrocarbon impregnated silica gel, RPS 20 x 20 cm, Analtech-uniplate^{TM}) was signed and aliquots from 10 to 20 µl of the ethyl acetate extracts were loaded on TLC plate. The extract was loaded on TLC drop by drop of 2 µl and dried in cold air step by step. TLC plates were developed with 60% (v/v) methanol-water solvent mixture in a saturated glass chamber. After elution, the plates were dried in cold air.

LB soft agar (150 ml) at 42 °C was mixed with 30 ml of the activated monitor strains *E. coli* MT102 (pSB403), *C. violaceum* CV026, or *A. tumefaciens* NTL4 according to the AHLs to be detect. For *A. tumefaciens* NTL4, 30 ml of this culture was mixed with 110 ml AB soft agar added of 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After supplementation with the appropriated monitor strain, the soft agar was dispensed on a dried TLC plate to produce a 2 to 3 mm thick layer. After 20 min, the plate was put in an airproof box with a wet paper inside and incubated overnight at 30 °C.

The documentation system was dependent on the monitor strain used. When *C. violaceum* CV026 was used as a monitor, the signal molecules could be identified by forming violet pigments after 48 h incubation. For *E. coli* MT102 pSB403, after incubation overnight at 30 °C, the TLC plates were put into a dark box and

bioluminescence was detected with a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics Herrsching, Germany). For *A. tumefaciens* NTL4, the material was incubated until 48 h and AHLs could be identified by visualizing blue pigments. In all cases the documentation was done by photographing.

3.2.6. DNA manipulations, PCR reactions and sequencing

3.2.6.1. DNA manipulations

All DNA manipulations were developed as described in chapter 2, item 2.2.6.1.

3.2.6.2. Amplification of the AHL synthase (*phzI* and *mupI*) genes of *P. fluorescens* by PCR

The PCR reaction consisted of 2.0 mM $MgCl_2$, 5.0 µl of 10X buffer Ex *Taq*, 2.5 mM deoxynucleotide triphosphates (dNTPs), 25 pmol of each primer, 1 U Ex *Taq* DNA polymerase, and 40 ng of DNA of *P. fluorescens* 07A and 041 in a final volume of 50 µl. Primers based on the sequences of the *phzI* (GenBank accession number L48616) and *mupI* gene (GenBank accession number AF318063) of *P. fluorescens* were constructed (Table 2) and synthesized by Microsynth (Zürich, Switzerland). PCR reactions were carried out in a T3 thermocycler (Biometra®, Biolabo Scientific Instruments, Zürich, Switzerland).

Table 2 – Primers used to amplify the *phzI* and *mupI* genes by PCR

Primer	Sequence (5'-3')	Application
phzI-F	ATG CAC ATG GAA GAG CAC	*phzI* gene
phzI-R	GAG TTT GAT GGC GAG GAT	*phzI* gene
phzI-F new	GAA TGG GAT CAA TAC GAC AC	*phzI* gene
phzI-F new-1	TTC ACC ACC CGC GAA CCG C	*phzI* gene
phzI-R new	GCC GAG AGT TTG ATG GCG AGG	*phzI* gene
mup-F	TAA TAG ACA AAC GCG AGA A	*mupI* gene
mup-R	GTT AAC TTC AAC AGC GAT G	*mupI* gene

3.2.6.3. Sequencing of the AHL synthase genes

The M13 Forward and Reverse primers were used to sequence the fragments of *phzI* and *mupI* genes cloned into pCR2.1-TOPO.

3.2.7. Evaluation of *P. fluorescens* resistance against different antibiotics and tellurite

P. fluorescens strains were inoculated into 5 ml of LB broth containing 100 µg/ml tellurite or different antibiotics (100 µg/ml trimethroprim, 20 µg/ml chloramphenicol, 25 µg/ml gentamicin, 100 µg/ml ampicillin, 50 µg/ml kanamycin, 20 µg/ml tetracycline, and 50 µg/ml spectinomycin). Growth at 25 °C was observed at 600 nm after incubation for 48 h.

3.2.8. Cloning of the gentamicin-3-acetyltransferase gene on broad-host-range expression vector

The gentamicin-3-acetyltransferase gene (GenBank accession number U25061) of pBBR1MCS-5 was amplified using the primer pair Gem-F (5' ATT <u>ATG CAT</u> GAA CCT GAA TCG CCA GCG G 3') and Gem-R (5' ATT <u>ATG CAT</u> GTT GAA CGA ATT GTT AGG TGG C 3'). The introduced restriction site *Nsi*I is underlined. The amplicon was digested with *Nsi*I and ligated directionally into the broadhost-range expression vector pMLBAD-aiiA-Trm^r (WOPPERER et al., 2006) cut with the same enzyme, yielding pMLBAD-aiiA-Trm^r-Gm^r. This plasmid containing the *aiiA* gene which encodes the lactonase enzyme was transferred to *E. coli* XL1-Blue by transformation.

3.2.9. Conjugative plasmid transfer

Plasmids were delivered to *P. fluorescens* strains by triparental mating as described previously (DE LORENZO and TIMMIS, 1994). Briefly, donor (*E. coli*

XL1-Blue pMLBAD-aiiA-Trm^r-Gm^r) and recipient strains, as well as the helper strain *E. coli* HB101 (pRK600), were grown overnight in 5 ml of LB medium supplied with the appropriate antibiotics. After subculturing to an optical density of 0.9 at 600 nm, the cells from 2 ml of the culture were harvested, washed, and resuspended in 500 µl of LB medium. Donor and helper cells (100 µl each) were mixed and incubated for 10 min at room temperature. Then, 200 µl of the recipient cells was added and the mixture was spot inoculated onto the surfaces of pre-heated LB agar plates. After overnight incubation at 30 °C, the cells were plated on *Pseudomonas* Isolation Agar (PIA) (Becton Dickinson Biosciences, Sparks, MD) containing antibiotics for counter selection of the donor, helper, and untransformed recipient cells.

After identity confirmation of the transconjugants, quorum sensing signals were extracted from supernatants of different media as described in chapter 3, item 3.2.3, and the detection of signal molecules by Thin Layer Chromatography (TLC) were developed as described in chapter 3, item 3.2.5.

3.2.10. Phenotypic characterization of wild type and transconjugant strains

Biofilm formation in polystyrene microtiter dishes was assayed essentially as described previously (PRATT and KOLTER, 1998) with a few modifications. Cells of *P. fluorescens* 07A and 041 wild-type and transcojugant were grown in the wells of microtiter dishes in 100 µl of LB, minimal medium salt (MMS) or AB medium supplemented with 10 mM citrate (ABC) for 48 h at 25 °C. Thereafter, the medium was removed, and 100 µl of a 1% (wt/vol) aqueous solution of crystal violet (CV) was added. After staining at room temperature for 20 min, the dye was removed, and the wells were washed thoroughly. For quantification of attached cells, the CV was solubilized in an 800:120 (vol/vol) mixture of ethanol and dimethyl sulfoxide, and the absorbance was determined at 570 nm.

The ability to form a swarming colony was tested by point inoculating strains into ABC minimal medium supplemented with 0.1% Casamino Acids and solidified with 0.4% agar as previously described (HUBER et al., 2001).

Proteolytic activity was determined by streaking strains on LB agar supplemented with 2% (w/v) skim milk (WOPPERER et al., 2006), and in cultures

of *P. fluorescens* in ABC, MMS, and TYEP using azocasein assay as described in chapter 2, item 2.2.3.2.

3.2.11. Identification of signal molecules by mass spectrometry

Culture supernatant of TYEP medium was extracted as described in chapter 3 item 3.2.3 using ultra pure dichloromethane stabilized with ethanol and analyzed by gas chromatograph-mass spectrometry (GC-MS).

3.2.12. Detection of AI-2 in supernatant of LB medium inoculated with *P. fluorescens*

P. fluorescens 07A, 041, 097, and 0109 were grown overnight with aeration at 28 °C on LB medium. Cell-free culture supernatants were prepared by removing the cells from the growth medium by centrifugation at 10,000 *g* for 20 min. The cleared culture supernatants were passed through 0.2 μm filters and stored at -20 °C. *Vibrio harveyi* BB120 was used as a positive control, and it was grown overnight at 30 °C with aeration in AB *Vibrio* medium. Cell-free culture fluids from *V. harveyi* BB120 were prepared from overnight culture exactly as described before for *P. fluorescens*. Aliquots of 10 μl of cell-free culture fluids were added to 96-well microtiter dishes. The monitor strain, *V. harveyi* BB170, was grown with aeration for 16 hours at 30 °C in AB medium and diluted 1:5,000 into fresh AB medium. Aliquots of 90 μl of diluted cells were added to wells containing the *P. fluorescens* cell-free culture fluids. Positive control wells contained 10 μl of cell-free culture fluid from *V. harveyi* BB120 and negative control wells contained 10 μl of sterile growth medium (LB or AB). Microtiter dishes were shaken in a rotary shaker at 175 RPM at 30 °C. Bioluminescence was measured using the program KC4 (Bio-Tek Instruments, Highland Park, Box 998, Vermont, USA).

3.3. RESULTS AND DISCUSSION

3.3.1. Detection of signal molecules produced by *P. fluorescens*

P. fluorescens strains isolated from refrigerated raw milk did not induce the biosensor strains *C. violaceum* CV026, *E. coli* MT102 pSB403, *P. putida* F117 pAS-C8, and *P. putida* F117 pKR-C12 (Table 3).

Table 3 – Activation of the AHL monitor strains in cross-streak experiments

Bacteria	Result obtained with					
	CV 026	pSB403	F117 (pAS-C8)	F117 (pKR-C12)	A 136	NTL4
P. fluorescens 07A	-	-	-	-	+	+
P. fluorescens 041	-	-	-	-	+	+
P. fluorescens 097	-	-	-	-	+	+
P. fluorescens 0109	-	-	-	-	+	+
B. cepacia H111	Nd	+++	+++	Nd	Nd	Nd
B. vietinamensis	Nd	Nd	Nd	+++	Nd	Nd
P. aeruginosa PAO1	+++	Nd	Nd	Nd	+++	+++

The six monitor strains were cross-streaked against *P. fluorescens* on LB agar plates. Following up to 48 h of incubation at 30 °C, the production of violacein by *C. violaceum* CV026, bioluminescence by *E. coli* pSB403, green fluorescent protein gfp(ASV) by *P. putida* F117, and β-galactosidase activity by *A. tumefaciens* A136 and NTL4 was visualized as described in material and methods. Levels of activation are indicated as follows: +++: strong activation, diffusion of AHL > 1 cm; +: activation, diffusion of signal of 0.3 cm; -: no detectable activation. Nd: not determined.

However, they were able to induce weakly *A. tumefaciens* A136 and NTL4 (Table 3) which indicates that signal molecule(s) produced by *P. fluorescens* strains were in low concentration or they are different from the cognate autoinducer that activates TraR protein in *A. tumefaciens*. Once *A. tumefaciens* strains responded to substances present into culture media, a false positive result should be considered, since in some experiments these strains were able to induce themselves (Figure 1). Other molecules besides AHLs such as diketopiperazines (DKPs) could activate biosensors, underlining the importance of chemical characterization of the molecules identified in such bioassays (HOLDEN et al., 1999). The development and use of ultrasensitive biosensors must be done with caution once the reality of the results obtained can be compromised because they can detect compounds that are not used as signal molecule in nature. The biological significance of these compounds like cyclic dipeptides (diketopiperazines) as putative signal molecules is discussed, with evidence presented that these compounds are capable of activating or antagonizing *lux*-based AHL biosensors and AHL-dependent phenotypes (HOLDEN et al., 1999).

Previously, many *Pseudomonas* spp. have been shown to produce and secrete signal molecules such as AHLs (SHAW et al., 1997; CHA et al., 1998; KIEVIT and IGLEWSKI, 2000; LAUE et al., 2000; PARSEK and GREENBERG, 2000; ANDERSEN et al., 2001; EL-SAYED et al., 2001; STEIDLE et al., 2001; WHITEHEAD et al., 2001; CUI et al., 2005; JUHAS et al., 2005; KHAN et al., 2005). Cui et al. (2005) suggested that AHL production seems to be more common among *P. fluorescens* closely associated with plants than among their soilborn counterparts. Because of the specificity requirements of the R protein, most of the detection systems are limited in the range of AHLs to which they respond (SHAW et al., 1997). Therefore, it represents a limitation of the bioassay since bacteria often produce more than one AHL molecule. This limitation can be overcome by using multiple monitor systems (PINTO et al., 2007).

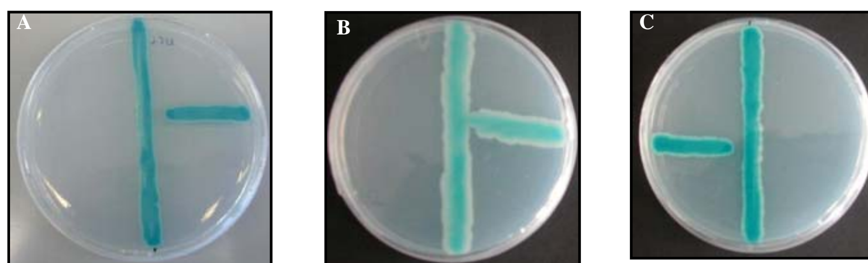

Figure 1 – Activation of the AHL monitor strain *A. tumefaciens* in cross-streak experiments in AB medium after 48 h at 30 °C. A) *A. tumefaciens* NTL4 in cross-streak to *A. tumefaciens* NTL4; B) *A. tumefaciens* KYC55 in cross-streak to *A. tumefaciens* KYC55; C) *A. tumefaciens* A136 in cross-streak to *A. tumefaciens* A136.

3.3.2. Detection of bioluminescence induced by *P. fluorescens*

Signal molecule extract obtained from *P. fluorescens* 07A and 041 did not induce *E. coli* MT102 pSB403 in the assay developed to detect the bioluminescence production (Table 4). Therefore, this result suggests that *P. fluorescens* 07A and 041 isolated from cooled raw milk did not produce AHLs able to induce a high sensitive biosensor as *E. coli* MT102 pSB403 and reinforce that the molecules able to induce *A. tumefaciens* in the cross-streak assays are other compounds present in the growth media. According to Winson et al. (1998), there is a significant advantage of using *lux* sensors since the sensitivity to AHL is in a range of picomol and nanomol concentrations over a large linear range in real time. By combining the results from *lux* sensor the activity profiles can be compared with those of known standards to give a preliminary identification of the nature of the AHL under investigation. This information can then be used to aid the development of appropriate extraction and identification procedures.

Table 4 – Values of bioluminescence produced by *E. coli* MT102 pSB403 at 175 nm after growth in LB broth supplemented with supernatant of *P. fluorescens* and supplemented with 3-oxo-C6-HSL. Data represent average of triplicate experiments.

Dilution rate	*P. fluorescens* 07A	*P. fluorescens* 041	LB Negative control	3-oxo-C6-HSL Positive control
1/2	13532	15823	14820	Nd*
1/4	15261	13831	15003	Nd
1/8	13967	15360	13230	Nd
1/16	14401	16580	15340	Nd
1/32	16977	16036	16720	51802
1/64	15862	14159	14579	28723
1/128	17092	17619	16220	21817
1/256	15716	13420	14943	20942

*Nd – not detected. The intensity of the signal was higher than the limit of detection of the equipment.

3.3.3. Supplementation of LB inoculated with *E. coli* MT102 pSB403 with extracts obtained from different media

As no activity of signal molecules was found in the supernatant of LB medium inoculated with *P. fluorescens* 07A and 041 against *E. coli* MT102 pSB403 using the microtiter dishes assay, it was tried to find correlation between the growth media and the production of signal compounds, but no influence on AHL production was found (Figure 2).

However, Mcphee (2001) found that synthesis of AHLs by *Pseudomonas* was influenced by the composition of the growth medium. It was suggested that AHL production is not only influenced by cell density, but environmental factors such as growth medium also are important. According to Mcphee (2001), *P. fluorescens* was found to up-regulate enzyme synthesis when it grew in a spent culture supernatant, presumably having already high levels of synthesized AHL.

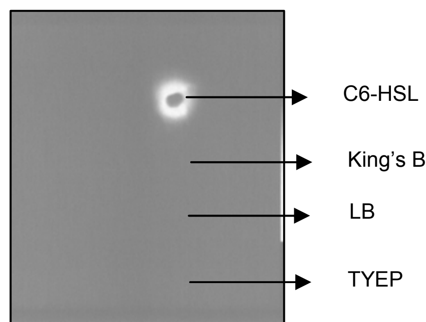

Figure 2 – Activation of the AHL monitor strain *E. coli* MT102 pSB403 after supplementation of the medium with 6 µL of signal molecule extracts obtained from the supernatant of different media inoculated with *P. fluorescens* 07A. The plate was incubated at 30 °C for 18 h.

3.3.4. Detection of signal molecules using TLC

The extracts of different media inoculated with *P. fluorescens* 07A and 041 induced *A. tumefaciens* NTL4 when it was used as a reporter strain on TLCs (Figures 3, 4, 5, 6 and 7). The only exception was observed in ABC minimal medium inoculated with *P. fluorescens* 07A wild type and transconjugant (Figure 3). This result confirms that in a pour medium, which contains only minerals and the carbon source was sterilized by filtration, compounds like DKPs is not produced. Then, there are no molecules able to induce *A. tumefaciens*.

Besides, AHL biosensors as *E. coli* MT102 pSB403 and *C. violaceum* CV026 did not detect any signal from the extracts obtained from LB medium inoculated with *P. fluorescens* 07A and 041.

Extracts obtained from different media without inoculation that were used as a negative control were able to induce *A. tumefaciens* NTL4 (Figures 4B, 5AB, 6, and 7AB). However, it can be verified that the extract of culture of *P. fluorescens* resulted in a signal with intensity and position on TLC plates different from the signal obtained with extracts of negative controls (Figures 4B, 5AB, 6, and 7AB). This indicates that *P. fluorescens* might produce some substance that is able to induce *A. tumefaciens*, but this substance needs to be characterized to confirm its identity and its biological potential as an autoinducer compound.

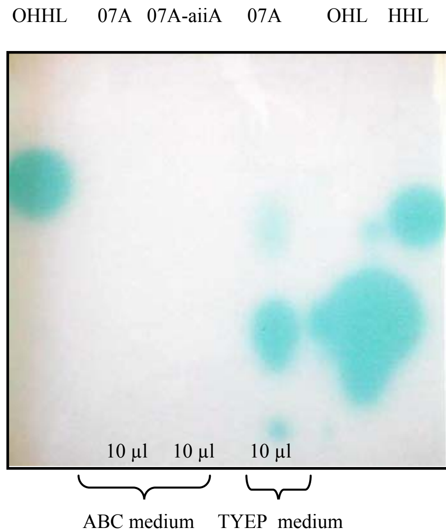

Figure 3 - A representative thin-layer chromatogram of the signal molecules present in cell free supernatants of *P. fluorescens* 07A. The spots were detected with the *A. tumefaciens* NTL4 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(octanoyl)-L-homoserine lactone (OHL); and N-(hexanoyl)-L-homoserine lactone (HHL).

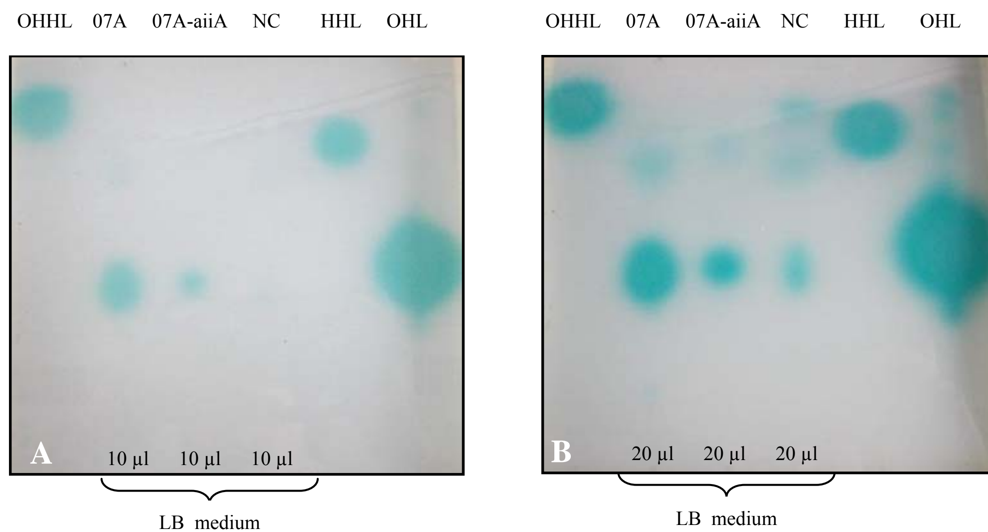

Figure 4 - Representative thin-layer chromatograms of the signal molecules present in cell free supernatants of *P. fluorescens* 07A. The spots were detected with *A. tumefaciens* NTL4 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(octanoyl)-L-homoserine lactone (OHL); and N-(hexanoyl)-L-homoserine lactone (HHL); (NC) negative control, extract from LB not inoculated. (A) 10 µl of extract obtained from LB medium. (B) 20 µl of extract obtained from LB medium.

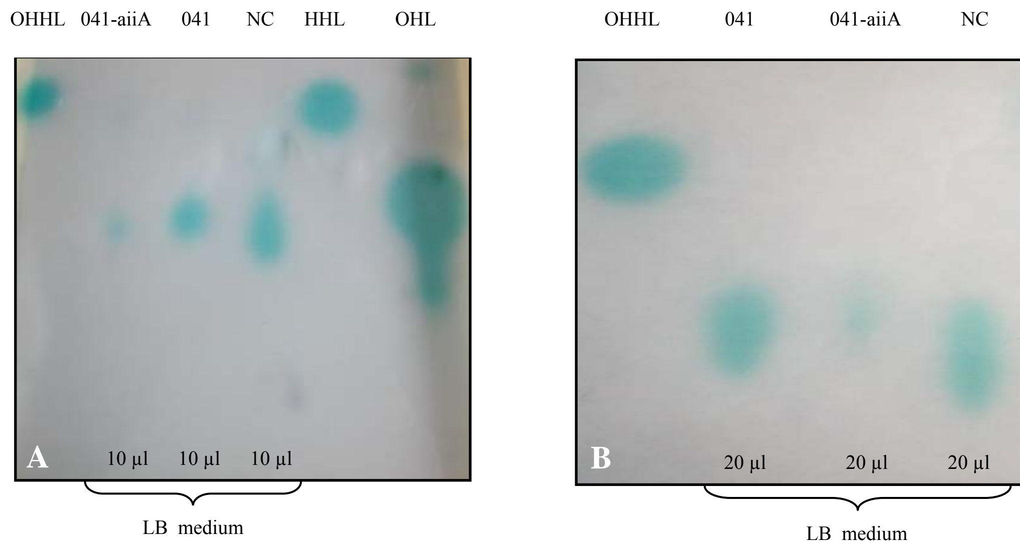

Figure 5 - Representative thin-layer chromatograms of the signal molecules present in cell free supernatants of *P. fluorescens* 041. The spots were detected with the *A. tumefaciens* NTL4 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(hexanoyl)-DL-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); (NC) negative control, extract from LB not inoculated. (A) 10 µl of extract obtained from LB medium. (B) 20 µl of extract obtained from LB medium.

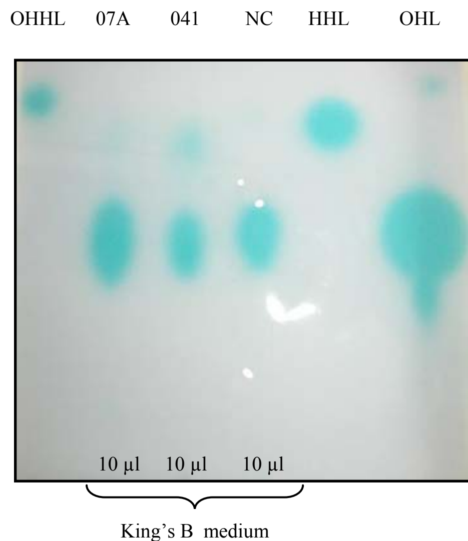

Figure 6 - A representative thin-layer chromatogram of the signal molecules present in cell free supernatants of *P. fluorescens* 07A and 041. The spots were detected with the *A. tumefaciens* NTL4 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(hexanoyl)-L-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); (NC) negative control, extract from LB not inoculated.

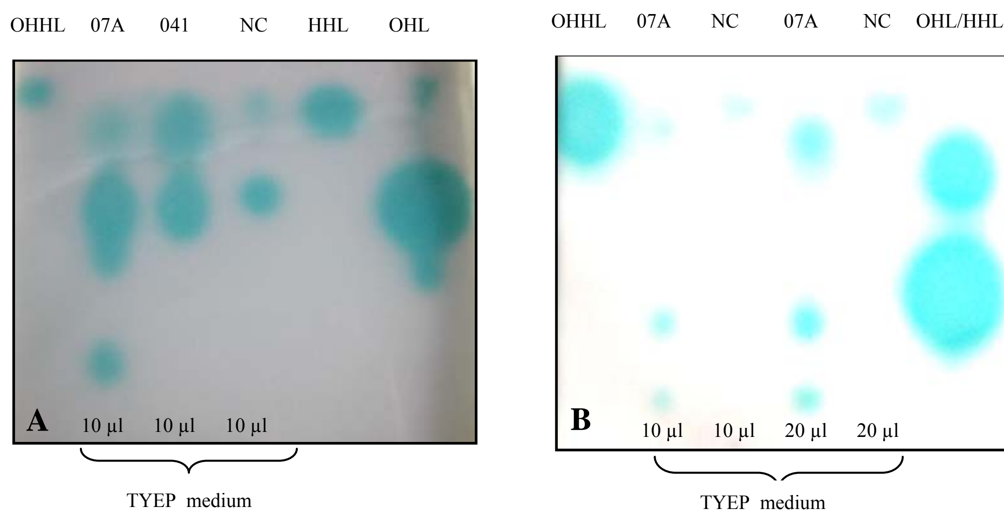

Figure 7 - Representative thin-layer chromatograms of signal molecules present in cell free supernatants of *P. fluorescens* 07A and 041. The spots were detected with *A. tumefaciens* NTL4 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(hexanoyl)-L-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); (NC) negative control, extract from LB not inoculated. (A) 10 µl of extract obtained from TYEP medium. (B) 10 µl or 20 µl of extract obtained from TYEP medium. The sample used in the last TLC was obtained using ultra pure dichloromethane and characterized by gas chromatograph-mass spectrometry.

The involvement of quorum sensing in growth of *P. fluorescens* was suggested by Whan et al. (2000). N-benzoyloxycarbonyl-L-homoserine lactone and N-3-oxyhexanoyl-DL-homoserine lactone significantly reduced the lag phase duration and increased the exponential growth rate of three strains of *P. fluorescens* (DUNSTALL et al., 2005). However, no signal molecules were found on TLC by Pinto et al. (2007) and by Viana (2006).

3.3.5. Amplification and sequencing of *phzI* and *mupI* genes by PCR

Since El-Sayed et al. (2001) and Khan et al. (2005) pointed out a relationship between the quorum sensing mechanism and antibiotic production by *P. fluorescens* NCIMB 10586 and *P. fluorescens* 2-79, and characterized the AHL synthases as MupI and PhzI, respectively, primers based on these sequences were developed and

used to amplify these genes by PCR in *P. fluorescens* 07A and 041. It was obtained unspecific PCR products when it was used primers to amplify *mupI* and *phzI* genes. However, bands of right size (530 bp for *mupI* and 510 bp for *phzI*) were extracted from the gels, cloned into pCR2.1-TOPO and sequenced. The results obtained were sequences of *P. fluorescens* not related to any AHL synthase, confirming that these strains 07A and 041 isolated from cooled raw milk do not have genes homologues to *phzI* and *mupI*.

3.3.6. Resistance of *P. fluorescens* against some antibiotics and selective agent

Although *P. fluorescens* strains isolated from raw milk were not able to grow in LB supplemented with tellurite, a selective agent, and in LB supplemented with gentamicin (Table 5), they grew well in LB supplemented with other antibiotics. The wide spectra of antibiotic resistance observed among *P. fluorescens* (Table 5) may demonstrate high selective pressure into raw milk. This datum confirms the data obtained by other authors who observed a high patter of antibiotic resistance among bacteria isolated from milk in Brazil (ARAÚJO, 1998; CARNEIRO and JÚNIOR, 2006).

