Investigation of quorum sensing process in Bacillus licheniformis.

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INVESTIGATION OF QUORUM SENSING PROCESS IN *BACILLUS LICHENIFORMIS*

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE UNIVERSITY OF WESTMINSTER FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MAY 2011

ABSTRACT

Quorum sensing is a well-established system of communication adopted by a number of bacterial, and some fungal, populations. This cell density dependent phenomenon is based on the accumulation of small diffusible molecules, termed as quorum sensing molecules, in the extracellular milieu until a threshold concentration triggers alteration in the expression of specific genes culminating in variety of responses including virulence, bioluminescence, sporulation, biofilm formation and secondary metabolites production.

In Bacilli, quorum sensing is mediated by small peptides that control competence, sporulation, and the production of certain secondary metabolites in a cell density dependent fashion. Two divergent pathways, triggered by the ComX pheromone and the Competence and Sporulation Factor (CSF), are engaged in the control of these processes.

B. licheniformis NCIMB 8874 is a bacterium with industrial relevance for the production of the antimicrobial agent bacitracin. This organism is genetically related to *Bacillus subtilis*, whose quorum sensing is regulated by the *comQXPA* operon. This study aimed to investigate the role of the *comQXPA* locus in *B. licheniformis* NCIMB 8874 and the production of potential signalling molecules in this bacterium.

Production of signalling molecule/s in *B. licheniformis* NCIMB 8874 was confirmed by the significant increase ($p>0.05$) in *srfA* expression in response to the addition of supernatants of *B. licheniformis* NCIMB 8874 cultures in their late exponential phase to low cell density cultures of *B. subtilis* reporter strains, carrying a *srfA-lacZ* fusion. The investigation of quorum sensing-regulated secondary metabolites production established production of lichenysin, γ-polyglutamic acid and extracellular proteases, whose biosynthesis is impaired in bacteria with disrupted *comQXPA* clusters. Bioinformatics studies on *B. licheniformis* NCIMB 8874 genome sequence confirmed the presence of essential quorum sensing-related genes, such as the *comQXPA* gene cluster, *comK*, *mecA* and *comS*. Moreover, *in silico* analysis allowed the identification of members of the Rap and Phr families, which aid the regulation of cell density dependent phenomena in *B. subtilis*.

The results presented in this work positively indicate that *B. licheniformis* NCIMB 8874 cell-cell communication operates in analogy with the well established *comQXPA*-controlled pathway of *B. subtilis*.

ACKNOWLEDGMENT

I would like to express my sincere gratitude to my Director of studies, Prof. Taj Keshavarz for his guidance and support throughout this experience and for his inspiring passion for research. His extraordinary help and continuous encouragement during the writing up of this thesis have been invaluable. In the course of this journey he has been not just a mentor, but a friend. I would also like to extend my gratitude to Dr Mark Clements for his precious help and support thoughout the project. And to Prof. Giovanni Sannia, for having confidence in me.

I would like to thank all the members of the lab, past and present, with whom I have shared these past four years: I owe it to all of them to have made this experience unique. Special thanks go to Patricia, Maryam, Rana and Eustace, who have made my time in the the lab more enjoyable (and the work less stressful!) with their help and company. To all the fellow PhD students with whom I have shared this experience, especially Dr Victor Ujor, for his friendship, and Myrsini, for the comforting words during the stressful period of write up. To Harpreet, for his unconditional emotional support and incessant encouragement, and for listening to my endless chattering when I needed to clarify my thoughts.

Many thanks to all the members of the technical staff of University of Westminster, who, to different extent, have helped me during the reaserch work.

Finally, I would like to thank my parents, for their love and support: this thesis would not have been a reality without them. I will be always grateful for the time spent at the phone, sharing a bit of this PhD with me even at a distance. To Rosa for always being there when I needed a friend, and Federica, for helping me in my thoughest times.

AUTHOR'S DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed: Date:

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACP	Acyl Carrier Protein
AIP	Autoinducing peptide
AHL	N-acyl-homoserine lactone
AI	Autoinducer
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	Base pair
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CSF	Competence and sporulation factor
Dha	Didehydroalanine
Dhb	2,3-didehydrobutyrine
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPD	4, 5-dihydroxy-2, 3-pentanedione
EDTA	Ethylenediaminetetraacitic acid
g	Gram
HPLC	High pressure liquid chromatography
HSL	Homoserine lactone
IPP	Isopentenylpyrophosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	Insertion sequence
LB	Lysogeny Broth
m	Meter or milli (10^{-3})
M	molar
MCS	Multiple cloning site
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial and Marine Bacteria
NMR	Nuclear Magnetic Resonance
NTP	Nucleoside triphosphate
OD	Optical density
ONPG	ortho-Nitrophenyl-β-galactoside
Opp	Oligopeptide permease
ORF	Open reading frame
PCR	Polymerase chain reaction
PGA	Polyglutamic acid
ppm	Part per million
RAP	RNAIII-Activating Protein
RNA	Ribonucleic acid
rpm	Rotations per minute
SAH	S-adenosyl-homocysteine

SAM	*S*-adenosylmethionine
SRH	S-ribosylhomocysteine
TRAP	Target of RNAIII-Activating Protein

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AIM

The overall aim of this work is to study the potential quorum sensing process in the Gram-positive bacterium *Bacillus licheniformis* NCIMB 8874 and to investigate the role of putative extracellular diffusible (quorum sensing) molecule(s) in this bacterium. To this end the following objectives were addressed:

- Investigation of the effect of *B. licheniformis* spent medium containing potential signalling molecule(s) on the expression of quorum sensing-regulated genes in *B. subtilis* reporter strains.
- Investigation of the production of secondary metabolites under the control of the quorum sensing cluster *comQXPA*.
- Investigation of the effect of *B. licheniformis* spent medium containing potential signalling molecule(s) on the production of secondary metabolites such as the antimicrobial agent lichenisyn, γ-polyglutamic acid and proteases.
- Genetic characterization of the cell-cell communication in *B. licheniformis* NCIMB 8874.

Chapter I

Introduction

INTRODUCTION

1.1 Quorum Sensing: Discovery and Definition

"A few words are necessary on the mass-action of bacteria. It is a common observation, one made by the writer at least a hundred times, that in culture media not exactly adapted to the needs of the organism, a scanty inoculation may not give any growth—not even after a long time—whereas a copious one will lead to a growth which gradually clouds the fluid or covers the solid....The only explanation I can think of is that a multitude of bacteria are stronger than a few, and thus by union are able to overcome obstacles too great for the few (Smith, 1905)."

For centuries, recognition and co-operation between cells in the unicellular bacterial world have been considered very unlikely, although co-operative behaviour between bacterial cells can be highly advantageous in their life cycle, particularly when they are involved in processes such as conjugation, symbiosis and niche adaptation, production of secondary metabolites, and population migration. However, the notion that bacteria are simple organisms with little capacity to interact with one another has been dramatically challenged by the discovery in bacterial populations of a complex system of communication consisting of diffusible chemical signals (Nealson *et al.*, 1970a). The first indication of bacterial cell-cell communication was introduced in 1965, when Tomasz suggested that the regulation of competence in *Streptococcus pneumoniae* was aided by a hormone-like extracellular product (Tomasz and Beiser, 1965). This hormone-like molecule was later recognized as a peptide, a widespread signal in cell-cell communication amongst Gram-positive bacteria (Dunny and Leonard, 1997, Lazazzera and Grossman, 1998).

However, cell-cell signalling and coordinated microbial group behaviour was officially ascertained by Nealson and co-workers, who reported that the bioluminescence developed by the marine bacterium *Vibrio fischeri* (formerly *Photobacterium fischeri*) in its symbiotic relationship with the Hawaiian squid *Euprymna scolopes* (*E. scolopes*) was controlled by one or more signalling molecules accumulating in the extracellular

milieu as a function of cell growth (Nealson *et al.*, 1970a). The relationship between *E. scolopes* and *V. fischeri* represents a noticeable example of cooperative development and growth for both the fish and the bacterium: whilst the luminescence provided by *V. fisheri* is advantageous for the squid as it serves as a system for communication, defence, and/or attraction, the bacterium, in turn, exploits its host as a stable source of nutrients (McFall-Ngai and Ruby, 1991, Ruby and McFall-Ngai, 1992). *V. fischeri* cells do not emit light in their free-living state, since the low cell densities occurring in seawater (100 cells mL^{-1}) do not allow the signal accumulation up to the threshold level. However, when *V. fischeri* infects the light organ of the squid, where the cell density reaches 10^{10}-10^{11} cells mL^{-1}, the signal molecules can accumulate to an adequate concentration to trigger the transcription of genes encoding the luminescence enzymes (Eberhard, 1972, Eberhard *et al.*, 1981a, Eberhard *et al.*, 1986). Figure 1.1 depicts the various stages of the infection and colonisation of *Euprymna scolopes* light organ by *V. fisheri*.

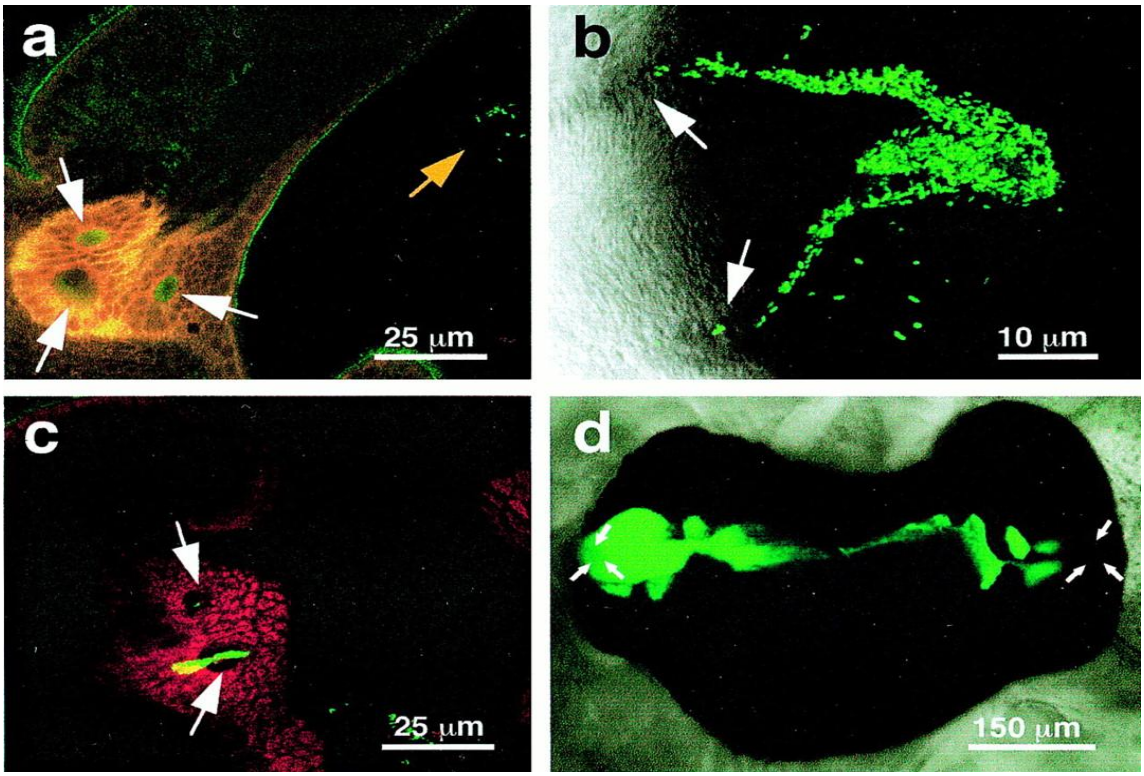


Figure 1.1: Infection and colonization of *E. scolopes* light organ by *V. fischeri* cells. (a) 1 hour post-inoculation: a small aggregate (orange arrow) of GFP-labelled *V. fischeri* cells (green) forms above a pore of the light organ (b) 2-4 hours post-

inoculation: *V. fisheri* cells migrate from the aggregate to the pores. (c) 4-6 hours post-inoculation: *V. fischeri* cells migrate through a pore and into a duct of the light organ (Cells within the duct appear yellow). (d) The light organ of *E. scolopes* is fully colonized by *V. fisheri* cells. White arrows indicate the location of the pores in all panels (Taken from Nyholm *et al.*, 2000).

The mechanism by which *V. fischeri* regulates bioluminescence was originally called “autoinduction” (Nealson, 1977), but later research on the mechanism of cell communication suggested that autoinduction was only one of the common features characterising the phenomenon. Microbial cell-cell signalling has become known as “Quorum Sensing” (Fuqua *et al.*, 1994), upon the principle that the communication among the population can only be successful when the concentration of external signal reaches a threshold or "quorum." Once a critical cell mass is reached, a number of target genes is activated or repressed for the development of processes that necessitate the cooperation of a large number of cells in order to be effective (Surette and Bassler, 1998).

Since its discovery cell to cell communication has been established in numerous microbial species, where it has been associated with processes such as bioluminescence, antibiotic production, conjugative DNA transfer, sporulation, virulence, biofilm formation and biosurfactant production (Swift *et al.*, 2001).

Signal molecules implicated in cell-to-cell communication are known as autoinducers or quorum sensing molecules and their function is to regulate gene expression in other cells of the community which in turn control a number of bacterial responses (Nealson *et al.*, 1970b) .

1.2 Quorum sensing mechanisms and regulation

1.2.1 Characteristics of a quorum sensing molecule

Bacterial cells produce and secrete a wide variety of small molecules with diverse specific roles in the modulation of metabolic activities characterising natural microbial communities, which might potentially be involved in cell-cell signalling and

communication (Yim *et al.*, 2006). The term “quorum sensing molecule”, however, can only be assigned to those small, diffusible molecules with an established role in cell communication. Based on the literature it is possible to enlist some important factors that are common amongst all the quorum sensing molecules that have been reported to date.

Therefore, for a molecule to be classified as a quorum sensing molecule, it needs to fulfil all of the following characteristics:

- The molecule is produced at a basal level throughout the cell growth, but the quorum sensing response is only initiated at a certain stage, which is species specific. This is usually determined by the cell density of the organism, or certain physiological conditions, or in response to changes in the environment (Winzer *et al.*, 2002b). Examples are secondary metabolite production (Bainton *et al.*, 1992b), sporulation (Weinrauch *et al.*, 1990), competence regulation (Magnuson *et al.*, 1994), virulence determination (Zhu *et al.*, 2002a) and morphological differentiation (Ochi, 1987).
- The molecule must be synthesised inside the cell and later secreted in the extracellular milieu where it is recognised by a specific receptor, such as the ComX pheromone regulating competence development in *B. subtilis* (Winzer *et al.*, 2002b, Magnuson *et al.*, 1994).
- Accumulation of the compound after a critical threshold concentration should produce a concerted response from all the cells belonging to the microbial population under study, such as the development of bioluminescence in *V. fisheri* cells after colonisation of the light organ of the squid (Winzer *et al.*, 2002a, Nealson *et al.*, 1970, Winzer *et al.*, 2002b).
- The molecule must be able to elicit a similar response when added exogenously to the null mutant cultures as it would do when endogenously expressed by the producer organism, as shown for the reinstatement of carbapenem production in *Erwinia carotovora* by exogenous addition of N-(3-oxohexanoyl) homoserine lactone (Bainton *et al.*, 1992a, Winzer *et al.*, 2002b).

- The molecule must exert a response in the host organism which is not only related to metabolising or detoxifying the molecule itself. An example is the activation of virulence determinants by N-(3-oxododecanoyl)-acyl-homoserine lactone in *Pseudomonas aeruginosa* (Winzer *et al.*, 2002a, de Kievit and Iglewski, 2000, Winzer *et al.*, 2002b).

The extent of the response generated by a chemical signal is the most important trait that needs to be taken into account when assigning a quorum sensing function to a given molecule (Winzer *et al.*, 2002b), as the other criteria are met by many other molecules. Molecules such as toxic metabolites or by-products, for example, build up in the extracellular milieu throughout the growth and might trigger a stress response once they reach a critical concentration. In such circumstances the molecules cannot be considered intercellular signals, as the cell population is purely acting in response to the toxic effect generated by the accumulation of such compounds in the environment. Likewise, certain compounds secreted in the extracellular medium, such as antibiotics, are able to induce their specific uptake machinery, thereby influencing the expression of genes involved in unrelated metabolic pathways. Indeed it has been suggested that antibiotics might be considered as quorum sensing molecules due to their capacity to modulate gene expression at sub-growth-inhibitory concentrations (Yim *et al.*, 2006). However, though some antibiotics, such as nisin from *Lactobacillus lactis* (Quadri, 2002) or subtilisin from *Bacillus subtilis* (Kleerebezem *et al.*, 2004, Kleerebezem *et al.*, 1997), are well established quorum sensing molecules, there exist a wide number of antibiotics whose involvement in cell-to-cell communication has not been determined. In conclusion quorum sensing molecules are involved in the regulation of processes whose purpose is far more complex than the mere metabolising toxins or nutrients; they enable the entire bacterial population to benefit from their number (cell density) or to prepare for the problems connected with it (Winzer *et al.*, 2002b).

1.2.2 Quorum sensing in Gram negative bacteria

1.2.2.1 N-acyl-homoserine lactones

The first Quorum Sensing molecule to be elucidated was N-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) the autoinducer responsible for the activation of bioluminescence in *V. fischeri* (Eberhard *et al.*, 1981b). The process regulating bioluminescence in *V. fischeri* is well established and can be simplified as depicted in Figure 1.2.

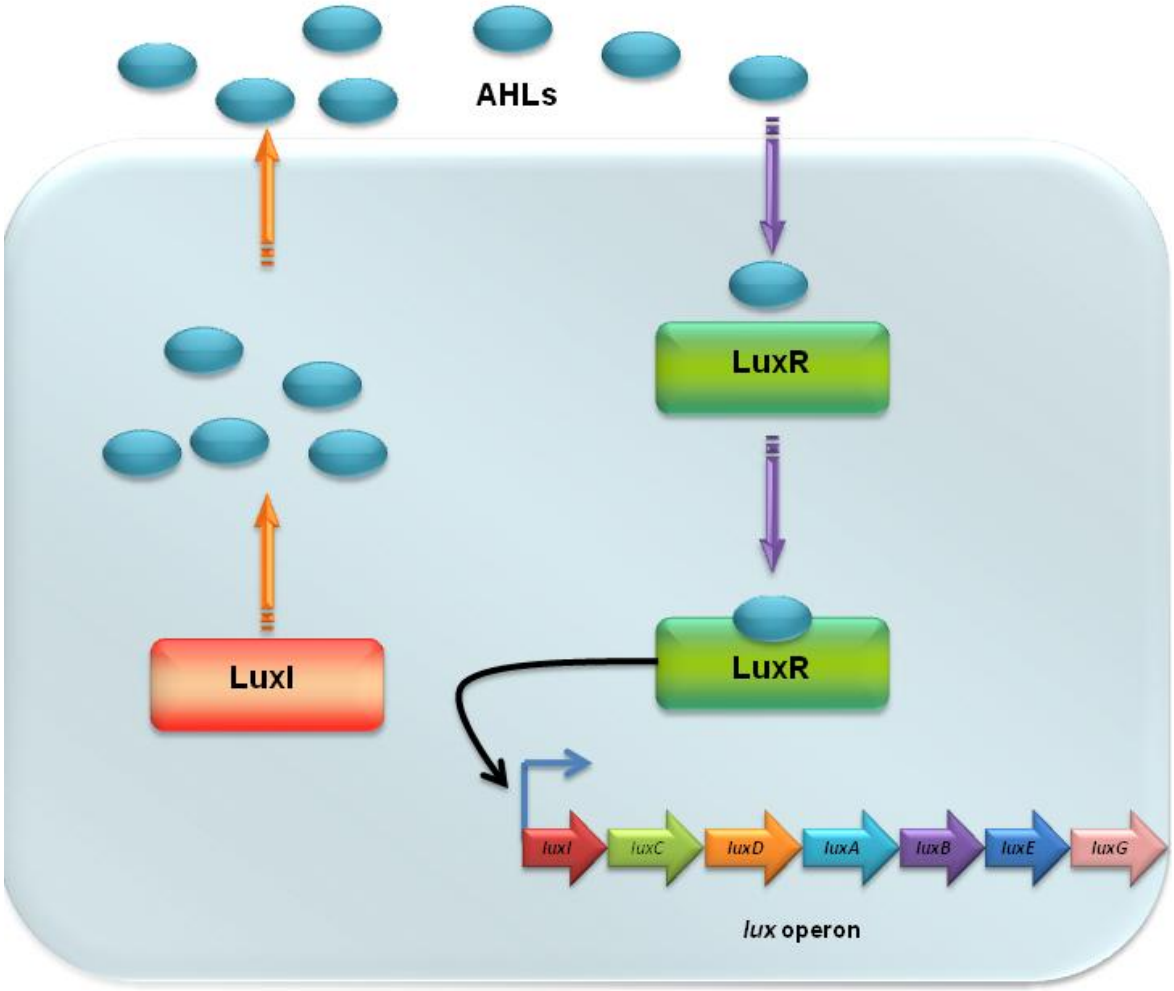


Figure 1.2: Quorum sensing process in Gram negative bacteria. Acyl homoserine lactones (AHLs) are produced by LuxI and are detected by LuxR-type protein**.** AHLs diffuse into the extracellular space in response to the changes in the surrounding

environment. AHLs bind to LuxR and activate the transcription of the *lux* operon (*luxICDABEG*) (Adapted from Miller and Bassler, 2001).

The LuxI protein catalyses the synthesis of 3-oxo-C6-HSL which upon interaction with the transcriptional regulator LuxR, triggers the transcription of the *lux* operon (*luxICDABEG*), thus leading to the activation of luciferase gene (Engebrecht *et al.*, 1983).

Since 3-oxo-C6-HSL discovery many quorum sensing molecules have been identified, which vary within different groups of micro-organisms. It has been widely accepted that Gram-negative bacteria utilise various AHLs to regulate the mechanisms which help them to adapt to changes in the environment (Withers *et al.*, 2001). AHL signals appear to be dedicated molecules produced with the sole purpose of mediating specific quorum sensing processes.

Most Gram-negative bacteria synthesise more than one AHL which are characterised by a homoserine lactone (HSL) ring whose β- and γ-positions are unsubstituted, whilst the α-position features an *N*-acylation with a fatty acyl group. Different AHLs are usually characterised by acyl chains with variable length, saturation level and oxidation state. The chemical structure of some AHLs regulating quorum sensing in Gram-negative bacteria is listed in Table 1.1. Although in the majority of cases the acyl chain has an even number of carbon atoms (C4–C18), AHLs with acyl chains containing 5 or 7 carbons have also been identified (Horng *et al.*, 2002, Lithgow *et al.*, 2000). In AHL-dependent quorum sensing systems the specificity of the transcriptional activator protein (usually a LuxR homologue) for its cognate AHL depends on both the length of the acyl side chain and chemical modification at the β-position of the HSL ring (Horng *et al.*, 2002, Lithgow *et al.*, 2000).

AHL signals are amphipathic molecules which can diffuse through the phospholipid bilayer of the cell membrane as well as move through the aqueous intracellular and extracellular milieus: this property derives from the balance between the hydrophilicity of the HSL ring and the hydrophobicity of the acyl side chain (Pearson *et al.*, 1994, Kaplan and Greenberg, 1985).

The shortest AHL signals identified to date are *N*-butanoylhomoserine lactone (C4-HSL) and *N*-hydroxybutanoylhomoserine lactone (3-hydroxy-C4-HSL) (Cao and Meighen, 1989, Winson *et al.*, 1995) and the future discovery of natural HSL-derived signals with shorter acyl chains is considered unlikely due to the AHLs chemical properties.

Yates and co-workers showed that the AHLs concentration in cultures of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa* (two human pathogens producing AHLs with acyl side chains ranging from 4 to 12 carbons in length) peaked during the exponential phase of growth and suddenly dropped at the onset of the stationary phase. However, no enzyme responsible for the inactivation of AHLs was identified in the extracellular medium of these organisms (Yates *et al.*, 2002).

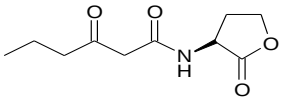
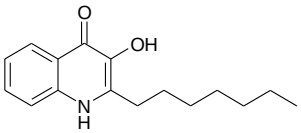
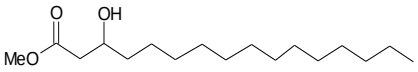
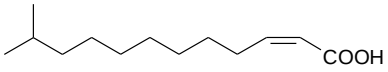
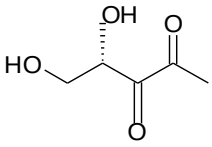
The rapid decrease in AHLs concentration was found to be caused by pH-dependent lactonolysis (hydrolysis of the HSL ring) due to the increase in pH registered at the onset of stationary phase and caused by release of ammonia from aerobic degradation of peptides as carbon and energy sources (Yates *et al.*, 2002).This phenomenon was found to be dependent on the length of the acyl side chain, as AHLs with longer chains are less susceptible to ring opening. Therefore, AHLs require an *N*-acyl side chain of at least four carbons in length in order to be functional under physiological conditions in mammalian tissue fluids, and the longer the acyl side chain the more stable the AHL signal molecule (Yates *et al.*, 2002).

AHL signal molecules are employed by several bacteria, where they regulate a number of processes. Examples are: serine protease production in *Aeromonas hydrophila* regulated by N-butanoyl-HSL; carbapenem synthesis by *Erwinia carotovora* controlled by N-(3-oxohexanoyl)-HSL and virulence determinants in *Pseudomonas aeruginosa* activated by N- (3-oxododecanoyl)-HSL (Miller and Bassler, 2001a).

Each system consists of an AHL receptor and signal transducer, homologue to *V. fisheri* LuxR, and a cognate AHL signal molecule which is synthesized by a *luxI* homologue. Once a threshold extracellular concentration of the autoinducer is reached, the regulatory protein binds to its cognate signal molecule in order to

modulate target gene(s) expression. Several AHL-dependent quorum sensing systems have been shown to possess multiple LuxR/LuxI/AHL modules

Table 1.1: *Chemical structure of some quorum sensing signals present in Gram-negative bacteria.*

Signal	Molecule	Organism
Acyl-homoserine lactones (Eberhard *et al.*, 1981a)	**3-oxo-C6-HSL** 	*Vibrio fischeri*
Alkyl quinolones (Pesci *et al.*, 1999)	**2-heptyl-3-hydroxy-4(1*H*)-quinolone** 	*Pseudomonas aeruginosa*
Fatty acid methyl esters (Flavier *et al.*, 1997)	**3-Hydroxylpalmitic acid methyl ester** 	*Ralstonia solanacearum*
Long chain fatty acids (Huang and Lee Wong, 2007)	***cis*-Δ2-11-methyl-dodecenoic acid** 	*Stenotrophomonas maltophilia*
Autoinducer-2 (Schauder *et al.*, 2001)	**DPD** 	*Vibrio harveyi*

1.2.2.2 AHLs biosynthesis by LuxI homologues

As previously highlighted, the enzymes responsible for AHLs synthesis are usually members of the LuxI protein family**.** These enzymes function as AHL synthases when provided with *S*-adenosylmethionine (SAM) as the amino donor (source of HSL ring moiety) and an appropriate acyl–Acyl Carrier Protein (acyl-ACP) as an acyl chain donor (Moré *et al***.** 1996; Jiang *et al***.** 1998; Parsek *et al***.** 1999). SAM binds to LuxI, followed by the appropriate acyl-ACP. Subsequently, SAM forms an amide bond with the acyl group, thus leading to the release of holo-ACP. Lactonization of the HSL ring occurs and the product, AHL, is liberated together with methylthioadenosine (Fuqua and Winans, 1996, Sitnikov *et al.*, 1995, Parsek *et al.*, 1997, Salmond *et al.*, 1995).