Table 5 – Tellurite and antibiotic susceptibility of *Pseudomonas fluorescens* in LB broth

Antibiotic	*P. fluorescens*			
	07A	041	097	0109
Tellurite	-	-	-	-
Trimethoprim	+	+	+	+
Chloramphenicol	+	+	+	+
Gentamicin	-	-	-	-
Ampicillin	+	+	+	+
Kanamycin	+	+	+	+
Tetracycline	+	+	+	+
Spectinomycin	+	+	+	+

Growth is indicated as follows: +, growth; -, no detectable growth.

3.3.7. Cloning of gentamicin-3-acetyltransferase gene in pMLBAD-aiiA-Trm^r and mobilization to *P. fluorescens* 07A and 041

Since *P. fluorescens* 07A and 041 were able to grow in LB supplemented with many antibiotics, it was necessary to obtain a new donor strain. First, *E. coli* XL1-Blue pMLBAD-aiiA-Tel^r was used as donor, but as *P. fluorescens* transconjugant was not able to grow well in LB supplemented with tellurite, it was necessary to clone the gentamicin-3-acetyltransferase gene in pMLBAD-aiiA-Trm^r.

To express *aiiA* lactonase gene in *P. fluorescens* strains, a fragment of gentamicin-3-acetyltransferase gene (860 bp) was amplified by PCR as described in Material and Methods and cloned directionally into the *Nsi*I site (Figure 8) of a broad-host-range expression vector pMLBAD-Trm^r (LEFEBRE and VALVANO, 2002) that had the *aiiA* gene (797 bp) cloned into *Nco*I and *Hind*III sites by Wopperer et al. (2006), yielding pMLBAD-aiiA-Trm^r-Gem^r. In pMLBAD-aiiA-Trm^r-Gem^r, the *aiiA* gene is transcribed from P_{BAD} promoter of *E. coli*, and thus expression is inducible with arabinose. Thus, routinely it was used 0.02% arabinose in all further experiments, as described by Wopperer et al. (2006).

To test the functionality of the construct and the applicability of the quorum-quenching approach, pMLBAD-aiiA-Trm^r-Gem^r was conjugated into *P. fluorescens*. To confirm that *P. fluorescens* strains contained the plasmid with the right inserts, this plasmid was extracted, and restricted with *Nco*I and *Hind*III (Figure 9).

It was observed that the signal extract obtained from strains of *P. fluorescens* transconjugant inoculated in LB induced *A. tumefaciens* NTL4 (Figure 4AB, and 5AB). This data confirm that the molecule(s) that induced *A. tumefaciens* was not compromised by lactonase expression.

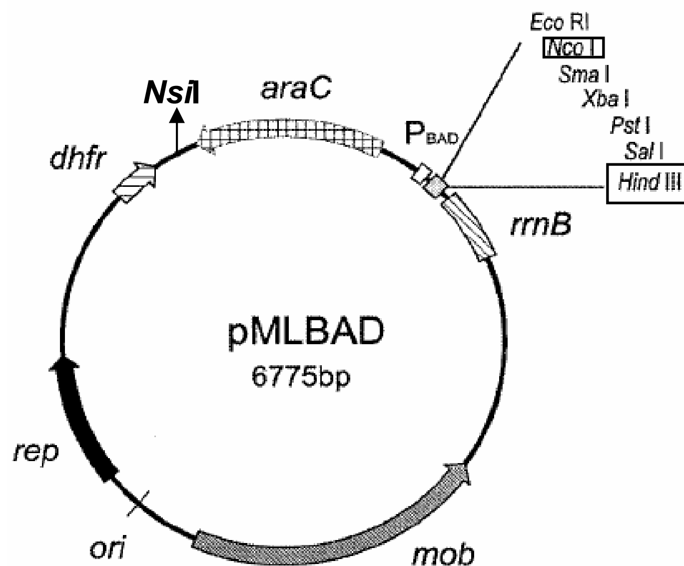

Figure 8 – Map of pMLBAD showing the *Nco*I and *Hind*III sites used by Wopperer et al. (2006) to clone the *aiiA* gene and the site to *Nsi*I used to clone gentamicin-3-acetyltransferase gene (this work). *dhfr*, dihydrofolate redutase gene encoding trimethoprim resistance; P_{BAD}, arabinose-inducible promoter; *araC*, transcriptional regulator gene; *rrnB*, transcriptional terminator; MCS, multiple cloning site; *mob*, gene required for conjugal transfer of plasmid; *rep*, replication protein gene; *ori*, origin of replication. Adapted from Lefebre and Valvano (2002).

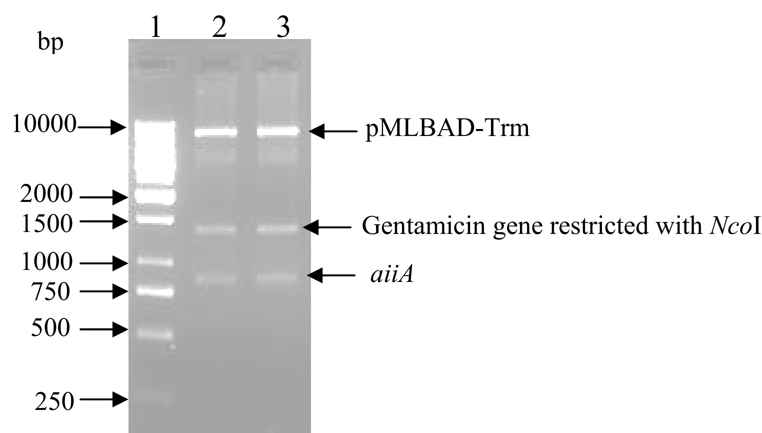

Figure 9 – Agarose gel electrophoresis of restriction reactions of pMLBAD-aiiA-Trm^r-Gem^r containing the *aiiA* and gentamicin-3-acetyltransferase genes cloned. Line 1, Standard; line 2, *P. fluorescens* 07A-2 transconjugant; line 3, *P. fluorescens* 041-3 transconjugant.

3.3.8. Phenotypic characteristics of *P. fluorescens* wild type and transconjugants

3.3.8.1. Biofilm

After 48 hours of incubation, it was observed that *P. fluorescens* 07A and 041 produced less biofilm in LB and MMS than in ABC medium. The strain 041 was able to bind better than 07A in polystyrene microtiter dishes (Figure 10). Viana (2006) also observed that different strains of *P. fluorescens* had different ability to bind in polystyrene and that minimal medium enhanced attachment. The ABC minimal medium is rich in divalent ions as phosphate, Ca^{+2}, Mg^{+2}, and Fe^{+2}. According to Fletcher et al. (1988), divalent ions as Ca^{+2} and Mg^{+2} can influence directly biofilm formation due to electrostatic interactions, and indirectly via process of adhesion dependent of microorganism physiology because they can act as cofactors of enzymes. The presence of ions as Ca^{+2} improve cross-binding between cells and between cells and surfaces (KOERSTGENS et al., 2001). Maybe, because the abundance of these compounds in ABC minimal medium, better biofilm formation in this medium was observed compared to LB and MMS.

It was not verified significant difference ($p>0.01$) on the ability to produce biofilm when wild type strains were compared to transconjugants (Figure 10). This result shows that the quorum quenching mechanism did not influence this phenotype in these strains of *P. fluorescens* isolated from raw milk. However, Allison et al. (1998) suggested the quorum sensing systems to be involved in promoting cell attachment and biofilm formation in *P. fluorescens* B52, but that do not involved short chain HSLs.

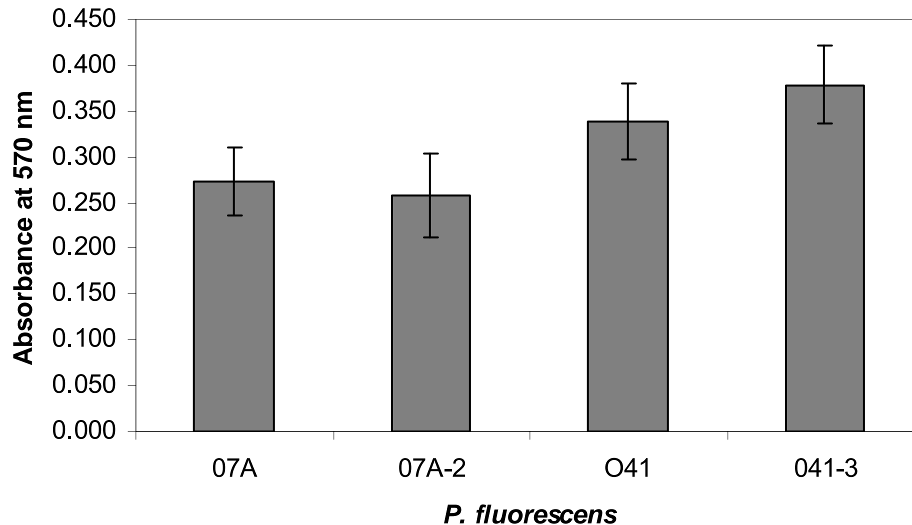

Figure 10 – Biofilm formation by *P. fluorescens* wild type (07A and 041) and transconjugant (07A-2 and 041-3) in minimal medium ABC after incubation for 48 h at 25 °C, determined as absorvance of crystal violet at 570 nm.

3.3.8.2. Swarming motility

To test swarming motility, the strains 07A and 041 were point inoculated into medium containing 0.4% agar. Under the conditions used, only *P. fluorescens* 07A was capable of swarming (Figure 11). When AiiA was expressed in *P. fluorescens* 07A, swarming motility was reduced, indicating that a factor required for swarming was compromised in this strain or that this assay was compromised due to the sensibility of this methodology since *P. fluorescens* 07A transconjugant was grown previously in LB supplemented with gentamicin.

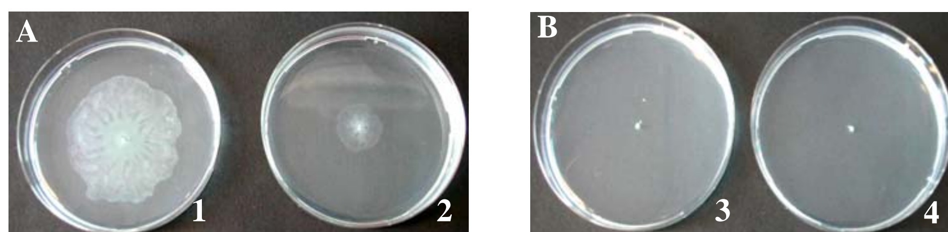

Figure 11 – Ability to form a swarming colony on ABC after incubation for 18 h at 25 °C. (A) 1, *P. fluorescens* 07A wild type; 2, *P. fluorescens* 07A-2 transconjugant. (B) 1, *P. fluorescens* 041 wild type; 2, *P. fluorescens* 041-3 transconjugant.

3.3.8.3. Extracellular protease

AHL-dependent quorum sensing systems control the production of extracellular proteolytic activity in many gram-negative bacteria (WHITEHEAD et al., 2001). To test whether expression of proteolytic activity is generally AHL-regulated in *P. fluorescens* 07A and 041, wild type and transconjugant strains were streaked onto LB agar plates supplemented with 2% skim milk. Clearing zones, which are indicative of protease activity, were observed for both wild type and transconjugant strains after 18 h of incubation (Figure 12). Besides, heterologous expression of AiiA did not decrease proteolytic activity in both strains when they were grown into different broth media (Figure 13), indicating that the AHL-dependent regulation of this phenotype is not conserved in these strains of *P. fluorescens* isolated from refrigerated raw milk.

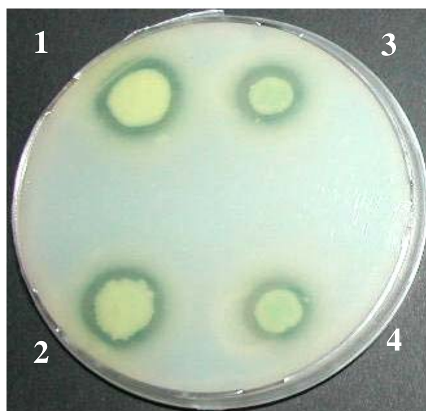

Figure 12 – Proteolytic activity on LB agar plates supplemented with skim milk powder 2% after incubation for 18 h at 25 °C. 1, *P. fluorescens* 07A wild type; 2, *P. fluorescens* 07A transconjugant; 3, *P. fluorescens* 041 wild type; 4, *P. fluorescens* 041 transconjugant.

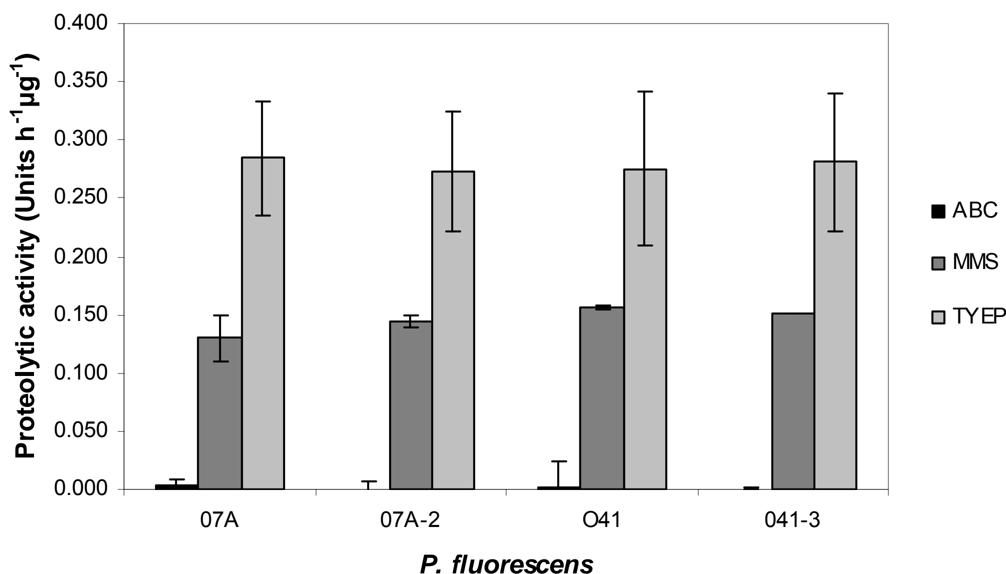

Figure 13 – Proteolytic activity on supernatant of ABC (minimal medium), MMS (minimal medium) and TYEP medium inoculated with *P. fluorescens* wild type (07A and 041) and transconjugants (07A-2 and 041-3) after 24 h of incubation at 25 °C.

3.3.9. Chemical characterization of signal molecules produced by *P. fluorescens* 07A into TYEP medium

The extracts of signal molecules obtained from TYEP medium were analysed using mass spectrometry. AHLs molecules were not detected in the samples (Table 6). However, two compounds (4-Dimethylaminobenzaldehyde and Cyclo(ile-val)) were present into the extract obtained from TYEP medium inoculated with *P. fluorescens* 07A and absent into TYEP medium not inoculated (Table 6).

A variety of cyclic dipeptides (DKPs) in the signal molecule extract were obtained from TYEP medium inoculated and not inoculated with *P. fluorescens* 07A (Table 6). Prasad (1995) showed that DKPs existed in protein hydrolysates as well as in fermentation broths and cultures of yeast, fungi, and bacteria. According to Holden et al. (2000), DKPs were identified as a consequence of their ability to activate biosensors previously considered specific for AHLs. Although DKPs are structurally quite distinct, at high concentrations they are able to cross-activate AHL-dependent reporter constructs based on several different LuxR homologues (HOLDEN et al., 1999). The detection of these DKPs appears to be an example of

fortuitous chemical crosstalk and raises the obvious question as to their origin and biological function(s). Therefore, although it is quite feasible that the DKPs do not function as bacteria-to-bacteria signaling molecules *per se*, they might have a role in modulating prokaryotic-eukaryotic interactions. In bacteria, the production of DKPs is not limited to *P. aeruginosa*, as other gram-negative bacteria, including *E. coli* and *P. fluorescens* (HOLDEN et al., 1999; HOLDEN et al., 2000). According to Cui (2004), these molecules demonstrated the complexity of quorum sensing and the existence of cross-communication in signalization systems of *P. fluorescens*.

Table 6 – Results of chemical characterization of signal molecule extract obtained from TYEP medium not inoculated and inoculated with *P. fluorescens* 07A.

Compound	Negative control TYEP medium	TYEP medium inoculated with *P. fluorescens* 07A
Benzaldehyde	+	-
Caprylic acid	+	-
Decanoic acid	+	-
Hexanoic acid	+	-
Tributylphosphate	+	+
Quinaldaldehyde	+	-
1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	+	+
3-Benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	+	+
4-Dimethylaminobenzaldehyde*	-	+
Cyclo(ala-pro)	+	+
Cyclo(ile-val)*	-	+
Cyclo(met-pro)	+	+
Cyclo(pro-leu)	+	±
Cyclo(pro-val)	+	+

*Compound present into TYEP inoculated with *P. fluorescens* 07A and absent into TYEP not inoculated. +: present; -: absent; ±: doubt.

3.3.10. Detection of AI-2

The results demonstrated in Table 7 suggest that *P. fluorescens* isolated from cooled raw milk produces AI-2 since the sterilized supernatant of these strains inoculated into LB broth was able to induce bioluminescence production by the monitor strain *V. harveyi* BB170.

Table 7 – Detection of auto-inducer two in supernatant of LB broth inoculated with *P. fluorescens*. *V. harveyi* BB170 was used as a monitor strain and *V. harveyi* BB120 was used as a positive control.

Strains and medium	Luminescence at 175 nm*
P. fluorescens 07A	9766 ± 728
P. fluorescens 041	12567 ± 2145
P. fluorescens 097	9933 ± 634
P. fluorescens 0109	10420 ± 652
V. harveyi BB120	6360 ± 1643
LB medium (negative control)	6776 ± 553
AB medium (negative control)	3111 ± 644

*Average and standard deviation of data is shown. n: number of repetitions equal 8.

The occurrence of *luxS*-dependent AI-2 signaling is widespread among both gram-negative and gram-positive bacteria including *E. coli* (pathogenic and non-pathogenic varieties), *Salmonella* Typhimurium, *Shigella flexneri*, *Helicobacter pylori*, *Streptococcus pyrogenes*, *Neisseria meningitides*, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (SURETTE et al., 1999; MILLER and BASSLER, 2001). Thus, AI-2 has evolved several diverse, species specific roles while simultaneously remaining a universal signal recognizable across numerous species of bacteria. It is clear that AI-2 signaling regulates the expression of numerous genes and is involved in determining phenotypes, but exactly what AI-2 is signaling is a murky subject (LERAT and MORAN, 2004). Emerging evidence indicates that AI-2 may not be a density-dependent signal, but rather a waste product.

3.4. CONCLUSIONS

Strains of *P. fluorescens* isolated from cooled raw milk do not produce AHL and do not have the genes *mupI* and *phzI*. On the other hand, these strains might produce AI-2.

P. fluorescens 07A and 041 produces less biofilm in LB and MMS than in ABC minimal medium, and the strain 041 binds better than 07A in polystyrene microtiter dishes.

Cyclic dipeptides found into the medium induced *A. tumefaciens* AHL sensor strains and caused false positive results in the TLC and cross-streak assays.

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CHAPTER 4

QUORUM SENSING IN PSYCHROTROPHIC STRAINS ISOLATED FROM REFRIGERATED RAW MILK

4.1. INTRODUCTION

Product contamination with psychrotrophic microorganisms is a particular concern for the dairy industry, as dairy products are maintained and distributed at temperatures permissive for the growth of these organisms. The diverse microorganisms categorized as psychrotrophic are ubiquitous in nature and can be isolated from soil, water, and vegetation (DOGAN and BOOR, 2003).

Gram-negative bacteria usually account for more than 90% of the microbial population in cold raw milk that has been stored (COUSIN, 1982; PINTO 2004; MUNSCH-ALATOSSAVA and ALATOSSAVA, 2006). The gram-negative microbiota is composed mainly of psychrotrophic species of *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Hafnia*, *Chromobacterium*, *Flavobacterium*, and *Enterobacter* (GARCÍA-ARMESTO and SUTHERLAND, 1997; SØRHAUG and STEPANIAK, 1997; RYSER, 1999; PINTO, 2004). Most of these bacteria produce extracellular proteolytic and lipolytic enzymes that are secreted into the milk. Many of these enzymes are not inactivated by pasteurization

or by ultra-high temperature treatment (GRIFFITHS et al., 1981). The residual activities of these enzymes can reduce the sensorial quality and shelf-life of processed milk products (FAIRBAIRN and LAW, 1986; DOGAN and BOOR, 2003; PINTO, 2004; MARTINS et al., 2006).

Among the most common psychrotrophs isolated from raw milk, *Aeromonas hydrophila* and *Hafnia alvei* are considerated opportunistic foodborne pathogens associated with cases of gastroenteritis. *A. hydrophila* is a widespread bacterium found in water, domestic animals, and foods (fish, shellfish, poultry, milk, and raw meat). This pathogen produces virulence factors, including exotoxins and cytotoxins, and multiple resistance of the bacterium to many antimicrobials is a fact of high significance (DASKALOV, 2006).

H. alvei is a gram-negative facultatively anaerobic bacillus that belongs to the family of *Enterobacteriaceae* (RODRÍGUEZ et al., 1999). It possesses several different virulence mechanisms, which are similar or identical to those of other gram-negative enteropathogens. It is suspected to cause a variety of intestinal disorders and other illnesses, including pneumonia, meningitis, abscesses, and septicemia (ALBERT et al., 1992; RODRÍGUEZ et al., 1999).

Another important representative of *Enterobacteriaceae* isolated from milk is *Enterobacter* sp. (COUSIN, 1982; PINTO, 2004). This bacterium produces extracellular enzymes that can compromise the sensorial quality of dairy products.

Gram et al. (1999) showed that strains of *Enterobacteriaceae* isolated from foods produce acyl-homoserine lactones (AHLs) as signal molecules and regulate the expression of some genes in response to density in a mechanism known as quorum sensing (QS). According to Gram et al. (1999), the production of signal molecules was detectable from naturally contaminated foods and from samples to which pure cultures have been added and the *Enterobacteriacea* reached 10^5 to 10^7 CFU/g. This high number of bacteria is not uncommon in foods, which indicates that AHLs could be implicated in regulating phenotypes important in food spoilage and thus possibly play a role in food quality deterioration.

According to Christensen et al. (2003), several hydrolytic enzymes produced by a typical member of a food spoilage flora are regulated by QS. These authors demonstrated that QS is involved in the production of spoilage characteristics *in situ* on food products.

Besides the control of spoilage enzyme expression, QS is also related to expression of virulence genes. A useful host model for studying innate immune responses to bacterial pathogens is the nematode *Caenorhabditis elegans*, which lacks an adaptive immunity (CARDONA et al., 2005). This model is genetically tractable from the perspectives of both host and pathogen, and thus, serves to investigate evolutionary conserved mechanisms of microbial pathogenesis and innate immunity (KURZ et al., 2003; CARDONA et al., 2005).

As QS is related to regulation of several phenotype characteristics, the control of this mechanism, usually referred to quorum quenching, has been studied and proved to be successful in microorganisms isolated from non-food sources (DONG et al., 2000; ULRICH, 2004; WOPPERER et al., 2006). Enzymatic cleavage, specifically lactone ring hydrolysis, of AHL molecules by numerous *Bacillus* species has been reported (DONG et al., 2002). These enzymes, termed lactonases AiiA, hydrolyze the lactone bond within the AHL moiety, thus changing the relative conformational structure of the signaling molecule, which prevents binding to the LuxR transcriptional regulator (ULRICH, 2004). Rasmussen et al. (2005) found that garlic extract was one of the most effective inhibitors of QS since it reduced *P. aeruginosa* biofilm tolerance to tobramycin treatment and virulence of this bacterium against *C. elegans*.

The understanding of the role of the QS mechanism in the regulation of spoilage phenotypes in bacteria from milk is relevant and may be used to create new strategies to preserve dairy products. Therefore, the purpose of the present work was to elucidate which signal molecules are produced by proteolytic pychrotrophic bacteria isolated from cooled raw milk and to relate the QS mechanism to the spoilage potential and pathogenicity of these strains.

4.2. MATERIAL AND METHODS

4.2.1. Strains and growth conditions

The psychrotrophic strains and other bacteria used in the present study are listed in Table 1. Unless otherwise stated, these strains were grown at 30 °C in Luria-Bertani (LB) medium or AB minimal medium (CLARK and MAALOE, 1967) supplemented with 10 mM glucose (ABG). *Vibrio harveyi* strains were grown at 30 °C in AB *Vibrio* medium (BASSLER et al., 1994). Solid media were routinely solidified with 1.4% agar. Antibiotics were added as required at final concentrations of 20 µg/ml for gentamicin and tetracycline, 50 µg/ml for trimethoprim and spectinomycin, 100 µg/ml for ampicillin, and 10 µg/ml for chloramphenicol. Kanamycin was used at 30 µg/ml for *V. harveyi* and at 50 µg/ml for *E. coli* S17-1 and psychrotrophic strains. Besides, tellurite, a selective agent, was added when required at a final concentration of 100 µg/ml. Growth of liquid cultures was monitored spectrophotometrically as described in chapter 3, item 3.2.1.

Table 1 - Bacterial strains and plasmids used in this study

Strain	Plasmid	Description	Reference or source
Aeromonas hydrophila 099		Wild type, psychrotrophic isolated from cooled raw milk	Pinto, 2004
Agrobacterium tumefaciens A136	pCF373, pCF218, Tc^r, Spc^r	As described in chapter 3, item 3.2.1	
A. tumefaciens NTL4	pZLR4, Gm^r	As described in chapter 3, item 3.2.1	
Burkholderia cepacia H111		As described in chapter 3, item 3.2.1	
Burkholderia vietinamensis		As described in chapter 3, item 3.2.1	
Chromobacterium violaceum CV026		As described in chapter 3, item 3.2.1	
Enterobacter sp. 067		Wild type, psychrotrophic isolated from cooled raw milk	Martins et al., 2005
Enterobacter sp. 067-7	pBHR1-aiiA-km^r	Transconjugant, express the lactonase enzyme	This study
Escherichia coli HB101	pRK600, Cm^r	As described in chapter 3, item 3.2.1	
E. coli MT102	pSB403, Tc^r	As described in chapter 3, item 3.2.1	
E. coli S17-1	pBHR1-aiiA-km^r	Donor of pBHR1-aiiA, km^r that codify the lactonase enzyme	Ulrich, 2004
E. coli XL1-Blue	pMLBAD-aiiA-Trm^r-Gm^r	Donor of pMLBAD-aiiA, Trm^r Gm^r that codify the lactonase enzyme	This study
E. coli XL1-Blue	pQE30-Xa	As described in chapter 2, item 2.2.1	This study
E. coli XL1-Blue	pQE30-Xa-halI068	Express AHL synthase, HalI, from *H. alvei* 068	This study
Hafnia alvei 059		Wild type, psychrotrophic isolated from cooled raw milk	Martins et al., 2005
H. alvei 068		Wild type, psychrotrophic isolated from cooled raw milk	Martins et al., 2005
H. alvei 068-1	pBHR1-aiiA-km^r	Transconjugant, express the lactonase enzyme	This study
H. alvei 071		Wild type, psychrotrophic isolated from cooled raw milk	Martins et al., 2005
H. alvei 071-1	pBHR1-aiiA-km^r	Transconjugant, express the lactonase enzyme	This study
Pantoea sp. 039		Wild type, psychrotrophic isolated from cooled raw milk	Martins et al., 2005
Pseudomonas aeruginosa PAO1		As described in chapter 3, item 3.2.1	
Pseudomonas putida F117	pAS-C8, Gm^r	As described in chapter 3, item 3.2.1	
P. putida F117	pKR-C12, Gm^r	As described in chapter 3, item 3.2.1	
Serratia liquefaciens MG1		Positive control in the chitinase assay	Laboratory of Microbiology, University of Zürich
Vibrio harveyi BB120		As described in chapter 3, item 3.2.1	
Vibrio harveyi BB170		As described in chapter 3, item 3.2.1	

4.2.2. Amplification and sequencing of 16S rDNA from psychrotrophic strains

The identification of psychrotrophic isolates from cooled raw milk was initially done by Pinto (2004) and in order to confirm their identities API ID32E (BioMérieux, Marcy-l'Etoile, France) was used for phenotypic characterization. Thereafter, 16S rDNA was sequenced as described by Juretschko et al. (1998).