All LuxI-type proteins appear to be AHL synthase orthologues, which catalyse the formation of the amide bond between the acyl side chain and the amino group of SAM. However, given that several AHLs have been identified with structurally different acyl side chains, the members of the LuxI family of protein must be selective towards the acyl-ACPs involved in the catalysis (Andersson *et al.*, 2000, Choi and Greenberg, 1991, Devine *et al.*, 1989); LuxI homologues are characterised by protein sequence ranging from 194 to 226 amino acids and although sequence alignment revealed that most of them have low sequence homologies, a sequence of ten invariant residues was found in the N-terminal region of each polypeptide (Fuqua *et al.*, 1995, Parsek *et al.*, 1997). Therefore it has been proposed that the less conserved C-terminal region might be involved in binding of the appropriate acyl-ACP and delivery of the acyl group to the active site (Fuqua *et al.*, 1995, Parsek *et al.*, 1997).

Two LuxI homologues, EsaI from *Pantoea stewartii* and LasI from *Pseudomonas aeruginosa* were found to belong to the GCN5-related *N***-**acetyltransferase protein family through analysis of their crystal structures (Gould *et al.*, 2004, Watson *et al.*, 2002). Although EsaI and LasI produce AHLs characterised by different side-chain lengths (3-oxo-C_6-HSL and 3-oxo-C_{12}-HSL respectively) studies conducted on their structures has revealed a common binding site for the

acyl-ACP phosphopantetheine prosthetic group. In EsaI a threonine residue (Thr140) of the acyl-chain binding site was found to block the activity of the enzyme against unsubstituted acyl-ACPs (Gould *et al.*, 2004, Pearson *et al.*, 1994, Watson *et al.*, 2002, von Bodman *et al.*, 1995). EsaI mutants with a threonine 140-to-alanine substitution showed a dramatic shift in AHL production from 3-oxo-C_6-HSL to C6-HSL (Watson *et al.*, 2002). Loss of specificity was also observed in the LasI mutants obtained by converting the threonine residue to a number of other amino acids identified in the same position in different AHL synthases. This study confirmed that a specific threonine residue in the acyl-chain binding site has an important role in determining the specificity showed by the members of the AHL synthase family towards 3-oxo-acyl-ACPs. Comparison of specific AHL synthase sequences and their product AHLs suggested that whereas the Thr140 is related to the production of 3-oxo-HSLs, alanine and glycine residues at the same position correlate with unsubstituted AHLs whilst serine is associated with the production of 3-hydroxy-HSLs (Watson *et al.*, 2002).

The production of specific AHLs by a given LuxI-type is dependent on the available acyl-ACP pools in the cell, which in turn is susceptible to metabolic changes. Bacteria produce fatty acids by extending the chain length of acyl-ACP through the addition of two carbons from malonyl-coenzyme A. As a consequence, it has been shown that AHLs production in a bacterium can be altered by modulating the fatty acid biosynthetic pathway. *P. aeruginosa* mutant strains with reduced FabG (β-ketoacyl-ACP reductase) activity have an impaired ability to elongate acyl-ACPs, resulting in the production of AHLs with shorter chains when compared with the wild-type (Hoang *et al.*, 2002).

LuxI homologues are not the only enzymes involved in AHL(s) production, as another AHL synthase, namely LuxM, has been identified in *Vibrio harveyi*, where it is positively involved in bioluminescence regulation (Bassler *et al.*, 1993). Further studies on this enzyme have led to the discovery of LuxM homologues in other *Vibrio* species, e.g. AinS in *V. fisheri* (Hanzelka *et al.*, 1999, Milton *et al.*, 2001).

Although the amino acid sequences of both AinS and LuxM do not show any similarity to LuxI, both the enzymes have been implicated in the synthesis of AHL(s)

using similar precursors to those identified for LuxI homologues (Bassler *et al.*, 1993, Gilson *et al.*, 1995). However, in contrast with LuxI-type proteins, whose primary sources for AHL side chains are acyl-ACPs, AinS was shown to use indifferently octanoyl-ACP or octanoyl-CoA conjugates as precursors for octanoyl-HSL biosynthesis. Acyl-ACP conjugates and acyl-CoA derivatives have different functions in bacterial cells, the formers being intermediates in fatty acid synthesis and the latter employed as intermediates of fatty acid oxidation. It has been hypothesized that *V. fisheri* cells might benefit from AinS flexibility towards its fatty acyl substrates under conditions that lead to an imbalance between these precursors pools. Whether this mechanism of action is shared by LuxM in the synthesis of 3-hydroxy-butanoyl-HSL has yet to be determined (Hanzelka *et al.*, 1999).

While the involvement of LuxM in the activation of bioluminescence has been established, no target gene has been identified for the AinS/octanoyl-HSL module so far. The identification of a DNA sequence resembling the *lux* box (a promoter element required to activate *lux* gene expression) upstream to *ainS* seems to indicate that *ainS* gene expression might be regulated by the LuxI-LuxR quorum sensing system (Gilson et al., 1995). Furthermore, it has been postulated that octanoyl-HSL might compete with 3-oxohexanoyl-HSL (LuxI product) for binding to LuxR, thus modulating the activity of the LuxI-LuxR quorum sensor (Kuo *et al.*, 1996).

Autoinducers produced via LuxM-type proteins, on the other hand, do not interact with a LuxR-type transcriptional regulator. In *V. harveyi*, for example, the sensor for 3-hydroxy-C4-HSL, named LuxN, is a histidine-kinase protein (Bassler *et al.*, 1993, Bassler *et al.*, 1994a), whose interaction with AHLs in the periplasm triggers a phosphorelay cascade which results in the activation of the target quorum sensing dependent genes (Camara *et al.*, 2002, Croxatto *et al.*, 2004).

A third potential AHL synthase (HdtS) was identified in *P. fluorescens*, which does not belong to either the LuxI or LuxM family. This lysophosphatidic acid acyltransferase is a protein of approximately 33 kDa capable of directing the synthesis of *N*-(3-hydroxy-7-*cis*-tetradecenoyl)homoserine lactone (3OH,$C_{14:1}$-HSL),

N-decanoylhomoserine lactone (C_{10}-HSL) and *N*-hexanoylhomoserine lactone (C_6-HSL) (Laue *et al.*, 2000).

1.2.2.3 AHLs recognition and response: LuxR-type proteins

Genetic and biochemical studies on *V. fischeri* LuxR protein and its homologues, mostly performed in *E. coli*, have contributed to the identification of these proteins as AHL receptors in Gram negative bacteria quorum sensing systems (Gilson *et al.*, 1995, Hanzelka and Greenberg, 1995). LuxR from *V. fisheri* contains 250 amino acid residues and it was found to be a two-domain polypeptide, whose folding into active confirmation is aided by the chaperone GroEL/ES complex (Adar *et al.*, 1992, Adar and Ulitzur, 1993, Dolan and Greenberg, 1992). Members of the LuxR family are characterised by sequence identity of 18–23%, though two clusters of higher sequence conservation have been identified which represent the AHL(s) interaction domain in the N-terminus of the protein and the DNA binding motif located on the C-terminal domain. Specific functions have been assigned to specific LuxR regions through analysis of a number of *luxR* products comprising either single-amino-acid alterations or deletions in the N- or C- terminus (von Bodman *et al.*, 1998, Parsek and Greenberg, 2000, Parsek *et al.*, 1999, Chai *et al.*, 2001, Chancey *et al.*, 1999).

The best evidence for the receptor activity of LuxR-type proteins has been obtained through analysis of purified preparations of these proteins. When purified TraR from *A. tumefaciens* and CarR from *Erwinia carotovora* were mixed with their cognate acyl-HSLs (3-oxo-octanoyl-HSL and 3-oxo-hexanoyl-HSL, respectively) in an equimolar ratio of acyl-HSL to protein, the formation of a stable complex could be observed (Welch *et al.*, 2000, Zhu and Winans, 1999).

Studies performed on truncated LuxR proteins have confirmed that the N-terminal region mediates the binding to recognized AHL signals, as derivatives of LuxR from *V. fisheri* and LasR from *P. aeruginosa* lacking their N-terminal domanins led to constitutive transcription of quorum sensing target genes (Hanzelka and Greenberg, 1995). A model has been postulated whereby amino acids located at the extreme N-

terminus might be involved in the repression of the *luxR* gene in the absence of AHL signal. Interaction with the cognate AHL(s) causes a change in LuxR conformation which leads to exposure of the C-terminal domain, thereby relieving it from inhibition (Choi and Greenberg, 1991, Pesci *et al.*, 1997, Anderson *et al.*, 1999). Binding to the specific AHL signal has been suggested to also stimulate LuxR multimer formation and association to the *lux* boxes upstream of the *lux* operon (Chancey *et al.*, 1999). Both the HSL ring and the acyl chain might be involved in interactions with LuxR (Passador *et al.*, 1996, Schaefer *et al.*, 1996)

The C-terminal region of LuxR homologues, comprising an essential helix-turn-helix motif (HTH), is required for DNA binding and transcriptional activation (Shadel *et al.*, 1990, Slock *et al.*, 1990). LuxR-type proteins belong to the FixJ-NarL superfamily (Kahn and Ditta, 1991), consisting of two-component-type response regulators, whose DNA binding activity is regulated by phosphorylation of a conserved aspartate residue in the amino-terminal domain of the proteins. Therefore, it is not surprising that no significant sequence similarity has been detected between the N-terminal regions of LuxR homologues and members of the FixJ-NarL group (Kahn and Ditta, 1991), as the specific function of the N-terminus of LuxR-type proteins is to interact with AHL(s) (Fuqua *et al.*, 1994). Despite the lack of similarity in their N-terminal regions LuxR-type proteins and other members of the FixJ-NarL superfamily show common features in their mechanisms of action.

A helix-turn-helix (HTH) motif which characterizes the DNA-binding regions of most transcription factors has been identified in the C-terminal region of LuxR-homologues (Fuqua and Greenberg, 1998), whilst the so-called C-terminal tail was shown to be critical for activation of *luxICDABEG* but not for *luxR* autoregulation, thus indicating that this region might be involved in contacting RNA polymerase necessary for activation rather that in DNA binding (Chai *et al.*, 2001).

LuxR-type proteins usually activate transcription by interacting with DNA sequences associated with their target genes. A 20-bp palindromic sequence, known as *lux* box, located at 42.5 bp upstream of *V. fischeri luxICDABEG* operon has been identified as the binding site for LuxR (Devine *et al.*, 1989, Gray *et al.*, 1994, Egland and Greenberg, 1999). Trascriptional activation mediated by LuxR homologues occurs

when the AHL/LuxR-type protein complex binds upstream of the transcriptional start site and recruits RNA polymerase through direct contact (Vannini *et al.*, 2002, Zhang *et al.*, 2002). DNA sequence elements characterized by sequence identity with the original *lux* box have been detected upstream of promoters of genes regulated by LuxR-type proteins in a number of bacteria. These *lux*-type boxes are characterized by lengths ranging from 18 to 22 bp and are usually located just upstream of the -35 promoter element (Egland and Greenberg, 1999, Stevens and Greenberg, 1999).

The process undertaken by LuxR homologues in response to the accumulation of AHL signals to the threshold concentration abides by specific steps which can be summarized as follows (Fuqua *et al.*, 1994):

1. Bind specifically with cognate AHLs;
2. Conformational changes and alterations in multimerization of the protein upon binding of the signal molecule;
3. Interaction with specific regulatory sequences upstream of target genes;
4. Activation of transcription.

1.2.3 Autoinducer-2 and LuxS mediated quorum sensing

It has been recently discovered that bioluminescence in *Vibrio harveyi* is controlled by a complex mechanism which shares characteristics found in both typical Gram-negative and typical Gram-positive quorum sensing systems. Analogous to other Gram-negative bacteria, *V. harveyi* secretes and responds to an AHL-type signal called Autoinducer 1 or AI-1 (Bassler *et al.*, 1993). Similar to Gram-positive bacteria the AHL-mediated processes in *V. harveyi* employ a two-component signal transduction system. Moreover, a novel signalling molecule, named Autoinducer 2 or AI-2 was found in *V. harveyi* (Schauder *et al.*, 2001, Chen *et al.*, 2002).

Both AI-1 and AI-2 play a part in regulation of the transcription of the luciferase operon *luxCDABEGH*. Whilst the AI-1 is a conventional AHL (N-(3-hydroxybutanoyl)-L-homoserine lactone) encoded by *luxLM*, the AI-2, encoded by *luxS*, does not show

similarity with other known signalling molecules. AI-2 is a furanosyl borate diester synthesised from SAM (the same substrate involved in AHLs synthesis) after a number of enzymatic reactions, described in Figure 1.3. The enzymatic reaction involving a SAM-dependant transmethylase deprives SAM of a methyl group leading to the formation of S-adenosyl-homocysteine. In the presence of enzyme Pfs (S-adenosylhomocysteine/5'-methylthioadenosine nucleosidase) an adenosine molecule is released by the toxic S-adenosyl-homocysteine (SAH) which gets hydrolysed to S-ribosylhomocysteine (SRH). LuxS then catalyses the cleavage of SRH, which results in the formation of homocysteine and the AI-2 precursor, known as DPD (4, 5-dihydroxy-2, 3-pentanedione). DPD is a highly unstable compound that readily cyclizes to form various furanone ring structures such as the AI-2 (Bacon Schneider *et al.*, 2002, Bassler *et al.*, 1997b, Bassler *et al.*, 1997a, Surette *et al.*, 1999a).

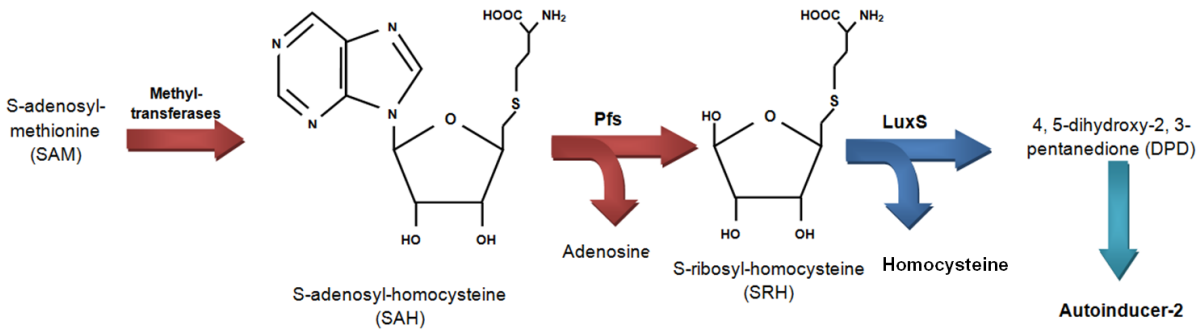


Figure 1.3: Autoinducer-2 biosynthetic pathway**:** a SAM-dependant transmethylase removes a methyl group from SAM leading to the formation of SAH. The toxic SAH releases an adenosine molecule through a reaction catalysed by Pfs, which result in SRH formation. LuxS then catalyses the cleavage of SRH in homocysteine and the AI-2 precursor, known as DPD (4, 5-dihydroxy-2, 3-pentanedione). DPD readily cyclizes to form the AI-2 (Bassler *et al.*, 1997b, Surette *et al.*, 1999b).

The detection of AI-1 and AI-2 signals occurs via the cognate hybrid sensor kinases called LuxN and LuxPQ which convey information to a common phosphorelay protein called LuxU, which in turn transfers the signal to the response regulator protein LuxO. At low cell densities, the concentration of both the autoinducers is lower than the

threshold required for the activation of quorum sensing mechanism. At these concentrations, the LuxN and LuxQ sensors autophosphorylate on their conserved His residues and then phosphorylate the conserved Asp residues in their response regulator domains (Freeman and Bassler, 1999, Freeman *et al.*, 2000). Subsequently, both sensors transfer their phosphate groups to LuxU which is responsible for LuxO phosphrylation. The activated LuxO triggers repression of the *luxCDABEGH* operon. However at high cell-densities a high concentration of AI-2 leads to the dephosphorylation of LuxQ by a phosphatase thereby leading to activation the luciferase gene operon (Freeman and Bassler, 1999, Martin *et al.*, 1989, Showalter *et al.*, 1990, Bassler *et al.*, 1994a, Bassler *et al.*, 1994b). A schematic representation of this mechanism is depicted in Figure 1.4.

Although originally described in *V. harveyi* AI-2 is now known to be produced by more than 70 bacterial species, and homologous of the enzyme LuxS have been found in several bacteria whose genomes have been sequenced. Moreover, AI-2 molecules produced by heterologues organisms were found able to activate luminescence in a *V. harveyi* reporter strain. These results taken together suggest the hypothesis that the AI-2 may be a universal signal molecule, which intriguingly, appears to be used by different organisms for diverse purposes (Schauder *et al.*, 2001, Bassler *et al.*, 1997b).

Microarray analysis performed on a *luxS* null mutant of enterohaemorrhagic *E. coli* (EHEC) showed how AI-2 signalling affects a large regulon consisting of 5–10% of the *E. coli* genome. In particular, genes involved in cell division, along with ribosomal and tRNA genes resulted to be downregulated by LuxS, whereas upregulated genes included several virulence factors such as genes involved in flagella biogenesis, motility and chemotaxis. Later, 242 genes of *E. coli* were found to respond to the presence of AI-2, when it was added to conditioned medium. These genes are either related to virulence or to processes such as cell division and morphogenesis (DeLisa *et al.*, 2001). LuxS is also concerned in the production of the type III secretion apparatus responsible for cell adherence to the intestinal epithelia in both EHEC and enteropathogenic *E. coli* (Sperandio *et al.*, 1999).

Regulation of virulence development in *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, two Gram-negative oral pathogens (Chung *et al.*, 2001, Fong *et al.*, 2001, Burgess *et al.*, 2002), and *Salmonella typhimurium* (Taga *et al.*, 2001, Taga *et al.*, 2003) has been shown to be a cell density-dependent phenomenon controlled by the AI-2/LuxS complex (Fong *et al.*, 2001, McNab *et al.*, 2003).

Virulence gene expression and biofilm formation in *Vibrio cholerae* are triggered by both AI-2 and the species-specific AI-1. *V. cholerae* cells use their signalling molecules to induce the production of virulence factors at low cell density and repress them at higher cell density (Miller *et al.*, 2002). In particular, genes involved in biofilm formation and the production of cholera toxin, together with several other virulence-associated genes are expressed at low cell density when the concentration of the autoinducer is too low to be detected (Zhu *et al.*, 2002b). All these gene products allow *V. cholerae* cells to adhere to intestinal epithelial cells and cause the diarrheal disease cholera. As the autoinducer concentration increases at high cell density, the expression of virulence-related genes is inhibited. Simultaneously, the accumulation of quorum aensing molecules induces the expression of the *hap* gene encoding the Hap protease. The role of this protease is suggested to be that of a “detachase”, allowing *V. cholerae* to be freed from the intestine walls in order to re-enter the environment (Finkelstein *et al.*, 1992).

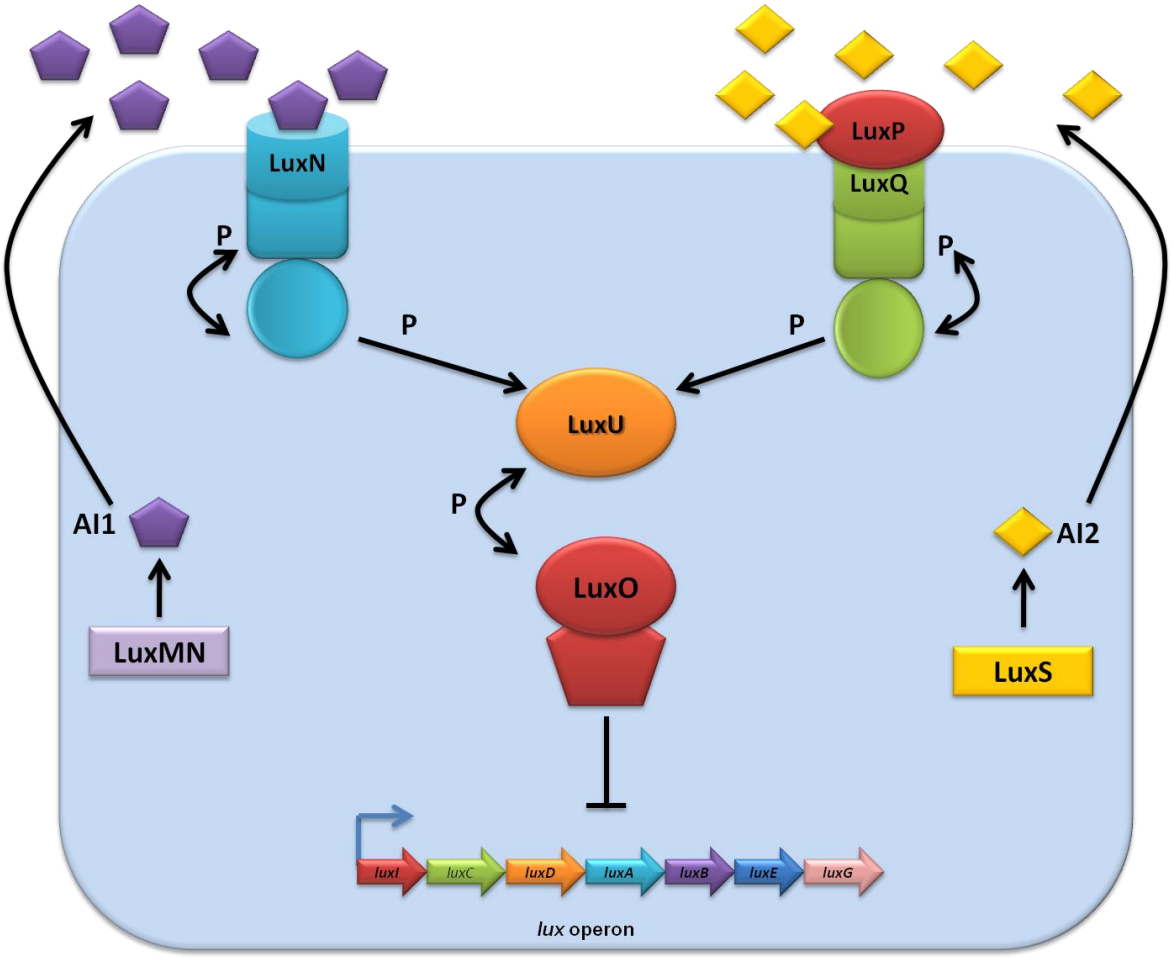


Figure 2.4: Model for quorum sensing process in *V. harveyi* regulated by both AI-1 and AI-2. A complex two-component signal transduction system is responsible for detection of the autoinducers and signal transmission to the luciferase structural operon (*luxCDABEGH*).

The AI-2 has been also identified in a number of Gram-positive bacteria, such as *Streptococcus pyogenes* where it regulates the expression of two virulence factors. Lyon and co-workers showed how mutation in *luxS* of *S. pyogenes* caused a decrease in the secretion and processing of a virulence-associated cysteine protease and an increase in the production of the virulence factor streptolysin S. These results suggest a similar level of complexity in the AI-2 control of signalling pathway for *V. cholerae* and *S. pyogenes*, both including either positive and negative regulation (Lyon *et al.*, 2001).

The detection of a LuxS-dependent signalling in *Bacillus anthracis* and *Bacillus cereus* has been recently reported and the regulation of density-dependent gene expression and pathogenesis can be related to this “universal” cell communication pathway. A putative *luxS* gene located on the chromosome of *B. subtilis* 168 showed a high level of identity with both *luxS* orthologues from *B. cereus* and *B. anthracis.* The *luxS* gene from *B. subtilis* has been overexpressed in *E. coli* and the purified LuxS unambiguously proven to be a ribosylhomocysteinase catalyzing the conversion of S-ribosylhomocysteine to homocysteine and 4, 5-dihydroxy-2, 3-pentanedione, which is the precursor of all known active forms of AI-2 (Hilgers and Ludwig, 2001, Pei and Zhu, 2004, Ruzheinikov *et al.*, 2001, Schauder *et al.*, 2001, Winzer *et al.*, 2002b). These results demonstrate that *B. subtilis* possesses an active *luxS* active gene, which codes for an active AI-2 recognized by the AI-2-dependent signalling system of a *V. harveyi* reporter strain. LuxS role in the regulation of *B. subtilis* multicellular behaviour is still unknown. The potential relation between AI-2-dependent quorum sensing and sporulation has been investigated and the expression of the regulatory protein SpoOA was not affected by LuxS activity (Lombardia *et al.*, 2006). Moreover, a *luxS*-negative mutant strain has been shown to retain a typical spore development (Hamon and Lazazzera, 2001, Chu *et al.*, 2006). Additionally, LuxS did not affect the expression of *abrB* and *sinR*, two other genes which have a key role in biofilm formation and multicellular behaviours in *B. subtilis*. On the other hand, SpoOA had a negative effect on *luxS* expression, implying that the regulation of *luxS* expression in *B. subtilis* is a complex process controlled by both the AI-2-dependent feedback loop and the master regulatory proteins SpoOA and SinR (Lombardia *et al.*, 2006).

1.2.4 Quorum sensing in Gram positive bacteria

Quorum sensing in Gram-positive bacteria is neither regulated by a LuxI/LuxR system, nor involves an N-acyl homoserine lactone-like signal molecule. Cell-cell communication in these bacteria is controlled by a two-component system, consisting of a histidine kinase and a response-regulator, which triggers signal transduction by using phosphorylation to convey information (Kleerebezem *et al.*, 1997, Bassler, 1999). Signal molecules employed by Gram-positive bacteria are usually small, either unmodified or post translationally modified peptides (Bassler, 2002) secreted via an ATP-binding cassette (ABC) exporter protein. Signalling peptides synthesised by Gram positive bacteria are ribosomally generated, but the pathways employed for the biosynthesis of the functional, extracellular end product are characterised by a considerable diversity. The signalling peptides are assumed to be produced constitutively throughout the growth, subsequently reaching a threshold concentration at a certain cell density. Accumulation of the signal in the extracellular medium is "sensed" by the histidine kinase, which auto-phosphorylates on an invariant histidine residue in its cytosolic transmitter domain and then gives the phosphate group to a conserved aspartate residue in the receiver domain of the response regulator (Parkinson and Kofoid, 1992, Parkinson, 1995). Development of competence for uptake of DNA by *Bacillus subtilis* and *Streptococcus pneumoniae*, microcin production by *Lactobacillus sake* and virulence determination in *Staphylococcus aureus* are some of the processes regulated by quorum sensing in Gram-positive bacteria, as listed in Table 1.2 (Bassler, 1999, Dunny and Leonard, 1997) . The general mechanism for peptide-mediated quorum sensing in Gram positive bacteria is represented in Figure 1.5.

Table 1.2: *Chemical structure of some quorum sensing signals present in Gram-positive bacteria.*

Signalling molecule	Functions	Organism	Mechanism
Peptide	Bacteriocin production	*Lactic acid* bacteria *spp.* (Brurberg *et al.*, 1997)	Two-component system-biosynthetic promoters
Peptide	Competence	*Streptococcus pneumonia* (Havarstein *et al.*, 1995a)	Two-component signal transduction.
Modified Peptide	Nisin production	*Lactococcus lactis* (Kuipers *et al.*, 1995a)	Two-component system-biosynthetic promoters
Modified peptide	Virulence	*Staphylococcus aureus* (Balaban and Novick, 1995)	Two-component system-regulatory RNA promoter
γ-Butyrolactone	Secondary metabolism	*Streptomyces griseus* (Miyake *et al.*, 1990)	Internalisation/Promoters for antibiotic biosynthetic genes.