DNA manipulations were conduced as described in chapter 2, item 2.2.6.1. For amplification of the 16S rDNA, the PCR reaction consisted of 25 mM $MgCl_2$, 5.0 µl of 10X buffer Ex *Taq*, 25 mM deoxynucleotide triphosphates (dNTPs), 25 pmol of each primer, 1 U Ex *Taq* DNA polymerase, and 40 ng of DNA in a final volume of 50 µl. Primers described by Juretschko et al. (1998) (616V 5'AGAGTTTGATYMTGGCTC 3' and 630R 5'CAKAAAGGAGGTGATCC 3') were synthesized by Microsynth (Zürich, Switzerland). PCR reactions were carried out in a T3 thermocycler (Biometra®, Biolabo Scientific Instruments, Zürich, Switzerland). The M13 Forward and Reverse primers were used to sequence the rDNA 16S genes cloned into pCR2.1-TOPO. Thereafter, the obtained sequences were used to search for similarity using the Ribosomal Database Project II (http://rdp.cme.msu.edu/seqmatch/seqmatch_result.jsp?qvector=204&depth=0¤tRoot=419&num=20. Access on June 4th, 2007).

4.2.3. Milk spoilage potential and production of exoenzymes by psychrotrophic strains

To evaluate the milk spoilage potential, samples of reconstituted 12% (w/v) skim milk powder were inoculated with approximately 1 x 10^4 CFU/ml of strains 059, 067, 068, 071, or 099. The samples were incubated at 30 °C for 18 h and their sensorial quality was checked.

Prior to the enzymatic assays, the method of Bradford (BRADFORD, 1976) was used for quantitative protein determination using bovine serum albumin as a standard. Proteolytic activity was determined by streaking the strains on LB agar plates supplemented with 2% (w/v) skim milk powder (WOPPERER et al., 2006) and on azocasein as described in chapter 2, item 2.2.3.2.

Supernatant proteins from the crude extracts of psychrotrophic strains in LB were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; LAEMMLI, 1970). After electrophoresis, the gels were stained with Coomassie brilliant blue. Exoprotease activities of culture supernatants from psychrotrophic strains were analysed by SDS-PAGE with 0.2% (w/v) azocasein incorporated into the gel matrix (12% polyacrylamide) as described in chapter 2, item 2.2.4.

Lipolytic activity was determined by streaking the strains on medium 884 (Tween 80-Agar). Besides, the lipolytic activity on p-nitrophenylpalmitate was investigated using 100 µl bacterial supernatant from overnight cultures in LB or TYEP as described in chapter 2, item 2.2.3.3.

Chitinase activity was determined by streaking the strains on ABC minimal medium supplemented with 2% chitin solution.

4.2.4. Detection and quantification of AHL

AHL production was investigated by cross-streaking psychrotrophic strains that were grown overnight on LB agar plates against monitor strains as described in chapter 3, item 3.2.2.

Escherichia coli MT102 pSB403 was used for quantification of bioluminescence induced by AHL molecules as described in chapter 3, item 3.2.2.

4.2.5. Extraction of AHLs from supernatants

Psychrotrophic strains (10^4 CFU/ml) were inoculated in 250 ml of LB or 400 ml of AB minimal medium supplemented with 10 mM citrate (ABC medium). Samples were incubated at 30 °C for 20 h skaking at 300 RPM or until the population reach 10^9 CFU/ml. Then, the cells were harvested by centrifugation at 10,000 *g* for 20 min and cell free supernatants were used to extract AHLs as described in chapter 3, item 3.2.3.

4.2.6. Detection of AHL using Thin Layer Chromatography (TLC)

The extracts were loaded on TLC plates as described in chapter 3, item 3.2.5. To 150 ml of soft agar at 42 °C, 30 ml of the monitor strain *E. coli* MT102 (pSB403) or *C. violaceum* CV026 was mixed. The soft agar supplemented with the appropriated monitor strain was dispensed on a TLC plate to produce a 2 to 3 mm thick layer. After 20 min, the plate was transferred to an airproof box with a wet paper inside and incubated overnight at 30 °C.

The documentation was dependent on the monitor strain used. For *C. violaceum* CV026, the material was incubated up to 48 h and the signal molecules were identified by formation of violet pigments. When *E. coli* MT102 pSB403 was the monitor, the material was incubated overnight at 30 °C, transferred to a dark box and bioluminescence was detected with a highly sensitive photon-counting camera (C2400-40; Hamamatsu Photonics Herrsching, Germany).

4.2.7. LC-MS analysis of AHL extracts from bacterial supernatants

One hundred and twenty microliters of dichloromethane extracts from 400 ml of culture supernatant in ABC or ABG minimal medium were evaporated under a gentle stream of nitrogen. The residue was re-dissolved in 120 µl of 60% (v/v) aqueous methanol and separated by reversed-phase LC-MS (C18 column, Grom-Sil 120 ODS-4 HE, 4.6 x 250 mm, Stagroma, Germany) under the following conditions: a flow rate of 1 ml/min; solvent A UV-treated H_2O and 0.1% formic acid; solvent B acetonitrile (ACN) and 0.1% formic acid. After separation, the mixture was analyzed by mass spectrometry (LCQ Duo Mass Spectrometer, Thermoquest, Finnigan) using an electrospray source. The following gradient was applied: solvent B from 25% ACN to 100% in 20 min, isocratic 5 min.

4.2.8. Resistance of psychrotrophic strains against different antimicrobials

Psychrotrophic strains were inoculated into 5 ml of LB broth containing 100 µg/ml tellurite or antibiotics (100 µg/ml trimethoprim, 20 µg/ml chloramphenicol, 25

µg/ml gentamicin, 100 µg/ml ampicillin, 50 µg/ml kanamycin, 20 µg/ml tetracycline, and 50 µg/ml spectinomycin). Growth at 30 °C was observed at 600 nm after incubation for 48 h.

4.2.9. Conjugative plasmid transfer and confirmation of identity of transconjugant strains

Plasmids were delivered to psychrotrophic strains (067, 068 and 071) by triparental mating as described previously in chapter 3, item 3.2.9. Briefly, donor (*E. coli* S17-1 pBHR1-aiiA, km^r) and recipient strains, as well as the helper strain *E. coli* HB101 (pRK600), were grown overnight in 5 ml of LB medium supplied with the appropriate antibiotics. After subculturing to an optical density at 600 nm of 0.9, the cells from 2 ml of culture were harvested, washed, and resuspended in 500 µl of LB medium. Donor and helper cells (100 µl each) were mixed and incubated for 10 min at room temperature. Then, 200 µl of the recipient cells was added and the mixture was spot inoculated onto the surfaces of preheated LB agar plates. After overnight incubation at 30 °C, the cells were plated on LB containing 100 µg/ml tetracycline and 50 µg/ml kanamycin for counter selection of the donor, helper, and untransformed recipient cells of 067. Tetracycline at 100 µg/ml and kanamycin at 25 µg/ml were used for counter selection of the donor, helper, and untransformed recipient cells of 068 and 071.

The transconjugant strains were characterized by restriction digest of pBHR1-aiiA with *Nco*I and *Eco*RI according to Ulrich (2004) and the identity of these strains was checked using the indol test since *E. coli* is indol positive and *H. alvei* and *Enterobacter* are indol negative. In addition, the AHL synthase gene *halI* of the transconjugant strains was amplified by PCR as described in 4.2.12.

4.2.10. Phenotypic characterization of wild type and transconjugant strains

Proteolytic activity was determined by inoculating the strains into pasteurized milk, by streaking them on LB agar supplemented with 2% (w/v) skim milk, and with the azocasein assay as described in chapter 2, item 2.2.3.2.

4.2.11. Detection and sequencing of a native plasmid from *H. alvei* 068 and 071

The native plasmid DNA of *H. alvei* 068 and 071 was isolated using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and named pMLM. After isolation, it was loaded on an agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and digested with restriction enzymes (*Apa*I, *Ava*I, *Bam*HI, *Bln*I, *Bsi*WI, *Bsm*I, *Bst*BI, *Bst*XI, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Mlu*I, *Msp*I, *Nco*I, *Not*I, *Nsi*I, *Nsp*I, *Pvu*I, *Rsa*I, *Rpn*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Sph*I, *Stu*I, *Xba*I, *Xho*I, and *Xho*II) in order to generate fragments to clone and sequence.

The fragments obtained were cloned into Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, United Kingdom) and the M13 Forward and Reverse primers were used for sequencing. Thereafter, primers based on the sequences of pMLM068 were constructed (Table 2) and synthesized by Microsynth (Zürich, Switzerland).

Table 2 - Primers used to sequence pMLM

Primer	Sequence (5’-3’)
pMLMA	CCT ATC CTG CAT CGT GTT
pMLMB	GGT AGC GTA AAA ATT TGC GG
pMLMC	GTA GAG GCA TTT ACG GCG TTT
pMLMCD	CAG TGG GTC AGT TCA TGC AA

4.2.12. DNA manipulations, PCR reactions and sequencing of *halI* and *halR* genes

DNA manipulations were developed as described in chapter 2, item 2.2.6.1. To amplify the AHL synthase gene (*halI*) and the gene *halR* that encodes the AHL receptor (HalR) by PCR, the reaction consisted of 2.0 mM $MgCl_2$, 5.0 µl of 10X buffer Ex *Taq*, 2.5 mM deoxynucleotide triphosphates (dNTPs), 25 pmol of each primer, 1 U Ex *Taq* DNA polymerase, and 40 ng of DNA in a final volume of 50 µl. Primers based on the sequences of *halI* and *halR* genes (GenBank accession number AF503776) of *H. alvei* were constructed (Table 3) and synthesized by Microsynth

(Zürich, Switzerland). PCR reactions were carried out in a T3 thermocycler (Biometra®, Biolabo Scientific Instruments, Zürich, Switzerland).

The M13 Forward and Reverse primers were used to sequence the *halI* and *halR* genes cloned into pCR2.1-TOPO.

Table 3 - Primers used to amplify *halI* and *halR* genes by PCR

Primer	Sequence (5'-3')	Application
halI-F	AACTGATTACACCAATGCAGT	Amplification and sequencing of *halI*
halI-R	GGAATGCTTGAACTATTTGATG	Amplification and sequencing of *halI*
halI-bam	ATTGGATCCTACACCAATGCAGTCTTAATT	Amplification of *halI* gene and preparation for cloning in pQE-30Xa
halI-sac	ATTGAGCTCATGCTTGAACTATTTGATGTC	Amplification of *halI* gene and preparation for cloning in pQE-30Xa
halR-F	CTT CAG GGA TGC CAT ATG TTT	Amplification and sequencing of *halR*
halR-R	ACT GCA TTG GTG TAA TCA GTT	Amplification and sequencing of *halR*

The introduced restriction sites for *Bam*HI and *Sac*I are underlined.

4.2.13. Cloning and heterologous expression of AHL synthase (*halI*) of *H. alvei* 068 in pQE-30Xa

Once the complete sequence of the *halI* gene was obtained, primers (Table 3) were designed to amplify the open reading frame (ORF) by PCR using the bacterial genomic DNA as a template and TaKaRa Ex Taq polymerase. Primers generated *Bam*HI and *Sac*I sites at the 5' and 3' ends of the amplificates, respectively. The DNA amplificate, 660 bp, containing the *halI* structural gene, was digested with *Bam*HI and *Sac*I and ligated into vector pQE-30Xa (Qiagen) cut with the same restriction enzymes. A plasmid harbouring the ORF of *halI* inserted downstream of the T5 promoter was selected and named pQE-30Xa-halI068. The plasmid was transformed into the expression strain *E. coli* XL1-Blue.

For overproduction of HalI, *E. coli* XL1-Blue cells carrying pQE-30Xa-halI068 was grown in dYT medium as describe previously in chapter 2, item 2.2.7. At an optical density at 600 nm of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG)

was added to the culture to a final concentration of 1 mM. After 5 h incubation at 37 °C, the cells were collected by centrifugation at 10,000 *g* for 30 min and resuspended in 50 mM Tris-HCl (pH 8.0). Then, 3 µl of cell suspension was loaded on SDS-PAGE (15%) to detect HalI.

4.2.14. Detection, extraction, and characterization of AHLs encoded by *halI*

AHL production was investigated by cross-streaking *E. coli* XL1-Blue pQE-30Xa-halI068 cells that were grown overnight on dYT agar plates supplemented with 1 mM IPTG against *E. coli* pSB403 or *C. violaceum* CV026, as described in chapter 3, item 3.2.2.

To extract AHLs, 10^4 CFU/ml of *E. coli* XL1-Blue pQE-30Xa-halI068 were inoculated into 250 ml of dYT or in 400 ml of AB minimal medium supplemented with 15 mM glucose (ABG). At an optical density at 600 nm of 0.5, IPTG was added to the culture to a final concentration of 1 mM. The samples were incubated at 30 °C up to 48 h with skaking at 350 RPM. Then, the cells were harvested by centrifugation at 10,000 *g* for 20 min and cell free supernatants were used to extract AHLs as described in chapter 3, item 3.2.3.

The extract was loaded on TLC plates drop by drop of 2 µl and the TLC was dried in cold air step by step as described in chapter 3, item 3.2.5. Aliquots of 150 ml of soft agar at 42 °C were mixed with 30 ml of the monitor strain *E. coli* MT102 pSB403 or *C. violaceum* CV026. The soft agar supplemented with the appropriated monitor strain was dispensed on a TLC plate to produce a 2 to 3 mm thick layer. After 20 min, the plate was put in an airproof box with a wet paper inside and incubated overnight at 30 °C. The documentation was developed as described in chapter 4, item 4.2.6.

Chemical characterization of AHL molecules encoded by *halI* was performed in extracts from bacterial supernatants obtained from AB minimal medium supplemented with 15 mM glucose by LC-MS as described in chapter 4, item 4.2.7.

4.2.15. Detection of AI-2 in supernatant of LB medium inoculated with psychrotrophic strains

Psychrotrophic strains were grown overnight with aeration at 30 °C on LB medium. The autoinducer two was detected as described in chapter 3, item 3.2.12.

4.2.16. Pathogenesis of psychrotrophic strains against *Caenorhabditis elegans*

4.2.16.1. Maintenance and cultivation of *C. elegans*

C. elegans was sustained on NGM I plates covered with *E. coli* OP50 untill three weeks at 20 °C. Plates of NGM I were inoculated with 100 µl of a fresh overnight culture of *E. coli* OP50 and incubated at 37 °C overnight. Then, overgrown plates were stored at 4 °C. During the assays, the nematodes were transferred every two days onto fresh *E. coli* plates. For this, a piece of agar covered with *E. coli* and *C. elegans* was cut with a sterile scalpel and put onto a new *E. coli* plate.

4.2.16.2. Egg preparation of *C. elegans*

To synchronize all *C. elegans* at the same developmental stage, plates with plenty of eggs were used. Worms and eggs were rinsed from plates four times with 1 ml of sterile water and the suspension was dispersed in three tubes of 2 ml. The suspension was mixed with 500 µl of 12% sodiumhypochlorite solution and mixed by vortexing for approximately 8 min or until all worms had dissolved. The suspension was centrifuged for 1 min at 4 °C and 3,200 RPM. The supernatant was carefully discarded and the pellet was washed with 1 ml of sterile water. Then, it was centrifuged for 1 min and the supernatant was discarded. The pellet of tube one was resuspended with 100 to 200 µl of M9 buffer and the suspension was used to resuspend the pellets of the other tubes. Thereafter, this solution was transferred to a NGM I plate with *E. coli* and incubated at 20 °C.

4.2.16.3. Nematode assays

Assays of pathogenesis against *C. elegans* were performed essentially as described by Wopperer et al. (2006). Briefly, 100 µl of the suspensions of overnight cultures of psychrotrophic strains were plated on six-well plates containing nematode growth medium (NGM II) for slow killing assays. After 24 h of incubation at 30 °C, a bacterial lawn was formed and approximately 25 hypochlorite-synchronized L4 larvae of *Caenorhabditis elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis, MN) were used to inoculate the plates. The actual number of worms was determined by using a Stemi SV6 microscope (Zeiss, Oberkochen, Germany) at a magnification of 50X. Plates were then incubated at 20 °C and scored for live worms; nematodes were considered dead when they failed to respond to touch. The percentage of live worms and their morphological appearance were registered after two days. After five days, the total number of nematodes, including parental and progeny nematodes, if present, was scored. All experiments were carried out five times, and *E. coli* OP50 was used as a negative control in the assays. A psychrotrophic strain was considered to be pathogenic for *C. elegans* if one of the following criteria described previously by Cardona et al. (2005), and Wopperer et al. (2006) was met: (i) a sick appearance at day two, including reduced locomotive capacity and the presence of a distended intestine; (ii) percentage of live worms after two days of $\leq$ 50%; and (iii) total number of worms after five days of $\leq$ 100. For differentiating mild from severe infections, the presence of one, two, or three of these criteria was scored as 1, 2, or 3, respectively. A strain was considered pathogenic when at least one criterion was observed. A strain was described as nonpathogenic when no symptoms of disease were observed during the course of the infection experiment (pathogenicity score 0).

4.3. RESULTS AND DISCUSSION

4.3.1. Confirmation of identity of psychrotrophic strains isolated from cooled raw milk

The strains used in this work were previously identified by Pinto (2004) as described in Table 4. In order to confirm the identity of these strains, API ID32E was used and the obtained results were different from the data described by Pinto (2004) (Table 4). Therefore, it was necessary to use an accurate assay in order to identify the strains since both identifications were used phenotypic methods that may give false positive results. Therefore, the 16S rDNA was sequenced since it is universally distributed and highly conserved among all organisms and it is known as a gold standard for discerning evolutionary relationships among prokaryotes (ZHANG et al., 2002).

Analysis of 16S rRNA gene sequencing and biochemical tests were used by Janda et al. (2005) to separate *Hafnia* into different groups. Based upon 16S rRNA gene sequencing results, two genetic groups were identified and the biochemical test of malonate utilization was found to be the most differential. The test results of the malonate utilization assay alone correctly assigned 90% of *Hafnia* isolates to their correct DNA group (JANDA et al., 2005).

Table 4 – Identification of psychrotrophic strains isolated from cooled raw milk

Strain	API 20NE *	API ID32E	rDNA 16S
039	*Serratia liquefaciens*	Nd**	*Pantoea* sp.
059	*Hafnia alvei*	Nd	*Hafnia alvei*
067	*Hafnia alvei*	*Enterobacter cloacae*	*Enterobacter* sp.
068	*Hafnia alvei*	*Hafnia alvei*	*Hafnia alvei*
071	*Serratia odorifera*	*Hafnia alvei*	*Hafnia alvei*
099	*Aeromonas hydrophila*	*Aeromonas hydrophila*	*Aeromonas hydrophila*

* Determined by Pinto (2004).
** Nd: not determined.

According to the results obtained, the strains 039 and 067 need further investigations of their identities since the sequencing of 16S rDNA did not achieve conclusive results about the identity of these species. A polyphasic approach should be used to identify these strains in the future.

4.3.2. Spoilage potential and production of exoenzymes by psychrotrophic strains isolated from cooled raw milk

4.3.2.1. Potential to spoil milk samples

Bacterial spoilage causes significant economic losses for the dairy industry (COUSIN, 1982; DATTA and DEETH, 2001) and different psychrotrophic strains can show different spoilage potentials (WIEDMANN et al., 2000; DOGAN and BOOR, 2003; PINTO, 2004). The strains evaluated in this study showed different abilities to spoil milk samples: *A. hydrophila* 099 was the most deteriorative, whereas *H. alvei* 059 had the least ability to spoil milk (Figure 1).

The proteolytic activity of some extracellular enzymes of *A. hydrophila* was recognized and these enzymes are considered to play a major role in the virulence and pathogenicity of the bacterium (MEDINA-MARTÍNEZ et al., 2006). Besides, Vivas et al. (2004) showed that this bacterium can produce and secrete proteases able to cleave milk proteins. According to Cousin (1982), proteases produced by *Aeromonas* are able to degrade α-, β-, κ-, and γ-casein, as well as the whey proteins.

H. alvei strains 059, 068 and 071 presented different spoilage potentials (Figure 1). According to Bruhn et al. (2004), this bacterium was the dominant

member of the *Enterobacteriaceae* in vacuum-packed meat and it may induce food quality-relevant phenotypes in other bacterial species in the same environment. Then, it may influence the spoilage of food products in which *Enterobacteriaceae* participate in the spoilage process.

Although *Enterobacter* sp. is normally isolated from raw and pasteurized milk and butter, this bacterium is not a potent bacterium for spoilage of dairy products (COUSIN, 1982); however, in this study, it was verified that *Enterobacter* sp. 067 had a large potential to spoil samples of reconstituted skim milk powder (Figure 1).

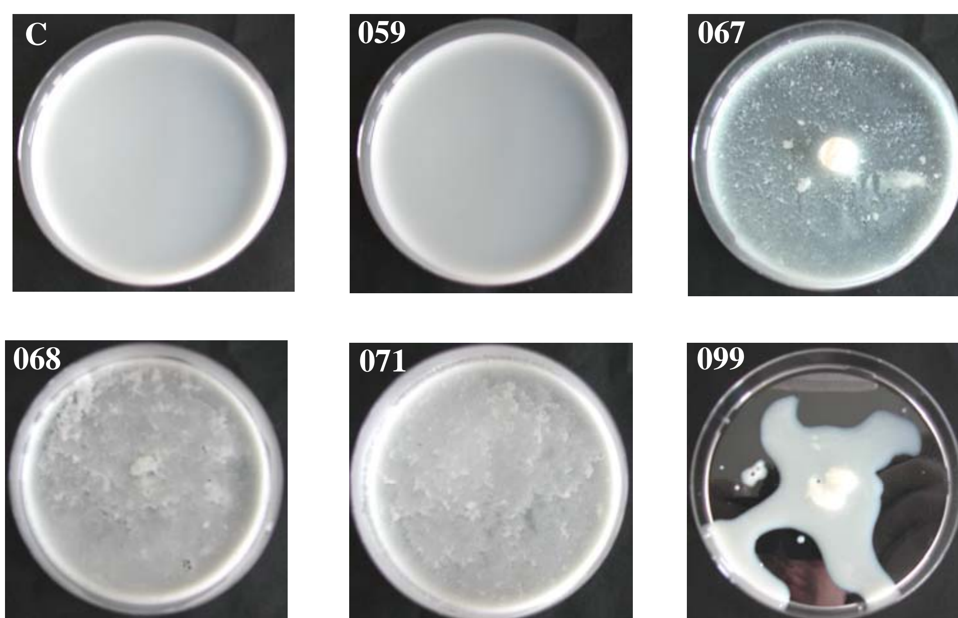

Figure 1 – Spoilage ability of psychrotrophic strains inoculated in reconstituted skim milk powder 12% (w/v) after incubation for 18 h at 30 °C. (C) Negative control, milk sample not inoculated, (059) *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (071) *H. alvei*, (099) *A. hydrophila*.

4.3.2.2. Production of extracellular enzymes

a) Protease

Psychrotrophic strains were streaked on LB agar plates supplemented with 2% skim milk powder and it was verified that they had different abilities to produce proteolytic enzymes able to cleave casein (Figure 2). These results confirmed the data obtained in the evaluation of spoilage potential and showed that *A. hydrophila* produced a high amount of exoproteases compared to the other strains (Figure 2).

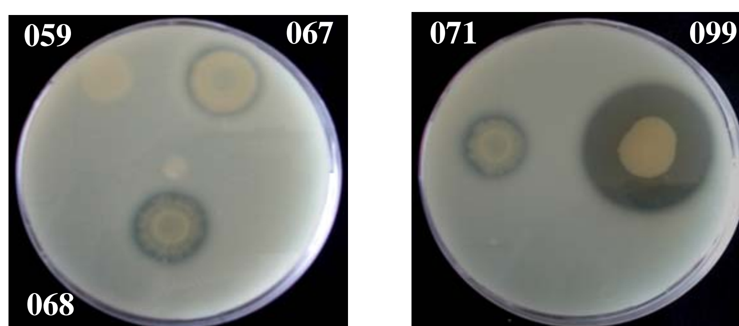

Figure 2 – Proteolytic activity on LB agar supplemented with 2% (w/v) skim milk powder after incubation for 24 h at 30 °C. Clearing zones are indicative of protease activity. (059) *H. alvei*, (067) *Enterobacter* sp, (068) *H. alvei*, (071) *H. alvei*, (099) *A. hydrophila*.

Proteolytic activity was not detected in the supernatants of LB and TYEP broth media cultured with strains 039, 059, 067, 068, and 071. However, the strain 099 showed a proteolytic activity of 0.131 units/h/μg protein in TYEP. Viana (2006) also did not detect proteolytic activity in supernatants of TYEP medium inoculated with 067 and 071 when it was used the azocasein assay. Maybe the azocasein is not the best substrate for the determination of proteolytic activity produced by these strains.

The presence of extracellular proteins was not observed in the supernatants of LB medium inoculated with 059, 068, and 071 by SDS-PAGE (Figure 3A). Small extracellular proteins were detected in the supernatants obtained from 039 and 067 (Figure 3A). Although the strains 067, 068, and 071 were proteolytic in LB supplemented with skim milk powder (Figure 2), proteolytic activity on zymogram was not detected (Figure 3B). Maybe these enzymes were in low concentration,

azocasein was not the best substrate for them, or they were not renaturated after the development of the zymogram.

On the other hand, many extracellular enzymes were detected in the supernatant obtained from *A. hydrophila* 099 (Figure 3A) and two of them had proteolytic activity on SDS-PAGE supplemented with 2% azocasein (Figure 3B). Production of both serine- and metalloprotease activities in *A. hydrophila* is under the control of quorum sensing mechanism (SWIFT et al., 1999), which also occurs for protease production in *Aeromonas salmonicida* (SWIFT et al., 1999) and other bacterial pathogens (ZHU et al., 2003).

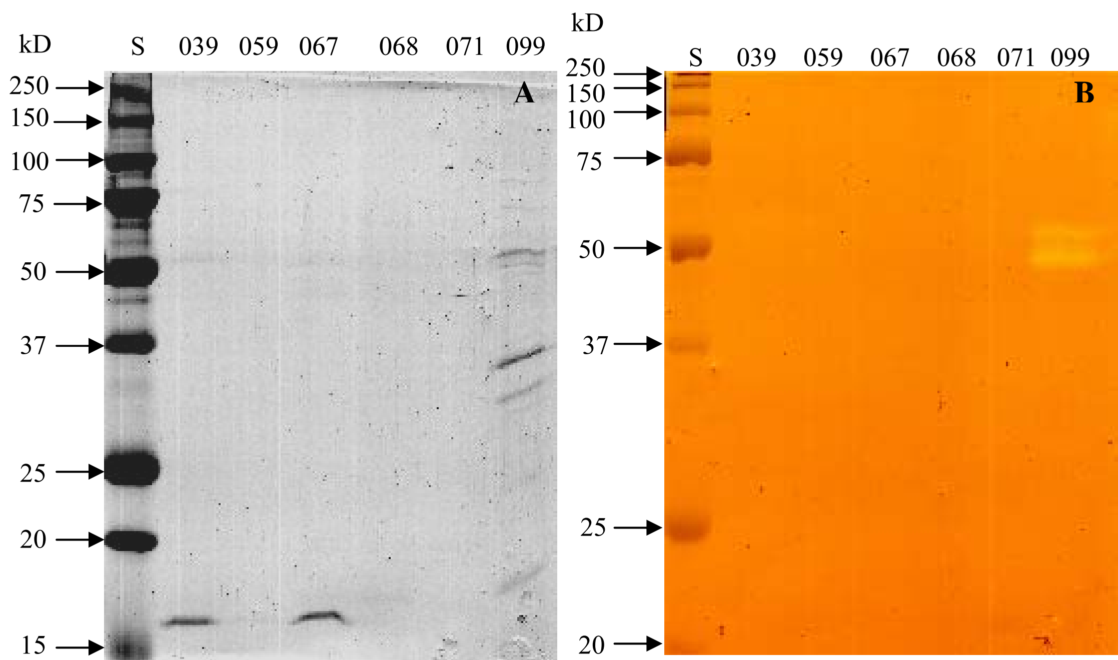

Figure 3 - SDS-PAGE (A) and zymogram azocasein (B) gels (12%) showing protease production by psychrotrophic strains after growth in LB medium for 18 h at 30 °C. Lanes: (S) molar mass standards (BioRad), (039) *Pantoea* sp (059), *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (071) *H. alvei*, (099) *A. hydrophila*.

b) Lipase

Lipolytic activity of gram-negative glucose fermenting isolates from cooled raw milk was generally associated with lecithinase activity (PINTO, 2004). In this study, lipolytic activity was not detected in the supernatants of LB and TYEP media

after growth of strains 039, 059, 067, 068, and 071 overnight. Only the strain 099 showed a lipolytic activity of 1.104 units/h/μg protein in TYEP. This activity was confirmed on Tween 80-Agar (Figure 4). According to Brumlik and Buckley (1996), among extracellular enzymes released by *A. hydrophila*, a glycerophospholipid-cholesterol acyltransferase (GCAT) has been described and characterized. This enzyme is analogous to the important mammalian plasma enzyme lecithin-cholesterol acyltransferase and, like most of the lipases found in the microbial world, is a member of the lipase superfamily (ANGUITA et al., 1993).