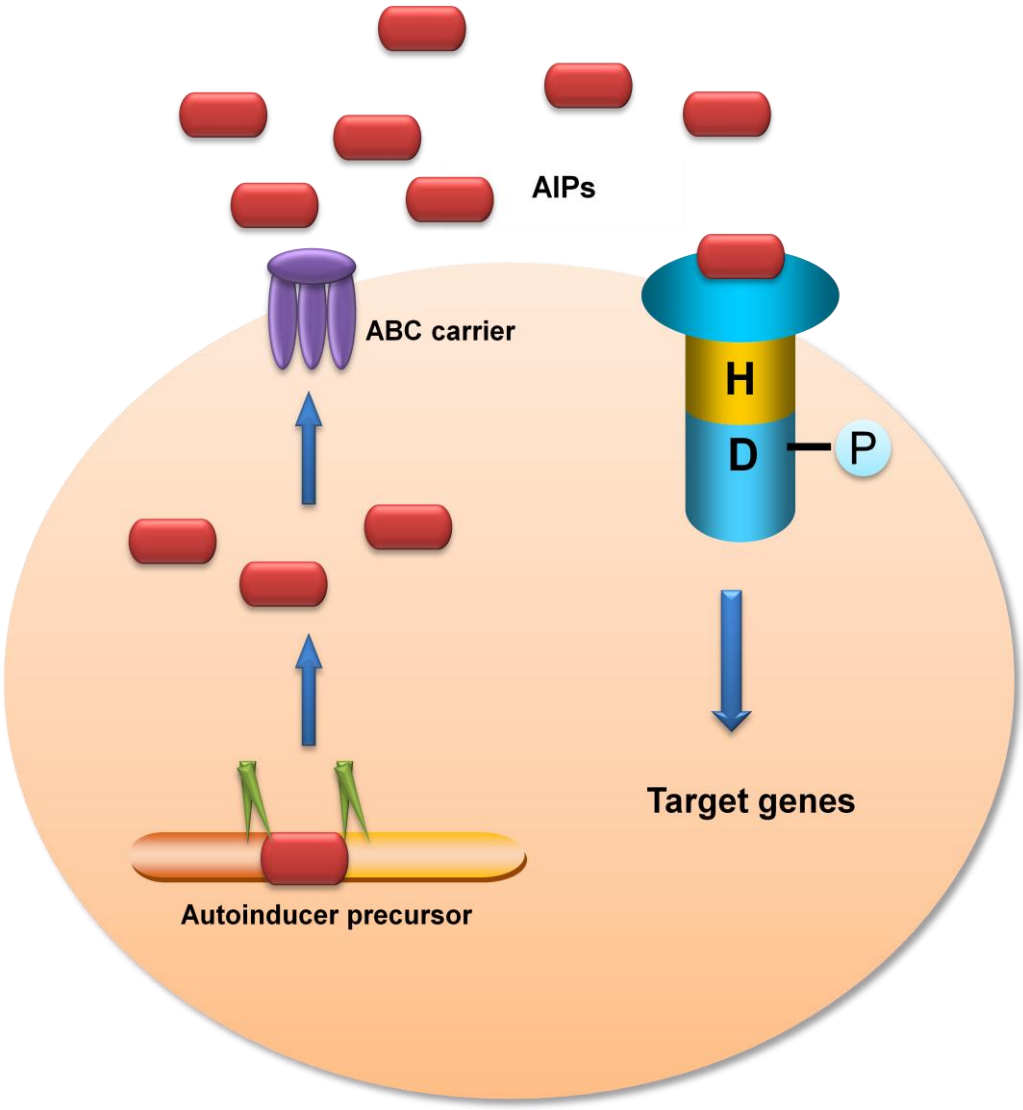


Figure 1.5: Quorum sensing mechanism in Gram-positive bacteria. Cell-cell communication is mediated by small peptides in response to changes in cell-density. The peptide is encoded as a polypeptide precursor, which, after cleavage by a specific enzyme, is exported via an ABC transporter system. The mature peptide accumulates in the extracellular milieu and is detected by a two-component signal transduction system, which in turn activates or represses gene expression (Adapted from Dunny and Leonard, 1997).

1.2.4.1 Quorum sensing mediated by modified peptides

Lactic acid bacteria produce a wide range of peptides with antimicrobial activity, known as bacteriocins which target the cell envelope using non-enzymatic mechanisms to either disturb the integrity of the cell membrane and/or inhibit cell wall synthesis. Most members of this class of peptide antibiotics are synthesised ribosomally as precursor peptides and are then modified post-translationally to acquire their biologically active forms. The structure of bacteriocins varies from linear, unmodified peptides containing one or more disulphide bridges (Class II bacteriocins) to highly post-translationally modified compounds, classified as lantibiotics or Class I bacteriocins, characterised by the occurrence in their sequence of the unusual amino acids lanthionine and 3-methyllanthionine residues (Jack and Jung, 2000, Jung, 1991a, Jung, 1991b, Jack *et al.*, 1997, Jack *et al.*, 1995, Jack and Sahl, 1995, Sahl *et al.*, 1995). These unusual (not genetically encoded) amino acids are formed following the dehydration of either serine or threonine residues to form didehydroalanine (Dha) or 2,3-didehydrobutyrine (Dhb), respectively. The development of intra-molecular thioether rings between Dha or Dhb and free cysteine residues leads to the formation of either lanthionine or β-methyllanthionine. The relative positions of the dehydrated amino acid and its specific target cysteine determine the size and position of the resulting ring. Lantibiotics show a high degree of structural diversity and can be classified in sub-groups, according to a series of criteria, including the structure of the mature peptide or the pre-peptide, the biosynthetic machinery and the mode of action (Jung, 1991b, de Vos *et al.*, 1995). Nisin and lacticin 481 from *Lactococcus lactis* (Siezen *et al.*, 1996) and lactocin S, produced by *Lactobacillus sake* (Skaugen and Nes, 1994) are the best characterized Class I bacteriocins amongst the numerous lantibiotics described in the literature. Nisin biosynthesis and regulation, in particular, provide the best model for the study of post-translationally modified polypeptides (Kuipers *et al.*, 1995a). Being a natural antibacterial agent with well established safety and efficacy, nisin is extensively used as a food preservative and in a number of other applications (Vandenbergh, 1993). The most fascinating characteristic of nisin reside in its dual function, which allows

this molecule to act as antimicrobial agent against other organisms and at the same time serve as a quorum-sensing signal for regulation of nisin production and control of immunity genes in *L. lactis* (Kuipers *et al.*, 1995b, de Ruyter *et al.*, 1996).

Nisin auto-regulatory engagement in its own biosynthetic process was established with the discovery that addition of small amounts of nisin to *L. lactis* culture medium could restore *nisA* transcript production in a strain defective in the nisin structural gene (Kuipers *et al.*, 1993, Kuipers *et al.*, 1995a, Kleerebezem *et al.*, 1997). The product of *nisA* is a 57-residue peptide, known as pre-nisin, comprising an N-terminal leader region of 23-residue, which is subjected to hydrolysis, and a 34-residue C-terminal region (pro-nisin) that is post-translationally modified to yield mature nisin (Jung, 1991b, de Vos *et al.*, 1995). The genes involved in nisin biosynthesis have been sequenced and studies have been performed on nisin biosynthetic pathway and structure/function relationships of nisin and related compounds. Nisin biosynthesis and regulation is illustrated in Figure 1.6. The nisin pre-peptide is encoded by *nisA*, whilst the enzymes required for post-translational modifications, proteolytic processing and export of the mature lantibiotic are encoded by *nisB-C, nisP* and *nisT*, respectively (de Vos *et al.*, 1995). *L. lactis* immunity towards nisin is ensured by the *nisI* gene product, a lipoprotein attached to the cell membrane, together with a putative ABC carrier encoded by the *nisFEG* gene cluster (Siegers and Entian, 1995). Although the exact role of the ABC exporter is still unclear, it has been hypothesised that the transporter apparatus might be involved in the translocation of any nisin that has penetrated the membrane without being affected by NisI, thereby increasing immunity (de Vos *et al.*, 1995). The *nisK* and *nisR* genes encode a histidine kinase and a response regulator, respectively, comprising the two-component system which responds to extracellular accumulation of nisin (de Vos *et al.*, 1995). Three functional promoters have been identified in the nisin gene cluster, *nisA*, *nisF* and *nisR*. Transcription driven by the first two promoters appeared to be triggered by nisin-mediated signal transduction, the level of promoter activity being directly related to the concentration of extracellular nisin. The *nisR* promoter, on the other hand, is structurally distinct and exhibits nisin-independent, constitutive production of the regulatory proteins (de Ruyter *et al.*, 1996, Kleerebezem *et al.*,

1999, Kuipers *et al.*, 1995a). As the concentration of secreted nisin increases, the signal is recognised by the histidine kinase NisK, which starts a phosphorylation dependent signal transduction cascade, thereby leading to the activation of the *nisA* promoter (controlling transcription of nisin biosynthetic machinery and NisI gene clusters) and the *nisF* promoter (regulating transcription of the genes involved in immunity (Figure 1.6) (de Ruyter *et al.*, 1996, Kleerebezem *et al.*, 1999, Kuipers *et al.*, 1995a).

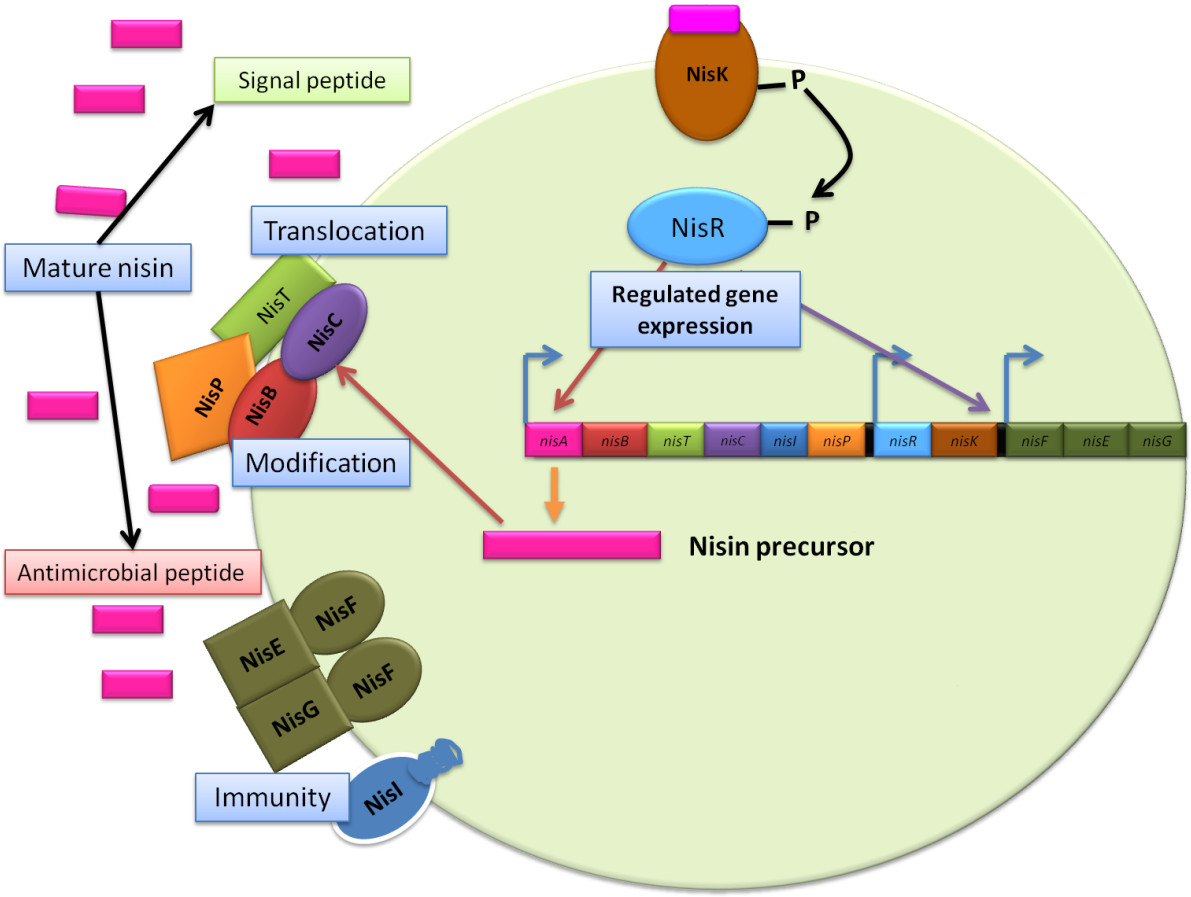


Figure 1.6: Nisin biosynthesis and regulation in *Lactobacillus lactis*. Nisin biosynthesis involves post-translational modification and export of the ribosomally derived peptide precursor by a membrane spanning complex comprising the enzymes NisB, NisC and NisT, which are encoded by *nisB, nisC* and *nisT*. Proteolytic processing of the precursor peptide is carried out by the protease encoded by NisP.NisI and NisFEG (encoded by gene *nisIFEG*) provide immunity to nisin-producing cells. Mature nisin is sensed by the sensor kinase NisK, which in turn activates the receiving domain of response regulator (NisR) thus leading to activation

of target genes. These genes include *nisA*, which codes for nisin precursor (de Vos *et al.*, 1995, Kuipers *et al.*, 1995a).

Lantibiotics are not the only modified inducers involved in cell density dependent processes in Gram positive bacteria. Another example is the RNA III-activating peptide (RAP), a heptapeptide modified to form a thiolactone-containing ring structure which is involved in the production of virulence factors in *Staphylococcus aureus*, a source of nosocomial infections through biofilm formation on medical devices (Giacometti *et al.*, 2003).

Currently two quorum sensing mechanisms have been explained in *S. aureus* (Figure 1.7). The first quorum sensing system consists of RAP (Balaban and Novick, 1995) and its target protein TRAP (Target of RNAIII-Activating Protein). The Agr system is composed of two divergently transcribed units, RNAII and RNAIII, whose transcription is under control of the P2 and P3 promoters, respectively (Morfeldt *et al.*, 1995b, Morfeldt *et al.*, 1995a). The RNAII unit encloses four genes: *agrB*, *agrD*, *agrC*, and *agrA* (Ji *et al.*, 1995). The quorum sensing peptide signal is contained in the middle of a 45 amino acid protein encoded by the *agrD* gene, which is cleaved and subsequently post-translationally modified by the *agrB* gene product (Mayville *et al.*, 1999, Otto *et al.*, 1998, Saenz *et al.*, 2000, Zhang *et al.*, 2004). However, the exact mechanism for secretion and maturation of the AgrD derived pheromone is still unknown. Autoinducers isolated by different staphylococcal strains and species show divergent primary amino acid sequences, but conserve the typical ring structure. They also show the unique phenomenon of cross inhibition (Otto *et al.*, 1999). The *agrC* gene product is a transmembrane protein which acts as the sensor kinase of the bacterial two-component regulatory system (Ji *et al.*, 1995). The staphylococcal autoinducer binds to AgrC, thus activating the response regulator, AgrA, which in sequence induces the transcription of RNAII and RNAIII (Ji *et al.*, 1995). A DNA-binding domain which recognizes a pair of direct repeats with a consensus sequence (5'-ACAGTTAAG- 3') separated by a 12-bp spacer region is responsible of AgrA interaction with DNA (Koenig *et al.*, 2004).

With its dual nature as both a regulatory RNA and the messenger RNA for the *hld* gene (coding for the delta toxin) RNAIII is considered the effector molecule of the Agr system (Novick *et al.*, 1995) Although it has been demonstrated that the 5'-end of RNAIII positively regulates the translation of the alpha hemolysin (Morfeldt *et al.*, 1995b, Novick *et al.*, 1995) while the 3' terminus represses protein A synthesis (Huntzinger *et al.*, 2005, Novick *et al.*, 1995), RNAIII involvement in other virulence factor genes expression remains still unclear. Production of the signal pheromone causes a double effect, as it decreases TRAP phosphorylation, thereby reducing cell-adhesion, whilst inducing AgrC phosphorylation and RNA III production, with a resulting increase in toxin production. The cell density dependent Agr system is vital for *S. aureus* infection as it causes up-regulation of the gene encoding for most of the exotoxins and virulence factors except enterotoxin A and K (Novick, 2003). During the first stages of infection Agr low activity allows colonization, but as the infection progresses, Agr triggers production of various virulence factors and exotoxins allowing bacterial proliferation and host-tissue damage (Otto, 2004).

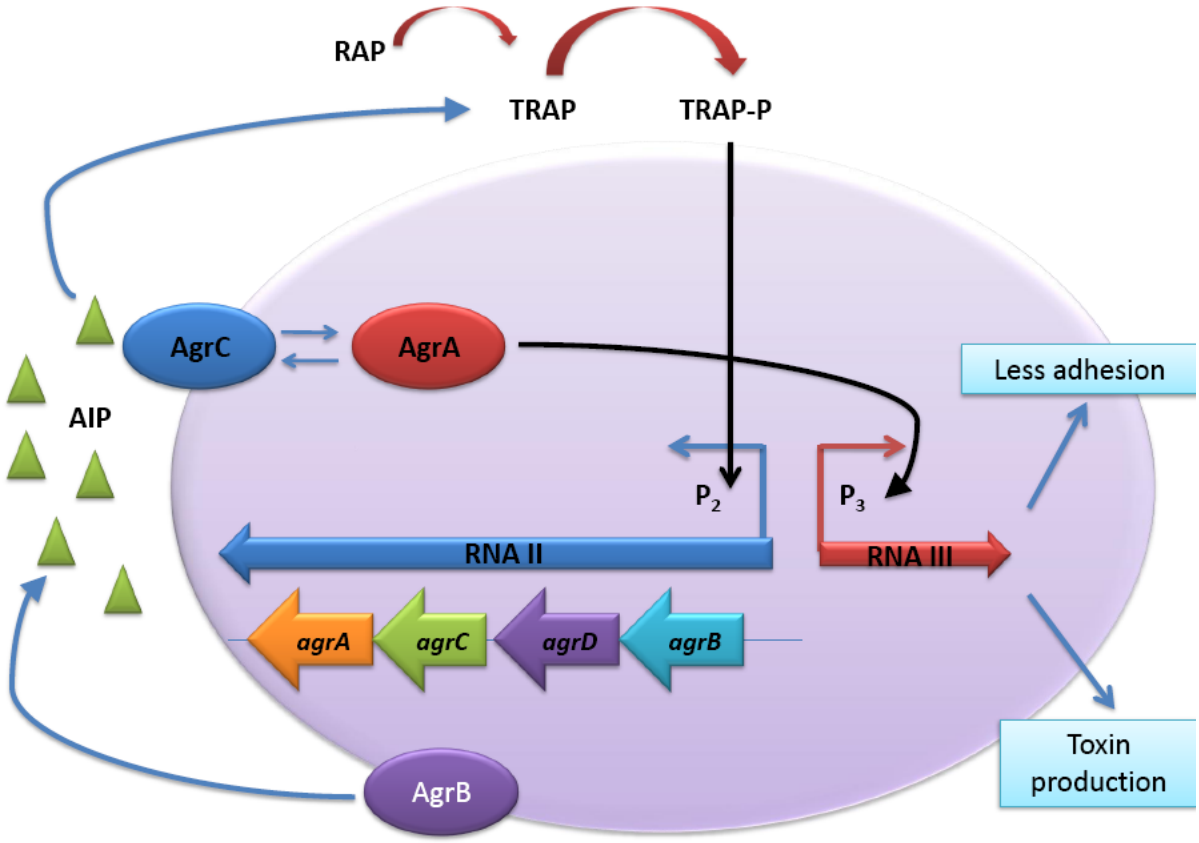


Figure 1.7: Schematic representation of the two quorum sensing systems of *Staphylococcus aureus*. The first quorum sensing system, regulated by the accessory gene regulator (*agr*) locus, comprises the autoinducer RNA III-activating peptide (RAP) and its target protein TRAP. The Agr system consists of two divergently transcribed units, RNAII and RNAIII, whose transcription is under control of the P2 and P3 promoters, respectively. Accumulation of RAP leads to TRAP activation, resulting in the transcription of RNA II. Four genes, *agrB*, *agrD*, *agrC*, and *agrA,* are enclosed in the RNAII unit. AgrB and AgrD are involved in the autoinducer production, while *agrC* and *agrA* encode the sensor kinase and the response regulator of the two-component transduction system, respectively. The interaction of the autoinducer with AgrA activates the signal transduction which leads to the transcription of both RNAII and RNAIII (Adapted from Raina *et al.*, 2009).

1.2.4.2 Quorum sensing mediated by unmodified peptides

Autoinducing peptides involved in the production of Class II bacteriocins and genetic competence are linear unmodified peptides, which are originally synthesised as

precursor polypeptides characterised by a typical double-glycine-type leader peptide. Cleavage of the leader sequence during export via a dedicated ABC-transporter (encoded by an autoregulated gene) yields the mature peptide. The cleavage is performed by the ABC exporter itself, which possesses an N-terminal extension which contains a characteristic peptidase domain (Havarstein *et al.*, 1995b).

This typical processing of autoinducing peptides is widely spread and has been reported for the competence-inducing peptide ComC in *Streptococcus pneumoniae* (Havarstein *et al.*, 1995a), and the bacteriocin-inducing peptides IP-673 in *Lactobacillus sake* LTH673 (Brurberg *et al.*, 1997), Plantaricin A in *L. plantarum* C11 (Diep *et al.*, 1995, Diep *et al.*, 1996), CbnB2 and CbnS in *Carnobacterium piscicola* LV17B (Quadri *et al.*, 1997, Kleerebezem *et al.*, 2001).

Quorum sensing systems regulating bacteriocin production in some lactic acid bacteria are characterized by a gene encoding a “bacteriocin-like” peptide, which is co-transcribed with genes that code for a sensor histidine kinase and a response-regulator of the two-component signal transduction family (Hoch and Silhavy, 1995). An unmodified proteolytic fragment of the “bacteriocin-like” peptide is exported in the extracellular milieu, where it acts as an induction factor for activation of bacteriocin production upon reaching a threshold concentration. The transcription of genes involved in bacteriocin biosynthesis and immunity is activated in response to the activation of the signal-mediated transduction pathway. The genetic organization described above can be observed in the gene clusters dedicated to Sakacin A and Sakacin P production in *Lactobacillus sake*, both comprising one operon for the production of the induction factor and a contiguous operon encoding bacteriocin biosynthetic genes (Diep *et al.*, 1996, Eijsink *et al.*, 1996, Huhne *et al.*, 1996). In *L. plantarum* C11 plantaricins production is controlled by plantaricin A, an induction factor whose coding gene (*plnA*) is located on the same operon as a two-component regulatory system (*plnBCD*) (Jimenez-Diaz *et al.*, 1995, Nes *et al.*, 1996b, Nissen-Meyer *et al.*, 1993). Plantaricin A biosynthesis, schematically represented in Figure 1.8, is regulated by a cluster of over 20 linked *pln* genes, containing at least 5 separate operons; the biosynthesis of at least 3 bacteriocins, including single peptide and two-peptide varieties, is also controlled by this gene cluster (Jimenez-Diaz *et al.*,

1995, Nes *et al.*, 1996a, Nissen-Meyer *et al.*, 1993).The product of *plnA* is a 48-amino-acid precursor peptide, which is then cleaved to generate two mature peptides of 22 and 23 amino-acid in length. Originally plantaricin A was believed to be a bacteriocin rather than an inducing factor, though recent evidence showed that bacteriocin production abolished in *L. plantarum* C11 cultures depleted for plantaricin A could be restored by addition of either synthetic or purified plantaricin A at a concentration of 1 ng mL^{-1} to non-producing cultures (Nissen-Meyer *et al.*, 1993).

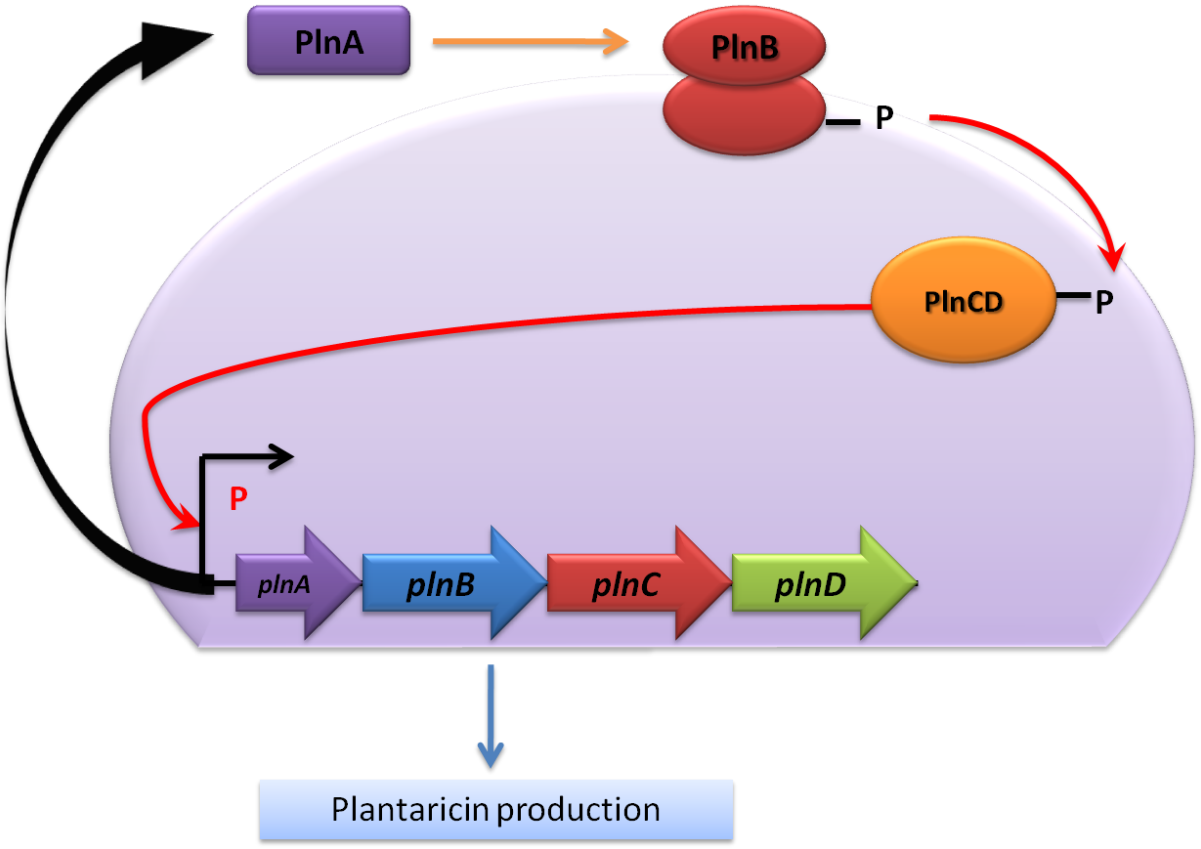


Figure 1.8: Quorum sensing-regulated Plantaricin production in *Lactobacillus plantarum*. The pln*ABCD* operon comprises the genes encoding the Inducing factor (*plnA*), the sensor kinase (*plnB)* and the response regulator protein (*plnC* and *plnD*). The production and accumulation of IF leads to stimulation of the histidine kinase and the response regulator, which in turn leads to activation of the *plnABCD* operon resulting in production of Plantaricin (Adapted from Nes *et al.*, 1996).

The extracellular signalling processes involved in the biosynthesis of Class II bacteriocins have been described as a three-component regulation, due to the widespread occurrence in bacteriocin-producing bacteria of gene operons coding for the induction factor, the response regulator, and the histidine kinase (Nes *et al.*, 1996a).

An analogous mechanism has been developed for the regulation of the pneumococcal competence system. Regulation of competence in *Streptococcus pneumoniae* was shown to be regulated by a heptadecapeptide, namely Competence Stimulating Peptide (CSP) (Havarstein *et al.*, 1995a). The precursor of CSP is encoded by the pneumococcal gene *comC*, whose product is a 17 amino acid long peptide subjected to cleavage to generate the mature peptide. Two CSP variants have been identified in *S. pneumoniae,* both comprising a conserved sequence fingerprint composed of a negatively charged N-terminal residue, an arginine in position 3, and a positively charged C-terminal tail. Also, a gene encoding an ABC transporter (*comA*) was identified in *S. pneumoniae* (Hui *et al.*, 1995), which is highly related to a family of proteins that is implicated in the simultaneous processing and export of peptide bacteriocins (Havarstein *et al.*, 1995b). The *comC* gene was revealed to be located on an operon together with *comD* and *comE*, which encode homologues of the histidine kinase and response regulator proteins of the two-component transduction system family (Pestova *et al.*, 1996). Extracellular CSP at the threshold concentration binds to the ComD receptor domain and triggers competence development in the pneumococcal culture, in a similar fashion to the one described for plantaricin A induction of bacteriocin production in *L. plantarum*. Once activated, ComD phosphorylates ComE, thus enabling the response regulator to activate transcription of the early competence genes. These include *comX*, which encodes an alternative sigma factor that controls the transcription of a large number of so-called late competence genes (Claverys and Havarstein, 2002, Ween *et al.*, 1999, Luo *et al.*, 2003, Luo and Morrison, 2003). In *S. pneumoniae* nearly 190 genes have been highlighted to be responsive to CSP, although only 23 appear to be involved in competence development and DNA uptake, thereby implying that CSP-

ComD signalling mechanism might regulate additional processes (Peterson *et al.*, 2004).

1.2.4.3 γ-butyrolactones as signalling molecules

Secondary metabolite production in many actinomycetes is regulated by diffusible molecules originally called autoregulators (Horinouchi and Beppu, 1992), whose activity has been later reported to be under quorum sensing control. *Streptomyces griseus* and *Streptomyces natalensis* quorum sensing molecules are respectively 2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone (A-Factor) (Miyake *et al.*, 1990, Recio *et al.*, 2004) and 2, 3-diamino-2, 3-bis (hydroxymethyl)-1, 4-butanediol (PI signalling factor) which are associated with production of the antibiotics streptomycin and pimaricin (Recio *et al.*, 2004).

The A-factor is required for production of secondary metabolites such as streptomycin and grixazone together with formation of aerial mycelia and, ultimately, sporulation (Bibb, 2005). The molecule is characterised by a butyrolactone moiety and its structure resembles the homoserine lactone (HSL) signalling molecules regulating quorum sensing in Gram-negative bacteria. Although A-factor shares structural similarities with AHLs, its mechanism of action does not imply LuxI/LuxR-like systems. Its cognate intracellular receptor protein ArpA acts as a repressor by binding to a specific target DNA sequence in the promoter region of one or more of the operons required for antibiotic production and differentiation. When A-factor concentration reaches a threshold, it binds to ArpA, which releases the promoter and allows the transcriptional activation of these operons. One of ArpA target promoters is *adpA* which regulates the transcription of StrR and GriR, two pathway-specific activators responsible for streptomycin and grixazone production, respectively (Figure 1.9). AdpA also regulates expression of other genes of the *adpA* regulon responsible for morphological differentiation (Natsume *et al.*, 2004).

Autoinducers showing a chemical structure analogous to the A-factor have been isolated in other members of *Streptomyces* sp.; Virginiae butanolides or VB regulate virginiamycin biosynthesis in *S. virginiae* (Nihira, 2002); IM2 [(2R,3R,1'R)-2- 10-

hydroxybutyl-3-hydroxymethyl γ-butanolide] elicits the production of antibiotics showdomycin and minimycin (Kitani *et al.*, 2001) in *S. lavendulae* and SCB1, which is related to pigmented actinorhodin production in *S. coelicolor.* Unlike A-factor the VB, IM2 or the SCB1 do not regulate the morphological differentiation (Takano *et al.*, 2001).

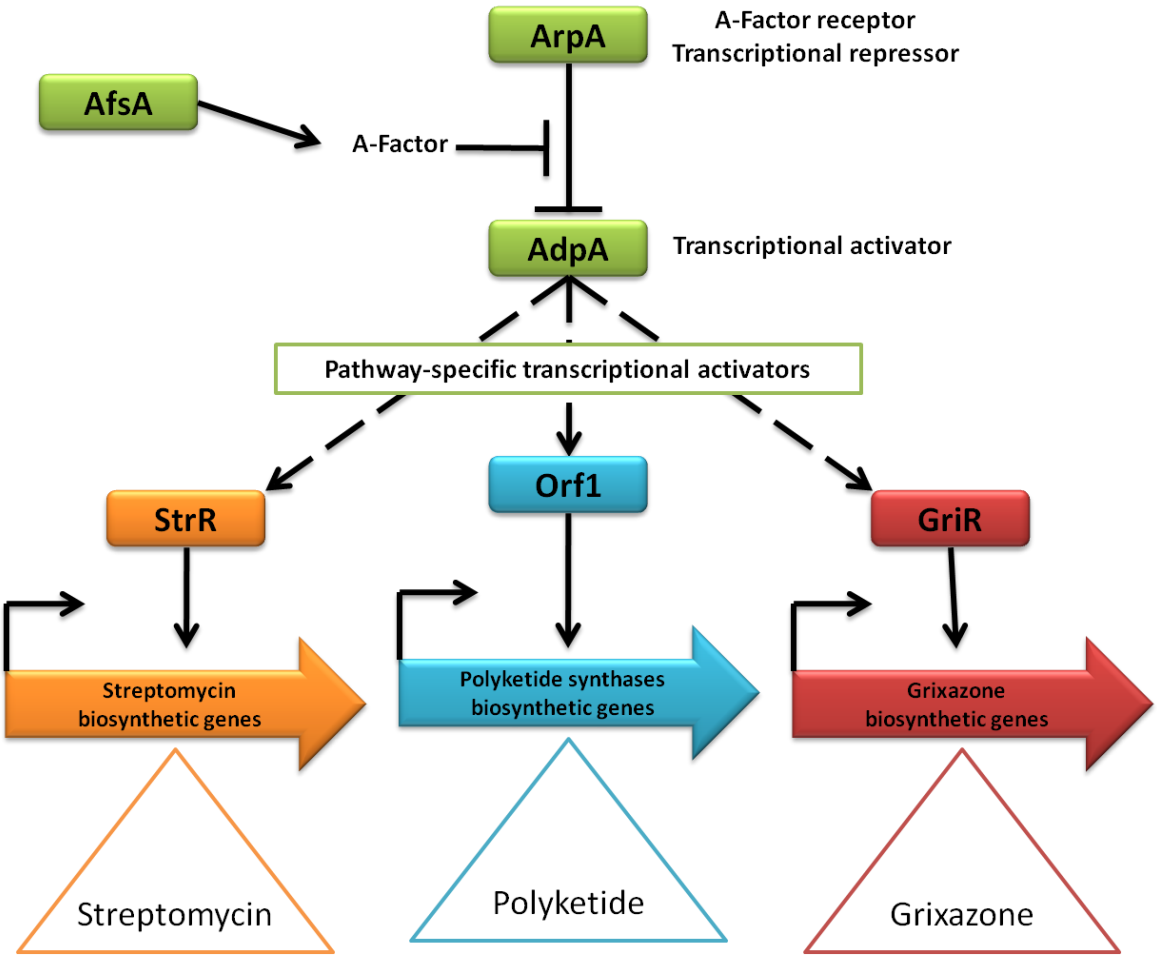


Figure 1.9: A-Factor-mediated regulation of secondary metabolite production in *Streptomyces griseus* (Adapted from Ohnishi *et al.,* 2005).

A similar mechanism has been described in *S. natalensis* for the production of the antifungal pimaricin, used in food industry for mould prevention. An autoinducer named PI factor appears to be responsible for primaricin production at high cell densities. It has been shown that nanomolar addition of either A-factor or PI-factor to

cultures of a *S. natalensis* strain deficient in pimaricin production was able to restore pimaricin biosynthesis in the mutant strain (Anton *et al.*, 2004). Since pimaricin biosynthesis was induced by both the factors, it has been suggested that quorum sensing signals might be exchangeable between members of different *Streptomyces* strains (Recio *et al.*, 2004).

1.3 Quorum sensing in *Bacillus subtilis*

The Gram-positive soil bacterium, *Bacillus subtilis,* initiates a range of responses which assists its survival in the increasingly adverse conditions characterising the onset of stationary phase. Examples of these responses are: sporulation; production of degradative enzymes and antibiotics, motility and development of genetic competence. Some of these processes, including competence and sporulation, are mutually exclusive, whilst others develop either sequentially or simultaneously (Dubnau *et al.*, 1994). Although degradative enzyme production, motility and competence are distinct processes their regulatory systems are all controlled by post exponential regulons activated by overlapping signal transduction pathways which end up in a co-ordinated set of transcriptional responses. Moreover, only a limited number of gene products involved in the sensing and transduction of environmental information are specific for a single form of response (Kunst *et al.*, 1994). A general scheme of the quorum sensing-regulated development of competence and sporulation in *B. subtilis* cells is illustrated in Figure 1.10.

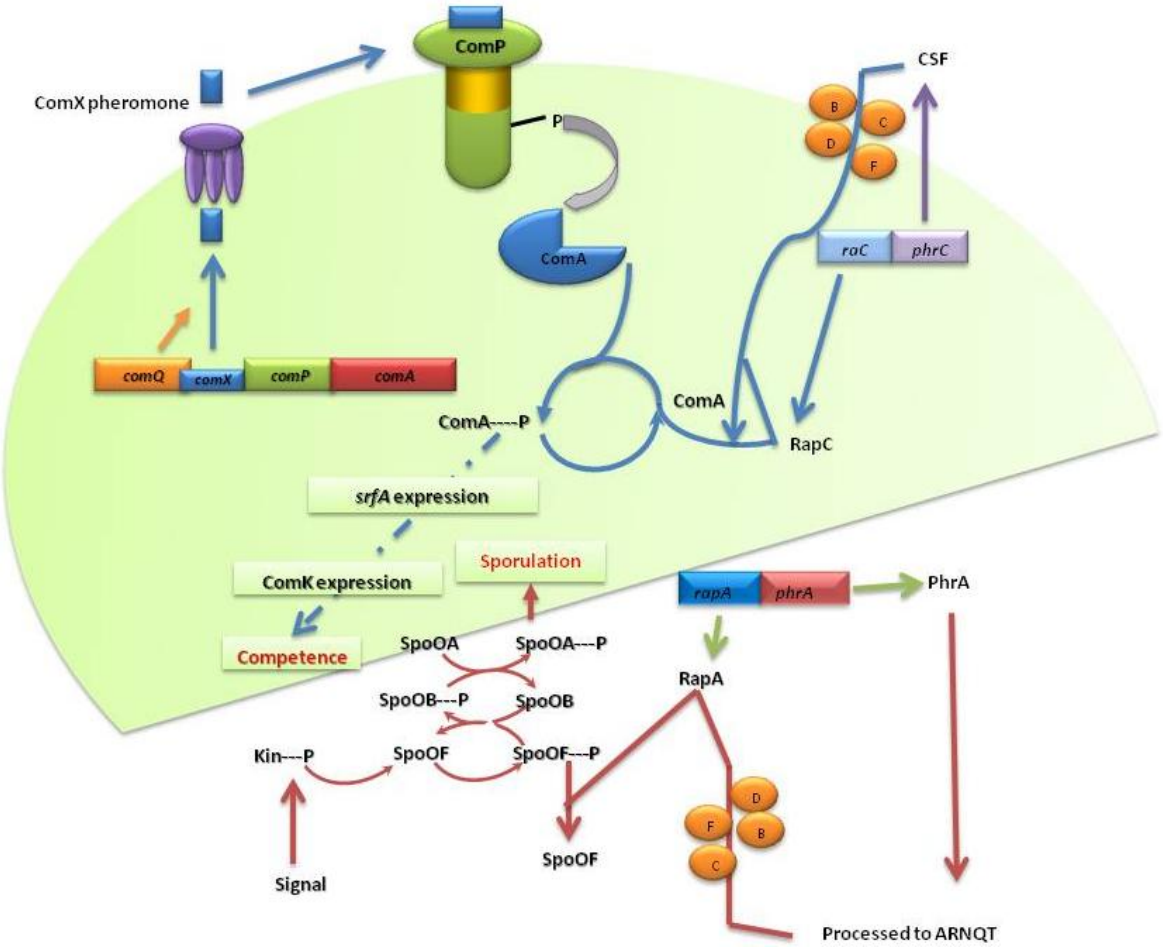


Figure 1.10: A simplified representation of the multiple peptides signalling in Bacillus subtilis for regulation of competence and sporulation. The histidine kinase sensor protein (ComP) interacts with ComX pheromone leading to phosphorylation of ComA response regulator. This in turn results in a multistep stimulation thereby regulating competence. CSF encoded by phrC is re-internalised via the Opp (Oligopeptide permease) system after it is processed to form a pentapeptide (EMRGT) whose role is to inhibit RapC phosphatase leading to increased accumulation of ComA-P which phosphorylates ComK inducing either competence or sporulation depending on CSF concentration in the extracellular medium (Adapted from Raina *et al*., 2009)

1.3.1 Quorum sensing-mediated regulation of competence

Numerous regulatory genes are required for the development of competence, which represents the natural ability of a microorganism to uptake exogenous DNA (Spizizen, 1958). Under specific growth conditions, naturally occurring at the onset of the stationary phase of the microbial growth, a sub-population of cells in the culture differentiates to become competent, thus leading to the production of specialized proteins involved in the uptake of DNA in a way that is independent from its nucleotide sequence (Spizizen, 1958).

Genes controlling competence in Bacilli can be classified into two groups: early and late. The early competency genes are involved in the quorum sensing regulation of competence development. Such gene products do not form any visible phenotypic proteins or enzymes, but they are required for the detection of extracellular conditions and transfer of the information to the late genes which are directly responsible for the development of proteins which make up the competence apparatus. (Hahn *et al.*, 1996, van Sinderen and Venema, 1994). Currently, five different loci have been identified which are involved in the formation of the DNA uptake apparatus in *B. subtilis*: *comC*, *comE*, *comF*, *comG* and *nucA,* The product of *comG* shows similarities with the type-IV pilins from *Pseudomonas* species and other pilin-like complexes. These similarities, confirmed by experimental data, suggested that a pilin-like structure, composed of several ComG subunits, is formed to transport DNA into *B. subtilis* cells (Chung *et al.*, 1998, Chung and Dubnau, 1998). The correct assembly of this structure is aided by ComC, whilst the product of *comE*, the polytopic transmembrane protein ComEC, is believed to form a pore that allows the DNA into the cell (Figure 1.11). Once the DNA is internalised it associates with the DNA-helicase-resembling protein encoded by the *comF* operon (Londono-Vallejo and Dubnau, 1994a, Londono-Vallejo and Dubnau, 1994b, Provvedi and Dubnau, 1999). The membrane localized nuclease NucA catalyses the endonucleolytic cleavage of the target DNA, which results in the formation of linear fragments up to 20 kb in size (Provvedi *et al.*, 2001).

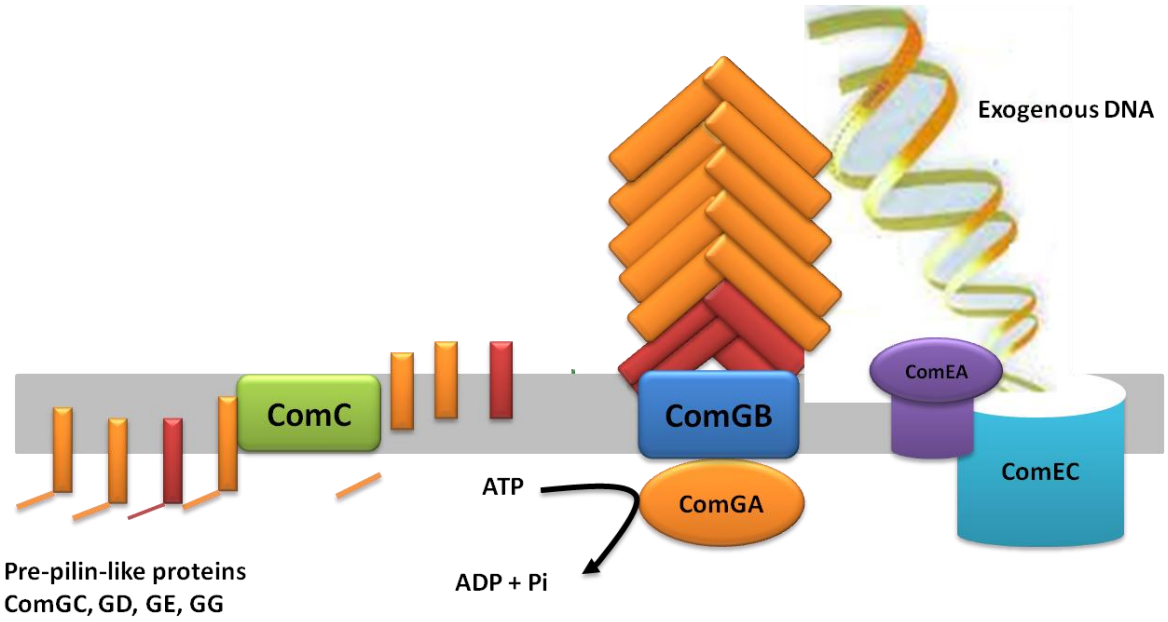


Figure 1.11: Model for the DNA uptake machinery in *Bacillus subtilis*. The prepilin peptidase ComC is involved in processing the prepilin-like proteins ComGC, GD, GE and GG by cleaving the short leader peptides at their N-terminus. The mature ComGC is translocated across the membrane and forms a polymeric complex which traverses the cell wall and allows the DNA-binding protein ComEA to access exogenous DNA. ComGA (a traffic NTPase), ComGB (a polytopic membrane protein) and the other pilin-like proteins are necessary for the complex formation. The major pilin-like protein (ComGC) is represented in orange and the minor pilin-like proteins in red.

Regulation of competence development, or early competence, in *B. subtilis* involves two peptide factors, both accumulating in the extracellular medium as the cells grow to high density. One peptide, known as the ComX pheromone, (Magnuson *et al.*, 1994), is strictly concerned with the control of competence development, whilst the second signalling molecule, CSF (Competence and Sporulation Factor) directs both competence and sporulation in *B. subtilis* cultures (Magnuson *et al.*, 1994, Solomon *et al.*, 1995b).

Two convergent pathways regulate the response of the cells to the two signalling factors: ComX requires the histidine kinase encoded by *comP*, a member of the two-component system family, for its activity (Solomon *et al.*, 1995a), while the oligopeptide permease (Opp) encoded by *spoOK* (Perego *et al.*, 1996a) is engaged

in the response to CSF (Solomon *et al.*, 1995b). Both CSF and the ComX pheromone act jointly to activate the response regulator/transcription factor ComA (Roggiani and Dubnau, 1993).

1.3.1.1 The ComX pheromone

The ComX pheromone, identified in 1994 by Magnuson and co-workers (Magnuson *et al.*, 1994), is encoded by *comX*, a gene located on the *comQXPA* cluster. The translation of *comX* results in a 55- residue precursor which is subsequently cleaved at the C-terminus to give a 9-10 amminoacid peptide, modified by the addition of a isoprene group on a tryptophan (Magnuson *et al.*, 1994). A partial overlapping has been observed between the 5' end of *comX* and the 3' end of *comQ*, which codes for a protein involved in the maturation and modification of the pheromone (Magnuson *et al.*, 1994). ComX production in *B. subtilis* 168 has been thoroughly investigated since its discovery, leading to the characterisation of several aspects of this process. Interestingly, accumulation of the pheromone in culture supernatant was found to be directly related to the cell growth, suggesting that the production of this signalling molecule is not autoinduced, in contrast with the majority of the AHL signals produced by Gram-negative bacteria (Bacon Schneider *et al.*, 2002). In modified strains overexpressing *comX* a 10-fold increase in the production of ComX has been detected when compared with the wild-type strain. These findings suggest that in wild-type cells *comX* expression limits ComX production, as the concentration of pheromone in the extracellular milieu plays a major role in determining the timing of expression of quorum-responsive genes, such as *srfA* (Bacon Schneider *et al.*, 2002).

The *comQXPA* locus has been identified in other members of the *Bacillus* genus closely related to *B. subtilis*, where it appears to have a high variability. The polymorphism is associated with the specificity of the quorum sensing response and therefore it is characteristic of ComQ, the ComX precursor polypeptide and the sensor domain of ComP, but not ComA (Tortosa *et al.*, 2001, Tran *et al.*, 2000).

Although the *comQXP* locus appears to be highly polymorphic, genetic and biochemical analysis performed on several natural isolates of *B. subtilis* allowed the classification of Bacilli possessing the *comQXPA* cluster into 4 different pherotypes, (each producing a specific variant of the ComX pheromone) according to their capability to activate a quorum sensing response in other strains (Ansaldi *et al.*, 2002). Both the sequence of the mature peptide and its modification residue can vary in pheromones produced by different pherotypes (Ansaldi *et al.*, 2002).

Characterization of the various ComX molecules is based on two main criteria: the N-terminal cleavage site and the mass of the post-translational modification. Alternative cleavage sites have been identified in different ComX precursors, which generate diverse mature peptides whose length can range from 5 to 10 amino acids, though each of them comprises a conserved tryptophan residue (Ansaldi *et al.*, 2002). The post-translational modification of the peptide occurs on this tryptophan residue, as highlighted for the ComX pheromone identified in *B. subtilis* 168 (Magnuson *et al.*, 1994). All the ComX variants investigated so far have been shown to be modified by isoprenylation, although the mass of the isoprene group varies among different pherotypes, thus suggesting that the modification may represent a major determinant of specificity. The mass of the isoprene group for each pheromone can be obtained simply by subtracting the calculated mass of the mature peptide from the actual mass measured by mass spectrometry (Ansaldi *et al.*, 2002). Based on this assumption it has been possible to determine that the ComX purified by *B.subtilis* 168 is subjected to a farnesylation addition because the mass (206 Da) matches that of a farnesyl group, whilst the 136 Da modifications associated with ComX from *B. subtilis* isolates RO-B-2 and RO-E-2 correspond to geranyl groups (Ansaldi *et al.*, 2002). Other pheromones have been isolated with a modification mass of 120 Da which does not correspond to any simple isoprenoid, thus indicating that the manner of modification of the tryptophan residue might not be a simple isoprenylation (Ansaldi *et al.*, 2002). As illustrated in Figure 1.12, the structure of ComX RO-E-2 has been investigated through mass spectrometry and NMR. The modification has been confirmed to be a substitution of a tryptophanyl proton with a geranyl group at position 3 of its indole

ring, which leads to the formation of a tricyclic structure (Okada *et al.*, 2005, Okada *et al.*, 2007b).

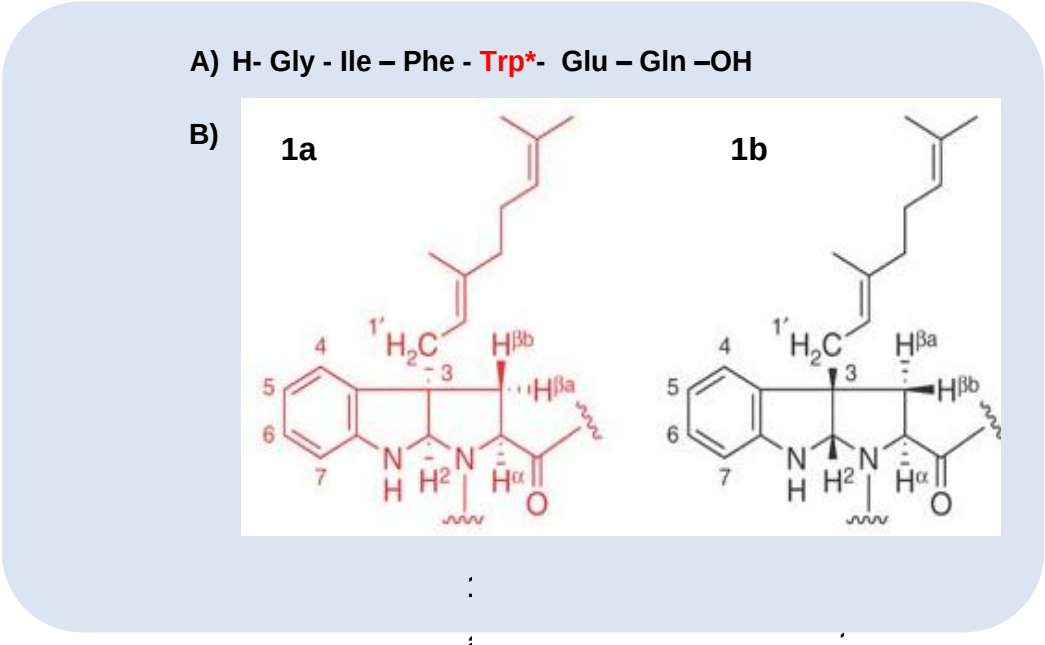


Figure 1.12: Structure of the ComX pheromone (A) Amino acid sequence of the ComX pheromone from *B. subtilis* strain RO-E-2. The Trp* residue, modified by a geranyl group is represented in red. (B) The putative structures of the modified tryptophan residue are shown in 1a and 1b (Taken from Okada *et al.*, 2005)

The ComX pheromone has been compared to the a-factor produced by the yeast *Saccharomyces cerevisiae*, which acts as inducer of the mating process in α-type cells, due to the analogies detected in their functions (they are both concerned with genetic exchange), and their biosynthesis (Chen *et al.*, 1997). Both peptide signals are synthesized as inactive precursors, which are then cleaved and modified by isoprenylations prior secretion into the extracellular medium. However, whilst the reaction leading to the formation of the active a-factor involves isoprenylation on a cystein residue (Chen *et al.*, 1997) the ComX pheromone undergoes a cyclization reaction prior to the attachment of the isoprene moiety to the conserved tryptophan residue, resulting in the formation of a unique post-translational modification (Okada *et al.*, 2005, Okada *et al.*, 2007a). Nonetheless, both the farnesylation of a-factor and the geranylation/farnesylation of the ComX pheromone appear to be absolutely essential for their biological activity (Gibbs, 2005).

The enzyme responsible for the maturation of ComX precursor is ComQ, the product of the *comQ* gene located directly upstream of *comX* in the chromosome as part of a peptide signalling cassette. The first indication that ComQ partakes in the production of the ComX pheromone was provided by a *comQ* knock-out which resulted in a reduced *srfA* (surfactin biosynthetic operon) expression compared to the wild-type (Magnuson *et al.*, 1994). Moreover, induction of quorum-sensing-related genes (*srfA*) at low cell density could be observed when *comQ* and *comX* alone were overexpressed (Shneider et al., 2002).

An isoprenoid binding site was recently identified in ComQ and the discovery that ComX maturation could be prevented by mutations in this region confirmed the role of ComQ in the processing of the ComX pheromone (Bacon Schneider *et al.*, 2002). Furthermore, homology research highlighted that ComQ shares high similarity with IdsA, the short-chain isoprenyl diphosphate synthase (IPPase) of *Methanobacterium thermoautotrophicum* (Bacon Schneider *et al.*, 2002). Isoprenyl diphosphate synthases are enzymes involved in the condensation reaction between isopentenylpyrophosphate (IPP) and allylic diphophates, which results in isoprenoid formation (Epstein *et al.*, 1991).