Figure 4 – Lipolytic activity after growth of *A. hydrophila* 099 on Tween 80-Agar for 48 h at 30 °C. Precipitation zones are indicative of lipase activity.

c) Chitinase

Only *A. hydrophila* 099 showed chitinase activity in ABG minimal medium supplemented with 2% chitin solution (Figure 5). According to Chen et al. (1991), *A. hydrophila* JP101 is able to use chitin as its carbon and nitrogen sources when grown on chitin medium and apparently it synthesizes the entire enzymatic system through which degradation of chitin occurs.

Chitinases cleave the β-1,4-glycosidic bonds of chitin, a β-1,4-linked, unbranched polymer of *N*-acetylglucosamine, which is a major component of insect exoskeletons, shells of crustaceans, and fungal cell walls. These enzymes have been detected in a variety of organisms, including organisms that do not contain chitin as a structural component, such as bacteria, plants, and animals. Bacteria utilize

chitinases for assimilation of chitin as a carbon and nitrogen source and these enzymes play an important ecological role in the degradation of chitin (WU et al., 2001).

Figure 5 – Chitinase activity after grouth for 5 days at 30 °C on ABG minimal medium supplemented with 2% (w/v) chitin solution. Clearing zones are indicative of chitinase activity. (MG1) Positive control: *S. liquefaciens* MG1, (059) *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (071) *H. alvei*, (099) *A. hydrophila*.

4.3.3. Detection of AHL molecules produced by *Enterobacter* sp., *H. alvei*, and *A. hydrophila*

Psychrotrophic proteolytic strains induced the biosensor strains *E. coli* MT102 pSB403, *C. violaceum* CV026, *P. putida* F117 pAS-C8, *P. putida* F117 pKR-C12, *A. tumefaciens* NTL4, and *A. tumefaciens* A136 (Table 5). Only *Pantoea* sp. 039 was not able to induce the biosensors. As we used a range of different AHL monitor systems in combination, it is possible that the entire range of known AHLs was detected. Members of *Enterobacteriaceae* isolated from foods have been shown to produce and secrete signal molecules, such as AHLs (GRAM et al., 1999; RAVN et al., 2001; GRAM et al., 2002; CHRISTENSEN et al., 2003; FLODGAARD et al., 2003; BRUHN et al., 2004).

Strains 059, 068, and 071 of *H. alvei* produced more AHLs than the others, since they were able to strongly induce the monitor strains (Table 5). Many authors reported that AHL is ordinarily produced by spoilage bacteria isolated from foods

(GRAM et al., 1999; RAVN et al., 2001; BRUHN et al., 2004). Pinto et al. (2007) demonstrated that AHL production is common among psychrotrophic bacteria isolated from milk and indicated that quorum sensing may play an important role in the spoilage of this product.

Table 5 – Activation of the AHL monitor strains in cross-streak experiments

Bacteria	Result obtained with					
	CV 026	pSB403	F117 (pAS-C8)	F117 (pKR-C12)	A 136	NTL4
Pantoea sp 039	-	-	-	-	-	-
H. alvei 059	+++	+++	++	-	+++	+++
Enterobacter sp 067	+	++	-	-	+	++
H. alvei 068	+++	+++	++	-	+++	+++
H. alvei 071	+++	+++	+	-	+++	+++
A. hydrophila 099	++	++	+	-	+	+++
B. cepacia H111	Nd	+++	+++	Nd	Nd	Nd
B. vietinamensis	Nd	Nd	Nd	+++	Nd	Nd
P. aeruginosa PAO1	+++	Nd	Nd	Nd	+++	+++

The six monitor strains were cross-streaked against different psychrotrophic strains on LB agar plates. Following up to 48 hours of incubation at 30 °C, the production of violacein by *C. violaceum* CV026, bioluminescence by *E. coli* pSB403, green fluorescent protein gfp(ASV) by *P. putida* F117, and β-galactosidase activity by *A. tumefaciens* A136 and NTL4 was visualized as described in material and methods. Levels of activation are indicated as follows: +++, strong activation, diffusion of AHL > 1 cm; ++, activation, diffusion of AHL of 0.5 to 1 cm; +, weak activation, diffusion of AHL < 0.5 cm; -, no detectable activation. Nd: not determined.

4.3.4. Characterization of AHL molecules using TLC

Enterobacter sp. 067 and *A. hydrophila* 099 produced less AHL compared to *H. alvei* 059, 068, and 071, since it was necessary to load high volumes of AHL extracts on TLC plates to detect production of bioluminescence by *E. coli* pSB403 (Figure 6). Besides, the extracts obtained from *Enterobacter* sp. 067 were not able to induce *C. violaceum* CV026 (Figure 7), which indicated that it does not produce C4-HSL. These results confirmed the data obtained in the cross-streak experiments and indicated that these bacteria produce different AHL molecules.

AHL was not detected on the TLCs loaded with supernatant of LB inoculated with *Pantoea* sp. 039 (Figure 6B and 7B). However, *Pantoea stewartii* subsp. *stewartii*, a bacterial pathogen of sweet corn and maize, used quorum sensing to

control the extracellular polysaccharide stewartan, a major virulence factor of this strain (BODMAN et al., 1998).

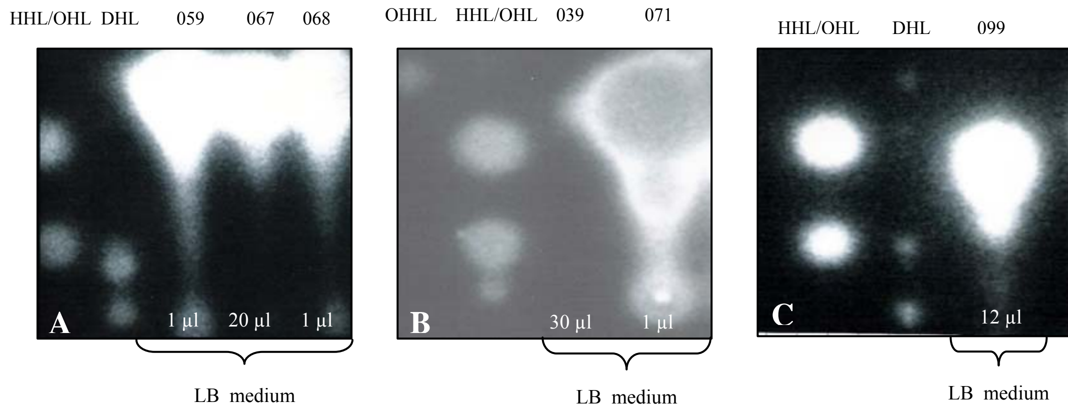

Figure 6 - A representative thin-layer chromatograms of the signal molecules present in cell free supernatants of *Enterobacteriaceae* isolated from cooled raw milk. The spots were detected with the *E. coli* pSB403 reporter strain. Standards: N-(hexanoyl)-DL-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); N-(dodecanoyl)-L-homoserine lactone (DHL); N-(3-oxohexanoyl)-L-homoserine lactone (OHHL). (059) *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (039) *Pantoea* sp., (071) *H. alvei*, (099) *A. hydrophila*.

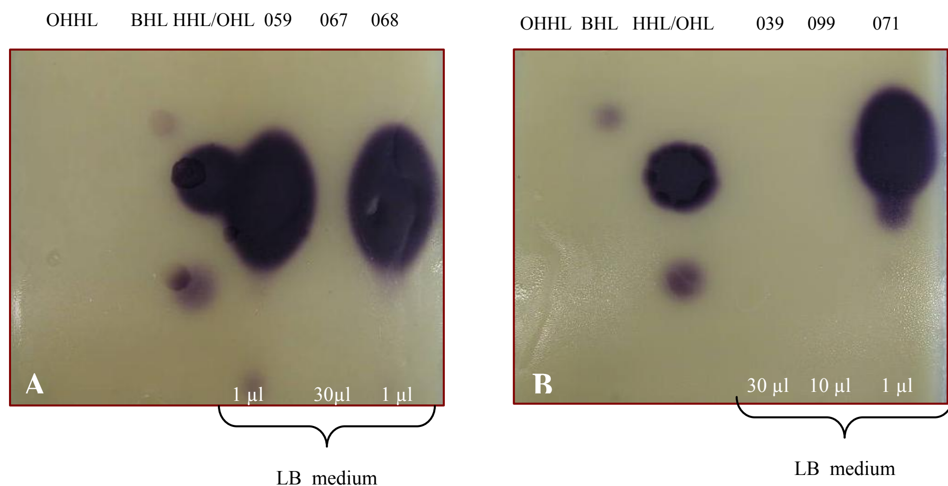

Figure 7 - A representative thin-layer chromatograms of the signal molecules present in cell free supernatants of *Enterobacteriaceae* isolated from cooled raw milk. The spots were detected with the *C. violaceum* CV026 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(butanoyl)-L-homoserine lactone (BHL); N-(octanoyl)-L-homoserine lactone (OHL); N-(hexanoyl)-DL-homoserine lactone (HHL). (059) *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (039) *Pantoea* sp., (099) *A. hydrophila*, (071) *H. alvei*.

Degradation products of N-(dodecanoyl)-L-homoserine lactone (DHL) were detected on TLC plates (Figure 6A and C). This suggested high sensitivity of the AHL molecule to manipulation and exposure to room temperature.

C. violaceum CV026 was unable to detect N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (Figure 7). Then, it is important to use multiple AHL sensor systems to detect a broad range of AHL molecules.

4.3.5. Characterization of AHL molecules by liquid chromatography-mass spectrometry (LC-MS)

AHL extracts obtained from cultures of psychrotrophic bacteria were analyzed by liquid chromatography and then pressed through a metal capillary at high potential in a positive-electrospray ionization high-resolution mass spectrometer. A mix of standards was used in order to determine the retention time of each compound and the light ions arrived at the detector sooner than the heavy ones (Figures 8 and 9).

AHL extracts from *Enterobacter* sp. 067, *H. alvei* 068, *H. alvei* 071, and *A. hydrophila* 099 were characterized by MS after calibration of the equipment as shown in Figure 8. For *H. alvei* 059, the AHL extract was also characterized by MS after recalibration of the equipment as shown in Figure 9.

LC-MS of dichloromethane extracts from 400 ml of culture supernatant in ABG minimal medium of psychrotrophic strains unambiguously detected different acyl-homoserine lactones with correct retention times. They were further validated by the accurate masses of the ions, which did not deviate more than 0.015 Da (Figures 10, 12, 13, and 14). Strains 059, 068, 071, and 099 produced different AHL molecules. However, it was not possible to detect AHL molecules in the AHL extract from *Enterobacter* sp. 067 inoculated in ABG minimal medium (Figure 11), although it induced the biosensors in the cross-streak assay and on the TLC plates.

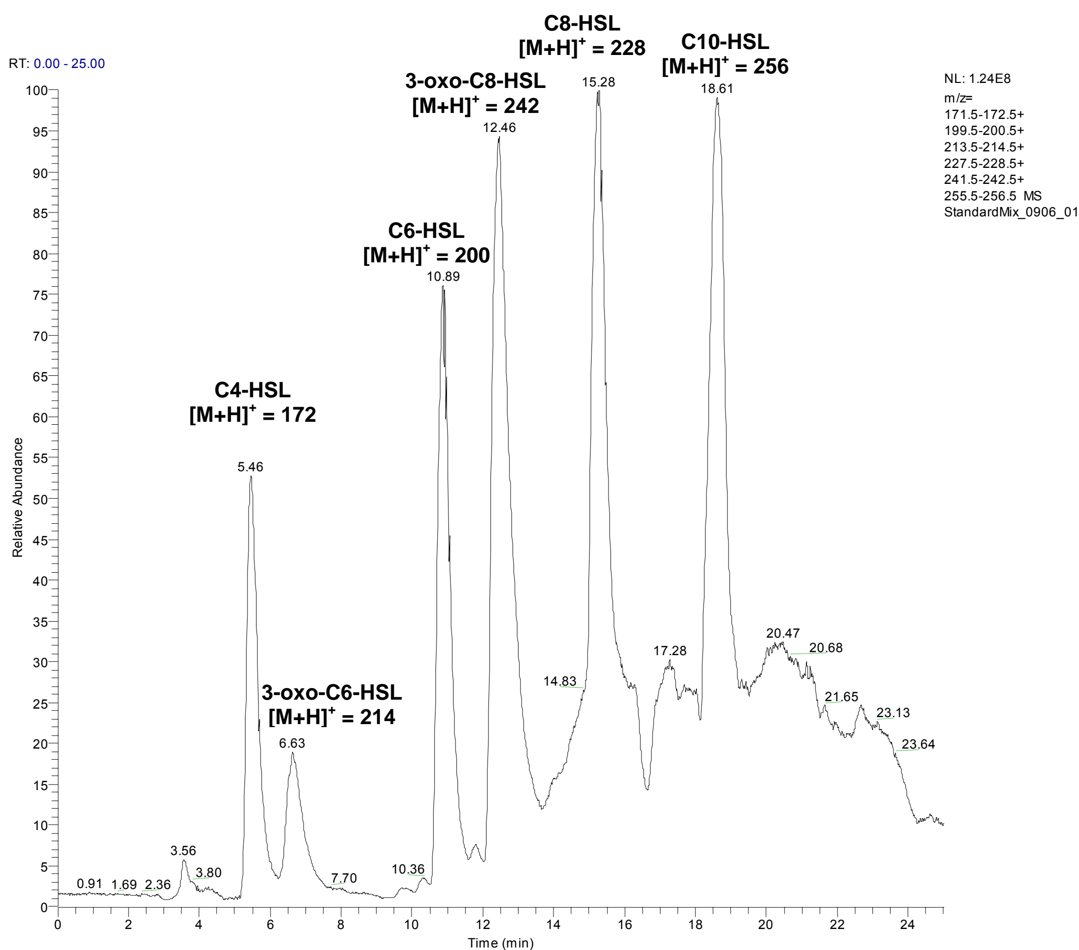

Figure 8 – High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the standards: N-(butanoyl)-L-homoserine lactone (C4-HSL), N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), N-(hexanoyl)-L-homoserine lactone (C6-HSL), N-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), N-(octanoyl)-L-homoserine lactone (C8-HSL), and N-(decanoyl)-L-homoserine lactone (C10-HSL).

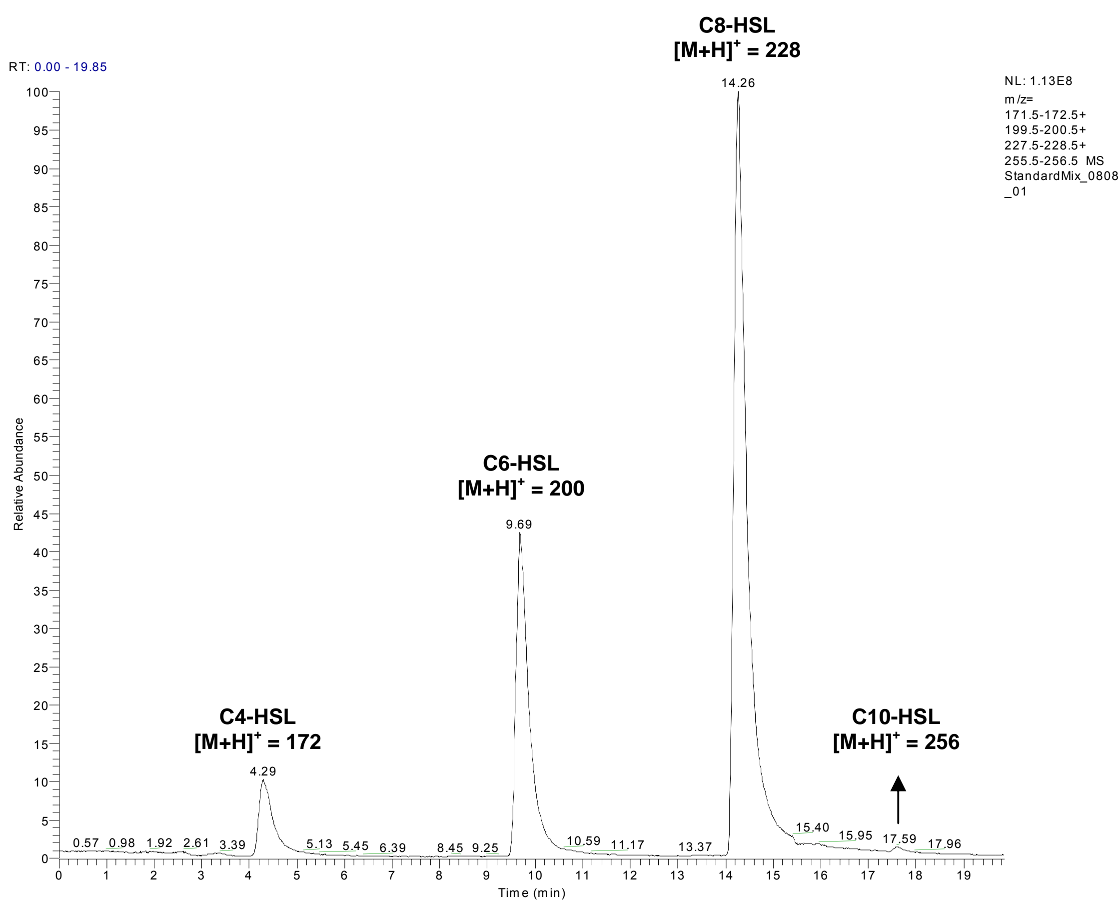

Figure 9 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the standards: N-(butanoyl)-L-homoserine lactone (C4-HSL), N-(hexanoyl)-L-homoserine lactone (C6-HSL), N-(octanoyl)-L-homoserine lactone (C8-HSL), and N-(decanoyl)-L-homoserine lactone (C10-HSL).

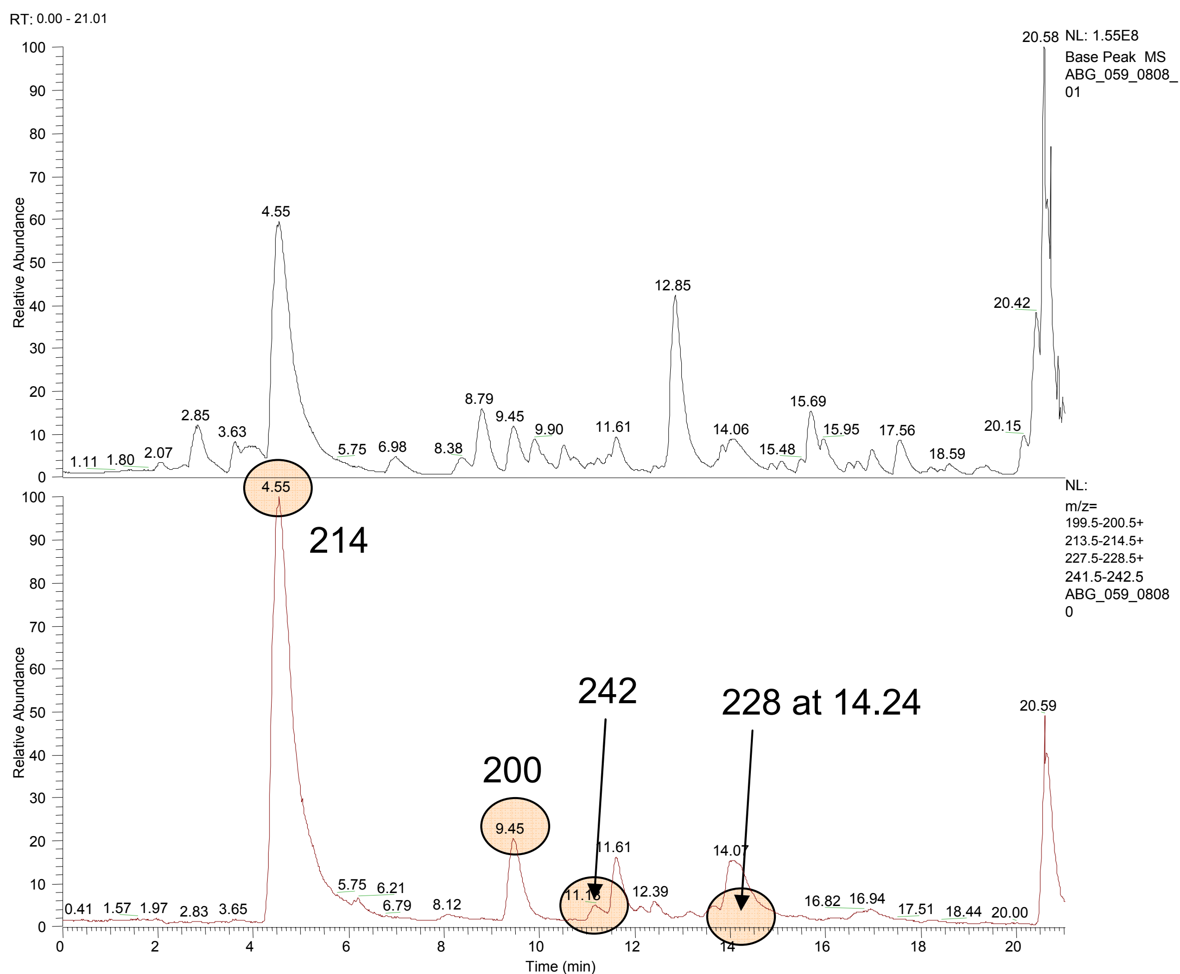

Figure 10 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *H. alvei* 059. Signal molecule extract was obtained from cell-free culture supernatant in ABG minimal medium. The upper panel shows the base peaks (total ion current chromatogram). The lower panel shows the extracted single chromatogram. The retention times as well as the extracted ion chromatograms for the $[M+H]^+$ adducts of four detected homoserine lactones are shown.

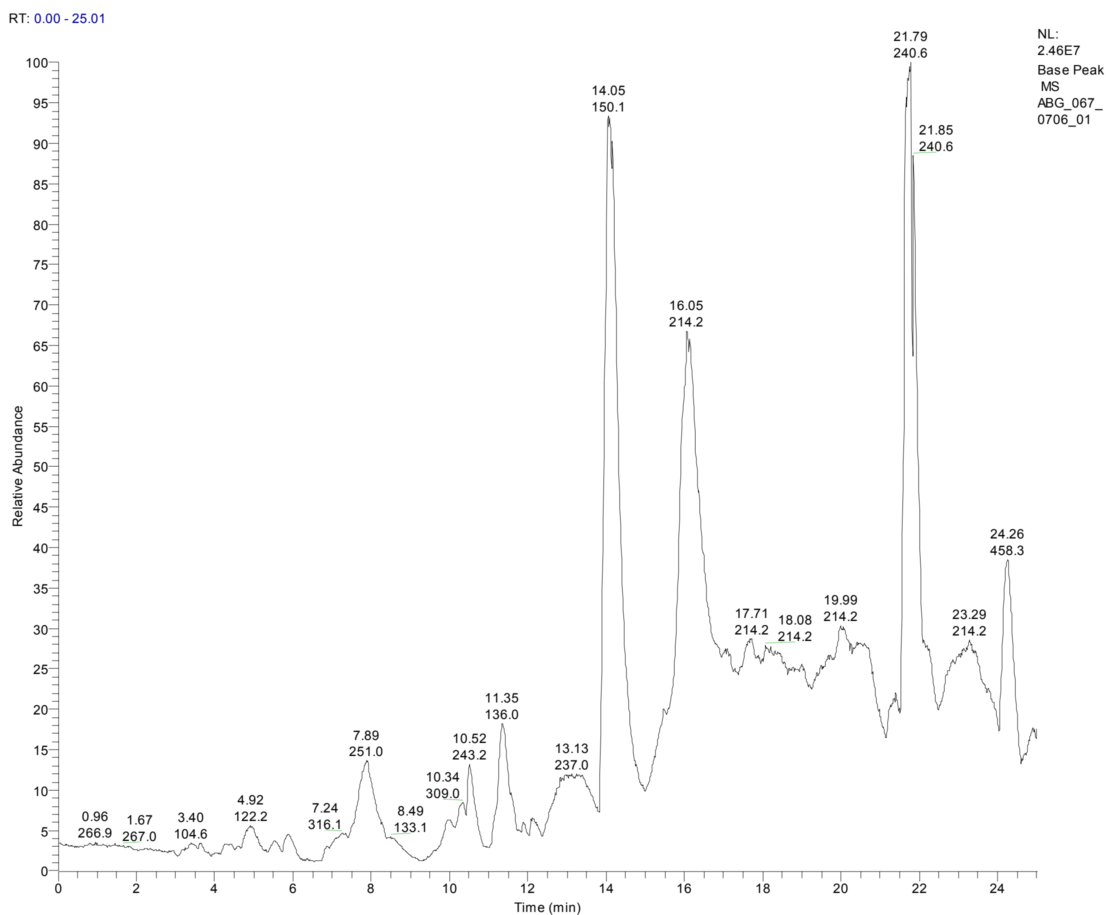

Figure 11 - High-performance liquid chromatography-positive electrospray ionization (ESI^{+})-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *Enterobacter* sp. 067. Signal molecule extract was obtained from cell-free culture supernatant in ABG minimal medium.

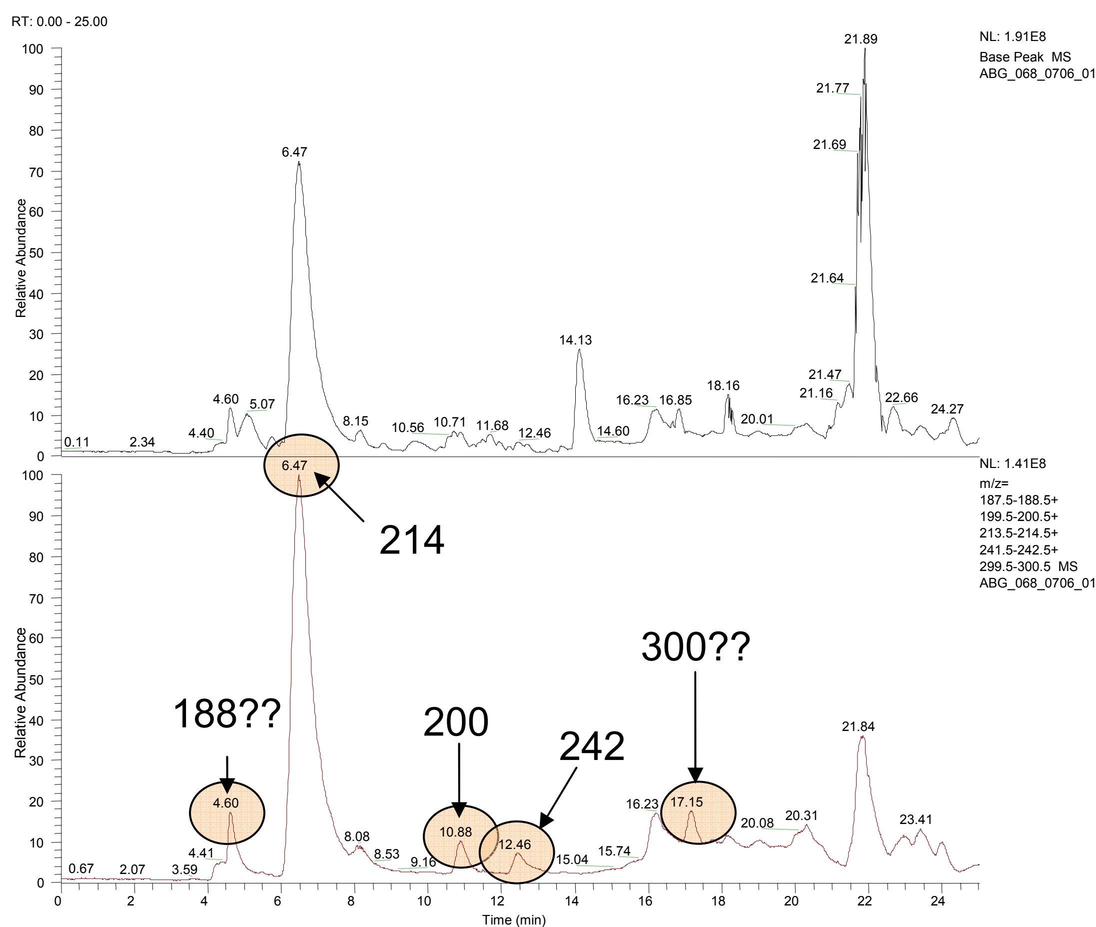

Figure 12 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *H. alvei* 068. Signal molecules extract was obtained from cell-free culture supernatant in ABG minimal medium. The upper panel shows the base peaks (total ion current chromatogram). The lower panel shows the extracted single chromatogram. The retention times as well as the extracted ion chromatograms for the $[M+H]^+$ adducts of three identified and two questionable homoserine lactones are shown.