1.3.1.2 The ComP-ComA two component transduction system

Two-component systems represent the core of prokaryotic signalling concerning phosphorylation transduction cascades (Alex and Simon 1994, West and Stock, 2001). The two-component system comprises several characteristic domains usually structured on two conserved proteins: a histidine kinase/sensor and a response regulator that are phosphorylated on histidine and aspartate residues, respectively.

External stimuli detected by the sensor domain of the histidine kinase activate the enzyme which in turn catalyzes an ATP-dependent *trans*-autophosphorylation reaction. A specific histidine residue within one subunit of the dimer is phosphorylated by the other subunit thereby generating a phosphoimidazole. The phosphoryl group is subsequently transferred by the response regulator to an

aspartate residue located on its own regulatory domain. Phosphorylation leads to the activation of the effector domain of the response regulator which then triggers the specific output response (Alex and Simon, 1994, West and Stock, 2001).

The catalytic domains of bacterial histidine kinases do not resemble those of serine-, threonine- or tyrosine kinases previously characterised, though they were shown to be related to ATPase domains of the type II topoisomerase gyrase B and the chaperone Hsp90 (Bilwes *et al.*, 1999, Tanaka *et al.*, 1998).

The histidine kinase ComP, involved in the regulation of competence in *B. subtilis*, is the 769 amino acid long polypeptide product of *comP*, located on the *comQXPA* gene cluster (Weinrauch *et al.*, 1989, Weinrauch *et al.*, 1990). The protein comprises a polytopic, membrane-localized sensor domain at the N-terminus of the protein, and a relatively conserved C-terminal cytoplasmic transmitter domain (Weinrauch *et al.*, 1990).

The sensor domain of ComP consists of eight transmembrane helices with two periplasmic linkers in the first four transmembrane regions (Weinrauch *et al.*, 1990). Sequence analysis performed on ComP homologues predicts the presence of ten transmembrane helices for three of these histidine kinases, suggesting that, although transmembrane regions are well conserved they might vary in number. The first and the second transmembrane regions of ComP are separated by a large loop which contains a PDZ domain with a length of about 70 amino acids (Weinrauch *et al.*, 1990). PDZ domains represent a common structural domain usually found in signalling proteins, where they are involved in binding of (poly)peptides, which suggests a putative role in the interaction with ComX. However, the exact function of the PDZ domain in peptide sensing in the ComP-like sensors has not been investigated yet (Piazza *et al.*, 1999).

ComA is the only product of the *comQXPA* cluster showing a relatively conserved sequence which reflects its role as a response/transcription regulator not concerned in the determination of specificity of the quorum sensing system (Tortosa et al., 2001). ComA comprises two domains: the response regulator, located at the N-terminus of the protein, containing an invariable aspartate residue at position 55 which is the site of phosphorylation by ComP (Guillen *et al.*, 1989, Weinrauch *et al.*,

1989) and the C-terminal effector region which exhibits high homology with the DNA-binding domain of several transcription factors (Roggiani and Dubnau, 1993). Sequence alignment and secondary structure prediction performed on the effector domain highlighted that ComA is a member of the NarL family of response regulator proteins. Proteins belonging to this family are characterised by a C-terminal domain which directs DNA interaction and activation on target sequences via a helix-turn-helix DNA-binding motif (Roggiani and Dubnau, 1993, Weinrauch *et al.*, 1989). Recent studies have revealed that ComA C-terminal domain forms a dimeric structure in solution which is held together by interactions running along the length of one of the α-helices located in each monomeric unit (Hobbs *et al.*, 2010). Analysis of ComA interaction with the *srfA* operon led to the identification of two DNA regions of dyad symmetry essential for the positive control of *srfA* transcription (Roggiani and Dubnau, 1993). A model has been created that indicates an inverted repeat sequence containing two 6 bp recognition elements separated by a 4 bp spacer as the binding site for ComA. Amongst the numerous genes regulated by ComA the majority is characterised by a single inverted repeat, but many others have been identified with multiple inverted repeats (Roggiani and Dubnau, 1993, Mueller *et al.*, 1992, Ogura *et al.*, 2001, Comella and Grossman, 2005).

Although the *srfA* operon is the best characterised binding target of ComA (Nakano and Zuber, 1991, Nakano *et al.*, 1991b, Nakano and Zuber, 1993, Roggiani and Dubnau, 1993, Hahn and Dubnau, 1991), this regulatory protein has been shown to be involved in the direct control of the several other operons. Microarray analysis identified at least 89 genes, in 35 operons not concerned with competence development, which showed to be affected by ComA (Comella and Grossman, 2005). Examples are: *degQ*, encoding a regulator protein involved in degradative enzyme production (Msadek *et al.*, 1991); and *rapA* (Mueller *et al.*, 1992), *rapC* (Solomon *et al.*, 1996b), *rapE* (Jiang *et al.*, 2000) and *rapF* (Jarmer *et al.*, 2001) each encoding a regulatory protein of the Rap family. Given the number of genes whose expression is controlled by ComA it is not surprising that the affinity of this regulator protein for its DNA binding site might represent the most important feature for the coordination of transcription regulated by population density (Griffith and Grossman,

2008). Degeneracy of the recognition elements in the ComA binding site has been shown to alter cell-density dependent gene expression as promoters with an optimized binding site have high activity at low culture density, whilst degenerate binding sites display activity only at higher culture densities (Griffith and Grossman, 2008). At low cell density, ComA is mainly present in an inactive non-phosphorylated state, and only a small amount of ComA is activated by phosphorylation. In this condition the active ComA is only able to bind to high affinity sites, such as the regulatory region of *rapA*, thus positively regulating the transcription of target genes at low population densities. Conversely, at high cell densities, phosphorylated ComA accumulates in the cytoplasm and its concentration allows binding to low affinity degenerate sites, such as those present in *srfA* (Griffith and Grossman, 2008).

1.3.1.3 The competence and sporulation factor CSF

The competence and sporulation factor CSF is the second signalling peptide involved in the cell density dependent control of competence development in *B. subtilis*. It was originally identified as a peptide with a molecular weight of 609 Daltons following ComX discovery (Solomon *et al.*, 1995b). CSF is a 5-amino acid peptide (ERGMT) encoded by the last 5 codons of a 40 codons open reading frame, named *phrC,* initially identified as the downstream region from a promoter recognized by RNA polymerase containing σ^H (Carter *et al.*, 1990). PhrC is a member of a family comprising 8 “Phr peptides”, which are cleaved from the C-terminus of Phr precursor polypeptides (Lazazzera, 2001, Auchtung *et al.*, 2006). All *phr* genes are clustered together with genes encoding a specific Rap aspartyl phosphatase, though three of the eleven known *rap* genes are followed by non functional *phr* genes, suggesting that these are not involved in quorum sensing regulatory pathways (Perego, 1997). Although Rap and Phr are co-trascribed, the phosphatase remains in the cytoplasmic space, whilst Phr peptides, characterised by an amino-terminal signal, are exported as pro-peptides, most likely via the Sec pathway (Tjalsma *et al.*, 2000). Once in the extracellular medium the pro-peptide is subjected to further processing resulting in

active Phr signals with a weakly conserved XRXXT sequence (Perego, 1997, Solomon *et al.*, 1996b). After reimport in the cell via an Opp system, Phr peptides act as inhibitors for the activity of their cognate Rap phosphatase (Perego, 1997, Perego, 1998, Solomon *et al.*, 1996b).

The product of *phrC* from *B. subtilis* has been characterised as a 40-aminoacid polypeptide which has a putative cleavage site indicating that an 11-25 aminoacid-long precursor is exported through Sec-dependent pathway (Lazazzera, 2001). Further processing steps that result in the formation of the active pentapeptide are yet to be elucidated. Whilst the ComX pheromone requires the histidine-kinase ComP for its recognition and signalling, the Opp encoded by *spoOK* (Perego *et al.*, 1991, Rudner *et al.*, 1991) was found to be essential for CSF sensing. SpoOK is a member of the ATP-binding cassette (ABC) carrier family that utilize ATP hydrolysis for the import and export of several compounds (Higgins, 1992), including oligopeptide transport through *B. subtilis* cell membrane (Rudner *et al.*, 1991, Perego *et al.*, 1991).

CSF was found to be an auxiliary competence pheromone, which acts as a modulator of the timing and levels of competence (Solomon *et al.*, 1996b). In order to investigate whether different pherotypes of CSF might be produced by diverse *Bacillus* strains, *rapC-phrC* operons from six strains previously shown to have different *comQXP* sequences have been analysed (Tortosa *et al.*, 2001, Pottathil *et al.*, 2008). A mature CSF peptide with a conserved sequence was identified for all the strains analysed and the correspondent PhrC polypeptide variants displayed both a functional signal sequence and peptidase cleavage sites (Becker *et al.*, 2004). Although differences were detected in RapC and PhrC amino acid sequences from different strains most of the amino acid substitutions are conservative and therefore cannot alter the function of these proteins (Pottathil *et al.*, 2008). These data suggest that *Bacillus* strains producing different ComX pherotypes are characterised by identical CSF peptides, indicative of CSF ability to mediate communication between strains that cannot communicate via ComX. This suggests that *Bacillus* developed a strain-level specificity for a secreted signalling molecule, while producing an additional peptide able to mediate communication between strains. It has been

shown that the competence and sporulation factor is neither strain specific nor species specific, as CSF homologues has been isolated from *B. mojavensis*, a species closely related to *B. subtilis* (Pottathil *et al.*, 2008).

The regulation of *phrC* transcription was found to be partially regulated by the RNA polymerase containing the alternate sigma factor σ^H. The concentration of σ^H in the cells is low in the course of the exponential growth, but its increase upon entry into stationary phase induces an enhancement in transcription from the *phrC* promoter P2 (Healy *et al.*, 1991, Weir *et al.*, 1991). After entry into stationary phase, when σ^H is believed to be most active, the extracellular concentrations of CSF have been detected to be as high as 100 nM, corresponding to the concentration of peptide signal which stimulates sporulation. A mutation in *spoOH* (the gene coding for the sigma factor) showed to have a more dramatic effect on the levels of CSF than can be explained by the effect of σ^H on transcription of *phrC*, implying that σ^H has an additional role in the production of CSF other than the induction of *phrC* transcription. It has been suggested that σ^H might affect the transcription of the gene encoding the specific peptidase that recognizes and cleaves pre-CSF (Lazazzera *et al.*, 1999).

At least three roles have been attributed to the pentapeptide CSF: stimulation of expression of genes activated by the phosphorylated ComA (at low concentrations), inhibition of those same genes after reaching a threshold concentration and activation of sporulation under specific conditions (Lazazzera *et al.*, 1997, Solomon *et al.*, 1996b). The expression of genes activated by phosphorylated ComA is modulated by CSF by inhibiting the activity of the phosphatase RapC. RapC is a negative regulator of *srfA* expression, most likely by inhibiting accumulation of the phosphorylated form of the ComA transcription factor, the direct activator of *srfA* expression (Lazazzera *et al.*, 1997, Solomon *et al.*, 1996b). Again, the contribution of CSF in the activation of sporulation, requires the inhibition of a phosphatase, in this case RapB, a negative regulator of the phosphorelay required for the initiation of sporulation (Perego, 1997).

Since the two extracellular competence factors identified in *B. subtilis* are involved in divergent processes, with the ComX pheromone stimulating a kinase and CSF inhibiting a phosphatase, it is not surprising that CSF is considered an auxiliary competence pheromone, whereas the ComX pheromone is known as the major competence pheromone (Lazazzera *et al.*, 1999).

1.3.1.4 Development of competence mediated by extracelluar peptide signals

In response to the cell density-dependent accumulation of phosphorylated ComA triggered by accumulation of both the ComX pheromone and CSF, the transcription of the *srfA* operon is stimulated (Figure 1.10). The large operon *srfA* constists of three Open Reading Frames (ORFs), whose gene products (Cosmina *et al.*, 1993, Fuma *et al.*, 1993, Nakano *et al.*, 1991b, van Sinderen *et al.*, 1993) are engaged in the nonribosomal biosynthesis of the lipopeptide antibiotic surfactin and development of competence (Nakano *et al.*, 1991b). A small ORF located in the *srfA* operon has been identified, which encodes a 46-amino-acid peptide, named ComS, involved in competence development (D'Souza *et al.*, 1994, Hahn and Dubnau, 1991, Hamoen *et al.*, 1995, Nakano *et al.*, 1991b, Nakano and Zuber, 1991, Nakano *et al.*, 1991a, van Sinderen *et al.*, 1990).

Interestingly, surfactin biosynthesis and competence development were shown not to be directly related, except for the presence of *comS* within *srfA*. This genetic organisation ensures that *B. subtilis* uses a single quorum-sensing pathway for two different adaptive processes (Hamoen *et al.*, 2003). The production of surfactin (a potent biosurfactant with antimicrobial activity) coordinated with competence development might be convenient for a dual reason: giving the cells selective advantage towards competitors whilst providing them with surfactin lytic activity for the integration of genetic material released by lysed micro-organisms (Hamoen *et al.*, 2003).

ComS appears to be essential for competence development (D'Souza *et al.*, 1994, Hamoen *et al.*, 1995), as it plays an essential role in the activation of the so called

“competence transcription factor” ComK and the production of proteins of the DNA uptake machinery (Hahn *et al.*, 1994, van Sinderen *et al.*, 1995, van Sinderen *et al.*, 1994, van Sinderen and Venema, 1994).The role of ComK is to block DNA replication and cell division when competence development starts (Haijema *et al.*, 2001), which makes accurate control of *comK* expression vital to *B. subtilis* (Hahn *et al.*, 1995). As illustrated in Figure 1.12, during the exponential growth the competence transcription factor is inhibited by direct interaction with a proteasome-like complex consisting of MecA; ClpC, and ClpP (Dubnau and Roggiani, 1990, Turgay *et al.*, 1998, Turgay *et al.*, 1997). At low cell densities, the adapter protein MecA binds to ComK therefore leading to the degradation of the transcription factor by the ClpCP proteases. At high cell densities, when the expression of the *srf* operon is activated, ComS binds to MecA, thus relieving ComK from inhibition (Magnuson *et al.*, 1994). ComK activation triggers *comK* expression, following an autoregulatory loop, and expression of the late competence genes (van Sinderen *et al.*, 1995, van Sinderen *et al.*, 1994, van Sinderen and Venema, 1994, Hahn *et al.*, 1994). ComK autoregulation has the purpose of committing cells to the competence pathway, which explains why high levels of expression *of comK* and the late *com* genes can only be detected in cells that develop competence (Albano *et al.*, 1987, Hahn *et al.*, 1994). The transcription factor ComK requires the response regulator DegU in order to be fully induced (Albano *et al.*, 1987, Hahn *et al.*, 1994, van Sinderen and Venema, 1994, Hahn *et al.*, 1996). DegU and the histidine protein kinase DegS form a two-component signal transduction system which is engaged in the biosynthesis of degradative enzymes and polyglutamate (Henner *et al.*, 1988, Kunst *et al.*, 1988). It has been shown that unphosphorylated DegU is involved in competence developments, whereas DegS-mediated phosphorylation of its target response regulator triggers the production of degradative enzymes (Dahl *et al.*, 1991, Mukai *et al.*, 1990, Tanaka *et al.*, 1991). The DegU-DegS two component system as well as two accessory regulatory polypeptides, DegQ and DegR act at the level of transcription (Klier *et al.*, 1992, Kunst *et al.*, 1994, Msadek *et al.*, 1991). The presence of a helix–turn–helix DNA binding motif in DegU sequence confirmed its activity at the transcription level, where it is suggested to aid the interaction of ComK

with its promoter. However, as Figure 1.13 illustrates, accumulation of phosphorylated ComA triggers phosphorylation of DegQ, thus promoting an increase in the levels of phosphorylated DegU, which facilitates the synthesis of extracellular enzymes and polyglutamate production (Comella and Grossman, 2005, Msadek *et al.*, 1991, Nakano *et al.*, 1991b, Stanley and Lazazzera, 2005, Nakano and Zuber, 1991) .

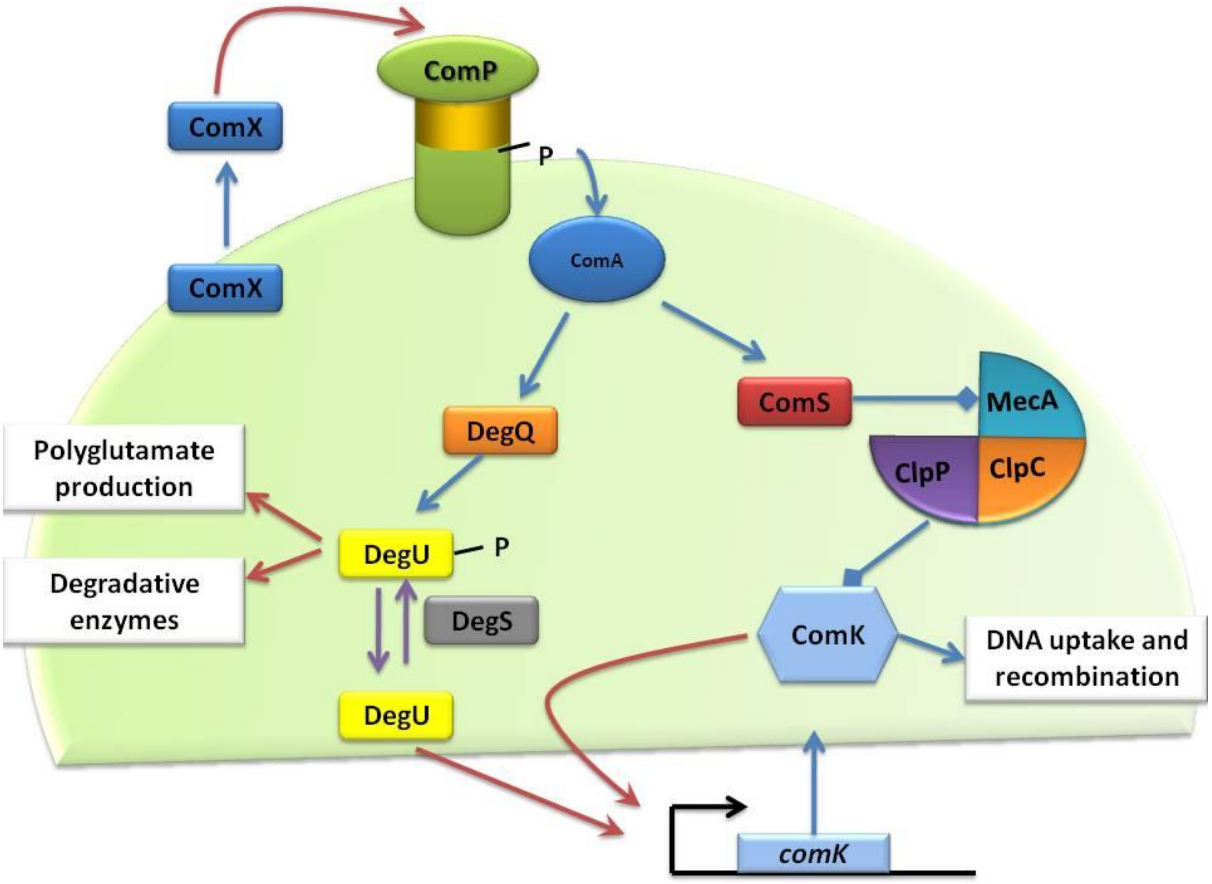


Figure 1.13: Regulation of competence and production of degradative enzymes and polyglutamate in *B. subtilis***.** The ComX pheromone activates the histidine kinase ComP, which in turn phosphorylates the response regulator ComA. Accumulation of ComA~P triggers *srfA* (comprising the small comS gene) transcription, thereby leading to ComS production. ComS relieves the transcription factor ComK from the inhibition operated by the MecA-ClpCP proteolytic complex. Through the action of the dephosphorylated response regulator DegU, the DegU/DegS two-component system facilitates the interaction of ComK to its cognate promoter. This results in the start of an autoregulatory loop, as ComK activates its own transcription. In its

phosphorylated state DegU promotes the synthesis of extracellular enzymes and polyglutamate for biofilm formation (Adapted from Hoffmann *et al.*, 2010).

Accumulation of phosphorylated ComA also triggers expression of the gene cluster containing *phrC* and co-transcribed *rapC* (Perego and Hoch, 1996). Both *rapC* and *phrC* influence their own expression by affecting transcription from the upstream operon promoter P1 (Solomon *et al.*, 1996a)**.** Although both the gene products appear to regulate their own expression by affecting the level of activated ComA they pursue divergent pathways. Whilst RapC negatively regulates its own transcription, thus creating a homeostatic loop, CSF acts positively on its own expression (Solomon *et al.*, 1996a). At low cell densities the extracellular CSF concentration is inadequate to inhibit RapC activity, therefore allowing RapC to affect accumulation of phosphorylated ComA by inhibiting the DNA-binding activity of the transcription factor (Bongiorni *et al.*, 2005). Since the accumulation of phosphorylated ComA controls *rapC* promoter, RapC inhibiting activity negatively regulates its own transcription (Solomon *et al.*, 1996a, Lazazzera *et al.*, 1997). Accumulation of CSF again causes an increase in transcription from the *rapC* promoter: this homeostatic loop guarantees the level of RapC to remain relatively constant while cells are at low densities. As cells grow, the extracellular concentration of CSF increases, partly due to the increasing number of cells producing CSF and partly due to ComX accumulation in the culture medium, which activates both ComA phosphorylation and transcription of *phrC* (Solomon *et al.*, 1996a, Lazazzera *et al.*, 1997). CSF reaches its threshold concentration (estimated to be between 2 and 5 nM) in mid-exponential growth phase. At this stage CSF is capable of trigger inhibition of the RapC phosphatase activity, which results in the accumulation of phosphorylated ComA and increase in transcription of the *rapC-phrC* operon (Lazazzera *et al.*, 1997, Solomon *et al.*, 1996b). As previously described, one of the operons activated by ComA~P, *srfA*, triggers activation of the competence transcription factor ComK and competence development (Hahn *et al.*, 1994, van Sinderen and Venema, 1994, van Sinderen *et al.*, 1994, van Sinderen *et al.*, 1995).

1.3.2 Quorum sensing and sporulation

For many years it has been postulated that sporulation in *B. subtilis* is related to carbon, nitrogen or phosphate starvation and to specific development signals. However, the addition of decoynine (an inhibitor of GMP synthetase that stimulates sporulation but not competence) to *B. subtilis* cultures was unable to trigger initiation of sporulation at low cell density. This evidence prompted the idea that quorum sensing and peptide signalling might contribute to the activation of sporulation in *B. subtilis* together with a wide range of environmental and physiological signals (Grossman and Losick, 1988). These signals, including nutrient depletion, cell density, the Krebs cycle, DNA synthesis, and DNA damage are directed towards a phosphorelay which leads to activation via phosphorylation of the transcriptional regulatory protein SpoOA.

SpoOA belongs to the response regulator family of two-component signal transduction systems, though this protein does not directly obtain its phosphate from a histidine protein kinase. Rather, phosphorylation of SpoOA concerns a complex phosphorelay system, where the phosphoryl group is initially transferred to the intermediate phosphoacceptor SpoOF, then to the phosphotransferase SpoOB, and finally to SpoOA, in a signal cascade involving three different histidine kinases, namely KinA, B and C (Grossman, 1995) (Figure 1.9). Accumulation of phosphorylated SpoOA activates the transcription of at least seven genes controlling the entry into sporulation by binding to their promoters, aided by RNA polymerases containing either the primary sigma factor σ^A (Baldus *et al.*, 1994, Bird *et al.*, 1996) or the alternative sigma factor σ^H (Bramucci *et al.*, 1995). In addition to its function as a transcriptional activator, SpoOA can also act as a transcriptional repressor, as is the case for a gene called *abrB* (Bird *et al.*, 1993, Strauch *et al.*, 1990).

Activation of the SpoOA transcription factor has been shown to be dependent on cell density, as a constitutively active form of SpoOA allowed efficient sporulation of cells at low density, thus completely bypassing the need for high cell density (Ireton *et al.*, 1993). This result implies that the quorum sensing mechanism involved in the

activation of sporulation is mediated by the control of production of the phosphorylated SpoOA (Lazazzera, 2000).

In *B. subtilis* there appear to be multiple extracellular peptide factors that affect sporulation, including the competence and sporulation factor CSF. This signal peptide generated by the modification of PhrC is involved in the activation of sporulation following its accumulation at concentrations estimated to be higher than 20 nM during the stationary phase (Lazazzera *et al.*, 1997). It has been shown that CSF is able to enhance sporulation of nutrient-deprived cells at low cell density, but only when supplemented at concentrations in the range of 50 to 100 nM (Lazazzera, 2000). CSF appears to activate sporulation by inhibiting the activity of histidine kinase ComP, thus preventing the formation of phosphorylated ComA and blocking the activation of *srfA* expression (Lazazzera *et al.*, 1997, Solomon *et al.*, 1996b). The *phrC* gene, which encodes CSF, is under the control of two promoters (Lazazzera *et al.*, 1999). One promoter, called P1, is involved in an autoregulatory loop involving both CSF and ComA (Lazazzera *et al.*, 1999). The second promoter (P2) is regulated by σ^H (Lazazzera *et al.*, 1999), whose levels of activity are stimulated by starvation and reach the maximum between the end of the exponential phase and the beginning of the stationary phase (Healy *et al.*, 1991). When cells enter stationary phase, the concentration of σ^H increases, thus activating the expression of *phrC* and inducing cells to sporulate rather than develop competence. The genes necessary for CSF to stimulate sporulation have not been identified so far, although it has been proposed that CSF might be involved in the inhibition of phosphatases, such as RapB (Lazazzera *et al.*, 1997, Perego, 1997, Solomon *et al.*, 1995b).

At least other two pentapeptides are involved in the regulation of sporulation in *B. subtilis*, namely PhrA and PhrE. The product of *phrA*, located in the *rap*A phosphatase gene operon (Solomon *et al.*, 1996b) is a 144 amino acid precursor polypeptide which is then cleaved resulting in a 19 amino acid peptide. However, the active form of the PhrA peptide is the pentapeptide ARNQT which inhibits the RapA phosphatase, after being re-internalised via the Opp system in *B. subtilis* cells (Perego *et al.*, 1996b). The pentapeptide derived from the product of *phrE* is generated from within the carboxy-terminal domain of the PhrE pre-pro-protein, thus

suggesting a processing event distinct from the one resulting in the production of ARNQT (Jiang *et al.*, 2000).The role of PhrE (if any) in the activation of sporulation has yet to be elucidated. The aspartyl phospatases co-transcribed with and repressed by PhrA and PhrE, (called RapA and RapE respectively) have an active role in activation of sporulation (Mueller *et al.*, 1992, Perego *et al.*, 1994). During the transition phase between exponential and post-exponential growth, sporulation is repressed due to the accumulation of phosphorylated ComA, which stimulates *rapA* and *rapE* expression. The resulting high cellular levels of RapA and RapE cause repression of SpoOF via dephosphorylation. At the onset of stationary phase, the accumulation of PhrA and PhrE pentapeptides results in RapA and RapE inhibition, therefore leading to accumulation of phosphorylated SpoOF, which in turn activates sporulation (Jiang *et al.*, 2000b, Perego *et al.*, 1996a).

1.4 *Bacillus licheniformis*

Bacillus licheniformis belongs, like *Bacillus subtilis*, to the genus *Bacillus*, comprising numerous rod-shaped Gram positive bacteria including both free-living and pathogenic species. Under stressful environmental conditions the cells produce endospores able to survive long periods of time in a dormant state, without nutrients and in adverse circumstances. *Bacillus* species are typically motile because of their flagella and are aerobic. *B. licheniformis* is ubiquitous in nature, is predominantly found in soil as endospores, and is a facultative anaerobe (Claus and Berkeley, 1986). Since 1972, different *B. licheniformis* strains have been safely used in the fermentation industry for production of proteases, amylases and specialty chemicals. Yields as high as 25 g L^{-1} have been obtained in the production of exoenzymes from some *B. licheniformis* strains (Schallmey *et al.*, 2004). The industrial relevance of *B. licheniformis* is also related to the production of several antimicrobial compounds, such as bacitracin (Johnson *et al.*, 1945) and the surfactin-resembling lichenysin (Yakimov *et al.*, 1995). *B. licheniformis* is genetically related to *B. subtilis,* whose regulation of competence and sporulation is controlled by a quorum sensing mechanism.

1.4.1 Quorum sensing in *B. licheniformis*

1.4.1.1 Identification of competence-related genes in *B. licheniformis*

Preliminary characterization of competence and quorum-sensing-related genes in *Bacillus licheniformis* was performed by establishing genetically co-linear regions with *B. subtilis* genome using physical mapping. This technique, named co-linear scaffolding, allowed Lapidus and co-workers (Lapidus *et al.*, 2002) to compare co-linear regions between *B. licheniformis* ATCC 14580 genome, whose sequence had not been published yet at the time, and the entirely sequenced genome of *B. subtilis* 168. As these two genomes are 70% identical at the nucleotide level the technique led to characterization of more than 60% of the *B. licheniformis* gene complement, comprising relevant genes which share significant homology with competence-regulating genes of *B. subtilis*, such as the *comQXPA* cluster, *clpC*, *comK*, *comC*, and the entire *comE*, *comF* and *comG* operons.

The gene encoding MecA, one of the proteins involved in the inhibition of the competence transcription factor ComK, could not be identified in any co-linear region. However, Southern hybridization analysis established the existence of this gene in *B. licheniformis* genome, most likely located on a non co-linear region. Similarly, no gene coding for ComS, the small protein responsible for ComK activation, was identified in *B. licheniformis*. In *B. subtilis* genome, *comS* is located inside the *srf* operon, encoding surfactin biosynthetic machinery. This operon consists of three long ORFs, which were not detected in any co-linear region. However, a high degree of similarity was established between the previously sequenced region comprising lichenysin biosynthetic genes in *B. licheniformis* ATCC 10716 (Konz *et al.*, 1999) and the *srf* operon of *B. subtilis* 168. The three genes for lichenysin biosynthesis in *B. licheniformis* ATCC 10716 are located on an operon (*lic*) which is almost identical to the *srf* operon of *B. subtilis*. However, as an ORF corresponding to *comS* could not be identified in the *lic* operon, it was initially suggested that a different protein with the same functions of ComS might be produced in *B. licheniformis* (Lapidus *et al.*, 2002).

Recently, *comS* identification was attempted by comparing the N-terminal region of the surfactin synthetase B of *B. subtilis* 168 with the corresponding sequence of the lichenysin synthetase B of *B. licheniformis.* However, the identification of a putative ComS protein in *B. licheniformis* was attained by searching for conserved ComS peptides in different *Bacillus* species. The putative ComS of *B. licheniformis* varies from its counterparts of *B. subtilis* and *B. amyloliquefaciens,* by an N-terminal extension of 16 amino acids, a four amino acids insertion at position 27, and a different core sequence for MecA binding (Hoffmann *et al.*, 2010).

1.4.1.2 The *comQXPA* cluster of *B. licheniformis*

The *comQXPA* gene cluster, which plays an essential role in the regulation of competence development in *B. subtilis*, was also identified in *B. licheniformis* genome. This gene cluster is only present in bacilli strictly related to *B. subtilis* (Nakano and Zuber, 1991, Magnuson *et al.*, 1994). At present, *B. licheniformis* is the species most remote from *B. subtilis* where the *comQXPA* cluster was detected, hence it is not surprising that the ComQ and ComX proteins of *B. licheniformis* ATCC 14580 show higher similarity to their counterparts in *B. mojavensis*, a closer related strain, than to those in *B. subtilis* 168 (Lapidus *et al.*, 2002). The response regulator ComA, which regulates the expression of the surfactin biosynthetic operon in *B. subtilis*, was shown to play a similar role in *B. licheniformis* by inducing transcription of the lichenysin synthase genes. This regulatory function was confirmed by the identification of a putative ComA Box in the promoter region of the lichenysin operon (Yakimov and Golyshin, 1997, Yakimov *et al.*, 1998).

In *B. licheniformis* genome a 1288-bp insertion sequence was identified in the gene encoding the histidine kinase ComP, dedicated to the recognition of the ComX pheromone and the phosphorylation of ComA in *B. subtilis*. The insertion sequence, named as *IS3Bli1*, encodes a 278 aa-protein belonging to the IS3 family of transposases of IS elements. Previously, a not-related IS insertion, *IS4Bsu1*, was identified in the ComP-encoding gene of *B. subtilis* (natto) NAF5 (Nagai *et al.*,

2000a). Apart from competence development the *comQXPA* system of *B. subtilis* is involved in the regulation of several cell-density-dependent phenomena, such as surfactin, poly-glutamic acid and degradative enzymes production and surface attachment (Magnuson *et al.*, 1994, Weinrauch *et al.*, 1990, Nakano and Zuber, 1991). The disruption of the sensor kinase of the system by the insertion of IS elements leads to impairment of all the aforementioned processes by interrupting communication between cells (Nagai *et al.*, 2000a). The finding that two natural isolates, such as *B. subtilis* (natto) NAF5 and *B. licheniformis* ATCC 14580, have their *comP* sequences interrupted by a similar transposon insertion, suggests a strong competition among bacilli for the benefits arising from communication between closely related neighbours. Recently, the genome sequences of *B. licheniformis* strains DSM13 (Veith *et al.*, 2004) and the isogenic ATCC 14580 (Rey *et al.*, 2004) have become available, thus allowing further research on cell-cell communication in these strains. The two genomes have been compared showing that they only differ in the orientation of the IS element accompanied by a breakdown in the GC content at the site of insertion in *comP*, possibly suggesting a flexible position within the genome (Hoffmann *et al.*, 2010).

Although quorum sensing is well established in *B. subtilis* and in spite of the above studies, no concrete report has been published on cell-cell communication in *B. licheniformis* and the production of signalling molecules in this organism has not been verified so far.

1.5 Lichenysin

A number of *Bacillus* species have been shown to produce lipopeptides with significant surface-active properties (Fiechter, 1992). The best characterized of these lipopeptides is surfactin, an acylated cyclic heptapeptide produced by several *Bacillus subtilis* strains. Surfactin was found capable of reducing water surface tension from 72 to 27 mN m^{-1} at concentrations lower than 0.05%. Also, this lipopeptide shows antimicrobial activity against both bacteria and fungi. Other lipoheptapeptides structurally related to surfactin, such as iturin (Peypoux *et al.*,

1978) and bacillomycin (Mhammedi *et al.*, 1982, Peypoux *et al.*, 1985), have been identified in some *B. subtilis* strains.

Certain strains of *B. licheniformis* produce a very effective biosurfactant, with structural properties similar to surfactin, named lichenysin. In addition to its surface activity, lichenysin shows some remarkable biological properties, such as inhibition of fibrin clot formation (Arima *et al.*, 1968); cholesterol-lowering effects (Imai *et al.*, 1971), antiviral and antitumoral activities (Vollenbroich *et al.*, 1997, Kameda *et al.*, 1972, Hosono and Suzuki, 1983, Nissen *et al.*, 1997). Furthermore, lichenysin has been shown capable of interacting with phospholipids and inducing selective cationic channels formation in artificial membranes (Sheppard *et al.*, 1991); it also inhibits enzymes such as cyclic adenosine monophosphate phosphodiesterase (Hosono and Suzuki, 1983) and phospholipase A_2 (Kim *et al.*, 1998).

The structure of lichenysin is characterised by the heptapeptide sequence L-Glx1-L-Leu2–D-Leu3–L-Val4–L-Asx5–D-Leu6–L-Ile7 linked to a β-hydroxy fatty acid chain ranging from 13 to 15 carbon atoms. The first amino acid is connected to the fatty acid by an amide bond, whilst the carboxy-terminal Ile residue forms a lactone ring with the β-OH group of the lipophilic part of the molecule (Grangemard *et al.*, 1999b, Yakimov *et al.*, 1999, Yakimov *et al.*, 1998). Lichenysin heptapeptide differs from surfactin only in two constituents amino acids, an amide residue at position 1 (L-Gln) or 5 (L-Asn) instead of an acidic one in surfactin, and an Ile residue in position 7 in place of Leu. However, it appears that the former variation is the most relevant, as it determines a significant change in the biosurfactant properties of the molecule. Also, the composition and length of the hydroxyl fatty acid chain varies between the two biosurfactants (Yakimov *et al.*, 1995, Grangemard *et al.*, 1999b). Lichenysin has been shown to have much higher surfactant power and antimicrobial activity of surfactin, although it is typically produced in much lower amounts (Yakimov *et al.*, 1996). Five different lichenysins, namely A, B,C, D, and G, have been isolated and characterised, revealing various distributions of branched and linear fatty acid moiety which depend on the producing strain (Yakimov *et al.*, 1996, Yakimov *et al.*, 1995, McInerney *et al.*, 1990, Jenny *et al.*, 1991). So far, research has been focussed mostly on lichenysin A, produced by *B. licheniformis* strains BAS50 and BNP29, a

complex comprising a mixture of 14 components with sizes ranging from 992.0 to 1034.0 Da with heterogeneous lipophilic chains (Yakimov *et al.*, 1996, Yakimov *et al.*, 1995).

In analogy with surfactin, lichenysin A biosynthesis is carried out non-ribosomally on a large multienzymatic complex, known as peptide synthetase, using a thiotemplate mechanism. The operon coding for lichenysin biosynthetic complex has been sequenced, thus leading to the identification of four distinct ORFs, *lchAA*, *lchAB, lchAC, lchA-TE* strictly resembling the organisation of the surfactin biosynthetic locus (Konz *et al.*, 1999, Yakimov *et al.*, 1998). Furthermore, a ComA-like box was identified upstream from the *lchA* promoter, therefore confirming that, similarly to surfactin, lichenysin biosynthesis is controlled by the response regulator ComA (Yakimov and Golyshin, 1997, Yakimov *et al.*, 1998).

The recent findings on the modular organisation of the peptide synthetases involved in surfactin and lichenysin biosynthesis facilitated the creation of new biosurfactants, produced by exchanging minimal modules between multi-modular peptide synthetases of different origins (Stachelhaus *et al.*, 1995, Schneider *et al.*, 1998).

1.6 γ-Poly Glutamic Acid (PGA)

Some Bacilli produce a polymer, known as γ-polyglutamic acid (PGA), as a capsular or an extracellular viscous material (Birrer *et al.*, 1994). This polymer, formed by glutamic acid residues linked by γ-glutamyl bonds, was originally isolated as a component of *B. anthracis* (Ivanovics and Erdos, 1937) and *B. mesentericus* (Ivanovics and Bruckner, 1937) capsule, whose function is to provide a protective barrier for the bacterium against hostile environments (Makino *et al.*, 1988, Makino *et al.*, 1989, Makino *et al.*, 2002). γ–PGA is found as an ingredient in a Japanese traditional food, natto, prepared with steamed soybeans fermented by *B. subtilis* (Hara *et al.*, 1982). Due to its polyanionic and nontoxic properties γ–PGA is widely used in industrial applications, such as production of food additives, cosmetics and natural biocides; and water treatment. Three stereochemically different variants of γ-PGA have been identified so far: a homopolymer comprising only D-glutamate, a

homopolymer composed solely of L-glutamate, and a copolymer of D- and L-glutamate units randomly arranged (Ashiuchi *et al.*, 2003, Tanaka and Ozaki, 1997). Following its discovery, PGA was identified in the culture medium of several Bacilli, such as *B. subtilis* (Ito *et al.*, 1996, Kubota *et al.*, 1993, Kunioka and Goto, 1994), *B. licheniformis* (Tabone and Jacobelli, 1961, Troy, 1973), and *B. megaterium* (Guex-Holzer and Tomcsik, 1956, Torii *et al.*, 1959).

In both *B. anthracis* and *B. subtilis*, the synthesis of the capsule polypeptide is catalysed by membrane-associated PGA-synthases, encoded by the *capBCA* and *pgsBCA* operons, respectively. Even though the organisation of γ–PGA biosynthetic genes is shared by a number of *Bacillus* species, their regulation of γ-PGA synthesis, and the stereochemistry of the biopolymer produced, differs depending on the producer organism (Ashiuchi *et al.*, 1999, Makino *et al.*, 1989, Urushibata *et al.*, 2002). In *B. anthracis*, for example, L-PGA is produced when the cells are grown in conditions mimicking host environments (Makino *et al.*, 1988, Makino *et al.*, 1989, Makino *et al.*, 2002), whereas in *B. subtilis* the production of DL-PGA copolymer is initiated specifically in early stationary phase. In particular, γ-PGA synthesis in *B. subtilis* is a cell-density-dependent process controlled by the *comQXPA* locus (Dubnau, 1999, Lazazzera *et al.*, 1999, Tran *et al.*, 2000). The ComPA two-component transduction system has been suggested to have a key role in the regulation of γ-PGA production by activating a second two-component system, comprising the histidine kinase DegU and the response regulator DegS. The DegUS system, in turn, promotes the formation of γ-PGA capsule formation. Although the exact mechanism of this regulation is yet to be elucidated, the relation between quorum-sensing and γ-PGA biosynthesis has been proven by reports that Bacillus strains with a non functional ComPA system, such as *B. subtilis natto* NAF4 (Nagai *et al.*, 2000b, Takahashi *et al.*, 2007) and *B. licheniformis* ATCC 14580 showed an impairment in production of the biopolymer (Hoffmann *et al.*, 2010).

1.7 Investigation of quorum sensing molecules based on bioassays

The use of reporter strains for the identification of quorum sensing molecules is a well established practise, which has been originally developed for the detection of AHLs in Gram negative bacteria. Examples are the *lux*-based biosensors, where *Escherichia coli* cells carrying the *luxCDABE* operon of *Photorhabdus luminescens* are used to detect AHLs through development of bioluminescence (Winson *et al.*, 1998a, Winson *et al.*, 1998b), and AHLs bioassays which exploit the production of purple pigment in *Chromobacterium violaceum* (Blosser and Gray, 2000, McClean *et al.*, 1997).

The first *B. subtilis PsrfA-lacZ* fusion-carrying reporter strain was developed by Magnuson and co-workers (1994) for the identification of the ComX pheromone and since then a number of different reporter strains have been created and used for the isolation of ComX variants in different Bacilli or for detection of the pheromone in the course of the purification process (Ansaldi *et al.*, 2002, Okada *et al.*, 2005, Okada *et al.*, 2004). All these studies report detection of the ComX pheromone from different strains or from natural isolates of *B. subtilis* and the use of *B. subtilis* reporter strains has never been extended to the investigation of the competence pheromone in supernatants of Bacilli as genetically distant as *B. licheniformis*. However, a similar approach has been reported for the investigation of AI-2 signal in *B. subtilis* where supernatants from the Gram positive *B. subtilis* cultures at high cell densities were tested on low cell density cultures of *V. harveyi* , a Gram negative bacterium (Lombardia *et al.*, 2006).

A diagram illustrating the mechanism for induction of β-galactosidase activity in *B. subtilis* reporter strains used in this study, namely JRL293 and LS27, is presented in Figures 1.14.

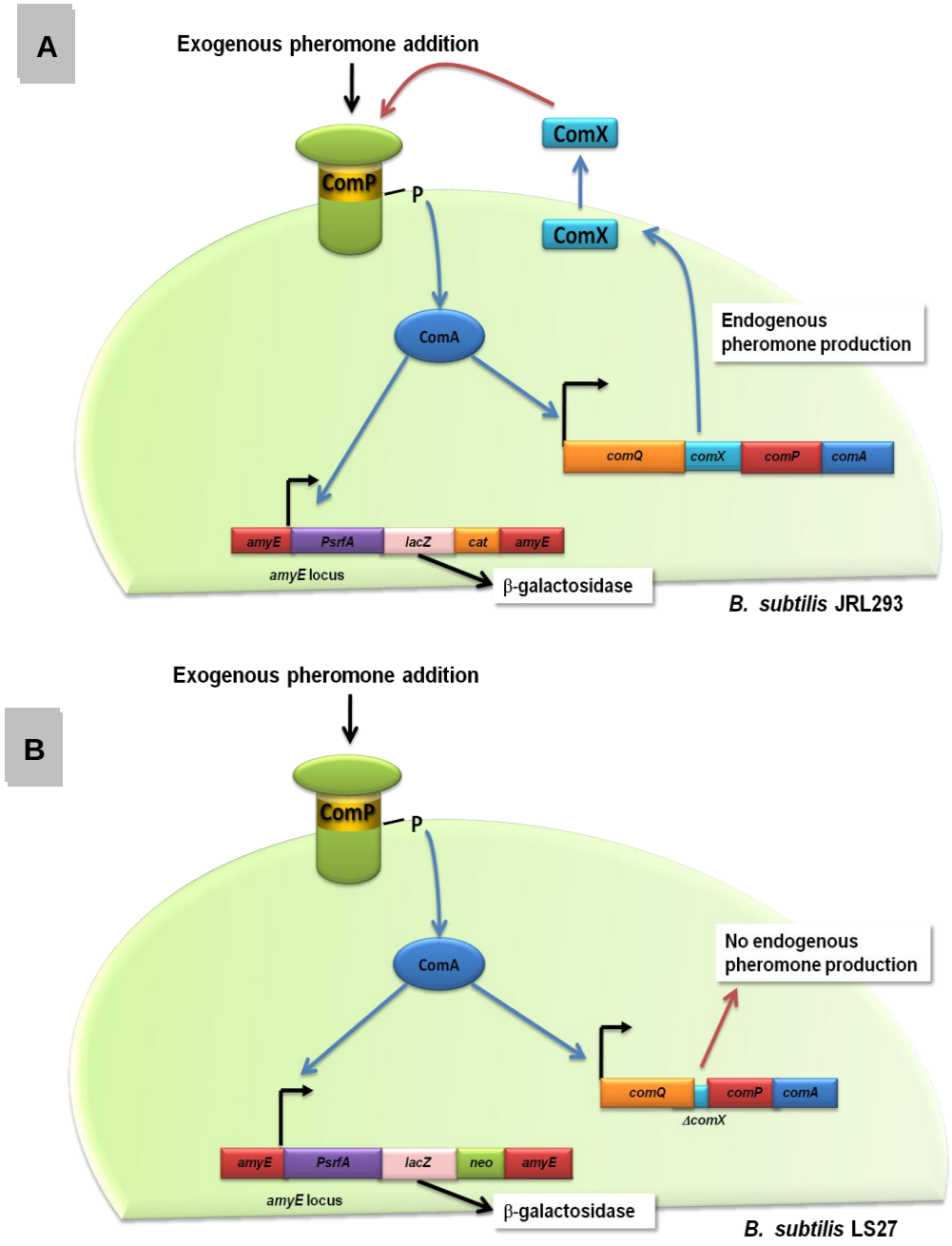


Figure 1.14: Illustration of the mechanism for induction of β-galactosidase activity in *B. subtilis* JRL293 (A) and *B. subtilis* LS27 (B) reporter strains.

As *srfA* is required for competence development and is induced by molecules accumulating in the extracellular medium, this reporter strain was constructed in order to monitor the expression of *srfA* operon through *lacZ* expression. In a minimal glucose medium, *B. subtilis srfA-lacZ* cultures at low cell densities show a basal level

of *srfA* expression. The addition of molecules capable to activate *srfA* to such cultures will induce an increase in *srfA* expression, which can be detected measuring the resulting β-galactosidase activity. Therefore, the level of β-galactosidase activity will be related to the increase in *srfA* expression induced by signalling molecules accumulated in the extracellular medium of the cultures to be tested (Magnuson *et al.*, 1994).

Chapter II

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Materials

All materials used in this study were obtained from Sigma-Aldrich Company Limited, Dorset, United Kingdom unless otherwise stated. All high-pressure liquid chromatographic (HPLC) assays were carried out using HPLC grade chemicals and HPLC grade water. All other qualitative and quantitative assays were carried out using reagents of analytical grade. Media were prepared using general purpose reagents. Materials for molecular biology studies were obtained from Qiagen (Crawley, UK), Promega (Southampton, UK) and Sigma (Dorset, UK). DNeasy Blood and Tissue Kit (Qiagen) was used for genomic DNA isolation. QIAquick Gel extraction kit from Qiagen was used for extraction of DNA from agarose gels. PCR Master Mix (Promega) was used for conventional polymer chain reaction. Primers for conventional PCR were purchased from Invitrogen (Renfrew, UK).

2.2 Microorganisms

Bacillus licheniformis NCIMB 8874 and *Bacillus subtilis* NCIMB 3610 were purchased from the National Collection of Industrial and Marine Bacteria, USA. *Bacillus subtilis* strains JRL293 [*amy::(srfA-lacZ, cat),trp, phe*]; TMH281[*comQ::spc*, *amy::(srfA-lacZ, neo), trp, phe)*] and LS27 [*amyE::(srfA-lacZ, neo), trp, phe,* ∆*comX*(*comX*∆1-45)] were a kind gift from Prof. Alan D. Grossman, Massachusetts Institute of Technology, USA.

2.3 Media

All media used in this study were sterilized at 121 °C for 15 minutes unless otherwise stated.

2.3.1 Media for maintenance of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type

Lysogeny broth (LB) (Table 2.1) and LB + agar were used for routine maintenance of both *B. licheniformis NCIMB 8874* and *B. subtilis* wild type strain. Medium (1 L) was prepared and autoclaved at 121 °C for 15 minutes. BactoAgar (BD, Oxford, UK) at a concentration of 15 g L^{-1} was added to the broth to make up LB agar.

***Table 2.1**: Lysogeny Broth (LB) composition*

Component	Concentration (g L^{-1})
Tryptone	10.0
Yeast Extract	5.0
NaCl	10.0

All the strains were routinely stored as glycerol stocks at -80 °C. The stocks were prepared by growing cells from a fresh colony in LB up to OD_{600} ~ 1 and adding 15% (v/v) pre-sterilised glycerol before freezing. Fresh colonies were obtained by streaking a loopful of the frozen material onto LB agar. The freshly inoculated plates were incubated at 37 °C for 18 hours and then stored at 4 °C for 5 days. Maintenance medium for *B. subtilis* JRL293 was supplemented with chloramphenicol (5 µg mL^{-1}), whereas neomycin (50 µg mL^{-1}) was added for selection of *B. subtilis* strains LS27 and TMH281.

2.3.2 Medium for growth of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type

Cultivation of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type for the development of competence and production of quorum sensing molecules was conducted in competence medium comprising S7 minimal salt solution (Table 2.2)

supplemented with Glucose 1% (w/v) and Sodium glutamate 0.1% (w/v). The S7 minimal salt solution was prepared in 1 L distilled water and autoclaved at 121 °C for 15 minutes. Glucose 10% (w/v) solution was prepared separately in 1 L distilled water and autoclaved at 110 °C for 10 minutes. Sodium glutamate 1% (w/v) solution was also made up separately and filter-sterilised through a 0.22 μm filter (Millipore).

Table 2.2: *S7 minimal salt solution composition*

Ingredient	Concentration (g L^{-1})
3-(N-morpholino) propanesulfonic acid (adjusted to pH 7.0 with 10M KOH)	10.4600
$(NH_4)_2SO_4$	1.3200
Potassium phosphate (pH 7)	0.6800
$MgCl_2$	0.2000
$CaCl_2$	0.0800
$MnCl_2$	0.0060
$FeCl_3$	0.0006
Thiamine	0.0005
$ZnCl_2$	0.0001

2.3.3 Medium for growth of *B. subtilis* reporter strains

Competence medium was also used for growth of *B. subtilis* reporter strains JRL293, LS27 and TMH281. The medium was prepared as described in Section 2.3.2 and supplemented with filter-sterilised tryptophan (50 μg mL^{-1}) and phenylalanine (50 μg mL^{-1}) and appropriate antibiotics (see Section 2.3.1).

2.3.4 Medium for production of biosurfactant

B. licheniformis NCIMB 8874 and *B. subtilis* wild type cultures were grown in Yeast Peptone Dextrose (YPD) broth for biosurfactant production and extraction (Thaniyavarn *et al.*, 2003). YPD medium composition is listed in Table 2.3.

Table 2.3: *YPD medium composition*

Component	**Concentration (g L^{-1})**
BactoPeptone	20
BactoYeast extract	10
Dextrose	20

2.3.5 Medium for γ-PGA production

B. licheniformis NCIMB 8874 cells were cultivated in PGA production medium for production of the biopolymer. The composition of the PGA production medium (Goto and Kunioka, 1992) is listed in Table 2.4. The medium was prepared in 1L distilled water and the pH was adjusted to 7 before it was autoclaved at 121 °C for 15 minutes. Sodium glutamate was made up separately and filter-sterilised through a 0.22 μm filter (Millipore).

Table 2.4: *PGA production medium composition*

Component	Concentration ($g\ L^{-1}$)
Citric acid	20.00
Sodium glutamate	30.00
Glycerol	20.00
$(NH_4)_2SO_4$	15.00
K_2HPO4 7H2O	1.00
$MgSO_4$	0.50
Na_2HPO_4	1.00
$FeCl_3$	0.05
$MnSO_4$	0.02
$CaCl_2$	0.20

2.3.6 Medium for proteolytic activity assay

Extracellular proteolytic activity of *B. licheniformis* NCIMB 8874 was analysed by disc diffusion assay on agar plates containing M9 salts supplemented with skimmed milk powder (10% w/v). Compositions of M9 salts (5X) and M9 agar are listed in Table 2.5 and 2.6, respectively (Sambrook and Russell, 2001).

Table 2.5: *M9 (5X) salts composition*

Component	Concentration ($g\ L^{-1}$)
$Na_2HPO_4.7H_2O$	64.0
KH_2PO_4	15.0
NH_4Cl	5.0
NaCl	2.5

***Table 2.6**: M9 agar composition for 1L*

Component	Concentration (g L^{-1}) or (volume, mL)
Skim milk	10.00
BactoAgar	15.00
$MgSO_4$	0.24
$CaCl_2$	0.01
M9 salts	(100)

2.4 Fermentation conditions

Stock cultures of both *B. licheniformis* NCIMB 8874 and *B. subtilis* strains were streaked separately on LB agar plates and incubated at 37 °C for 16 hours. The competence medium (5 mL) (as specified in Section 2.3.2) was inoculated with a single colony and incubated at 37 °C on a rotary shaker at 200 rpm (2 cm-throw) for 15 hours. Erlenmeyer flasks (500 mL) were used for the growth of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type for production of quorum sensing molecules in Shaken Flask (SF) fermentation.

For SF fermentations, the 15 hour old inoculum cultures were diluted in 100 mL of the competence medium to reach an OD_{600} value of 0.08-0.1. SF fermentations were carried out at 37 °C on a rotary shaker at 200 rpm (2 cm-throw) for 10-12 hours. SF studies were performed in triplicate.

2.5 Assays procedures

2.5.1 Optical density and pH measurements

The optical density (OD) of the samples collected in the course of fermentation was read at 600 nm wavelength, where competence medium was used as a

blank. Absorbance readings above 0.5 were diluted using fresh medium. The pH was also monitored.