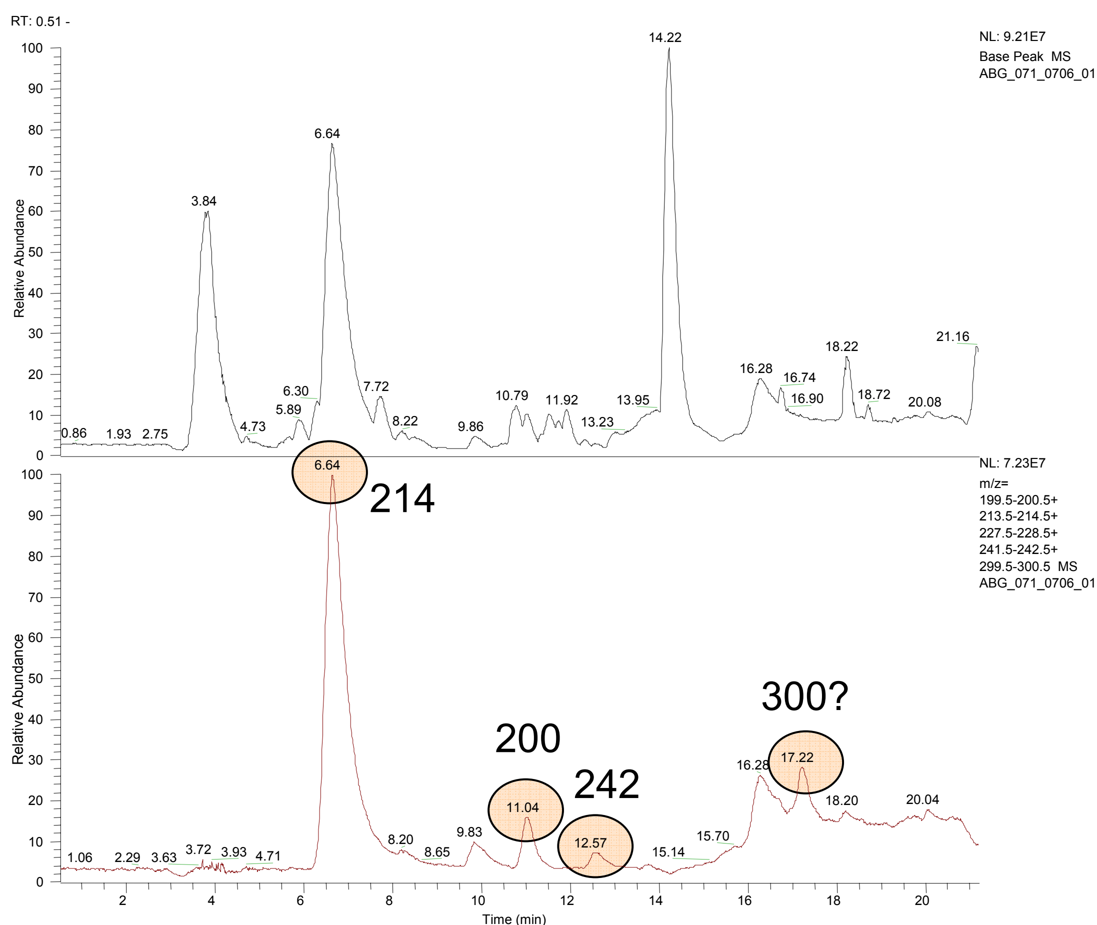

Figure 13 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *H. alvei* 071. Signal molecule extract was obtained from cell-free culture supernatant in ABG minimal medium. The upper panel shows the base peaks (total ion current chromatogram). The lower panel shows the extracted single chromatogram. The retention times as well as the extracted ion chromatograms for the $[M+H]^+$ adducts of three identified and one questionable homoserine lactones are shown.

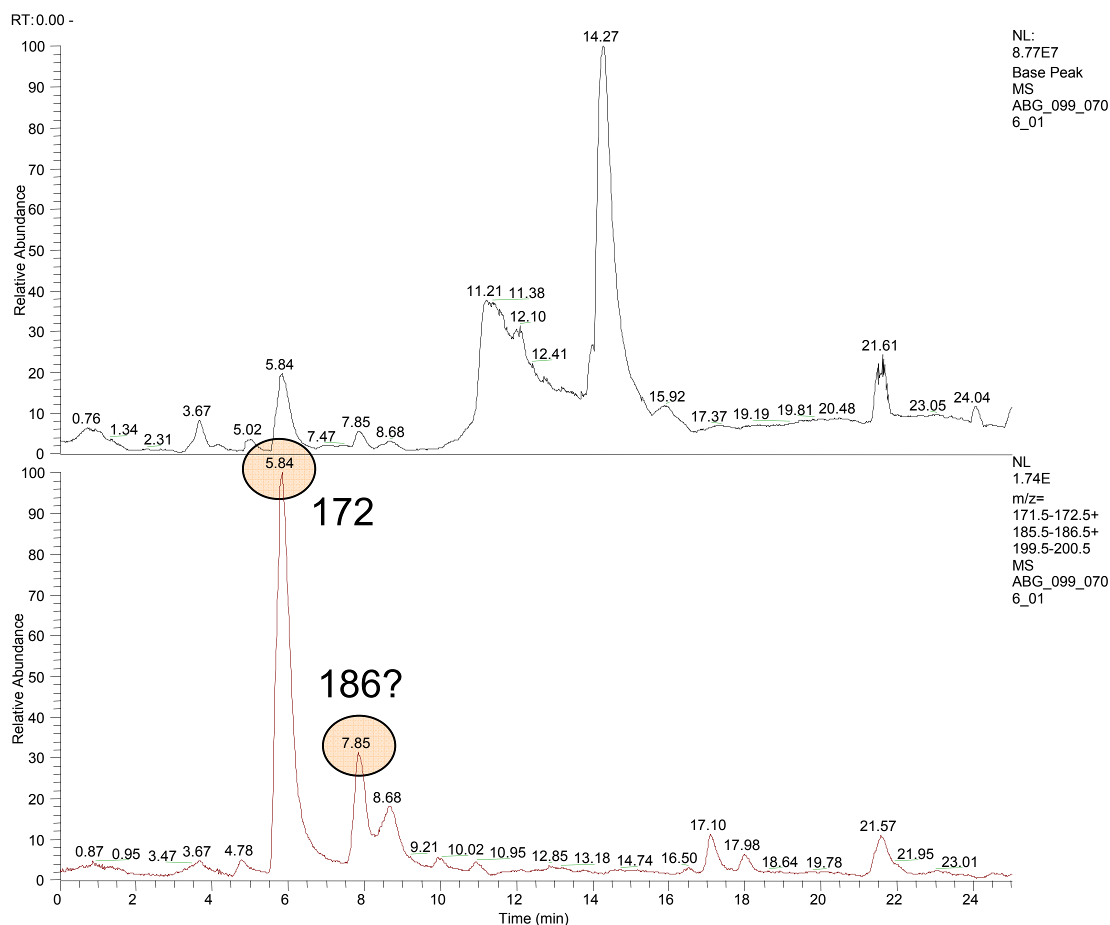

Figure 14 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *A. hydrophila* 099. Signal molecule extract was obtained from cell-free culture supernatant in ABG minimal medium. The upper panel shows the base peaks (total ion current chromatogram). The lower panel shows the extracted single chromatogram. The retention times as well as the extracted ion chromatograms for the $[M+H]^+$ adducts of one identified and one questionable homoserine lactones are shown.

The results obtained by LC-MS (Figures 8 - 14) are summarized in Table 6. *H. alvei* strains 059, 068, and 071 were able to produce 3-oxo-C6-HSL, C6-HSL, and 3-oxo-C8-HSL, whereas C8-HSL was produced by *H. alvei* 059 and 071 (Table 6).

In the experimental conditions adopted, 3-oxo-C6-HSL was the main AHL produced by *H. alvei* strains (Figures 10, 12, 13). This result is in agreement with Bruhn et al. (2004), who verified that this same HSL was predominant among the four AHLs produced by *H. alvei* isolated from vacuum-packed meat.

A. hydrophila 099 was able to produce C4-HSL and C6-HSL in ABG minimal medium (Figure 14, Table 6), which is in agreement with Swift et al. (1997), who demonstrated that *A. hydrophila* produces C4-HSL as the principal AHL molecule. A quorum sensing system in this pathogen has been associated with the regulation of biofilm development (LYNCH et al., 2002) and exoprotease production (SWIFT et al., 1999). Medina-Martínez et al. (2006) showed an effect of environmental conditions, such as temperature and glucose concentration, on C4-HSL production by *A. hydrophila* and this warrants further investigation to elucidate the effect of external conditions on the production of AHL signal molecules to reveal the relevance of quorum sensing in food storage.

H. alvei produced a molecule that presented a mass spectrum similar to 3-hydroxy-C4-HSL and 3-hydroxy-C12-HSL, whereas *A. hydrophila* 099 probably produced C5-HSL (Table 6). However, the identity of these molecules was not confirmed, since standards for these compounds were not available to determine their mass spectrums and retention times.

Characterization of different AHLs reinforce the data obtained in the cross-streak assay when the induction of different biosensor strains by *H. alvei* was verified. Each AHL biosensor relies on a particular LuxR family protein, thus displays specificity towards the cognate AHL, and in some cases, to closely related AHLs.

Table 6 – Summary of identification by high-performance liquid chromatography positive electrospray ionization (ESI^+)-MS of AHLs produced by *H. alvei* 059, 068, and 071, *Enterobacter* sp. 067, and *A. hydrophila* 099

Isolates	Acyl-homoserine lactones (AHLs)							
	C4-HSL	3-hydroxi-C4-HSL	C5-HSL	3-oxo-C6-HSL	C6-HSL	3-oxo-C8-HSL	C8-HSL	3-hydroxi-C12-HSL
H. alvei 059	-	-	-	+	+	+	+	-
Enterobacter sp. 067	-	-	-	-	-	-	-	-
H. alvei 068	-	±	-	+	+	+	-	±
H. alvei 071	-	-	-	+	+	+	+	±
A. hydrophila 099	+	-	±	-	+	-	-	-

- Absent; + Present; ± doubt.

4.3.6. Resistance of psychrotrophic strains against antibiotics and tellurite

The high incidence of antibiotic resistance detected among psychrotrophic isolates (Table 7) may demonstrate high selective pressure in raw milk. This datum was confirmed by Araújo (1998), who found that 95% of 201 strains of *Staphylococcus aureus* isolated from raw milk were resistent to different antibiotics, and by Carneiro and Júnior (2006), who demonstrated that *Aeromonas* spp. was resistant to several antimicrobials present in pasteurized milk in Brazil.

Table 7 – Tellurite and antibiotic susceptibility of psychrotrophic strains in LB broth

Antibiotic	Strain					
	039	059	067	068	071	099
Tellurite	-	-	+	+	+	-
Trimethoprim	+	+	+	+	+	+
Chloramphenicol	+	+	+	+	+	+
Gentamicin	-	-	-	-	-	-
Ampicillin	+	+	+	+	+	+
Kanamycin	-	-	-	-	-	-
Tetracycline	+	+	+	+	+	+
Spectinomycin	+	-	+	-	-	-

Growth is indicated as follows: + growth; - no detectable growth.

4.3.7. Quorum quenching mechanism in *Enterobacter* and *H. alvei*

4.3.7.1. Mobilization of pBHR1-aiiA

E. coli XL1-Blue pMLBAD-aiiA-Trm^r-Gm^r was used as the donor in the triparental mating assay, but since the transconjugant strains did not grow well in LB supplemented with gentamicin *E. coli* S17-1 pBHR1-aiiA was used as the donor in the triparental mating since it carries a plasmid that confers resistance against kanamycin and the wild type strains were not able to grow in LB supplemented with this antibiotic.

The plasmid pBHR1-aiiA-Km^r was successfully transferred to *Enterobacter* sp. 067 (Figure 15). However, it was not possible to detect the *aiiA* gene after extraction and digestion of pBHR1-aiiA-Km^r from *H. alvei* 068 and 071 transconjugants (Figure 16). When pBHR1-aiiA-Km^r obtained from these strains was digested with *Eco*RI and *Nco*I, some additional bands on the agarose gel were verified (Figure 16) and one of these bands was similar to a native plasmid that contains a high number of copies.

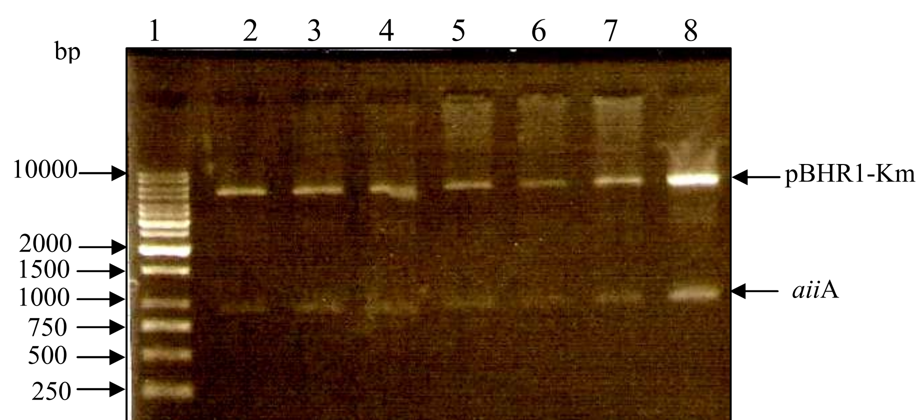

Figure 15 – Agarose gel electrophoresis of restriction reactions of pBHR1-aiiA-Km^r containing the cloned *aiiA* gene. Lane 1, Standard; lanes 2, 3, 4, 5, 6, and 7, 067 transconjugants; lane 8, positive control: *E. coli* XL1-Blue pBHR1-aiiA-Km.

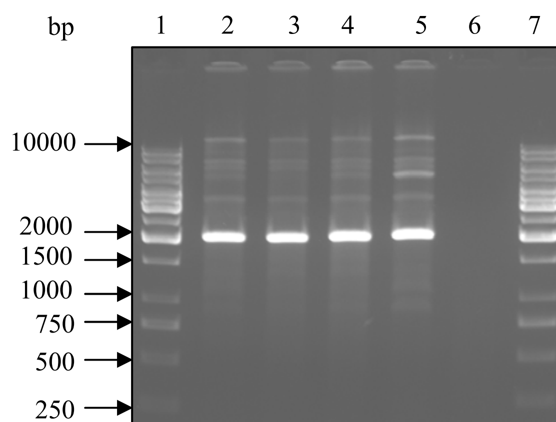

Figure 16 – Agarose gel electrophoresis of restriction reactions of pBHR1-aiiA-Km^r containing the cloned *aiiA* gene. Lanes 1 and 7, standards; lanes 2 and 3, 068 transconjugants; lanes 4 and 5, 071 transconjugants; lane 6, negative control.

4.3.7.2. AHL production by transconjugant strains

Enterobacter sp. 067 transconjugant was not able to induce *E. coli* pSB403 as the wild type did (Figure 17A and B, Table 8). This result indicates efficient expression of the *aiiA* gene, which did not allow the accumulation of AHL molecules. The bioluminescence sensitivity value determined by a photon-counting camara was of 9.6 for the *Enterobacter* sp. 067 transconjugant (Figure 17A), 6.7 for the *Enterobacter* sp. 067 wild type (Figure 17B), and 3.0 for the positive control, *B. cepacia* H111 (Figure 17C). Low sensitivity values of bioluminescence indicate the presence of AHLs in the medium, while high values indicate that these molecules are absent.

However, the mechanism of quorum quenching did not influence the AHL production by *H. alvei* 068 and 071. These transconjugants were able to grow in LB supplemented with kanamycin, but they accumulated AHLs. As they have a native plasmid, the triparental mating approach did not work well with these strains. It is probable that some recombination between pBHR1-aiiA-Km^r and the native plasmid occurred, resulting in inhibition of *aiiA* gene expression. Another possibility could be that these strains produced high amounts of AHLs and the concentration of lactonase in the cells was insufficient to completely degrade these molecules.

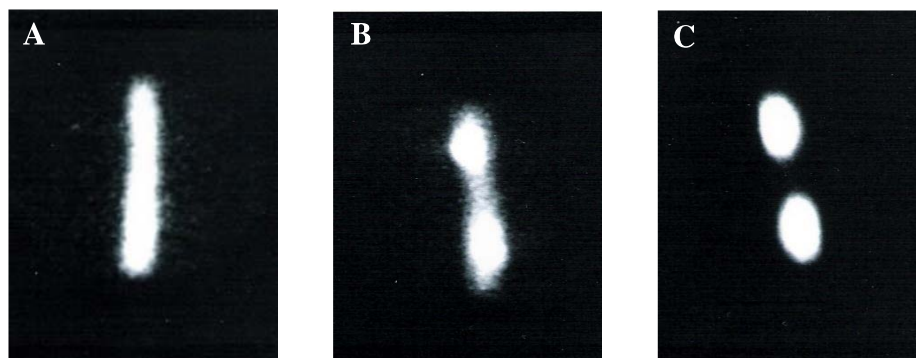

Figure 17 – Activation of the AHL monitor strain *E. coli* pSB403 in cross-streak experiment. (A) *E. coli* pSB403 cross-streaked to 067 transconjugant; (B) *E. coli* pSB403 cross-streaked to 067 wild type; (C) positive control, *E. coli* pSB403 cross-streaked to *B. cepacia* H111.

Table 8 – Values of bioluminescence produced by *E. coli* MT102 pSB403 at 100 nm after growth in LB broth supplemented with the supernatant of *Enterobacter* sp. 067 wild type, *Enterobacter* sp. 067 transconjugant, or with HHL standard. Data represent average of triplicate experiments.

Dilution rate	Bioluminescence values			
	067 Wild type	067-aiiA Transconjugant	HHL Positive control	H_2O Negative control
1/2	30041	134	26989	174
1/4	6288	123	22226	145
1/8	184	127	995	129
1/16	123	104	864	123
1/32	123	105	264	105
1/64	119	109	252	103
1/128	113	69	172	119
1/256	105	84	139	108

4.3.7.3. Proteolytic activity of wild type and transconjugants

Enterobacter sp. 067 transconjugant was more proteolytic than the wild type in reconstituted milk (Figure 18) and on LB agar supplemented with 2% skim milk powder (Figure 19). Higher protease activity by a transconjugant strain may occur due to the inability of the HalR protein to interfere with protease expression when it

is bound to an AHL. Another possibility is related to the production of more than one protease that is not negatively regulated by quorum sensing. Production of protease by *Enterobacter* sp. 067 wild type indicated that AHL did not completely repress HalR binding to the protease promoter. This result suggests a negative regulation of the quorum sensing mechanism in this strain, which needs further investigation.

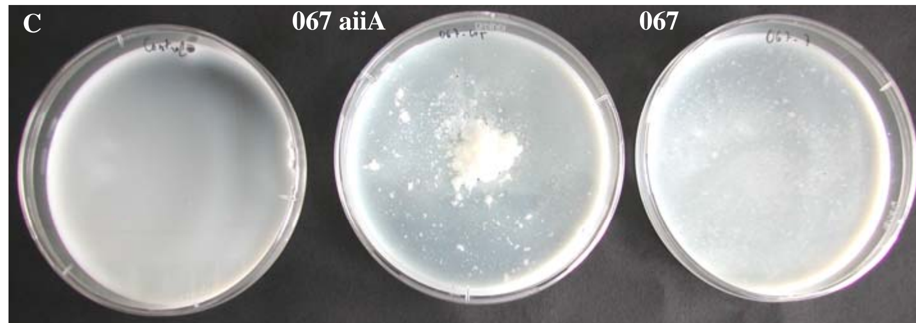

Figure 18 – Proteolytic activity in 12% (w/v) reconstituted skim milk powder inoculated with *Enterobacter* sp. after incubation for 24 h at 30 °C. (C) Negative control, milk sample not inoculated, (067 aiiA) *Enterobacter* sp. transconjugant, (067) *Enterobacter* sp. wild type.

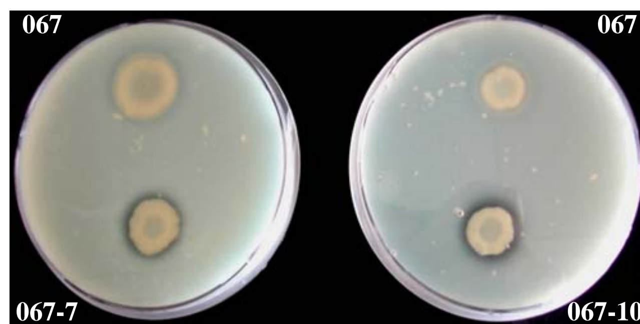

Figure 19 – Proteolytic activity on LB agar supplemented with 2% (w/v) skim milk powder after incubation for 24 h at 30 °C. Clearing zones are indicative of protease activity. (067) *Enterobacter* sp. wild type, (067-7) *Enterobacter* sp. transconjugant, (067-10) *Enterobacter* sp. transconjugant.

While most LuxR-type proteins act as AHL-dependent transcriptional activators, a few members appear to act as repressors. Their ability to repress

transcription is not surprising given that LuxR and TraR can act as repressors when their binding sites are moved. What is striking, however, is that AHLs abolish repression and, in at least one case, abolish DNA binding (VON BODMAN et al., 1998; NASSER et al., 1998). In this regard, these repressor proteins work in fundamentally different ways from the majority of LuxR-type proteins. The best characterized examples of repression by AHLs are the EsaR/EsaI system of *Pantoea stewartii* (VON BODMAN et al., 1998), a vascular pathogen of maize, and the ExpR/ExpI system of *Erwinia chrysanthemi*, another plant pathogen that macerate plant tissues by releasing pectinases and other hydrolytic enzymes. Mutations in *esaI* abolish exopolysaccharide production and prevent pathogenesis (VON BODMAN et al., 1998). In contrast, mutations in *esaR* cause hyperproduction of exopolysaccharides. The double mutant has the *esaR* phenotype. These data suggest that EsaR is a direct repressor of genes required for exopolysaccharide production. However, this protein could also act indirectly, for example, as an activator of an uncharacterized repressor gene. On the other hand, it seems inescapable that the AHL made by EsaI (3-oxo-hexanoyl-HSL) must oppose EsaR function rather than stimulate it. Evidence that EsaR is a direct repressor was obtained from studies of the esaR promoter, which is autorepressed by EsaR. Purified EsaR bound to the *esaR* promoter in the absence of AHL, but not in its presence. Like EsaR, ExpR of *E. chrysanthemi* also autorepresses its synthesis in the absence of AHL (also 3-oxo-hexanoyl-HSL) and this DNA binding is inhibited by AHL (NASSER et al., 1998). Gel shift assays and footprinting assays showed that ExpR acts directly upon its promoter. Both EsaR and ExpR could in principle act as activators of other promoters, but seems likely that AHL would oppose protein function (by abolishing DNA binding) at all target promoters. It is fascinating that AHLs could block this group of proteins from binding DNA, while stimulating DNA binding of other LuxR-type proteins.

In a preliminary assay, *H. alvei* 068 and 071 wild type showed the same proteolytic activity as the *H. alvei* 068 and 071 transconjugants. As the quorum quenching mechanism did not compromise accumulation of AHLs by *H. alvei* 068 and 071, it was not possible to evaluate the phenotypes controlled by quorum sensing in these strains. Bruhn et al. (2004) made comparisons between *H. alvei* wild type, the AHL-negative mutant, and the OHHL-complemented AHL-negative mutant in many assays and no difference was found between the strains in the following tests:

production of antibiotics, biogenic amines, adhesion and biofilm formation, motility, starvation survival, resistance to oxidative stress, or virulence against *Drosophila*. This suggests that these phenotypes are not upon quorum sensing regulation and more studies need to be done to understand the role of AHLs in this species.

On the other hand, Viana (2006) verified the inhibitory effect of furanones, a quorum sensing inhibitor, on biofilm formation by *H. alvei* 071. Then, the development of mutants of *H. alvei* is important in order to confirm the phenotypes regulated by quorum sensing in this bacterium.

4.3.7.4. Influence of quorum quenching mechanism in expression of extracellular proteins by *Enterobacter* sp. 067

As the transconjugant strain was more proteolytic than the wild type, SDS-PAGE and zymogram were developed to evaluate if there were differences in the pattern of extracellular proteins between them. Only one protein was missing in the transconjugant strain (Figure 20A) and this might have occurred due to the control of quorum sensing in expression of this protein.

Although three different proteins were detected in the concentrated supernatant of 067 wild type inoculated in TYEP medium (Figure 20A), none of them showed proteolytic activity in the SDS-PAGE supplemented with azocasein (Figure 20B). This confirmed the previous result of absence of proteolytic activity in the supernatant of TYEP medium inoculated with *Enterobacter* sp. 067. This might occur because this strain does not produce and secrete a protease into TYEP, azocasein is not the best substrate for protease, or the process of renaturation of this enzyme after development of SDS-PAGE was not sufficient.

The different proteins of wild type 067 present in the SDS-PAGE (Figure 20A) were purified from the gel and analysed by mass spectrometry. Only the protein of approximately 50 kDa was identified and showed identity to FliC, a bacterial flagellin, from *E. coli* (GenBank accession number Q842A7).

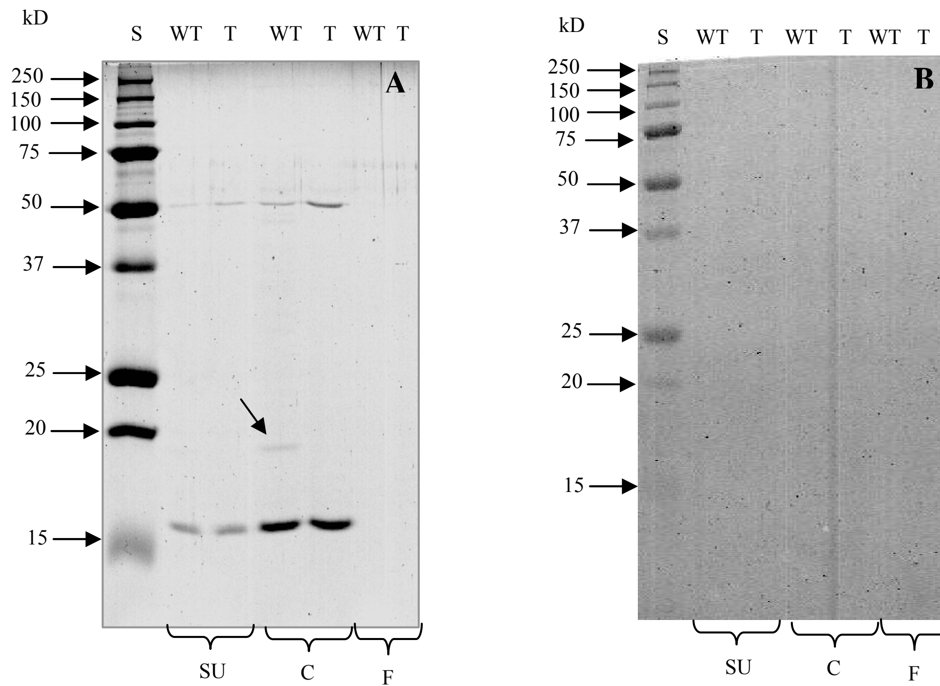

Figure 20 – SDS-PAGE (A) and zymogram azocasein (B) gels (12%) showing extracellular protein production by *Enterobacter* sp. 067 after growth for 18 h at 30 °C in TYEP medium supplemented with 0.25% $CaCl_2$. (S) molar mass standards (BioRad); (WT) wild type; (T) transconjugant; (SU) supernatant of TYEP medium; (C) supernatant of TYEP medium concentrated four times; (F) filtrate of TYEP medium after concentration.

4.3.8. Native plasmid of *H. alvei*

Once a native plasmid was detected in *H. alvei* 068 and 071 after restriction of pBHR1-aiiA-Km^r extracted from the transconjugant strains, wild type strains were grown and the plasmid was extracted and loaded on an agarose gel. It was confirmed that *H. alvei* 068 and 071 have a native plasmid that was named pMLM (Figure 21).

The size of this plasmid extracted from wild type *H. alvei* 068 and 071 was different from the size observed when it was extracted from the transconjugant strains. This might indicate that some rearrangement occurred in this molecule after the triparental mating.

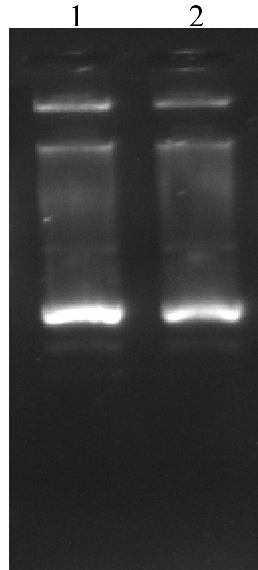

Figure 21 – Agarose gel electrophoresis of pMLM extraction. Lane 1, pMLM068; lane 2, pMLM071.