2.5.2 β-galactosidase assay

β-galactosidase activity was estimated by colorimetric assay. The cell density of the culture to be assayed was recorded by measuring the absorbance at 600 nm. The assay was carried out in plastic test tubes by adding warmed permeabilization solution (Table 2.7) to the sample to be assayed. When high levels of β-galactosidase were to be assayed, 0.9 mL of the permeabilisation solution was added to 0.1 mL of the culture to be assayed. When low levels of enzymatic activity were being determined, 0.5 mL of the permeabilisation solution was added to 0.5 mL of the culture. The test tubes were incubated at 28 °C for 5 minutes. The reaction was started by adding 0.2 mL of the substrate buffer (Table 2.8) to each tube and mixing thoroughly. The reaction was then stopped by addition of 0.5 mL of a 1M Na_2CO_3 solution after yellow colour was developed. Samples were read at 420 nm wavelength using a BioMate 3 spectrophotometer (Thermo Scientific, UK). β-Galactosidase specific activity was then calculated according to Miller (ΔA_{420}/min mL^{-1} of culture OD_{600}) x 1000 (Miller unit, MU) (Miller, 1972).

Table 2.7: *Composition of permeabilisation buffer for β-galactosidase assay*

Component	Concentration (M)
$Na_2HPO_4.7H_2O$	0.060
$NaH_2PO_4.H_2O$	0.040
KCl	0.010
$MgSO_4$	0.001
β -mercaptoethanol	0.050

***Table 2.8**: Substrate buffer composition for β-galactosidase assay*

Component	Concentration (M)
$Na_2HPO_4.7H_2O$	0.06
$NaH_2PO_4.H_2O$	0.04
o-nitrophenyl-β-galactoside	0.01

2.5.2.1 Pheromone assay (70 minutes assay)

Pheromone activity was assayed based on its ability to induce expression of *srfA-lacZ* in cells at low cell density. In the standard assay, 0.25 mL of *B. subtilis* cells carrying the *srfA-lacZ* fusion at an OD_{600} of 0.08-0.1 were added to 0.25 mL of spent medium to be assayed. BSA (50 μg ml^{-1}) was added to the sample potentially containing the ComX pheromone to prevent non-specific loss of activity (Magnuson *et al.*, 1994). The mixture was incubated at 37 °C for 70 minutes in a rotary shaker at 200 rpm (2 cm-throw) and then assayed for β-galactosidase specific activity. The pheromone assay is schematically presented in Figure 2.1.

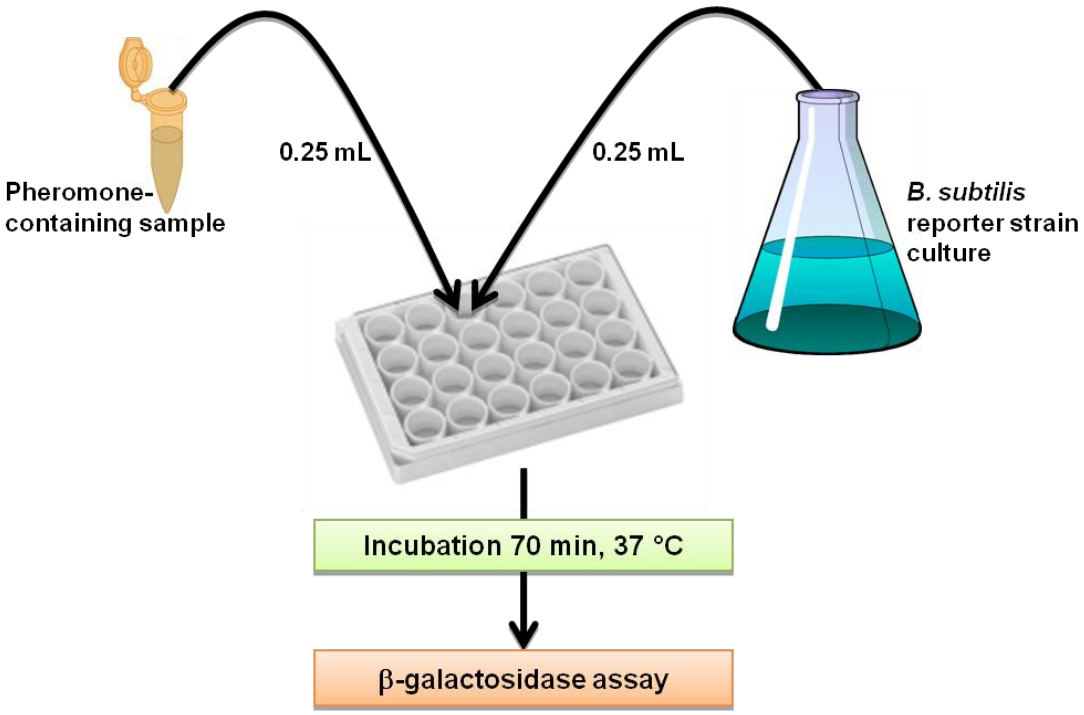


Figure 2.1: Diagram of the pheromone (70 minutes) assay.

2.5.2.2 Addition of spent medium for induction of quorum sensing response in *B. subtilis* reporter strains

Spent media for induction of quorum sensing response in *B. subtilis* reporter strains was prepared by growing *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type (used as control) in competence medium at 37 °C for 8 hours on a rotary shaker at 200 rpm (2 cm-throw). Cultures were harvested and centrifuged at 4700 rpm for 45 minutes. The supernatants were collected and filtered through a 0.22 μm syringe filter (Millipore). The spent media were stored at 4°C for future experiments.

The competence medium (50 mL) was diluted with 50 mL of spent medium and inoculated with *B. subtilis* reporter strain cells to OD_{600} 0.08. SF fermentation was performed as described in Section 2.4. Throughout the growth, 1 mL samples were collected at specific time intervals. The samples collected were homogenised

for 40 seconds at 4.5 m/s (meter/second) using a FastPrep-24 Instrument, (MP Biomedicals, Cambridge, UK) and then stored at 4°C and later assayed for β-galactosidase activity.

2.5.2.3 Addition of spent medium for induction of secondary metabolites production

For induction of secondary metabolite production in *B. licheniformis* NCIMB 8874 cultures, cell-free supernatants from *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type cultures at high cell densities were prepared by cultivating *B. licheniformis* NCIMB 8874 and *B. subtilis* (used as control) in 1L of the competence medium at 37 °C for 8 hours on a rotary shaker at 150 rpm (2 cm-throw). Cultures were harvested and centrifuged at 4700 rpm for 45 min. The supernatants were collected and filtered through a 0.22 μm syringe filter (Millipore). The spent media were lyophilized and then resuspended in distilled water to obtain a 25-fold concentrated solution. The concentrated spent media were ultra-filtrated using Ultra-15 centrifugal units (Millipore) with a 3kDa cut-off membrane, and centrifuged at 4000 rpm for 30 minutes. The filtrates were collected and stored at 4 °C for the later use.

2.5.2.4 Preparation of spent media for pheromone assay

Samples for the pheromone assay were prepared by growing *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type cells in the competence medium for 12 hours. Samples (1 mL) were collected from the growing cultures at specific time intervals and centrifuged at 13000 rpm for 10 minutes. The supernatants were collected and filter-sterilised through a 0.22 μm syringe filter (Millipore). Samples were stored at 4 °C overnight prior to the assay.

2.6 Molecular biology studies

2.6.1 Genomic DNA isolation

Genomic DNA isolation from *B. licheniformis* NCIMB 8874 cells was carried using DNeasy Blood and Tissue kit (Qiagen). *B. licheniformis NCIMB 8874* single colonies were inoculated in 5 mL LB and incubated overnight on a rotary shaker at 37 °C and 200 rpm for 15 hours. Cultures (1.25 mL) at an OD_{600} of 2 (corresponding to 2 x 10^9 cells) were transferred in a micro-centrifuge tube and centrifuged at 7500 rpm for 10 minutes. Genomic DNA extraction was carried out following the instructions provided by the manufacturer. After removal of the supernatant the bacterial pellets were resuspended in 180 µL enzymatic lysis buffer and incubated for 30 minutes at 37 °C. Proteinase K (25 µL) and Lysis Buffer (AL) (200 µL) were added and the mixture was incubated at 56 °C for 30 minutes. Samples were then treated with 200 µL ethanol (96-100%) and thoroughly mixed by vortexing. The mixture obtained was then transferred into a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minutes. The DNA was first washed with 500 µL Washing Buffer 1 (AW1), then with 500 µL Washing Buffer 2 (AW2), both containing ethanol. After the ethanol was removed from the DNeasy membrane by centrifugation at 8000 rpm for 3 minutes, the DNA was eluted in a clean micro-centrifuge tube by adding 200 µL Elution Buffer (AE) and spinning at 8000 rpm for 1 minute. Genomic DNA concentration was evaluated using Nanodrop 3300 (Thermo Scientific, UK) Full-Spectrum fluorospectrometer. DNA samples were stored at 4 °C for future use.

2.6.2 Primer design

2.6.2.1 Primers for *Bacillus licheniformis* NCIMB 8874

Primers for Polymerase Chain Reaction (PCR) for analysis of *B. licheniformis* NCIMB 8874 genes were designed using Primer 3 software. Primers were designed for the genes encoding the histidine kinase ComP and for the ComX pheromone, using the genome sequence from *B. licheniformis* strain ATCC 14580 (PubMed). Primers for the bacitracin biosynthetic gene *bacA* (Murphy, 2008) were used as a positive control for the PCR reaction. Sequences of the primers used in this project are listed in Table 2.9. Primers were custom prepared by Invitrogen (UK).

Table 2.9: *Primer sequences for the amplification of the quorum sensing-related genes comP (including the insertion element IS3Bli) and comX and the control gene bacA (bacitracin biosynthetic gene).*

Primer	Sequence	Tm (°C)	Product Size (bp)
comPN fw	5’ GCCTTTGCTCTTATCATTTATGG 3’	59.2	389
comPN rev	5’ GGACAGGCAGCATAGGTGTT 3’	60.1	
IS fw	5’ TTGAACTGACCCGTTAAAATGA 3’	59.5	329
IS rev	5’ CATAGCGACCAGGGATCTG 3’	60.1	
IS2 fw	5’ TTGGCCAAATTCTGATATGC 3’	58.6	395
IS2 rev	5’ GGGATAAATGTAAACCGCAAAA 3’	60.1	
IS3 fw	5’ TTCTTCTTAACTTGACACTGAATCTC 3’	57.5	400
IS3 rev	5’ TCGGAGGAAATGAGTGAGCTA 3’	60.0	
comPC fw	5’ GCCAATTGAGCCTCTAGCTG 3’	60.1	325
comPC rev	5’ AAAAACACCCCCTGAATCGT 3’	60.6	
comX fw	5’ TTAAATCCAAAATCCTCCTCCA 3’	59.8	150
comX rev	5’ AACTTTTTGGTCGAAAATCCTG 3’	59.5	
bacA fw	5’ AAGTGGCAAGGCTTTTGAGA 3’	60.0	140
bacA rev	5’CTCAGGATCAATCGGCAAAT 3’	60.0	

2

Conventional PCR was performed using genomic DNA isolated from *Bacillus licheniformis* cells and primers designed for amplification of *comP* and *comX* genes. This procedure was used to confirm that the size of the product obtained corresponded to the target sequence. Primers for *B. licheniformis* NCIMB 8874 were amplified using PCR master mix (Promega). The composition of the reaction mix and the PCR amplification cycle is shown in Table 2.10 and 2.11.

Table 2.10: *Reaction composition for PCR*

Component	**Volume (μL)**	**Final Concentration**
PCR Master Mix (2X)	12.5	1X
Forward primer (10μM)	2.5	1.0 μM
Reverse primer (10μM)	2.5	1.0 μM
DNA template	1-5	<250 ng
Nuclease-Free Water	1-5	NA

Table 2.11: *Amplification program for conventional PCR*

Stage	**Temperature (°C)**	**Time (min)**	
Initial denaturation	94	2	
Denaturation	94	1	30 cycles
Annealing	55	1	
Extension	72	2	
Extension	72	5	

PCR products between 100-500 bp were run on a 3% agarose gel using Hyperladder V (Bioline, London, UK) as a DNA size marker. Products with sizes >500 bp were visualised on a 1% agarose gel and 100 bp DNA ladder (New England Biolabs, Hitchin, UK) was used as the DNA marker. When the size of the expected PCR product exceeded 1 Kb a 0.8% agarose gel was prepared for electrophoresis and 1Kb ladder was selected as a size marker (New England Biolabs).

2.6.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for analysis of PCR products obtained after amplification. The same method was also used to purify DNA fragments generated via PCR. Different agarose concentrations were used for gel preparation depending upon the expected product size of the target DNA. During the course of this project 1 and 3 % agarose gels were prepared in 1X Tris Acetate EDTA (TAE) buffer (pH 8.5). The composition of 50X TAE buffer is given in Table 2.12.

PCR products to be analysed through electrophoresis (10 µL) were mixed with 2 µL of 6X Blue/Orange Loading Dye (Promega) before loading onto the gel wells. The gel was run at 100 V for 60 minutes to allow the DNA fragments to separate. After electrophoresis the gels were stained with ethidium bromide (10 mg mL^{-1}) and placed on a UV transilluminator to visualise the DNA fragments.

Table 2.12: *Composition of 50X TAE buffer (1 L)*

Components	**Weight or (volume)**
Tris Base	252 g
Glacial acetic acid	(57.1 mL)
EDTA (0.5 M, pH 8)	(100 mL)

2.6.3.2 DNA isolation for genome sequencing

Genomic DNA was isolated from the overnight culture of *B. licheniformis* NCIMB 8874 prepared by inoculating a single colony in 3 mL Brain Heart Infusion (BHI) broth. An aliquot of the culture (1.25 mL) was collected in a centrifuge tube and centrifuged at 13.000 rpm for 10 minutes. The supernatant was discarded and a Qiagen DNAeasy Blood and Tissue kit was used to extract the genomic DNA from the cell pellet as per manufacturers' instructions (Section 2.6.1). The samples were vortexed vigorously throughout the purification in order to shear the DNA for high quality sequencing data. Genomic DNA was eluted in 2 X 200 μL of Tris-EDTA (TE) buffer at pH 8 and treated with 40 μL of a 3M Sodium Acetate solution (pH 5.2) and 400 μL isopropanol. The sample was vortexed to mix and cenrtrifuged at 13.000 rpm for 5 minutes. The supernatant was discarded and pellet was washed with 0.5 mL of 70% Ethanol. The DNA pellet was air-dried before adding 0.25 mL of EB buffer from the Qiagen PCR gel extraction kit (Qiagen) and then stored at 4 °C for 3-5 days to allow for DNA complete re-suspension.

2.6.3.3 DNA Sequencing and analysis

PCR products obtained by amplification of genomic DNA from *B. licheniformis* NCIMB 8874 were sequenced by UCL (London, UK). The nucleotide sequences obtained were analysed using BLAST (NCBI). Genomic DNA isolated from *B. licheniformis* NCIMB 8874 (20 μg) was sent to the University of TURKU (Finland) for complete genome sequencing. The sequencing was carried out using two sequencers, 454 GS Junior and Illumina GAII. The outcomes of the sequencing were assembled using Velvet software. The assembly resulted in a single nucleotide sequence consisting of 304 contigs.

The phylogenetic analysis was carried out by aligning sequences obtained from NCBI nucleotide database with protein sequences derived by the genome of *B. licheniformis* NCIMB 8874 using ClustalW and Jalview.

2.7 Lichenysin extraction

Biosurfactant production and extraction was carried out by growing *Bacillus licheniformis* and *Bacillus subtilis* in 1 L YPD broth (Table 2.13) for 26 hours at 37 °C and 150 rpm on a rotary shaker (2 cm-throw). For confirmation of biosurfactant production in the competence medium, *B. licheniformis NCIMB 8874* NCIMB 8874 and *B. subtilis* wild type cells were also cultivated in 1 L competence medium for 13 hours at 37 °C and 150 rpm. Cells were harvested and centrifuged at 4.600 rpm for 45 minutes and the supernatants were collected. The pH of the spent medium was lowered to 2 by adding concentrated HCl. The acidified supernatant was then stored at 4 °C overnight to allow complete precipitation of the biosurfactant. The precipitate was centrifuged at 4.600 rpm for 20 minutes to obtain the crude biosurfactant as a pellet. This pellet was then resuspended in water and the pH was raised to 7.5 by adding 4M NaOH. The biosurfactant was stored at -80 °C for 14 hous and then placed in freeze-drier until complete lyophilisation. The lyophilised biosurfactant was solvent-extracted with methanol. The methanol-soluble fraction was allowed to dry at 60 °C. Following methanol evaporation the biosurfactant was dissolved in 5 mL of distilled water, which was again lyophilized and stored at -20 °C for further studies (Das *et al.*, 2008). Figure 2.2 schematically illustrates the protocol used for biosurfactant extraction.

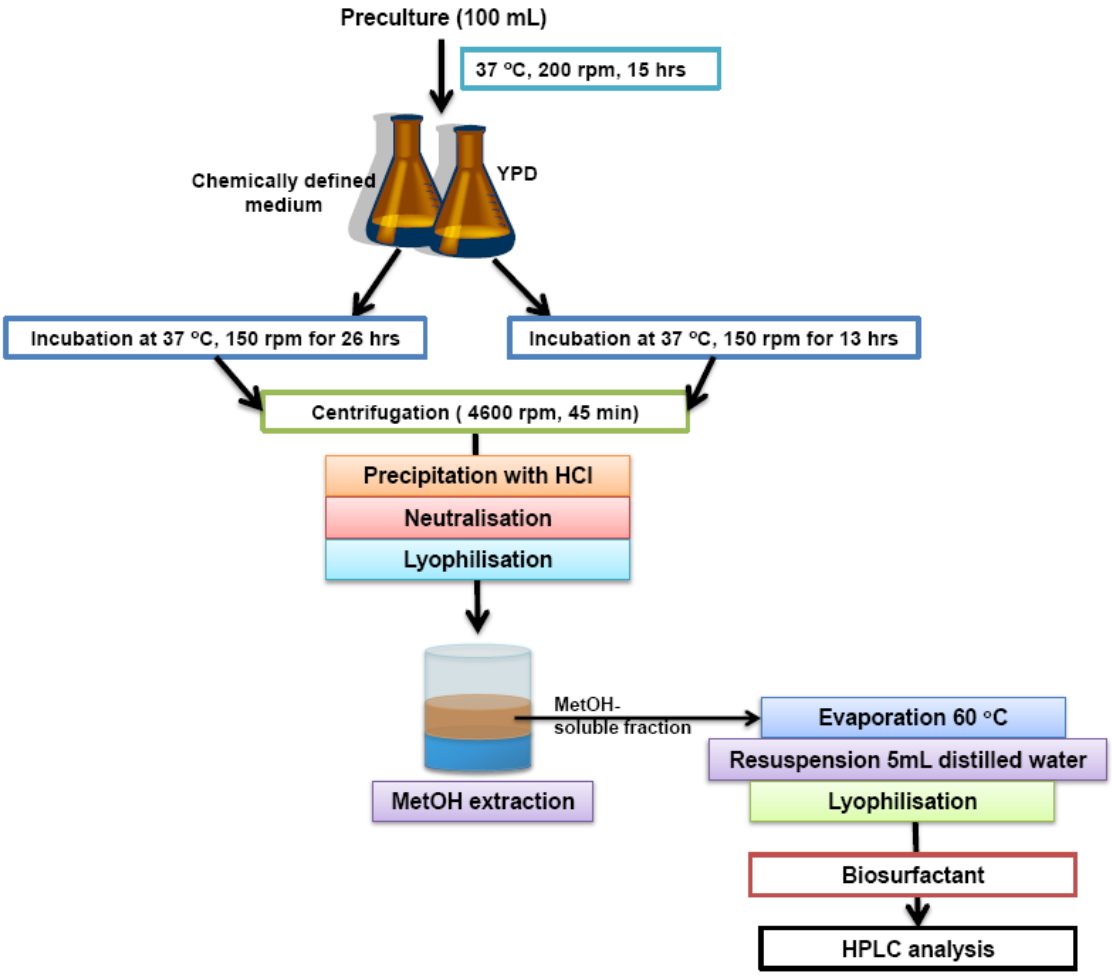


Figure 2.2: Schematic representation of lichenysin and surfactin extraction.

2.7.1 Lichenysin detection

Lichenysin and surfactin extracted from *B. lichenifomis* and *B. subilis* cultures, respectively, were detected through Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using a C18 column (Dionex, Acclaim ® 120 (C18, 5µm x 4.6mm x 150 mm), on a Dionex Ultimate-3000 HPLC instrument (Dionex, Dorset, UK). The lyophilized biosurfactant was re-dissolved in methanol to make a biosurfactant solution of 1 mg mL^{-1} which was passed through 0.22-µm filter (Millipore, Watford, UK). A surfactin standard (Sigma) was used for lichenysin quantification and also to confirm the retention time. The mobile phase, consisting

of 80% (v/v) Acetonitrile (CH_3CN, HPLC–far UV grade, Acros Organic, UK) and 20% (v/v) of a 38 mM Trifluoracetic acid (TFA, Fisher Scientific, UK) solution in water, was added isocratically at a flow rate of 1.4 mL min^{-1} for 40 minutes. Lichenysin was detected at 210 nm (Hsieh *et al.*, 2004).

2.7.2 Determination of lichenysin antimicrobial activity

Agar disc diffusion test was carried out to investigate the antimicrobial activity of lichenysin against different test organisms. Diagnostic Sensitivity Test (DST) agar (Oxoid) plates were prepared by dissolving 40 g of the powder in 1 L distilled water and autoclaving at 121 °C for 15 minutes. Each plate was inoculated with a different test organism. Different biosurfactant dilutions (0 mg mL^{-1}, 1mg mL^{-1}, 2 mg mL^{-1} and 4 mg mL^{-1}) were prepared by dissolving lyophilised lichenysin in distilled water. The solutions were filter-sterilised using a 0.22 μm syringe filter (Millipore). An aliquot (10 μL) of each dilution was applied to Sterile Whatman (N^o.1) filter paper discs. The plates were incubated overnight at 37°C. The test was run in triplicate.

2.8 Pheromone extraction

For the isolation of the ComX pheromone, *B. licheniformis* NCIMB 8874 and *B. subtilis* cells were grown for 8 hours in 5 L shaken flasks containing 1 L competence medium. Figure 2.3 provides a schematic representation of pheromone extraction.

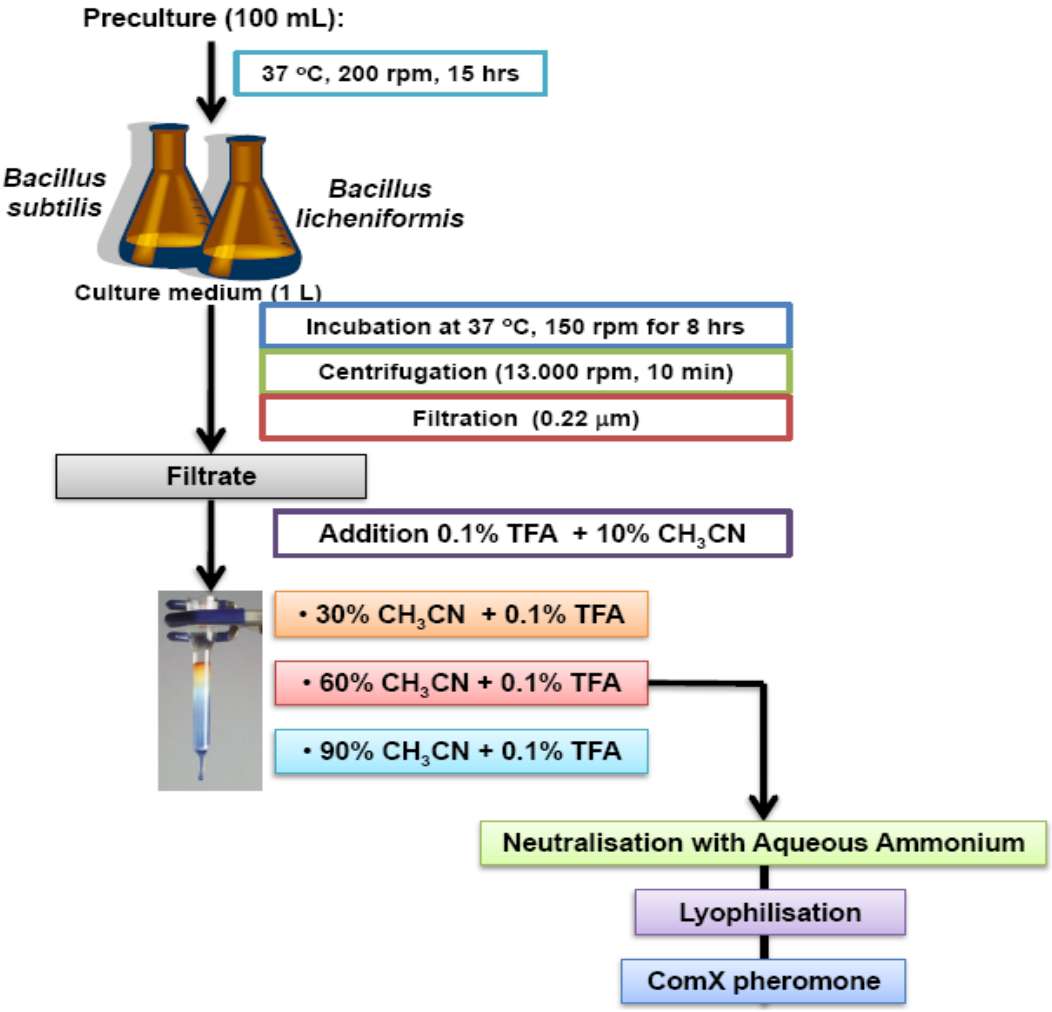


Figure 2.3: Diagram of the ComX pheromone extraction.

The supernatant was collected by centrifugation for 10 minutes at 13000 rpm at 4 °C and filtered through a 0.22 µm vacuum filtration unit (Millipore). The filtrate was then treated with 10% CH_3CN and 0.1% TFA. A 0.4 g pre-packed C18 Sep-Pak column (Waters, UK) was washed with 25 mL of 80% CH_3CN and 0.1% TFA in water and equilibrated with 25 mL of an aqueous solution containing 20% CH_3CN and 0.1% TFA. The filtrate (30 mL) was poured over the Sep-Pak column and allowed to flow by gravity at room temperature. The column was then washed with a step gradient of CH_3CN (30, 60, 90% + 0.1% TFA in water). The eluant of the 60% ACN wash was promptly neutralised with aqueous ammonium (30%) and stored at -80 °C overnight prior to lyophilisation (Okada *et al.*, 2005). The

lyophilised fraction was resuspended in 1-2 mL distilled water and store at -80 °C for future use. Pheromone activity was tested using the "70 minutes assay" described in section 2.5.2.1.

2.9 γ-PGA production

Bacillus licheniformis single colonies were inoculated into 100 mL of LB medium and cultured on a rotary shaker at 200 rpm at 37°C for 16 hours. The seed culture was inoculated into the production medium with a starting OD value of 0.1-0.2. Samples (1 mL) were taken from the cultures at regular intervals and tested for γ-PGA production. For this, the cells were spun down at 13000 rpm for 5 minutes and the polymer was extracted as indicated in Section 2.9.1 (Goto and Kunioka, 1992).

2.9.1 Extraction of γ-PGA

Cells were harvested by centrifugation at 46000 rpm for 25 minutes at 4 °C. The supernatant containing the polymer was separated from the cell pellet and subjected to a second round of centrifugation at 4600 rpm for 25 minutes at 4 °C to remove any cell debris. The polymer was precipitated by adding methanol in a ratio of 1:4 to the culture supernatant. The precipitation mixture was incubated at 4 °C for 24 hours and the polymer was collected by centrifugation at 4700 rpm for 45 minutes and was subsequently lyophilized (Goto and Kunioka, 1992).