A total of 32 restriction enzymes were used to digest pMLM and only *Dde*I, *Hinf*I, *Msp*I, and *Rsa*I were able to cut this plasmid. In order to clone and sequence the DNA fragments, *Rsa*I was used to digest pMLM since it produces blunt ends after digestion and because, afterwards, the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, United Kingdom) was used.

A fragment of 1,857 bp of pMLM was sequenced and sites for three endonucleases, *Cla*I, *Sca*I, and *Kpn*I, which were used to digest this plasmid previously, were found (Figure 22). However, these restricition enzymes did not cleave pMLM, probably due to methylation in the sites of these endonucleases.

The plasmid pMLM appears to be a new molecule and its complete sequence needs to be elucidated. It did not show similarity with the plasmids pAlvA and pAlvB, which contain genes that encode alveicin, a bacteriocin from *H. alvei* (Wertz and Riley, 2004). Plasmids pAlvA and pAlvB resemble the ColE1-type replicons and carry mobilization genes, as well as colicin-like bacteriocin operons. Plasmid pAlvA is 5113 bp, 46.18% G+C, and was sequenced from five of six *H. alvei* strains examined by Wertz and Riley (2004). Plasmid pAlvB is 5216 bp, 46.97% G+C, and was isolated from strain MISC261 (WERTZ and RILEY, 2004).

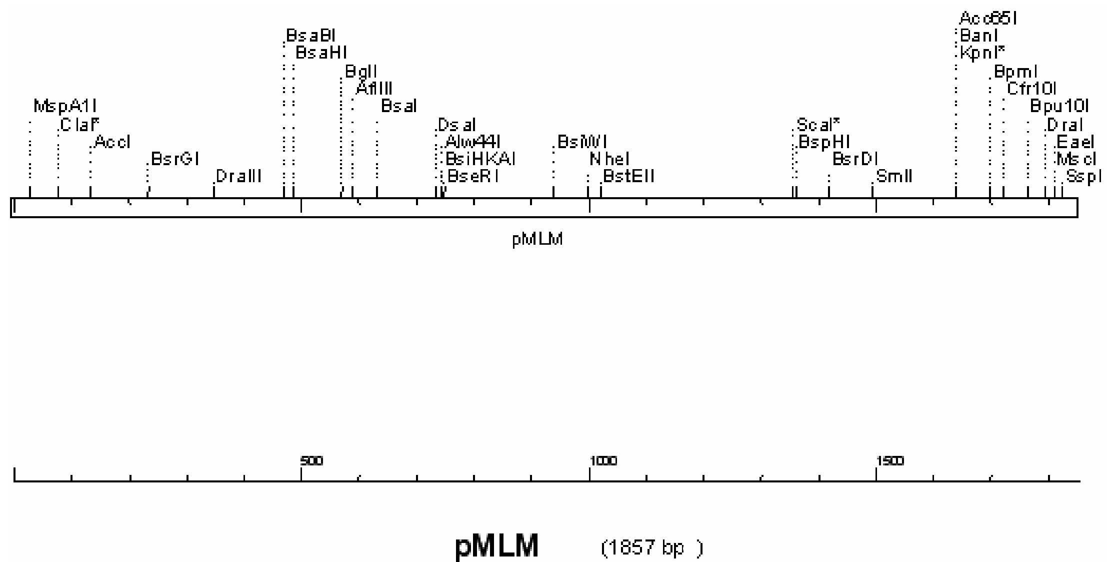

Figure 22 – Restriction map of a fragment of 1,857 bp fragment of pMLM constructed using Clone Manager 5.0. *Enzymes previously used to digest pMLM that did not cleave it.

4.3.9. Amplification of AHL synthase (*halI*) and AHL receptor (*halR*) genes by PCR

Primers based on the sequences of the *halI* and *halR* genes of *H. alvei* were constructed and used to amplify these genes by PCR. Amplified products of the expected sizes, 660 bp or 751 bp, were obtained for the *halI* (Figure 23) and *halR* (Figure 24) genes, respectively.

The *halI* gene was detected in all strains of *H. alvei* used in this study, as well as in *Enterobacter* sp. 067 (Figure 23). The *halI* gene of *H. alvei* 068 and 071 showed 99% identity with each other. The same result was observed when the *halI* and *halR* genes of *H. alvei* 068 were compared to the *halI* and *halR* genes of *Enterobacter* sp. 067. However, when sequences of the *halI* gene of *H. alvei* 068 and *H. alvei* 059 were aligned, they showed only 75% identity with each other.

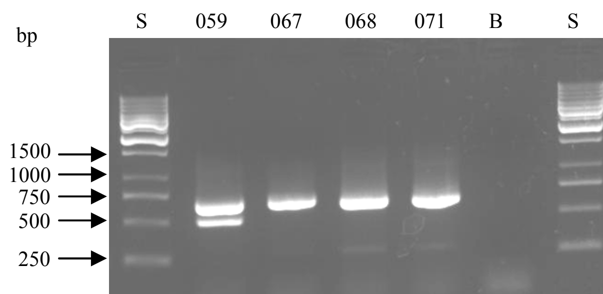

Figure 23 – Agarose gel electrophoresis of PCR products obtained from *halI* gene amplification. (S) standard, (059) *H. alvei*, (067) *Enterobacter* sp., (068) *H. alvei*, (071) *H. alvei*, (B) negative control: reaction mix without DNA.

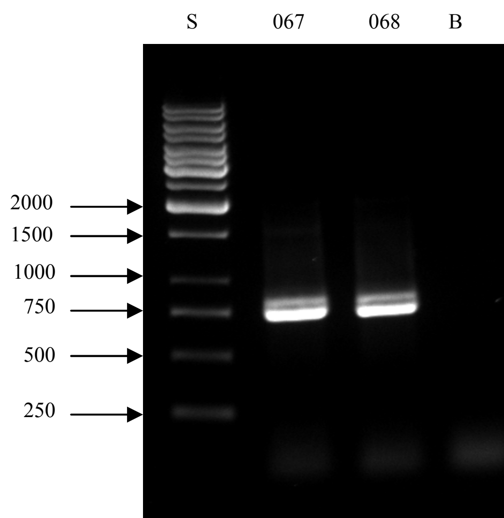

Figure 24 – Agarose gel electrophoresis of PCR products obtained from *halR* gene amplification. (S) standard, (067) *Enterobacter* sp., (068) *H. alvei*, (B) negative control negative control: reaction mix without DNA.

Amino acid sequence alignments of LuxI-type proteins revealed ten completely conserved amino acids, most of these within the amino-terminal halves of the proteins. Of the ten conserved amino acid residues, seven are charged. The observation that the amino-terminal portions of LuxI-type proteins are the best conserved, whereas the carboxy-termini are more divergent, suggests that the C-termini may provide recognition of the different acyl chains on precursor acyl-ACPs (FUQUA et al., 2001).

On the other hand, according to Fuqua et al. (2001), LuxR-type proteins share an end-to-end sequence identity of 18-23%. An acyl-HSL interaction region (residues 66 to 138) and a DNA binding motif (residues 183-229) are defined by two clusters of stronger sequence conservation. LuxR-type proteins are members of the larger FixJ-NarL superfamily. Most members of the FixJ-NarL superfamily are two component-type response regulators that differentially control DNA binding activity by phosphorylation of a conserved aspartate residue in the amino-terminal halves of these proteins. There is no significant sequence similarity between the amino-terminal halves of LuxR-type proteins and other members of the FixJ-NarL group. This reflects the specific function of this region in the acyl-HSL interaction of LuxR-type proteins. However, several mechanistic features may be shared between LuxR-type proteins and other members of the FixJ-NarL superfamily. LuxR-type proteins facilitate responses to acyl-HSLs through a series of recognizable steps, including (1) specific binding of cognate acyl-HSLs, (2) conformational changes and alterations in multimerization of the protein following binding of the signal, (3) binding or release of specific regulatory sequences upstream of the target genes, and often, (4) activation of transcription.

4.3.10. Sequencing and overexpression of *halI* in *E. coli* XL1-Blue

In order to know which AHL molecules are synthesized by HalI, the *halI* gene was sequenced, cloned (Figure 25) and overexpressed in *E. coli* XL1-Blue. This gene comprised 660 bp and encoded a protein with 216 amino acids. The protein length of HalI was in agreement with LuxI-type proteins, which range from 194 to 226 amino acids long (FUQUA et al., 2001). This protein has a predicted molecular mass of 25 kDa but based on electrophoretic mobility, the molecular mass was estimated to be approximately 16 kDa (Figure 26). This result suggests possible degradation of HalI.

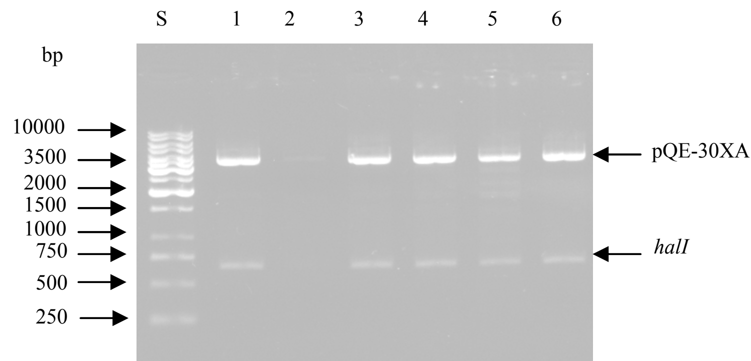

Figure 25 – Agarose gel electrophoresis of restriction reactions of pQE-30Xa-halI containing the cloned *halI* gene. (S) Standard; (1, 2, 3, 4, 5, 6) clones of *E. coli* XL1-Blue pQE-30Xa-halI.

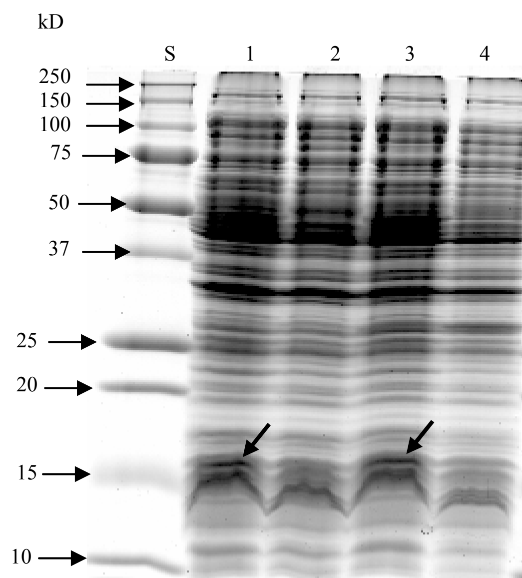

Figure 26 - SDS-PAGE (12%) showing AHL synthase production after expression of 6xHis-tagged HalI from *H. alvei* 068 in *E. coli* XL1-Blue. (S) molar mass standards (BioRad); (1 and 3) crude extracts of *E. coli* XL1-Blue pQE-30Xa-halI; (2 and 4) crude extracts of *E. coli* XL1-Blue containing the plasmid pQE-30Xa.

4.3.11. Detection of AHL molecules produced by *E. coli* XL1-Blue pQE-30Xa-halI

4.3.11.1. Cross-streak assay

A bioluminescence sensitivity of 3.9 detected when *E. coli* pSB403 was cross-streaked to *E. coli* XL1-Blue pQE-30Xa-halI indicates successfull heterologous expression of HalI in *E. coli* (Figure 27A). The positive control, *B. cepacia* H111, resulted in a bioluminescence sensitivity, measured by photon-counting camera, after induction of *E. coli* pSB403 equal to 3.8 (Figure 27B) and the negative control, *E. coli* XL1-Blue pQE-30Xa, resulted in a bioluminescence induction of 10, indicating that it did not produce AHLs (Figure 27C).

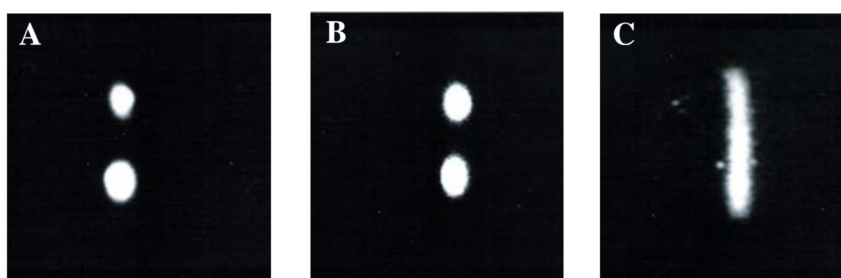

Figure 27 – Activation of the AHL monitor strain *E. coli* pSB403 in cross-streak experiments. (A) *E. coli* pSB403 cross-streaked to *E. coli* XL1-Blue pQE-30Xa-halI; (B) *E. coli* pSB403 cross-streaked to *B. cepacia* H111, positive control; (C) *E. coli* pSB403 cross-streaked to *E. coli* XL1-Blue pQE-30Xa, negative control.

4.3.11.2. Thin layer chromatography assay

Supernatant extracts of *E. coli* XL1-Blue harboring pQE-30Xa-halI cultured in ABG media induced *E. coli* pSB403 and *C. violaceum* CV026 on the TLC assays (Figures 28 and 29). The spots observed on TLC, on which *E. coli* pSB403 was used as a biosensor, presented the same retention factor (rf) as 3-oxo-C6-HSL (Figure 28), while a small spot close to C6-HSL was detected on TLC developed with *C.*

violaceum CV026 (Figure 29). These results indicated that the *halI* gene encodes the AHL synthase able to synthesize 3-oxo-C6-HSL and C6-HSL.

These results were confirmed with the supernatant extracts of dYT medium.

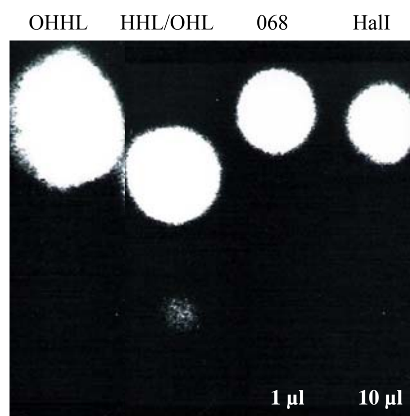

Figure 28 - A representative thin-layer chromatogram of HalI expression in *E. coli* XL1-Blue cultured in ABG minimal medium. The spots were detected with *E. coli* pSB403 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), N-(hexanoyl)-L-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); (068) *H. alvei* wild type, AHL extract diluted 50 times in ethyl acetate; (HalI) *E. coli* XL1-Blue harbouring pQE-30Xa-halI.

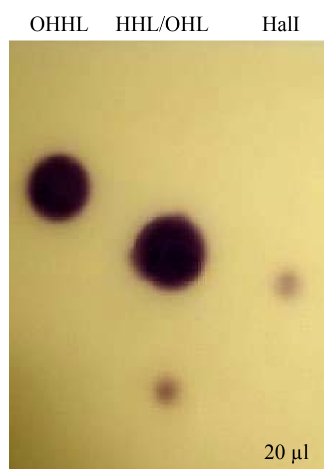

Figure 29 - A representative thin-layer chromatogram of HalI expression in *E. coli* XL1-Blue cultured in ABG minimal medium. Spots were detected with *C. violaceum* CV026 reporter strain. Standards: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL); N-(hexanoyl)-L-homoserine lactone (HHL); N-(octanoyl)-L-homoserine lactone (OHL); (HalI) *E. coli* XL1-Blue harbouring pQE-30Xa-halI.

4.3.11.3. Chemical characterization of AHL molecules by LC-MS

In heterologous expression of HalI protein, the extract of AHL molecules obtained from ABG minimal medium inoculated with *E. coli* XL1-Blue pQE-30Xa-halI was analysed by LC-MS and C6-HSL and 3-oxo-C6-HSL were detected (Figure 30), confirming the preliminary results of the TLCs. Interestingly, another noteworthy result is the observation that the *H. alvei* parental strain produces 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, and C8-HSL and the *halI* gene, when expressed in *E.coli*, directed the synthesis only of two of these molecules. This suggests that there is a gene encoding a second putative AHL synthase which is significantly different from *halI* or *E. coli* was not able to synthesize 3-oxo-C8-HSL and C8-HSL.

A known example of bacteria that has two AHL synthases is *P. aeruginosa*. The LasI/LasR system of this bacterium, which is homologue of LuxI/LuxR, initiates a cascade of signaling by the induction of transcription of virulence factors in high cell density. LasI synthesizes an AHL known as 3-oxo-C12-HSL (PASSADOR et al., 1993). Moreover, LasI/LasR activates expression of *rhlI* and *rhlR*, which activate genes that are already under the control of LasI/LasR, besides some additional genes. The protein RhlI synthesizes a second AHL molecule known as C4-HSL (LATIFI et al., 1995). The regulation of RhlI/RhlR by LasI/LasR ensures the establishment of two quorum sensing circuits that occur in a sequential way in *P. aeruginosa*.

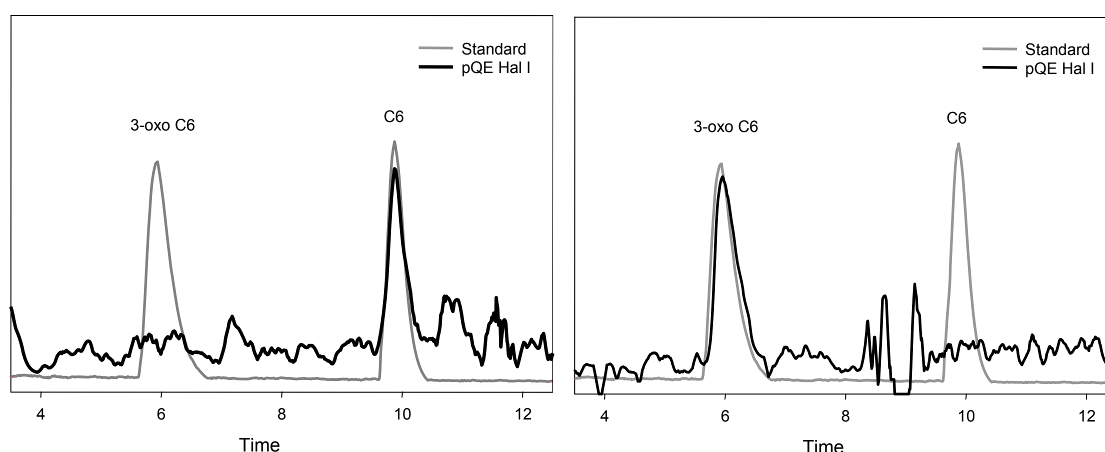

Figure 30 - High-performance liquid chromatography-positive electrospray ionization (ESI^+)-MS chromatogram showing the mass spectra for the signal molecules present in cell free supernatant of *E. coli* XL1-Blue pQE-30Xa-halI. Signal molecule extract was obtained from overnight cell free culture supernatant in ABG minimal medium.

4.3.12. Detection of auto-inducer two (AI-2)

Sterilized supernatant of overnight culture of *A. hydrophila* 099 in LB broth was able to induce bioluminescence production by *V. harveyi* BB170 (Table 9). This result indicates production of AI-2, although in the literature there isn't any data related to production of this molecule by *A. hydrophila*.

While the AHLs are confined to a reasonable range of bacteria, recent evidence has suggested the existence of a universal quorum sensing language. A family of molecules, termed AI-2, is common to many gram-negative and gram-positive pathogens. However, there is no direct evidence for the involvement of AI-2 in the regulation of pathogenic traits. Furthermore, whether AI-2 has a true role in quorum sensing signaling in general has been questioned, with suggestions that in most bacteria AI-2 is simply a metabolic byproduct, which casts doubt on its suitability as a target in the context of quorum sensing inhibition (CÁMARA et al., 2002).

Table 9 – Detection of auto-inducer two in supernatant of LB medium inoculated with psychrotrophic strains. *V. harveyi* BB170 was used as a monitor strain and *V. harveyi* BB120 was used as a positive control.

Strains and medium	Luminescence at 175 nm*
Pantoea sp. 039	1973 ± 345
H. alvei 059	2948 ± 810
Enterobacter sp 067	2087 ± 439
H. alvei 068	2899 ± 606
H. alvei 071	3708 ± 687
A. hydrophila 099	12903 ± 192
V. harveyi BB120	4478 ± 390
LB medium	2299 ± 384
AB medium	1927 ± 336

*Average and standard deviation of data is shown. n: number of repetitions equal 8.

4.3.13. Pathogenesis against *Caenorhabditis elegans*

As the bacteria used in this study are recognized as opportunistic pathogens, assays were developed using the nematode *C. elegans*, which is a valuable model for studying pathogenesis of various species of bacteria (EWBANK, 2002; CARDONA

et al., 2005). It was observed that only *A. hydrophila* was able to kill 100% of *C. elegans* (Figure 31). *H. alvei* 059, 068, and 071 were not pathogenic to this worm and *Enterobacter* sp. 067 and *Pantoea* sp. 039 were able to kill 25% and 28% of the nematodes, respectively, after 48 h of incubation (Figure 31).

The pathogenicity scores were based on the percent survival and appearance of worms at two days post-infection and the total number of parental and progeny nematodes after five days, as described in the material and methods. As controls, infections with the non-pathogenic *E. coli* strain OP50 and *B. cepacia* H111, whose pathogenic phenotypes were assigned scores of 0 and 3, respectively, were performed. Among the six psychrotrophic strains screened, only *A. hydrophila* 099 presented pathogenicity and the score of 3 was comparable to that of *B. cepacia* H111 (Table 10).

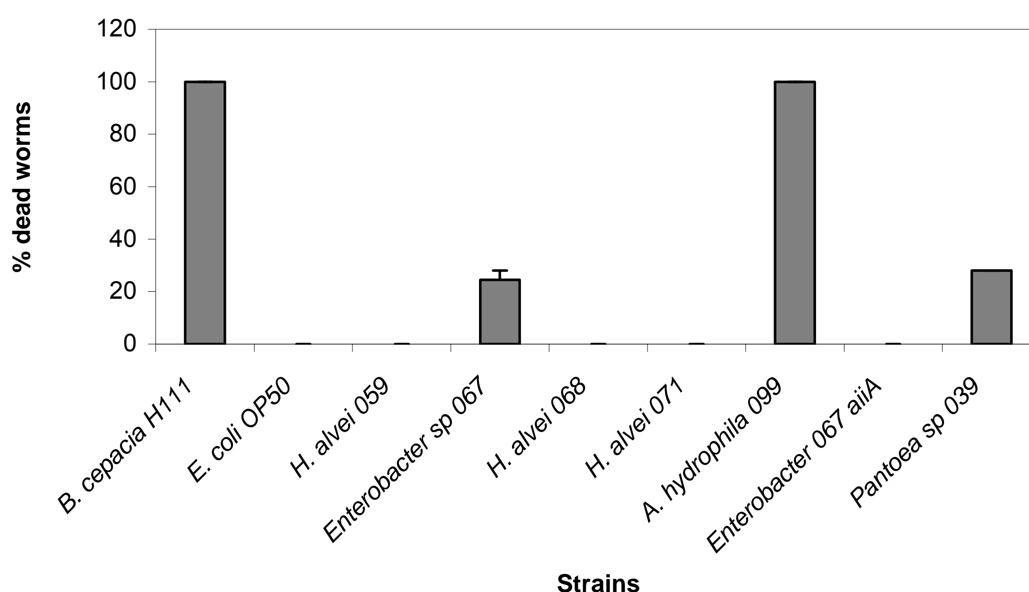

Figure 31 – Pathogenicity of psychrotrophic strains isolated from cooled raw milk in the *C. elegans* Bristol N2 model. The strains were grown on NGM agar plates at 30 °C and the mortality assay was performed.

Proteolytic activity of some extracellular enzymes of *A. hydrophila* was considered to play a major role in the virulence and pathogenicity of the bacterium. These enzymes provide nutrients by breaking down host proteins into small

molecules capable of entering the bacterial cell. *A. hydrophila* is known as a pathogenic bacterium to *C. elegans* (EWBANK, 2002). In addition, according to Kirov (2003), this bacterium is associated with gastroenteritis and its pathogenicity and virulence to humans depend on the ability to produce factors, such as exotoxins, cytotoxins, endotoxins, siderophores, invasins, adhesins, S-layers and flagella.

Table 10 – Scores obtained by plate mortality assay

Strain	Pathogenicity score
B. cepacia H111	3
E. coli OP50	0
H. alvei 059	0
Enterobacter sp 067	0
H. alvei 068	0
H. alvei 071	0
A. hydrophila 099	3
Enterobacter sp 067-7	0
Pantoea sp 039	0

4.4. CONCLUSIONS

The strains 039, 059, 067, 068, 071, and 099 were identified as *Pantoea* sp., *H. alvei*, *Enterobacter* sp., *H. alvei*, *H. alvei*, and *A. hydrophila*, respectively, based on 16S rDNA sequencing. These strains showed different potentials to spoil milk and only *A. hydrophila* 099 presented proteolytic and lipolytic activities in supernatants of TYEP and LB.

H. alvei possesses a native plasmid, the *halI* and *halR* genes, and produces 3-oxo-C6-HSL, C6-HSL, C8-HSL, and 3-oxo-C8-HSL. Besides, the *aiiA* transconjugants of *H. alvei* 068 and 071 still accumulate AHL molecules and the overexpression of HalI in *E. coli* suggests the presence of an additional AHL-based quorum sensing system in *H. alvei*.

Enterobacter sp. 067 contains the *halI* and *halR* genes, produces 3-oxo-C6-HSL, and C6-HSL, and the transconjugant is more proteolytic than wild type suggesting that AHL-mediated quorum sensing negatively regulates protease expression.

A. hydrophila 099 produces C4-HSL, C6-HSL, AI-2 and proved to be pathogenic in the *C. elegans* model system.

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APPENDICES

CHAPTER 2

Table 2.1 - Identification of proteins in ammonium sulfate precipitated supernatant of *P. fluorescens* 041 by MALD-TOF mass spectrometry.

Band[1]	**Accession**	**Mass**	Identified protein	**Score**	**Description**
1	Q7X4S5	49440	Extracellular alkaline metalloprotease	64	*P. fluorescens*
2	Q7X4S5	49440	Extracellular alkaline metalloprotease	254	*P. fluorescens*
3	Q7X4S5	49440	Extracellular alkaline metalloprotease	239	*P. fluorescens*
4	Q7X4S5	49440	Extracellular alkaline metalloprotease	148	*P. fluorescens*
5	Q7X4S5	49440	Extracellular alkaline metalloprotease	89	*P. fluorescens*
6	Q7X4S5	49440	Extracellular alkaline metalloprotease	123	*P. fluorescens*
7	Q7X4S5	49440	Extracellular alkaline metalloprotease	56	*P. fluorescens*
8	Q7X4S5	49440	Extracellular alkaline metalloprotease	221	*P. fluorescens*
9	Q7X4S5	49440	Extracellular alkaline metalloprotease	91	*P. fluorescens*

1 – Bands were extracted from SDS-PAGE, line 3, figure 2.

```
AprX07A    MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR    60
AprX041    MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR
AY298902   MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR
DQ146945   MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR
           .......... .......... .......... .......... .......... ..........

AprX07A    DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK   120
AprX041    DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK
AY298902   DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK
DQ146945   DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK
           .......... .......... .......... .......... .......... ..........

AprX07A    ASGGDGHMTF GNYSSGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT   180
AprX041    ASGGDGHMTF GNYSGGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT
AY298902   ASGGDGHMTF GNYSSGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT
DQ146945   ASGGDGHMTF GNYSSGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT
           .......... ....*..... .......... .......... .......... ..........

AprX07A    LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSP MSYWSESNTN QNLSKGGVEA   240
AprX041    LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA
AY298902   LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA
DQ146945   LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA
           .......... .......... .......... .......... .......... ..*.......

AprX07A    YASGPLIDDI AAIQKLYGAN FNTRATDTTY GFNSNTGRDF LSATSNADKL VFSVWDGGGN   300
AprX041    YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSATSNADKL VFSVWDGGGN
AY298902   YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSASSNADKL VFSVWDGGGN
DQ146945   YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSATSNADKL VFSVWDGGGN
           .......... .......... **........ .......... ...*...... ..........

AprX07A    DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGSGNDL IIGNQVANTI   360
AprX041    DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGAGNDL IIGNQVANTI
AY298902   DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGSGNDL IIGNQVANTI
DQ146945   DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGSGNDL IIGNQVANTI
           .......... .......... .......... .......... .....*.... ..........

AprX07A    KGGAGNDLIY GGGGADQLWG GAGSDTFVYG ASSDSKPGAA DKIFDFTSGS DKIDLSGITK   420
AprX041    KGGAGNDLIY GGGGADQLWG GAGSDTFVYG ASSDSKPGAA DKIFDFTSGS DKIDLSGITK
AY298902   KGGAGNDLIY GGGGADQLWG GTGSDTFVYG ASSDSRPGAA DKIFDFTSGS DKIDLSGITK
DQ146945   KGGAGNDLIY GGGGADQLWG GTGSDTFVYG ASSDSRPGAA DKIFDFTSGS DKIDLSGITK
           .......... .......... .*........ .....*.... .......... ..........

AprX07A    GAGVTFVNAF TGHAGDAVLT YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA      477
AprX041    GAGVTFVNAF TGHAGDAVLS YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA
AY298902   GAGVTFVNAF TGHAGDAVLT YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA
DQ146945   GAGVTFVNAF TGHAGDAVLT YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA
           .......... .........* .......... .......... .......... .......
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Figure 2.1 – Multiple sequence alignment of deduced protease AprX from *P. fluorescens* 07A and 041 (this study), *P. fluorescens* A506 (Genbank accession number AY298902), and *P. fluorescens* strain F (Genbank accession number DQ146945). The differences of similarity in amino acid residues are indicated by gray shading and the catalytic domain of neutral zinc metalloprotease is underlined.

```
LipM07A     MGVFDYKNLG TEGSKALFAD AMAITLYSYH NLDNGFAVGY QNNGLGLGLP ATLVSALIGG    60
LipM041     MGMFDYKNLG TEDSKALFAD AMAITLYSYH NLDNGFAVGY QNNGLGLGLP ATLVSALIGG
DQ305493    MGIFDYKNLG TEGSKALFAD AMAITLYSYH NLDNGFAVGY QHNGLGLGLP ATLVGALLGS
AY694785    MGIFDYKNLG TEGSKTLFAD AIAITLYSYH NLDNGFAVGY QHNGLGLGLP ATLVGALLGS
AF216702    MGIFDYKNLG TEGSKTLFAD AMAITLYSYH NLDNGFAVGY QHNGLGLGLP ATLVGALLGS
            ..*....... ..*..*.... .*........ .......... .*........ ....*..*.*

LipM07A     SNAQGVIPGI PWNPDSEKAA LEAVQAAGWT PISASTLGYS GKVDARGTYF GEKFGYGTAQ    120
LipM041     SNAQGVIPGI PWNPDSEKAA LEAVQAAGWT PISASTLGYS GKVDARGTFF GEKFGYGTAQ
DQ305493    TNSQGVIPGI PWNPDSEKAA LEAVQNAGWT PISASTLGYG GKVDARGTYF GEKAGYTTAQ
AY694785    TDSQGVIPGI PWNPDSEKAA LEAVQKAGWT PISASDLGYG GKVDGRGTFF GEKAGYTTAQ
AF216702    TDSQGVIPGI PWNPDSEKAA LEAVQKAGWT PISASALGYA GKVDARGTFF GEKAGYTTAQ
            ***....... .......... .....*.... .....*...* ....*...*. ...*..*...

LipM07A     AEVLGKYDDA GKLLEIGISF RGTSGPRESV ITDTIGDVIN DLLAAFGPKD YAKNYAGEAF    180
LipM041     AEVLGKYDDA GKLLEIGISF RGTSGPRESV ITDSIGDVIS DLLAAFGPKD YAKNYAGEAF
DQ305493    VEVLGKYDDA GKLLEIGIGF RGTSGPRETL ISDSIGDLVS DLLAALGPKD YAKNYAGEAF
AY694785    VEVLGKYDDA GKLLEIGIGF RGTSGPRESL ITDSIGDVIS DLLAAFGPKD YAKNYAGEAF
AF216702    VEVLGKYDDA GKLLEIGIGF RGTSGPRETL ISDSIGDLIS DLLAALGPKD YAKNYAGEAF
            *......... ........*. ........** .*.*...*** .....*.... ..........

LipM07A     GGLLKNVADY ATAQGLGGND VVVSGHSLGG MAVNSMADLS DSTWSGFYKD ANYVAYASPT    240
LipM041     GGLLKNVADY ATAQGLGGND VVVSGHSLGG LAVNSMADLS DSTWSGFYKD SNYVAYASPT
DQ305493    GGLLKNVADY AAAHGLTGKD VVVSGHSLGG LAVNSMADLS TNKWSGFYTD ANYVAYASPT
AY694785    GGLLKNVADY AGAHGLSGKD VVVSGHSLGG LAVNSMADLS NNKWSGFYKD ANYVAYASPT
AF216702    GGLLKNVADY AGAHGLTGKD VVVSGHSLGG LAVNSMADLS NYKWAGFYKD ANYVAYASPT
            .......... .*.*..*.*. .......... *......... ***.*...*. *.........

LipM07A     QSAGDKVLNV GYENDPVFRA LDGSSFNLSS LGVHDKAHES STDNIVSFND HYASSLWNVL    300
LipM041     QSAGDKVLNV GYENDPVFRA LDGSSFNLSS LGVHDKPHES STDNIVSFND HYASSLWNVL
DQ305493    QSAGDKVLNI GYENDPVFRA LDGSSFNLSS LGVHDKPHES TTDNIVSFND HYASTLWNVL
AY694785    QSAGDKVLNI GYENDPVFRA LDGSSFNLSS LGVHDKPHES TTDNIVSFND HYASTLWNIL
AF216702    QSAGDKVLNI GYENDPVFRA LDGSSFNLSS LGVHDKPHES TTDNIVSFND HYASTLWNVL
            .........* .......... .......... ......*... *......... ....*...*.

LipM07A     PFSILNLPTW VSHLPTGYGD GMTRILDSGF YEQMTRDSTV IVANLSDPAR ATTWVQDLNR    360
LipM041     PFSILNLPTW VSHLPTGYGD GMTRILDSGF YEQMTRDSTV IVANLSDPAR ATTWVQDLNR
DQ305493    PFSIVNLPTW VSHLPTAYGD GMTRILDSGF YDQMTRDSTV IVANLSDPAR ATTWVQDLNR
AY694785    PFSIVNLPTW VSHLPTGYGD GMTRILESGF YDQMTRDSTV IVANLSDPAR ATTWVQDLNR
AF216702    PFSIVNLPTW VSHLPTAYGD GMTRILESGF YDQMTRDSTV IVANLSDPAR ANTWVQDLNR
            ....*..... ......*... ......*... .*........ .......... .*........

LipM07A     NAEAHKGNTF IIGSDGDDLI KGGRGADFIE GGKGNDTIRD SSGYNTFLFS GQFGNDRVIG    420
LipM041     NAEAHKGNTF IIGSDGDDLI KGGRGVDFIE GGKGNDTIRD SSGHNTFLFS GQFGNDRVIG
DQ305493    NAEPHKGNTF IIGSDGNDLI QGGKGADFIE GGKGNDTIRD NSGHNTFLFS GQFGNDRVIG
AY694785    NAEPHKSNTF IIGSHGNDLI QGGKGADFIE GGKGNDTIRD NSGHNTFLFS GNFGNDRVIG
AF216702    NAEPHKGNTF IIGSDGNDLI QGGNGADFIE GGKGNDTIRD NSGHNTFLFS GHFGNDRVIG
            ...*..*... ....*.*... *..*.*.... .......... *..*...... .*........

LipM07A     YQATDKLVFN DVAGSNDYRD HIKVVGGDTV IGFGTDSVTL VGVS--SLSG EGIVIG        474
LipM041     YQATDKLVFN DVAGSTDYRD HAKVVGGDTV ISFGTDSVTL VGVS--SLSG EGIVIG        474
DQ305493    YQTTDKLVFQ DVQGSTDLRD HAKVVGADTV LTFGADSVTL VGVGHGGLWA DGVSIG        476
AY694785    YQTTDKLVFQ NVEGSTDLRD HAKVVGADTV LTFGADSVTL VGVGHGGLWA DGVSIG        476
AF216702    YQPTDKLVFK DVQGSTDLRD HAKVVGADTV LTFGADSVTL VGVGHGGLWT EGVVIG        476
            ..*......* *.*..*.*.. .*....*... **..*..... ...*--*.** *.**..
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Figure 2.2 - Multiple sequence alignment of deduced lipase LipM from *P. fluorescens* 07A and 041 (this study), Lip (Genbank accession number DQ305493), Lip68 (Genbank accession number AY694785), and LipA (Genbank accession number AF216702) from *P. fluorescens*. The differences of similarity in amino acid residues are indicated by gray shading and the catalytic domain of serine lipase is underlined.

CHAPTER 3

Table 3.1 – Composition of LB soft agar

Component	Weight (g) and volume (ml)
Tryptone	1.50
Yeast extract	0.75
NaCl	0.75
Agar	1.00
Water	150

Table 3.2 – Composition of AB soft agar for *Agrobacterium tumefaciens*

Component	Volume (ml) and weight (g)
20X AB salt[a]	5
20X AB buffer[b]	5
Manitol 10%	5
Water	88
Agar	1

(a) 20X AB salt: NH_4Cl (20 g); $MgSO_4$ x $7H_2O$ (6 g); KCl (3 g); $CaCl_2$ (0.2 g); $FeSO_4$ x 7 H_2O (0.05 g); water (1000 ml). (b) 20X AB buffer: K_2HPO_4 (60 g); NaH_2PO_4 (23 g); water (1000 ml). The pH was adjusted to 7.0.

Table 3.3 – Composition of the AB medium for *V. harveyi* (Bassler et al., 1994)

Component	Concentration
NaCl	0.30 M
$MgSO_4$	0.05 M
Vitamin-free casamino acids	0.2 %

The pH was adjusted to 7.5 with KOH. The medium was sterilized, cooled, and added of 10 ml of sterile 1 M potassium phosphate (pH 7.0), 10 ml of 0.1 M L-arginine, 20 ml of glycerol, 1 ml of 10 μg/ml riboflavin, and 1 ml of 1 mg/ml thiamine per litre.

CHAPTER 4

Table 4.1 – Composition of the ABC or ABG minimal medium

Component	Weight (g) and volume (ml)
A10	
$(NH_4)_2SO_4$	20
Na_2HPO_4	60
KH_2PO_4	30
NaCl	30
Destilled water	1000
B	
$MgCl_2$ x 6 H_2O (1M)	2.0
CaCl x 2 H_2O (0.5M)	0.2
$FeCl_2$ x 6 H_2O (0.01M)	0.3
Destilled water	900
C	
Citrate or glucose	1M

Sterilize the media A10 and B separately at 121 °C per 15 min. Wait cooling to approximately 50 °C. Sterilize by filtration the citrate or glucose solution. Combine 900 ml of medium B with 100 ml of medium A10 plus 10 ml of citrate. After preparation, the medium B needs to be maintained in a dark place.

Table 4.2 – Composition of medium 884 – Tween 80-Agar

Component	Weight (g) and volume (ml)
Solution A	
Peptone	10.0
NaCl	5.00
$CaCl_2$ x 2 H_2O	0.10
Agar	15.0
Destilled water	900
Solution B	
Tween 80	10.0
Destilled water	100.0

Autoclave solutions A and B separately and combine after cooling to approximately 50 °C. Tween 80 must be weight.

Table 4.3 – Composition of NGM I medium

Component	Weight (g) and volume (ml)
NaCl	1.50
Tryptone	1.25
Agar	8.50
Destilled water	500
Nystatine (10 mg/ml)	2.50
1 M KPO_4 buffer, pH 6.0	12.50
1 M $CaCl_2$	0.50
1 M $MgSO_4$	0.50
Uracile 2 mg/ml (sterile filtrated)	0.50
Cholesterine (10 mg/ml in ethanol)	0.25

Table 4.4 – Composition of NGM II medium

Component	Weight (g) and volume (ml)
NaCl	1.50
Bactopeptone	1.75
Agar	8.50
Destilled water	500
Nystatine (10 mg/ml)	2.50
1 M KPO_4 buffer, pH 6.0	12.50
1 M $CaCl_2$	0.50
1 M $MgSO_4$	0.50
Uracile 2 mg/ml (sterile filtrated)	0.50
Cholesterine (10 mg/ml in ethanol)	0.25

Table 4.5 – Composition of M9-buffer

Component	Weight (g) and volume (ml)
KH_2PO_4	3.0
Na_2HPO_4	6.0
NaCl	5.0
1 M $MgSO_4$	1.0
Destilled water	1000
pH	6.0

Table 4.6 – Composition of sodiumhypochlorite solution

Component	Volume (µl)
Sterile destilled water	600
Sodiumhypochlorite (12%)	500
NaOH 6N	400

```
039    AGAGTTTGAT TATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAAC    60
039    GGTAGCACAG AGAGCTTGCT CTCGGGTGAC GAGTGGCGGA CGGGTGAGTA ATGTCTGGGA    120
039    AACTGCCTGA TGGAGGGGGA TAACTACTGG AAACGGTAGC TAATACCGCA TAACGTCGCA    180
039    AGACCAAAGA GGGGGACCTT CGGGCCTCTT GCCATCAGAT GTGCCCAGAT GGGATTAGCT    240
039    AGTAGGTGGG GTAACGGCTC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA    300
039    GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG    360
039    CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTAT GAAGAAGGCC TTCGGGTTGT    420
039    AAAGTACTTT CAGCGGGGAG GAAGGTGTTG TGGTTAATAA CCACAGCAAT TGACGTTACC    480
039    CGCAGAAGAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC    540
039    GTTAATCGGA ATTACTGGGC GTAAAGCGCA CGCAGGCGGT CTGTCAAGTC GGATGTGAAA    600
039    TCCCCGGGCT CAACCTGGGA ACTGCATTCG AAACTGGCAG GCTAGAGTCT TGTAGAGGGG    660
039    GGTAGAATTC CAGGTGTAGC GGTGAAATGC GTAGAGATCT GGAGGAATAC CGGTGGCGAA    720
039    GGCGGCCCCC TGGACAAAGA CTGACGCTCA GGTGCGAAAG CGTGGGGAGC AAACAGGATT    780
039    AGATACCCTG GTAGTCCACG CCGTAAACGA TGTCGACTTG GAGGTTGTGC CCTTGAGGCG    840
039    TGGCTTCCGG AGCTAACGCG TTAAGTCGAC CGCCTGGGGA GTACGGCCGC AAGGTTAAAA    900
039    CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGATGCAA    960
039    CGCGAAGAAC CTTACCTACT CTTGACATCC AGAGAACTTT CCAGAGATGG ATTGGTGCCT    1020
039    TCGGGAACTC TGAGACAGGT GCTGCATGGC TGTCGTCAGC TCGTGTTGTG AAATGTTGGG    1080
039    TTAAGTCCCG CAACGAGCGC AACCCTTATC CTTTGTTGCC AGCGGTCCGG CCGGGAACTC    1140
039    AAAGGAGACT GCCAGTGATA AACTGGAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC    1200
039    CTTACGAGTA GGGCTACACA CGTGCTACAA TGGCGCATAC AAAGAGAAGC GACCTCGCGA    1260
039    GAGCAAGCGG ACCTCATAAA GTGCGTCGTA GTCCGGATTG GAGTCTGCAA CTCGACTCCA    1320
039    TGAAGTCGGA ATCGCTAGTA ATCGTAGATC AGAATGCTAC GGTGAATACG TTCCCGGGCC    1380
039    TTGTACACAC CGCCCGTCAC ACCATGGGAG TGGGTTGCAA AAGAAGTAGG TAGCTTAACC    1440
039    TTCGGGAGGG CGCTTACCAC TTTGTGATTC ATGACTGGGG TGAAGTCGTA ACAAGGTAAC    1500
039    CGTAGGGGAA CCTGCGGTTG GATCACCTCC TTT                                 1533
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Figure 4.1 – Nucleotide sequence of 16S rDNA of *Pantoea* sp. 039.

```
059    AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC    60
059    GGTAGCACAA GAGAGCTTGC TCTCTGGGTG ACGAGCGGCG GACGGGTGAG TAATGTCTGG    120
059    GAAACTGCCT GATGGAGGGG GATAACTACT GGAAACGGTA GCTAATACCG CATGACGTCT    180
059    TCGGACCAAA GTGGGGGACC TTCGGGCCTC ACGCCATCAG ATGTGCCCAG ATGGGATTAG    240
059    CTAGTAGGTG GGGTAATGGC TCACCTAGGC GACGATCTCT AGCTGGTCTG AGAGGATGAC    300
059    CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT    360
059    TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG CCTTCGGGTT    420
059    GTAAAGTACT TTCAGCGAGG AGGAAGGCAT TGTGGTAAAT AACCGCAGTG ATTGACGTTA    480
059    CTCGCAGAAG AAGCACCCGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGTGCAA    540
059    GCGTTAATCG GAATTACTGG GCGTAAAGCG CACGCAGGCG GTTGATTAAG TCAGATGTGA    600
059    AATCCCCGAG CTTAACTTGG GAACTGCATT TGAAACTGGT CAGCTAGAGT CTTGTAGAGG    660
059    GGGGTAGAAT TCCAGGTGTA GCGGTGAAAT GCGTAGAGAT CTGGAGGAAT ACCGGTGGCG    720
059    AAGGCGGCCC CCTGGACAAA GACTGACGCT CAGGTGCGAA AGCGTGGGGA GCAAACAGGA    780
059    TTAGATACCC CTGGTAGTCC ACGCTGTAAA CGATGTCGAC TTGGAGGTTG TGCCCTTGAG    840
059    GCGTGGCTTC CGGAGCTAAC GCGTTAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA    900
059    AAACTCAAAT GAATTGACGG GGGCCCGCAC AAGCGGTAGA GCATGTGGTT TAATTCGATG    960
059    CAACGCGAAG AACCTTACCT ACTCTTGACA TCCAGAGAAT TTGCTAGAGA TAGCTTAGTG    1020
059    CCTTCGGGAA CTCTGAGACA GGTGCTGCAT GGCTGTCGTC AGCTCGTGTT GTGAAATGTT    1080
059    GGGTTAAGTC CCGCAACGAG CGCAACCCTT ATCCTTTGTT GCCAGCGCGT AATGGCGGGA    1140
059    ACTCAAAGGA GACTGCCGGT GATAAACCGG AGGAAGGTGG GGATGACGTC AAGTCATCAT    1200
059    GGCCCTTACG AGTAGGGCTA CACACGTGCT ACAATGGCAT ATACAAAGAG AAGCGAACTC    1260
059    GCGAGAGCAA GCGGACCTCA TAAAGTATGT CGTAGTCCGG ATTGGAGTCT GCAACTCGAC    1320
059    TCCATGAAGT CGGAATCGCT AGTAATCGTA GATCAGAATG CTACGGTGAA TACGTTCCCG    1380
059    GGCCTTGTAC ACACCGCCCG TCACACCATG GGAGTGGGTT GCAAAAGAAG TAGGTAGCTT    1440
059    AACCTTCGGG AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT CGTAACAAGG    1500
059    TAACCGTAGG GGAACCTGCG GTTGGATCAC CTCCTTTCTG                          1540
```

Figure 4.2 – Nucleotide sequence of 16S rDNA of *H. alvei*. 059

067	AGAGTTTGAT	CATGGCTCAG	ATTGAACGCT	GGCGGCAGGC	CTAACACATG	CAAGTCGAGC	60
067	GGTAGCACAA	GAGAGCTTGC	TCTCTGGGTG	ACGAGCGGCG	GACGGGTGAG	TAATGTCTGG	120
067	GAAACTGCCT	GACGGAGGGG	GATAACTACT	GGAAACGGTA	GCTAATACCG	CATAACGTCG	180
067	CAAGACCAAA	GAGGGGGACC	TTCGGGCCTC	TTGCCATCAG	ATGTGCCCAG	ATGGGATTAG	240
067	CTAGTAGGTG	GGGTAACGGC	TCACCTAGGC	GACGATCCCT	AGCTGGTCTG	AGAGGATGAC	300
067	CAGCCACACT	GGAACTGAGA	CACGGTCCAG	ACTCCTACGG	GAGGCAGCAG	TGGGGAATAT	360
067	TGCACAATGG	GCGCAAGCCT	GATGCAGCCA	TGCCGCGTGT	ATGAAGAAGG	CCTTCGGGTT	420
067	GTAAAGTACT	TTCAGCGGGG	AGGAAGGTGT	TGTGGTTAAT	AACCACAGCA	ATTGACGTTA	480
067	CCCGCAGAAG	AAGCACCGGC	TAACTCCGTG	CCAGCAGCCG	CGGTAATACG	GAGGGTGCAA	540
067	GCGTTAATCG	GAATTACTGG	GCGTAAAGCG	CACGCAGGCG	GTCTGTCAAG	TCGGATGTGA	600
067	AATCCCCGGG	CTCAACCTGG	GAACTGCATT	CGAAACTGGC	AGGCTAGAGT	CTTGTAGAGG	660
067	GGGGTAGAAT	TCCAGGTGTA	GCGGTGAAAT	GCGTAGAGAT	CTGGAGGAAT	ACCGGTGGCG	720
067	AAGGCGGCCC	CCTGGACAAA	GACTGACGCT	CAGGTGCGAA	AGCGTGGGGA	GCAAACAGGA	780
067	TTAGATACCC	TGGTAGTCCA	CGCCGTAAAC	GATGTCGACT	TGGAGGTTGT	GCCCTTGAGG	840
067	CGTGGCTTCC	GGAGCTAACG	CGTTAAGTCG	ACCGCCTGGG	GAGTACGGCC	GCAAGGTTAA	900
067	AACTCAAATG	AATTGACGGG	GGCCCGCACA	AGCGGTGGAG	CATGTGGTTT	AATTCGATGC	960
067	AACGCGAAGA	ACCTTACCTA	CTCTTGACAT	CCAGAGAACT	TTCCAGAGAT	GGATTGGTGC	1020
067	CTTCGGGAAC	TCTGAGACAG	GTGCTGCATG	GCTGTCGTCA	GCTCGTGTTG	TGAAATGTTG	1080
067	GGTTAAGTCC	CGCAACGAGC	GCAACCCTTA	TCCTTTGTTG	CCAGCGGTCC	GGCCGGGAAC	1140
067	TCAAAGGAGA	CTGCCAGTGA	TAAACTGGAG	GAAGGTGGGG	ATGACGTCAA	GTCATCATGG	1200
067	CCCTTACGAG	TAGGGCTACA	CACGTGCTAC	AATGGCGCAT	ACAAAGAGAA	GCGACCTCGC	1260
067	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	1320
067	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCAGAATGCT	ACGGTGAATA	CGTTCCCGGG	1380
067	CCTTGTACAC	ACCGCCCGTC	ACACCATGGG	AGTGGGTTGC	AAAAGAAGTA	GGTAGCTTAA	1440
067	CCTTCGGGAG	GGCGCTTACC	ACTTTGTGAT	TCATGACTGG	GGTGAAGTCG	TAACAAGGTA	1500
067	ACCGTAGGGG	AACCTGCGGT	TGGATCACCT	CCTTTATG			1538

Figure 4.3 – Nucleotide sequence of 16S rDNA of *Enterobacter* sp. 067

068	AGAGTTTGAT	CATGGCTCAG	ATTGAACGCT	GGGGGCAGGC	CTAACACATG	CAAGTCGAGC	60
068	GGTAGCACAA	GAGAGCTTGC	TCTCTGGGTG	ACGAGCGGCG	GACGGGTGAG	TAATGTCTGG	120
068	GAAACTGCCT	GATGGAGGGG	GATAACTACT	GGAAACGGTA	GCTAATACCG	CATGACGTCT	180
068	TCGGACCAAA	GTGGGGGACC	TTCGGGCCTC	ACGCCATCAG	ATGTGCCCAG	ATGGGATTAG	240
068	CTAGTAGGTG	GGGTAATGGC	TCACCTAGGC	GACGATCTCT	AGCTGGTCTG	AGAGGATGAC	300
068	CAGCCACACT	GGAACTGAGA	CACGGTCCAG	ACTCCTACGG	GAGGCAGCAG	TGGGGAATAT	360
068	TGCACAATGG	GCGCAAGCCT	GATGCAGCCA	TGCCGCGTGT	ATGAAGAAGG	CCTTCGGGTT	420
068	GTAAAGTACT	TTCAGCGAGG	AGGAAGGCAT	TAAGGTTAAT	AACCTTGGTG	ATTGACGTTA	480
068	CTCGCAGAAG	AAGCACCGGC	TAACTCCGTG	CCAGCAGCCG	CGGTAATACG	GAGGGTGCAA	540
068	GCGTTAATCG	GAATTACTGG	GCGTAAAGCG	CACGCAGGCG	GTTTGTTAAG	TCAGATGTGA	600
068	AATCCCCGAG	CTTAACTTGG	GAACTGCATT	TGAAACTGGC	AAGCTAGAGT	CTTGTAGAGG	660
068	GGGGTAGAAT	TCCAGGTGTA	GCGGTGAAAT	GCGTAGAGAT	CTGGAGGAAT	ACCGGTGGCG	720
068	AAGGCGGCCC	CCTGGACAAA	GACTGACGCT	CAGGTGCGAA	AGCGTGGGGA	GCAAACAGGA	780
068	TTAGATACCC	TGGTAGTCCA	CGCTGTAAAC	GATGTCGACT	TGAGGTTGTG	CCCTTGAGGC	840
068	GTGGCTTCCG	GAGCTAACGC	GTTAAGTCGA	CCGCCTAGGG	AGTACGGCCG	CAAGGTTAAA	900
068	ACTCAAATGA	ATTGACGGGG	GCCCGCACAA	GCGGTGGAGC	ATGTGGTTTA	ATTCGATGCA	960
068	ACGCGAAGAA	CCTTACCTAC	TCTTGACATC	CAGAGAATTT	GCTAGAGATA	GCTTAGTGCC	1020
068	TTCGGGAACT	CTGAGACAGG	TGCTGCATGG	CTGTCGTCAG	CTCGTGTTGT	GAAATGTTGG	1080
068	GTTAAGTCCC	GCAACGAGCG	CAACCCTTAT	CCTTTGTTGC	CAGCACGTGA	TGGTGGGAAC	1140
068	TCAAAGGAGA	CTGCCGGTGA	TAAACCGGAG	GAAGGTGGGG	ATGACGTCAA	GTCATCATGG	1200
068	CCCTTACGAG	TAGGGCTACA	CACGTGCTAC	AATGGCATAT	ACAAAGAGAA	GCGAACTCGC	1260
068	GAGAGCAAGC	GGACCTCATA	AAGTATGTCG	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	1320
068	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCAGAATGCT	ACGGTGAATA	CGTTCCCGGG	1380
068	CCTTGTACAC	ACCGCCCGTC	ACACCATGGG	AGTGGGTTGC	AAAAGAAGTA	GGTAGCTTAA	1440
068	CCTTCGGGAG	GGCGCTTACC	ACTTTGTGAT	TCATGACTGG	GGTGAAGTCG	TAACAAGGTA	1500
068	ACCGTAGGGG	AACCTGCGGT	TGGATCACCT	CCTTTCTG			1538

Figure 4.4 – Nucleotide sequence of 16S rDNA of *H. alvei* 068

```
071    AGAGTTTGAT TATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC    60
071    GGTAGCACAA GAGAGCTTGC TCTCTGGGTG ACGAGCGGCG GACGGGTGAG TAATGTCTGG    120
071    GAAACTGCCT GATGGAGGGG GATAACTACT GGAAACGGTA GCTAATACCG CATGACGTCT    180
071    TCGGACCAAA GTGGGGGACC TTCGGGCCTC ACGCCATCAG ATGTGCCCAG ATGGGATTAG    240
071    CTAGTAGGTG GGGTAATGGC TCACCTAGGC GATGATCTTT AGCTGGTCTG AGAGGATGAC    300
071    CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT    360
071    TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG CCTTCGGGTT    420
071    GTAAAGTACT TTCAGCGAGG AGGAAGGCAT TAAGGTTAAT AACCTTGGTG ATTGACGTTA    480
071    CTCGCAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGTGCAA    540
071    GCGTTAATCG GAATTACTGG GCGTAAAGCG CACGCAGGCG GTTGATTAAG TCAGATGTGA    600
071    AATCCCCGAG CTTAACTTGG GAACTGCATT TGAAACTGGC AAGCTAGAGT CTTGTAGAGG    660
071    GGGGTAGAAT TCCAGGTGTA GCGGTGAAAT GCGTAGAGAT CTGGAGGAAT ACCGGTGGCG    720
071    AAGGCGGCCC CCTGGACAAA GACTGACGCT CAGGTGCGAA AGCGTGGGGA GCAAACAGGA    780
071    TTAGATACCC TGGTAGTCCA CGCTGTAAAC GATGTCGACT TGGAGGTTGT GCCCTTGAGG    840
071    CGTGGCTTCC GGAGCTAACG CGTTAAGTCG ACCGCCTGGG GAGTACGGCC GCAAGGTTAA    900
071    AACTCAAATG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGATGC    960
071    AACGCGAAGA ACCTTACCTA CTCTTGACAT CCAGAGAATT TGCTAGAGAT AGCTTAGTGC    1020
071    CTTCGGGAAC TCTGAGACAG GTGCTGCATG GCTGTCGTCA GCTCGTGTTG TGAAATGTTG    1080
071    GGTTAAGTCC CGCAACGAGC GCAACCCTTA TCCTTTGTTG CCAGCACGTG ATGGTGGGAA    1140
071    CTCAAAGGAG ACTGCCGGTG ATAAACCGGG GGAAGGTGGG GATGACGTCA AGTCATCATG    1200
071    GCCCTTACGA GTAGGGCTAC ACACGTGCTA CAATGGCATA TACAAAGAGA AGCGAACTCG    1260
071    CGAGAGCAAG CGGACCTCAT AAAGTATGTC GTAGTCCGGA TTGGAGTCTG CAACTCGACT    1320
071    CCATGAAGTC GGAATCGCTA GTAATCGTAG ATCAGAATGC TACGGTGAAT ACGTTCCCGG    1380
071    GCCTTGTACA CACCGCCCGT CACACCATGG GAGTGGGTTG CAAAAGAAGT AGGTAGCTTA    1440
071    ACCTTCGGGA GGGCGCTTAC CACTTTGTGA TTCATGACTG GGGTGAAGTC GTAACAAGGT    1500
071    AACCGTAGGG GAACCTGCGG TTGGATCACC TCCTTTATG                           1539
```

Figure 4.5 – Nucleotide sequence of 16S rDNA of *H. alvei* 071

```
099    AGAGTTTGAT TCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG CAAGTCGAGC    60
099    GGCAGCGGGA AAGTAGCTCG CTACTTTTGC CGGCGAGCGG CGGACGGGTG AGTAATGCCT    120
099    GGGAAATTGC CCAGTCGAGG GGGATAACAG TTGGAAACGA CTGCTAATAC CGCATACGCC    180
099    CTACGGGGGA AAGCAGGGGA CCTTCGGGCC TTGCGCGATT GGATATGCCC AGGTGGGATT    240
099    AGCTAGTTGG TGAGGTAATG GCTCACCAAG GCGACGATCC CTAGCTGGTC TGAGAGGATG    300
099    ATCAGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT    360
099    ATTGCACAAT GGGGGAAACC CTGATGCAGC CATGCCGCGT GTGTGAAGAA GGCCTTCGGG    420
099    TTGTAAAGCA CTTTCAGCGA GGAGGAAAGG TTGATGCCTA ATACGTATCA ACTGTGACGT    480
099    TACTCGCAGA AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGTGC    540
099    AAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCACGCAGG CGGTTGGATA AGTTAGATGT    600
099    GAAAGCCCCG GGCTCAACCT GGGAATTGCA TTTAAAACTG TCCAGCTAGA GTCTTGTAGA    660
099    GGGGGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGGAGGA ATACCGGTGG    720
099    CGAAGGCGGC CCCCTGGACA AAGACTGACG CTCAGGTGCG AAAGCGTGGG GAGCAAACAG    780
099    GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA TTTGGAGGCT GTGTCCTTGA    840
099    GACGTGGCTT CCGGAGCTAA CGCGTTAAAT CGACCGCCTG GGGAGTACGG CCGCAAGGTT    900
099    AAAACTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAT    960
099    GCAACGCGAA GAACCTTACC TGGCCTTGAC ATGTCTGGAA TCCTGTAGAG ATACGGGAGT    1020
099    GCCTTCGGGA ATCAGAACAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT    1080
099    TGGGTTAAGT CCCGCAACGA GCGCAACCCC TGTCCTTTGT TGCCAGCACG TAATGGTGGG    1140
099    AACTCAAGGG AGACTGCCGG TGATAAACCG GAGGAAGGTG GGGATGACGT CAAGTCATCA    1200
099    TGGCCCTTAC GGCCAGGGCT ACACACGTGC TACAATGGCG CGTACAGAGG GCTGCAAGCT    1260
099    AGCGATAGTG AGCGAATCCC AAAAAGCGCG TCGTAGTCCG GATCGGAGTC TGCAACTCGA    1320
099    CTCCGTGAAG TCGGAATCGC TAGTAATCGC AAATCAGAAT GTTGCGGTGA ATACGTTCCC    1380
099    GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTGGGT TGCACCAGAA GTAGATAGCT    1440
099    TAACCTCCGG GAGGGCGTTT ACCACGGTGT GATTCATGAC TGGGGTGAAG TCGTAACAAG    1500
099    GTAACCCTAG GGGAACCTGG GGTTGGATCA CCTCCTTTAT G                         1541
```

Figure 4.6 – Nucleotide sequence of 16S rDNA of *A. hydrophila* 099

```
GGGACGCAAA TTCAGCTTGT GTAAGCCCCG CTGAAAGCCT GATTTCTTTG AATTCTCTGT        60
TATTCATAGG GTTAGAATCG ATCCTCTCGT GATTTTAAGG ATGTGATATT ACATCACCTC       120
AACAAACCGA TGAGTCTACC CGCACGCTAC CAGAAGTTAG GTTTTAACTG GAAAACTCCG       180
TGTTTCCGTT GACTCTACCC CCGTGTTACA GGGCGGGGGT ATCGGTGCGG CGGTGTACAC       240
AATGCACATC CTGTCCTGCC TTTGATGTCG AAACCGGACA AGATGTGTTT TTGGAGTACC       300
GCCATTCGCG GCAACATCCA AAAACGGCTC ATCAGGGCGA TGGCCTCACT GCGTGAACCA       360
TCACCCGTTA GCAGTGTTAT TTCTCCGGCG TGTCGTCAAT CACTAAATCG GAACCCTGAA       420
AGGGAACCCC CGATTTAGCG TTTGACGAGT TCAGACCACA GAGATGCGGA TGAATATCCA       480
GAAAAGACGC CTCCAAATTC GCGCTCAGAA GCTCTATGAG GGGATGAAGG TCATCATCAA       540
GGGGATGGAG TGAGGCAAAC GCCGTAAATG CCTCTACGGC GTTCTGGTGA CGTGTAATCA       600
TGGCAAGCAT GGTAGGATTC GTGTCAGTCA TGGAGACCTC CGTAACAGGT TTTCGTGATA       660
GCAGGGGGCG GGAACTCGTA ATTCCTTCCC CCTGCGCCCA TCAGATCATA ATTCCGCTAC       720
ACGGGCAAGC CGCCACGGGA AGGGTGCACT CCTCGTGGGA ACTCGGTGCG CTCCCTTCCC       780
GTGCCCGCCT GCCAGCTCGT GCCATTCTGA CCCGTCCGGC ACAGACAGCC AGAAACCACG       840
CGAGAGAGTC CGCACCGCTC GCCGCAAGAC GTGATTAGCG AGCAACGCGA GTCTGTCACG       900
TCGAGGAAGC GGAATATCAC CTGAACGGGC ATAGAAAACC GTACGCACAG TACCCACAGA       960
AAAACACGAT GCAGGATAGG GCAAACGCCG CAAAATGCTA GCATTTTACT CGTTTGCCCG      1020
GTGACCTACA GCAGTTTCCA GAAGTGATGT TTTCGTGGCT GGCATCGCGC ACAGAGTTAC      1080
AAGATACAGA AGGGAACCTC CCGCAAGCCG CGCCACAAGC GGAATCATTT GACAGATAGT      1140
TATGTTCAGG CATAACTTAG TTATGTTCAG GCATAACTAT TTAAGGAATT TTCATGCGAA      1200
AAGTCACACA GGTTGACCTC GAAACCGGAG AGGATTTGGG CGGGTTTGTC GCCGTGATCC      1260
GTCCCAAGCA AAAATCATCG TTCGAGAGGC ATTTCACTAT GAATCAGGCA GCACTCAAAA      1320
TCATCGCTAC AGAACTGAAC CATGAGCAGA CAAAAGTACT CATGATGCTT CTCGCAGACC      1380
TGGACTACGA AAATTACATT CAGGTGGCAC AAATCGACAT TGCAGAATCA TTGGGAATGA      1440
AAAAACCAAA TGTTAGCAAA GCTGTTAAAA ACCTGATTGA GTTCGGAATA ATCCTTGAGG      1500
GGCCAAAGAT AGGCCGAAGC AAAACCTACC GCCTGAACCC TCAGTTCGGC TGGAAAGGCA      1560
CGGTAAGCAA CCACAAAAAA GCACTCAAAA ACGGCCTCAG TATCATTCAG GGTGGCAAGG      1620
TGTGAGCCGT ACCATCTTGG TACCCTTCCG ATCCAAACCT CCCTTTCATT TCACGATTTA      1680
AGCGGCTTTT CGCCCCGCTC CAGTGGGTCA GTTCATGCAA GCCGGTAGCG TAAAAATTTG      1740
CGGCATCGGC AAACAAATGA CGCTCAGGCA ACCAGATTTC ATCCGTTAGC GGTTTAAAAA      1800
ATGCCTGCTG GCCACGTTCT GTAATATTCG CTCCTGTAAG CCGAATTCCA GCACACT          1857
```

Figure 4.7 – Nucleotide sequence of pMLM extracted from *H. alvei* 068

```
halI/halR H.alvei AF503776    GTACTTAAGT ACACCGCTGC CGCCGAGACT GTAGAACAAA CTTCAGGGAT GCCATATGTT    60
halI/halR068                  ---------- ---------- ---------- ---------- CTTCAGGGAT GCCATATGTT    20
halI/halR067                  ---------- ---------- ---------- ---------- CTTCAGGGAT GCCATATGTT    20
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             .......... .......... .......... .......... CTTCAGGGAT GCCATATGTT

halI/halR H.alvei AF503776    TTCTATTTTC AATAAAAATC AGATAATAAC GGAAACGCTT CGTGATTATA TCGATAGAAA    120
halI/halR068                  TTCTATTTTC AATAAAAATC AGATAATAAC GGAAACGCTT CGTGATTATA TCGATAGAAA    80
halI/halR067                  TTCTATTTTC AATAAAAATC AGATAATAAC GGAAACGCTT CGTGATTATA TCGATAGAAA    80
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             TTCTATTTTC AATAAAAATC AGATAATAAC GGAAACGCTT CGTGATTATA TCGATAGAAA

halI/halR H.alvei AF503776    ACTGTCCCAG TTTGGTAGCC CTGAGTACGC TTACACCGTC GTCAATAAGA AAAACCCGTC    180
halI/halR068                  ACTGTCCCAG TTTGGTAGCC CTGAGTACGC TTACACCGTC GTCAATAAGA AAAACCCGTC    140
halI/halR067                  ACTGTCCCAG TTTGGTAGCC CTGAGTACGC TTACACCGTC GTCAATAAGA AAAACCCGTC    140
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             ACTGTCCCAG TTTGGTAGCC CTGAGTACGC TTACACCGTC GTCAATAAGA AAAACCCGTC

halI/halR H.alvei AF503776    AAAACTGCTT ATCATCTCAA GCTATCCTGA TGAATGGGTA AACCTGTACA TTGCGAATAA    240
halI/halR068                  AAAACTGCTT ATCATCTCAA GCTATCCTGA TGAATGGGTA AACCTGTACA TTGCGAATAA    200
halI/halR067                  AAAACTGCTT ATCATCTCAA GCTATCCTGA TGAATGGGTA AACCTGTACA TTGCGAATAA    200
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             AAAACTGCTT ATCATCTCAA GCTATCCTGA TGAATGGGTA AACCTGTACA TTGCGAATAA

halI/halR H.alvei AF503776    CCTGCAGCAC ATTGACCCGG TGATCCTGAC CGCGTTTAAA CGCACGTCCC CTTTCGTGTG    300
halI/halR068                  CCTGCAGCAC ATTGACCCGG TGATCCTGAC CGCGTTTAAA CGCACGTCCC CTTTCGTGTG    260
halI/halR067                  CCTGCAGCAC ATTGACCCGG TGATCCTGAC CGCGTTTAAA CGCACGTCCC CTTTCGTGTG    260
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             CCTGCAGCAC ATTGACCCGG TGATCCTGAC CGCGTTTAAA CGCACGTCCC CTTTCGTGTG

halI/halR H.alvei AF503776    GGATGAGAAC ATCACGTTGA TGTCTGACCT CAAGATCTCA AAGATTTTCT CTTTATCCAA    360
halI/halR068                  GGATGAGAAC ATCACGTTGA TGTCTGACCT CAAGGTCTCA AAGATTTTCT CTTTATCCAA    320
halI/halR067                  GGATGAGAAC ATCACGTTGA TGTCTGACCT CAAGGTCTCA AAGATTTTCT CTTTATCCAA    320
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             GGATGAGAAC ATCACGTTGA TGTCTGACCT CAAG.TCTCA AAGATTTTCT CTTTATCCAA

halI/halR H.alvei AF503776    GAAATACAAC ATCGCCAACG GCTATACTTT CGTCCTGCAC GATCATCTCA ACAATCTGGC    420
halI/halR068                  GAAATACAAC ATCGCCAACG GCTATACTTT CGTCCTGCAC GATCATCTCA ACAATCTGGC    380
halI/halR067                  GAAATACAAC ATCGCCAACG GCTATACTTT CGTCCTGCAC GATCATCTCA ACAATCTGGC    380
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             GAAATACAAC ATCGCCAACG GCTATACTTT CGTCCTGCAC GATCATCTCA ACAATCTGGC

halI/halR H.alvei AF503776    ACTACTATCA TTAATTATTG ATAGCAATAT GAAAGCGAAT CTGGAAGAGC AGTTCTCTTC    480
halI/halR068                  ACTACTATCA TTAATTATTG ATAGCAATAT GAAAGCGAAT CTGGAAGAGC AGTTCTCTTC    440
halI/halR067                  ACTACTATCA TTAATTATTG ATAGCAATAT GAAAGCGAAT CTGGAAGAGC AGTTCTCTTC    440
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             ACTACTATCA TTAATTATTG ATAGCAATAT GAAAGCGAAT CTGGAAGAGC AGTTCTCTTC

halI/halR H.alvei AF503776    AGAGAAAGGC AACTTACAGA TGTTACTCAT TGAGATTAAT GAGCAAATGT ATCGGCTCGT    540
halI/halR068                  AGAGAAAGGC AACTTACAGA TGTTACTCAT TGAGATTAAT GAGCAAATGT GTCGGCTCGT    500
halI/halR067                  AGAGAAAGGC AACTTACAGA TGTTACTCAT TGAGATTAAT GAGCAAATGT ATCGGCTCGT    500
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             AGAGAAAGGC AACTTACAGA TGTTACTCAT TGAGATTAAT GAGCAAATGT .TCGGCTCGT

halI/halR H.alvei AF503776    GCAGTCAGTT TCGGAAGATA AGGATGGTTC AGAGATGGGC GTAAGCAAAG CAACGTTTAC    600
halI/halR068                  GCAGTCAGTT TCGGTAGATA AGGATGGTTC TGAGATGGGC GTAAGCAAAG CAACGTTTAC    560
halI/halR067                  GCAGTCAGTT TCGGTAGATA AGGATGGTTC TGAGATGGGC GTAAGCAAAG CAACGTTTAC    560
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             GCAGTCAGTT TCGG.AGATA AGGATGGTTC .GAGATGGGC GTAAGCAAAG CAACGTTTAC

halI/halR H.alvei AF503776    AGCCAGAGAA CATGAAGTAC TTTACTGGGC GAGTATGGGG AATACTTACG CGGAGATCGC    660
halI/halR068                  AGCCAGAGAA CATGAAGTAC TTTACTGGGC GAGTATGGGG AAAACTTACG CGGAGATCGC    620
halI/halR067                  AGCCAGAGAA CATGAAGTAC TTTACTGGGC GAGTATGGGG AAAACTTACG CGGAGATCGC    620
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             AGCCAGAGAA CATGAAGTAC TTTACTGGGC GAGTATGGGG AA.ACTTACG CGGAGATCGC

halI/halR H.alvei AF503776    CACAATCATT GGTATTTCAG TAAGAACGGT TAAATTTCAC ATGGGCAACG TGGTAAGTAA    720
halI/halR068                  CACAATCATT GGGATTTCAG TAAGAACGGT TAAATTTCAC ATGGGCAACG TGGTAAGTAA    680
halI/halR067                  CACAATCATT GAGATTTCAG TAAGAACGGT TAAATTTCAC ATGGGCAACG TGGTAAGTAA    680
halI059                       ---------- ---------- ---------- ---------- ---------- ----------    0
halI071                       ---------- ---------- ---------- ---------- ---------- ----------    0
Clustal Consensus             CACAATCATT G..ATTTCAG TAAGAACGGT TAAATTTCAC ATGGGCAACG TGGTAAGTAA
```

Figure 4.8 - Multiple sequence alignment of *halI* gene of *H. alvei* 059, 068, 071, and *Enterobacter* sp. 067 (this study) with *halI* gene of *H. alvei* (Genbank accession number AF503776). The differences of identity are indicated by gray shading.

```
halI/halR H.alvei AF503776     ATTGGGTGTG AGTAACGCCC GTCAGGCGAT CAGGCTGGGC GTTGAACTCG AACTGATTAC    780
halI/halR068                   ATTGGGTGTG AGTAACGCCC GTCAGGCGAT CAGGCTGGGC GTTGAACTCG AACTGATTAC    740
halI/halR067                   ATTGGGTGTG AGTAACGCCC GTCAGGCGAT CAGGCTGGGC GTTGAACTCG AACTGATTAC    740
halI059                        ---------- ---------- ---------- ---------- ---------- AACTGATTAC    10
halI071                        ---------- ---------- ---------- ---------- ---------- AACTGATTAC    10
Clustal Consensus              ATTGGGTGTG AGTAACGCCC GTCAGGCGAT CAGGCTGGGC GTTGAACTCG AACTGATTAC

halI/halR H.alvei AF503776     ACCAATGCAG TCTTAATTAC CGCCACTGAC ATCGGCCAGT GCTCAAATTC AGGCAGTGAA    840
halI/halR068                   ACCAATGCAG TCTTAATTAC CGCCACTGAC ATCGGCCAGT GCTCAAATTC AGGCAGTGAA    800
halI/halR067                   ACCAATGCAG TCTTAATTAC CGCCACTGAC ATCGGCCAGT GCTCAAATTC AGGCAGTGAA    800
halI059                        ACCAATGCAG TCGTAA---C GGCTATCGAC ATTGGCCAAT TAACCAGCTG GGACTGAATA    67
halI071                        ACCAATGCAG TCTTAATTAC CGCCACTGAC ATCGGCCAGT GCTCAAATTC AGGCAGTGAA    70
Clustal Consensus              ACCAATGCAG TC.TAATTAC .GC.A..GAC AT.GGCCA.T ...C.A..T. .G.C.G...A

halI/halR H.alvei AF503776     CAACCTTCAA ATGCATTAAT TTTTGTCGCC ATTTTCGCCT GACTATCAGA GTCCGTAGGC    900
halI/halR068                   CAACCTTCAA ATGCATTAAT TTTTGTCGCC ATTTTCGCCT GACTATCAGA GTCCGTAGGC    860
halI/halR067                   CAACCTTCAA ATGCATTAAT TTTTGTCGCC ATTTTCGCCT GACTATCAGA GTCCGTAGGC    860
halI059                        CCACCGACTG AAGCGGTTAT TTTGGCTGCC ATTTTATTCT GGCTGACAGA GTCTGTCGGG    127
halI071                        CAACCTTCAA ATGCATTAAT TTTTGTCGCC ATTTTCGCCT GACTATCAGA GTCCGTAGGC    130
Clustal Consensus              C.ACC..C.. A.GC..T.AT TTT.G..GCC ATTTT...CT G.CT..CAGA GTC.GT.GG.

halI/halR H.alvei AF503776     AAATAGAGAA GATAAATCCT TTCCTCCTCA CTCAAATATG CCTCTTTAAG TACCCTCACC    960
halI/halR068                   AAATAGAGAA GATAAATCCT TTCCTCCTCA CTCAAATATG CCTCTTTAAG TACCCTCACC    920
halI/halR067                   AAATAGAGAA GATAAATCCT TTCCTCCTCA CTCAAATATG CCTCTTTAAG TACCCTCACC    920
halI059                        AGATAAACCA GATAAATTCG CTCATCCTCA CTCAGATATG CTTCCTTAAG CGGTTTAATT    187
halI071                        AAATAGAGAA GATAAATCCT TTCCTCCTCA CTCAAATATG CCTCTTTAAG TACCCTCACC    190
Clustal Consensus              A.ATA.A..A GATAAAT.C. .TC.TCCTCA CTCA.ATATG C.TC.TTAAG .....T.A..

halI/halR H.alvei AF503776     TGCCAGCCAC TGCGCTTTAG TATTGTCAGC ATAGCGCGGC TAACAATCGT ATAAATGCCG    1020
halI/halR068                   TGCCAGCCAC TGCGCTTTAG TATTGTCAGC ATAGTGCGGC TAACAATCGT ATAAATGCCG    980
halI/halR067                   TGCCAGCCAC TGCGCTTTAG TATTGTCAGC ATAGCGCGGC TAACAATCGT ATAAATGCCG    980
halI059                        TGCCAACCTG AACGTTTCAA TATTGTCAGC ATTGCTCGGC TGACGATAGT GTAAATACCG    247
halI071                        TGCCAGCCAC TGCGCTTTAG TATTGTCAGC ATAGCGCGGC TAACAATCGT ATAAATGCCG    250
Clustal Consensus              TGCCA.CC.. ..CG.TT.A. TATTGTCAGC AT.G..CGGC T.AC.AT.GT .TAAAT.CCG

halI/halR H.alvei AF503776     TTTAAGTTAT ATTGACGAGC GTAGTTGATC ATCGCCAGAA AAAGAACCTG ACTAACAGGA    1080
halI/halR068                   TTTAAGTTAT ATTGACGAGC GTAGTTGATC ATCGCCAGAA AAAGAACCTG ACTAACAGGA    1040
halI/halR067                   TTTAAGTTAT ATTGACGAGC GTAGTTGATC ATCGCCAGAA AAAGAACCTG ACTAACAGGA    1040
halI059                        TTGCAACCAT AGTGCCTAGC ATAATTTATC ATCGCTAAAA ATAGTACTTG GCTTAGCGGA    307
halI071                        TTTAAGTTAT ATTGACGAGC GTAGTTGATC ATCGCCAGAA AAAGAACCTG ACTAACAGGA    310
Clustal Consensus              TT..A...AT A.TG.C.AGC .TA.TT.ATC ATCGC.A.AA A.AG.AC.TG .CT.A..GGA

halI/halR H.alvei AF503776     TATCTCTCCC CCAGCAAATC TCGAGCGCGA CTTTTGTCGA CAAAGAATCG GCTTGATTCC    1140
halI/halR068                   TATCTCTCCC CCAGCAAATC TCGAGCGCGA CTTTTGTCGA CAAAGAATCG GCTTGATTCC    1100
halI/halR067                   TATCTCTCCC CCAGCAAATC TCGAGCGCGA CTTTTGTCGA CAAAGAATCG GCTTGATTCC    1100
halI059                        TATCGCTCGC CTAACAAATC CCTCGCGCGT GATTTATCAA CAAAAAATCG GCTTGATTCT    367
halI071                        TATCTCTCCC CCAGCAAATC TCGAGCGCGA CTTTTGTCGA CAAAGAATCG GCTTGATTCC    370
Clustal Consensus              TATC.CTC.C C.A.CAAATC .C..GCGCG. ..TTT.TC.A CAAA.AATCG GCTTGATTC.

halI/halR H.alvei AF503776     ACTTCACCGG CTGGCAATGA GACATCATGA AAACAAGAGT GGAAGGTATG AGTAATCATA    1200
halI/halR068                   ACTTCACCGG CTGGCAATGA GACATCATGA AAACAAGAGT GGAAGGTATG AGTAATCATA    1160
halI/halR067                   ACTTCACCGG CTGGCAATGA GACATCGTGA AAACAAGAGT GGAAGGTATG AGTAATCATA    1160
halI059                        GTTTCACCGG CAGGCAAAGG CACATCCGAA AAACAAGCCT GAAACGTGTG AGTAATCATA    427
halI071                        ACTTCACCGG CTGGCAATGA GACATCATGA AAACAAGAGT GGAAGGTATG AGTAATCATA    430
Clustal Consensus              ..TTCACCGG C.GGCAA.G. .ACATC...A AAACAAG..T G.AA.GT.TG AGTAATCATA

halI/halR H.alvei AF503776     TTTGGCAGTT CGAGCGGCAC AAAACGAACA CTGCAAATCA ACTGCCCTTC ATACAGACCC    1260
halI/halR068                   TTTGGCAGTT CGAGCGGCAC AAAACGAACA CTGCAAATCA ACTGCCCTTC ATACAGACCC    1220
halI/halR067                   TTTGGCAGTT CGAGCGGCAC AAAACGAACA CTGCAAATCA ACTGCCCTTC ATACAGACCC    1220
halI059                        TTAGGCTCAT CCAATGGAAC AAAGCGCACG CTGCAGACCA GCTGTCCCTC ATATAAACCT    487
halI071                        TTTGGCAGTT CGAGCGGCAC AAAACGAACA CTGCAAATCA ACTGCCCTTC ATACAGACCC    490
Clustal Consensus              TT.GGC...T C.A..GG.AC AAA.CG.AC. CTGCA.A.CA .CTG.CC.TC ATA.A.ACC.

halI/halR H.alvei AF503776     AAGATATAGC GCGTGTTTGG ATTATCAAAC TCATCAAACT CCATACCGCG GTTGCAAACG    1320
halI/halR068                   AAGATATAGC GCGTGTTTGG ATTATCAAAC TCATCAAACT CCATACCGCG GTTGCAAACG    1280
halI/halR067                   AAGATATAGC GCGTGTTTGG ATTATCAAAC TCATCAAACT CCATACCGCG GTTGCAAACG    1280
halI059                        AGGATGTAAC GTGTATTAGG ATTATCAAAC TCGTCAAACT CCATGTCTTT GTTGCAGACA    547
halI071                        AAGATATAGC GCGTGTTTGG ATTATCAAAC TCATCAAACT CCATACCGCG GTTGCAAACG    550
Clustal Consensus              A.GAT.TA.C G.GT.TT.GG ATTATCAAAC TC.TCAAACT CCAT..C... GTTGCA.AC.

halI/halR H.alvei AF503776     ACATCCCAAC CTAGTCGATC GCTGAAAGTT TTTTTTCTCA GTCGATAAAG TTCATCCGAA    1380
halI/halR068                   ACATCCCAAC CTAGTCGATC GCTGAAAGTT TTTTTTCTCA GTCGATAAAG TTCATCCGAA    1340
halI/halR067                   ACATCCCAAC CTAGTCGATC GCTGAAAGTT TTTTTTCTCA GTCGATAAAG TTCATCCGAA    1340
halI059                        ACATCCCATC CCAACCGGTC ACTGAACGTT TTTTTTCTCA GTCGATAGAG TTCATCAGAA    607
halI071                        ACATCCCAAC CTAGTCGATC GCTGAAAGTT TTTTTTCTCA GTCGATAAAG TTCATCCGAA    610
Clustal Consensus              ACATCCCA.C C.A..CG.TC .CTGAA.GTT TTTTTTCTCA GTCGATA.AG TTCATC.GAA

halI/halR H.alvei AF503776     CGCACTCCAT TTAGTTCATC ATAACTGACA TCAAATAGTT CAAGCATTCC ATCACCTTGA    1440
halI/halR068                   CGCACTCCAT TTAGTTCATC ATAACTGACA TCAAATAGTT CAAGCATTCC ----------    1390
halI/halR067                   CGCACTCCAT TTAGTTCATC ATAACTGACA TCAAATAGTT CAAGCATTCC ----------    1390
halI059                        CGGACCCCAT TCAGTTCTTC GTAACTGACA TCAAATAGTT CAAGCATTCC ----------    657
halI071                        CGCACTCCAT TTAGTTCATC ATAACTGACA TCAAATAGTT CAAGCATTCC ----------    660
Clustal Consensus              CG.AC.CCAT T.AGTTC.TC .TAACTGACA TCAAATAGTT CAAGCATTCC ATCACCTTGA
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Figure 4.8 (continued).