2.9.2 γ-PGA analysis by Nuclear Magnetic Resonance (NMR)

Crude γ-PGA extracted from culture supernatants was dissolved in d_6-DMSO (Fisher Scientific, UK) for NMR analysis. An aliquot of the extracted polymer (20 mg) was dissolved in 1 mL d_6-DMSO and vortexed to resuspend. The solution was then passed through a membrane with a 3kDa cut-off to eliminate any impurity. Samples were analysed NMR spectroscopy at UCL (London).

2.10 Proteolytic activity

Proteolytic activity in *B. licheniformis NCIMB 8874* and *B. subtilis* LS27 culture supernatants was qualitatively evaluated by performing disc diffusion assay on agar plates containing M9 salts supplemented with skimmed milk (10%). Sterile N°1 paper discs (Whatman) were positioned on the milk agar and inoculated with 10 µL aliquots of corresponding bacterial supernatants. The digested substrate formed clear proteolytic zones surrounding the discs. These areas were measured after 25 hours incubation at 37° C (Miedzobrodzki *et al.*, 2002).

Chapter III

RESULTS

Introduction to the Results Chapter

This chapter presents the results of the investigation into the potential quorum sensing in *Bacillus licheniformis* NCIMB 8874. This organism was chosen for its industrial relevance for biosynthesis of products, such as proteases and antimicrobial compounds, and for its genetic similarity to *Bacillus subtilis,* where quorum sensing-related regulation of competence and sporulation has been well established.

At the beginning, due to the lack of molecular and genetic information on quorum sensing process in *Bacillus licheniformis* NCIMB 8874, the work was designed initially on the use of *B. subtilis* reporter strains for determination of the production of potential signalling molecules. However, as, later on, the preliminary genome sequence of *B. licheniformis* NCIMB 8874 became available, the study was extended to the analysis of genes involved in cell-cell communication.

The results obtained for this project can be summarized as follows:

✓ Shaken flasks studies

Studies on *Bacillus licheniformis* were mainly performed in 500 mL shaken flasks. Cultures of *Bacillus subtilis* wild type strain were used for comparative analysis. During the course of each fermentation, samples were collected for the measurement of biomass (OD at 600 nm) and pH. Cell-free supernatants collected from *B. licheniformis* cultures were assessed for the presence of putative signalling molecules by using genetically modified *Bacillus subtilis* strains as reporters. These results are presented in Section 3.1. A diagram of shaken flask studies carried out for investigation of signalling molecule production in *B. licheniformis* is presented in Figure 3.0.

Shaken flasks (500 mL) studies were also carried out for the investigation of γ-polyglutamic acid production in *B. licheniformis* NCIMB 8874. These results are presented under Section 3.3. Investigation on the production of extracellular

proteases was carried out qualitatively on agar plates and the results are presented in Section 3.4.

Studies in 5 L shaken flasks were carried out for investigation of production of the antimicrobial biosurfactant lichenysin and for isolation of potential signalling molecules. Results are presented under Section 3.2.

✓ Molecular studies

Original studies on quorum sensing in *B. licheniformis* NCIMB 8874 at the molecular level were carried out by amplification of competence-regulating genes, namely *comX* and *comP*, of this organism using the annotated genome sequence of strain ATCC 14580 as a reference.

Subsequently, and after the genome sequence of *B. licheniformis* NCIMB 8874 became available (very recently through collaboration with the Centre of Biotechnology, University of Turku, Finland), bioinformatics studies were carried out to confirm the presence of cell-cell communication-related genes/proteins in this bacterium. Moreover, essential genes/proteins were analysed in comparison with other Bacilli to determine their conservation. Results obtained from these studies are presented under Section 3.3 and 3.4.

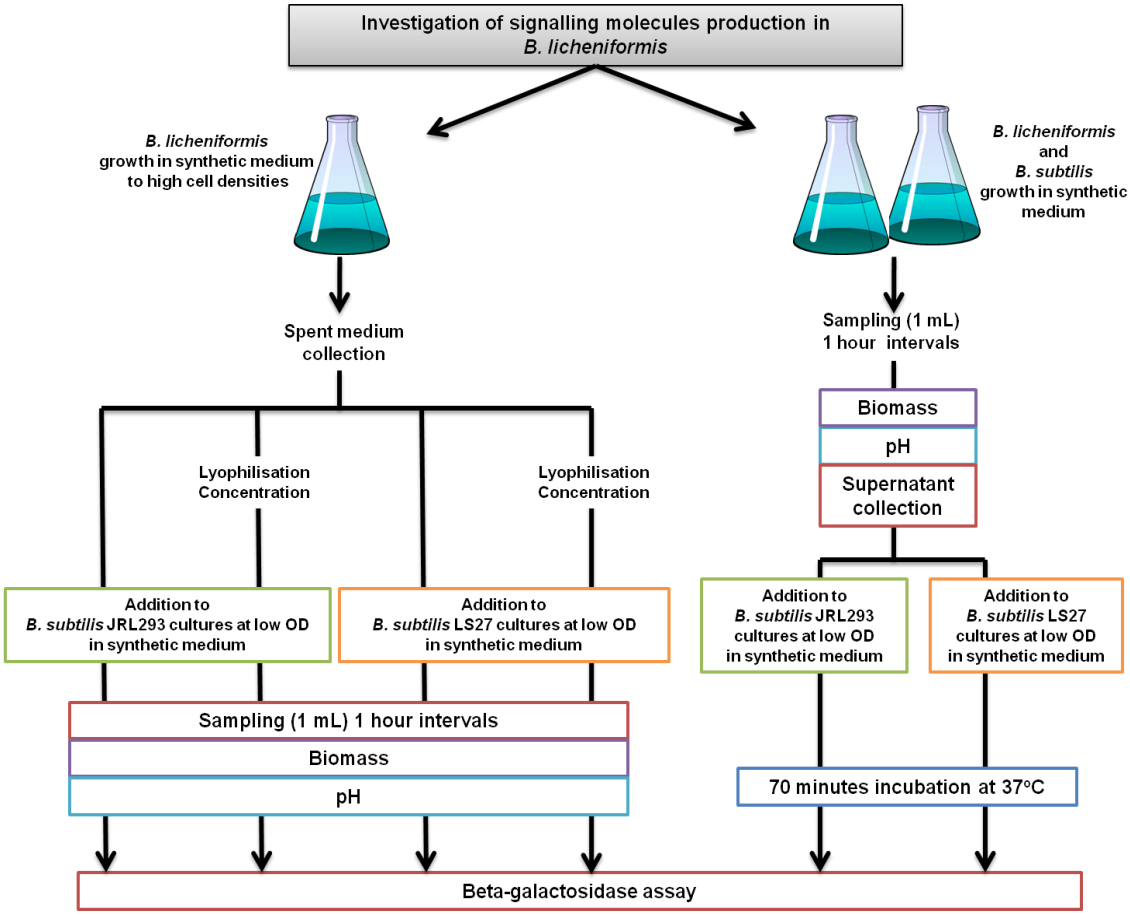


Figure 3.0: Overview of the experiments performed for the investigation of signalling molecules production in *B. licheniformis*. Studies on *B. licheniformis* production of potential quorum sensing molecules were performed at shaken flasks (500 mL) level in competence medium (on the left side of the diagram) Supernatants were collected from *B. licheniformis* cultures at high cell densities (late exponential phase) and their cell-free supernatants were collected and added to cultures of *B. subtilis* reporter strains JRL293 and LS27 at low cell densities. Effect of exogenous addition of spent medium on the growth and pH profiles of test cultures was investigated by collecting samples at specific time points for OD_{600} and pH determination. The results are presented under Section 3.1.1 of this thesis. On the right side of the diagram are represented the studies performed for determination of the production pattern of the potential signalling molecules in *B. licheniformis* in comparison with *B. subtilis* wild type strain. Cells were grown in competence medium until late exponential phase was reached. Samples were collected from these cultures at selected intervals and supernatants were harvested to assess their ability to induce β-galactosidase activity in *B. subtilis* JRL293 and LS27.

RESULTS

3.1 Production of potential signalling molecules

Studies on *Bacillus licheniformis* production of potential quorum sensing molecules were performed at shaken flask (500 mL) level in synthetic medium designed for induction of competence. Supernatants were collected from *B. licheniformis* cultures at high cell densities (late exponential phase) and their ability was investigated for the induction of β-galactosidase activity in *B. subtilis* reporter strains carrying a *srfA-lacZ* fusion on their genome. *B. subtilis* reporter strains JRL293 and LS27 were grown in synthetic medium in presence (test cultures) and absence (control cultures) of spent medium collected from *B licheniformis* cultures. Effect of exogenous addition of spent medium on the growth and pH profiles of test cultures was investigated by collecting samples at specific time points for OD_{600} and pH determination. The results are presented under Section 3.1.1 of this thesis.

For determination of the production pattern of the potential signalling molecules in *B. licheniformis* and comparison with *B. subtilis* wild type strain, cells were grown in synthetic medium until late exponential phase was reached. Samples were collected from these cultures at selected intervals and supernatants were harvested to assess their ability to induce β-galactosidase activity in *B. subtilis* JRL293 and LS27. These results are presented under section 3.1.2 of this thesis.

3.1.1 *B. licheniformis* and *B. subtilis* cultures in synthetic medium

Shaken flask fermentations were performed by growing *B. licheniformis* and *B. subtilis* cells in synthetic medium containing glucose as the only carbon source. Both *B. licheniformis* and *B. subtilis* cells were grown for 10 hours and samples were taken at 1 hour intervals throughout the course of fermentation for determination of OD_{600} and pH.

3.1.1.1 Growth curve

Growth curves for *B. licheniformis* and *B. subtilis* cultures in synthetic medium are displayed in Figure 3.1. No statistically significant difference was detected in the growth of the two organisms ($p>0.05$) whose specific growth rates were calculated to be 0.51 h^{-1} and 0.58 h^{-1} for *B. licheniformis* and *B. subtilis*, respectively. This implies that the average doubling time (t_d) for *B. licheniformis* cells grown in synthetic medium is 80 minutes, whereas *B. subtilis* cells duplicate every 71 minutes.

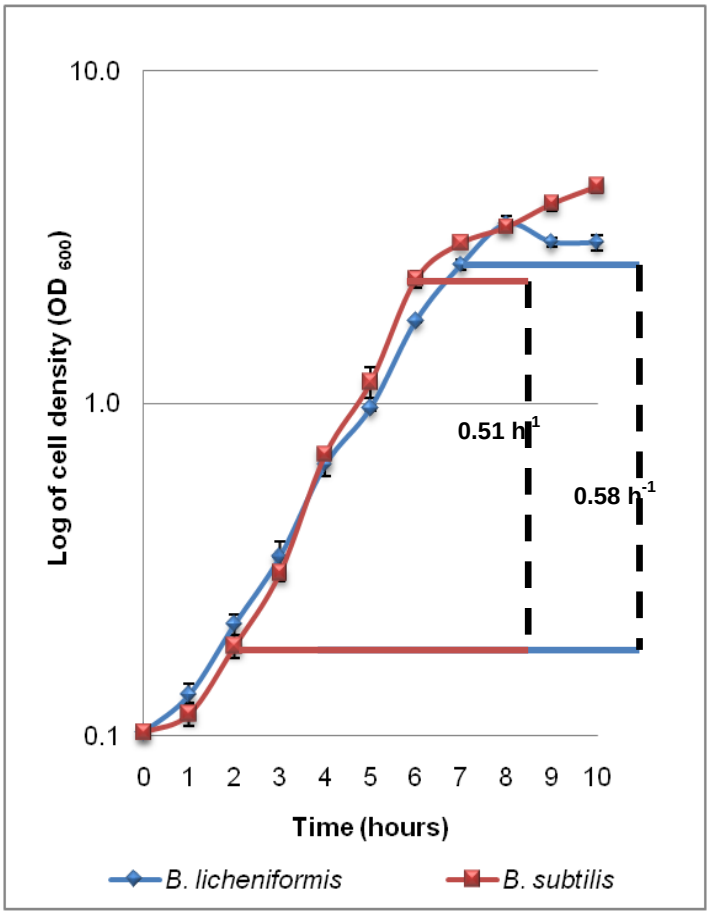


Figure 3.1: *B. licheniformis* (Blue) and *B. subtilis* (Red) growth curve during the course of shaken flask fermentation in competence medium. Cell density (OD_{600}) was measured at 1hour intervals throughout the fermentation. Specific growth rates for both the organisms are displayed. The experiments were carried out in triplicates and the error bars indicate the standard deviation.

B. licheniformis and *B. subtilis* pH profiles obtained during the course of shaken flask fermentation in synthetic medium show no significant differences ($p>0.05$) between the two organisms.

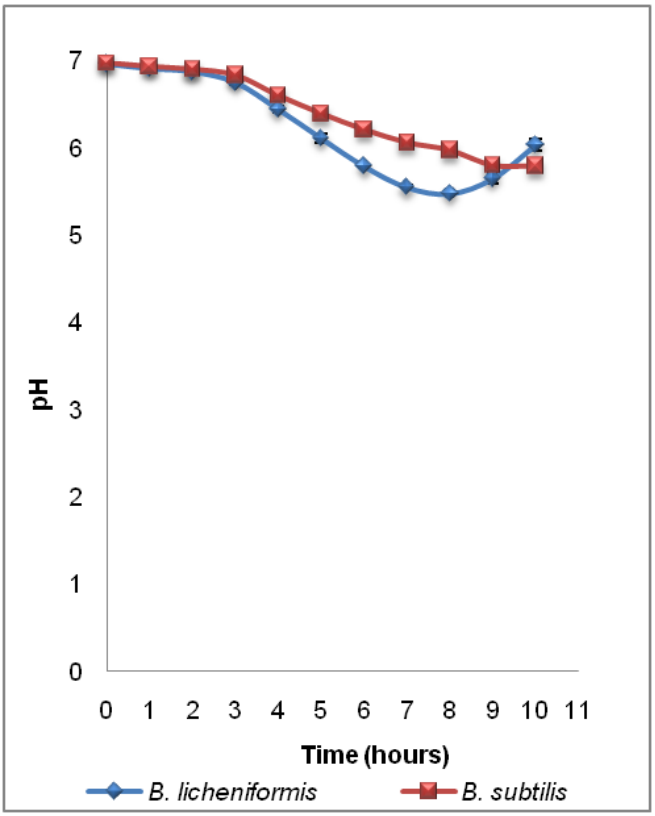


Figure 3.2: pH profiles for *B. licheniformis* and *B. subtilis* cultures in the course of shaken flask fermentation in competence medium. pH was measured at 1 hour intervals throughout the cell growth. The experiments were carried out in triplicates and the error bars indicate the standard deviation.

Production of potential signalling molecules in *B. licheniformis* was investigated by studying the effect of the spent medium collected from *B. licheniformis* cultures in late exponential phase on *B. subtilis* reporter strains JRL293 and LS27, both carrying an *srfA-lacZ* fusion. The genotype of *B. subtilis* reporter strains used in this study is illustrated in Figure 3.3.

Shaken flask fermentations of *B. subtilis* reporter strains were carried out by growing the cells in competence medium (Control) and competence medium diluted with equal volume of *B. licheniformis* spent medium (Test). Results obtained from studies on *B. subtilis* JRL293 and *B. subtilis* LS27 are presented in Section 3.1.2.1 and 3.1.2.2, respectively.

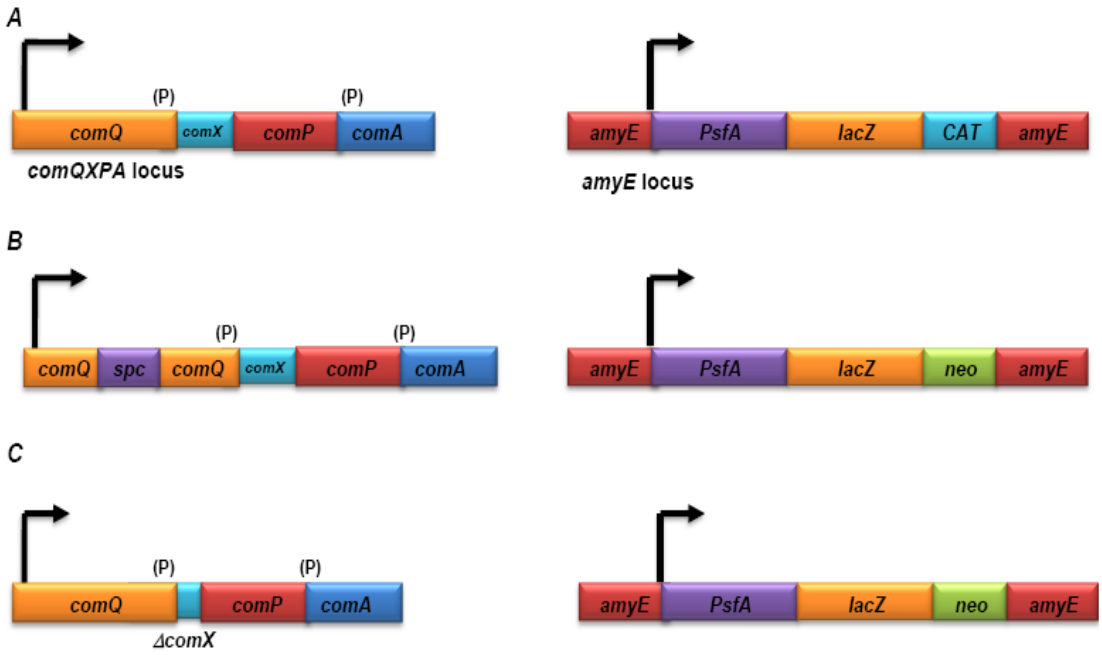


Figure 3.3: Schematic representation of the genotype of *B. subtilis* reporter strains used in this study**.** A) *B. subtilis* JRL293; B) *B. subtilis* TMH281: C) *B. subtilis* LS27. All the strains carry a *srfA-lacZ* fusion inserted into the *amyE* gene

(encoding α-amylase), which comprises the *srfA* promoter (-434 +10 region) and *lacZ* gene.

3.1.2.1 Effect of *B. licheniformis* cell-free supernatant on *B. subtilis* JRL293

Filter-sterilised spent medium collected from *B. licheniformis* cultures at high cell densities (~ 3.5 OD_{600}) was diluted into an equal amount of fresh medium and used to cultivate *B. subtilis* JRL293 cultures at low cell densities (0.08-0.1 OD_{600}). *B. subtilis* JRL293 cultures grown in competence medium without addition were used as a control. At 1 hour intervals samples from both control and test cultures were collected for OD_{600} and pH measurements and for determination of β-galactosidase activity.

3.1.2.1.1 Growth curve and pH profile

As indicated in Figure 3.4A, exogenous addition of spent medium to *B. subtilis* JRL293 cultures had a statistically significant effect on the cell growth ($p<0.05$) when compared to control cultures. More specifically, *B. licheniformis* spent medium appears to have an inhibitory effect on *B. subtilis* JRL293 culture viability. Addition of spent medium from *B. subtilis* wild type cultures at high cell densities did not produce any significant effect on *B. subtilis* JRL293 growth profile (data not shown).This implies that the growth inhibition was not caused by accumulation of toxic by-products in the culture broth, rather, the impairment in *B. subtilis* JRL293 cell growth might be due to compounds (e.g. antimicrobials) present in *B. licheniformis* spent medium.

The pH profiles for *B. subtilis* JRL293 test and control cultures are shown in Figure 3.4B. No significant difference ($p>0.05$) could be detected between the pH profiles of cultures grown in the absence (Control) and in the presence (Test) of spent medium from *B. licheniformis* cultures in late exponential phase.

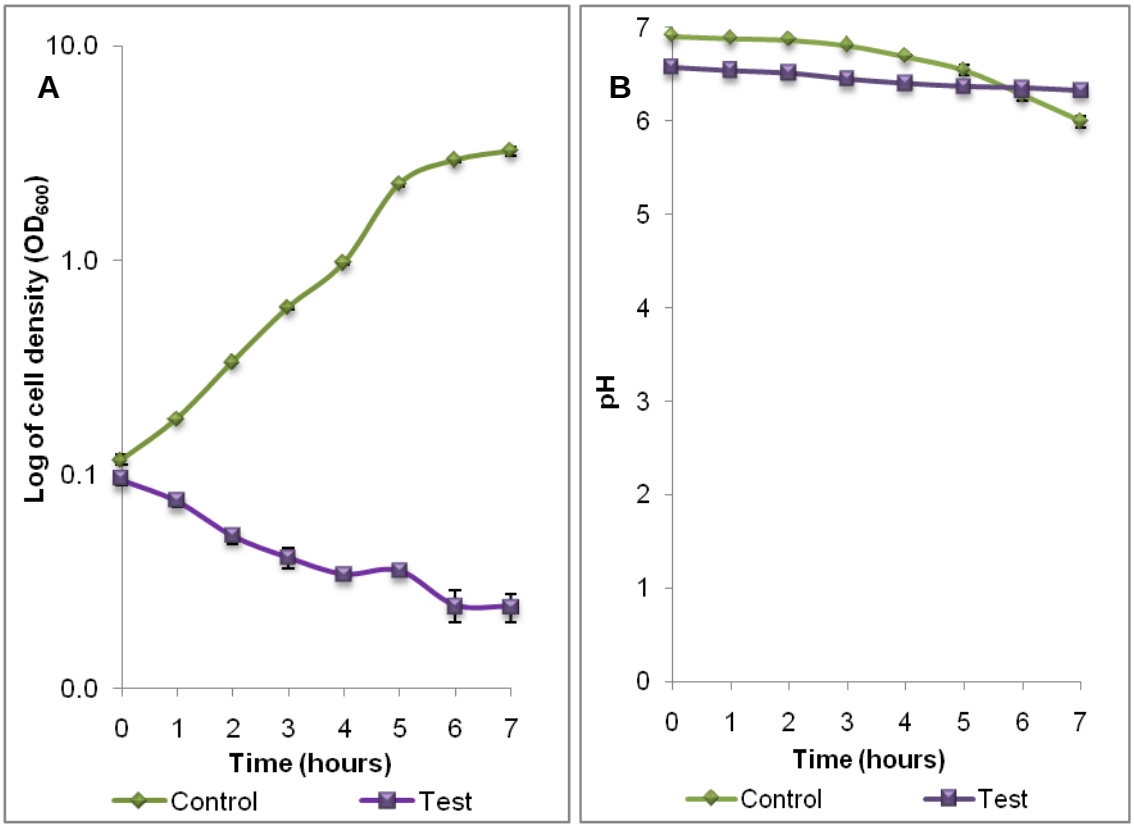


Figure 3.4: (A) Growth curves and (B) pH profiles for *B. subtilis* JRL293 cultures grown in both competence medium (Control) and competence medium supplemented with spent medium (50% v/v) collected from *B. licheniformis* cultures at high cell density (Test) for 7 hours. Samples were collected at 1 hour intervals for OD_{600} and pH measurement. The experiment was performed in triplicates and error bars show the standard deviation.

3.1.2.1.2 Induction of β-galactosidase activity

The growth-inhibiting effect caused by the exogenous addition of *B. licheniformis* spent medium to *B. subtilis* JRL293 cultures (Figure 3.4A) was accompanied by a statistically significant increase ($p<0.05$) in β-galactosidase activity in the test cultures with respect to control cultures. As Figure 3.5 shows, for the first 2 hours after induction of the cells with spent medium no significant difference could be detected between β-galactosidase activities produced by the test and the control cultures. The control cultures showed a constant enhancement in enzyme activity

throughout the fermentation in a cell density-dependant fashion. In the test cultures, however, β-galactosidase production increased steeply up to 5 hours after induction, with the highest 7.9-fold increase compared to the control cultures A decline in activity was detected after 5 hours (Figure 3.5).

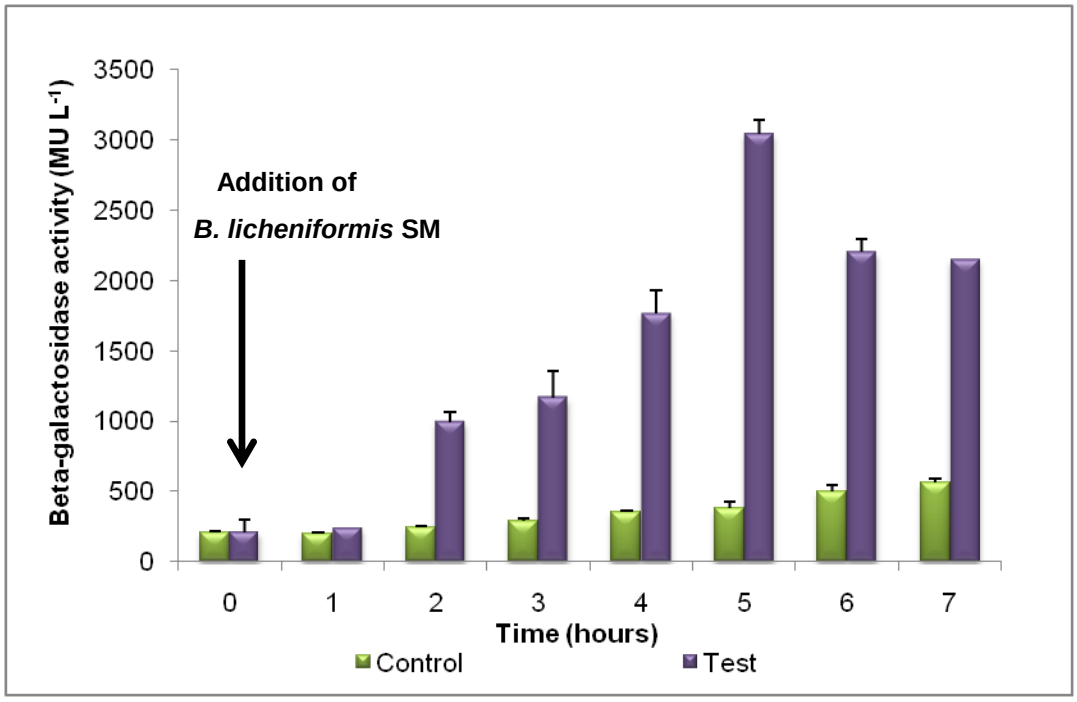


Figure 3.5: β-galactosidase activity in *B. subtilis* JRL293 cells grown in absence (Control) and in presence of 50% (v/v) spent medium from *B. licheniformis* cultures at high cell density (Test). Time of exogenous spent medium addition is indicated with a black arrow. Standard deviation of triplicates is represented by error bars.

3.1.2.2 Effect of *B. licheniformis* lyophilised cell-free supernatant on B. subtilis JRL293

Spent medium collected from *B. licheniformis* cultures at high cell densities was lyophilised and concentrated 25-fold prior addition to *B. subtilis* JRL293 cultures. Reporter strain cultures at 0.1 OD_{600} were supplemented with 1.25% (w/v) *B. licheniformis* spent medium. The amount of lyophilised spent medium added to the test cultures was calculated to have the same composition of 50% non-

concentrated spent medium. Samples collected at 1 hour intervals from both control and test cultures were assessed for changes in OD_{600}, pH and β-galactosidase activity.

3.1.2.2.1 Growth curve and pH profile

Figure 3.6A illustrates the growth curve for *B. subtilis* JRL293 control and test cultures. A decrease, albeit not significant ($p>0.05$), was detected in the growth rate of *B. subtilis* JRL293 test cultures, as compared to control cultures, upon addition of exogenous spent medium. The pH profile of *B. subtilis* JRL293 control and the test cultures is shown in Figure 3.6B. Addition of *B. licheniformis* lyophilised spent medium had no significant effect on the pH profile as no significant differences ($p > 0.05$) were observed between the control and the test cultures.

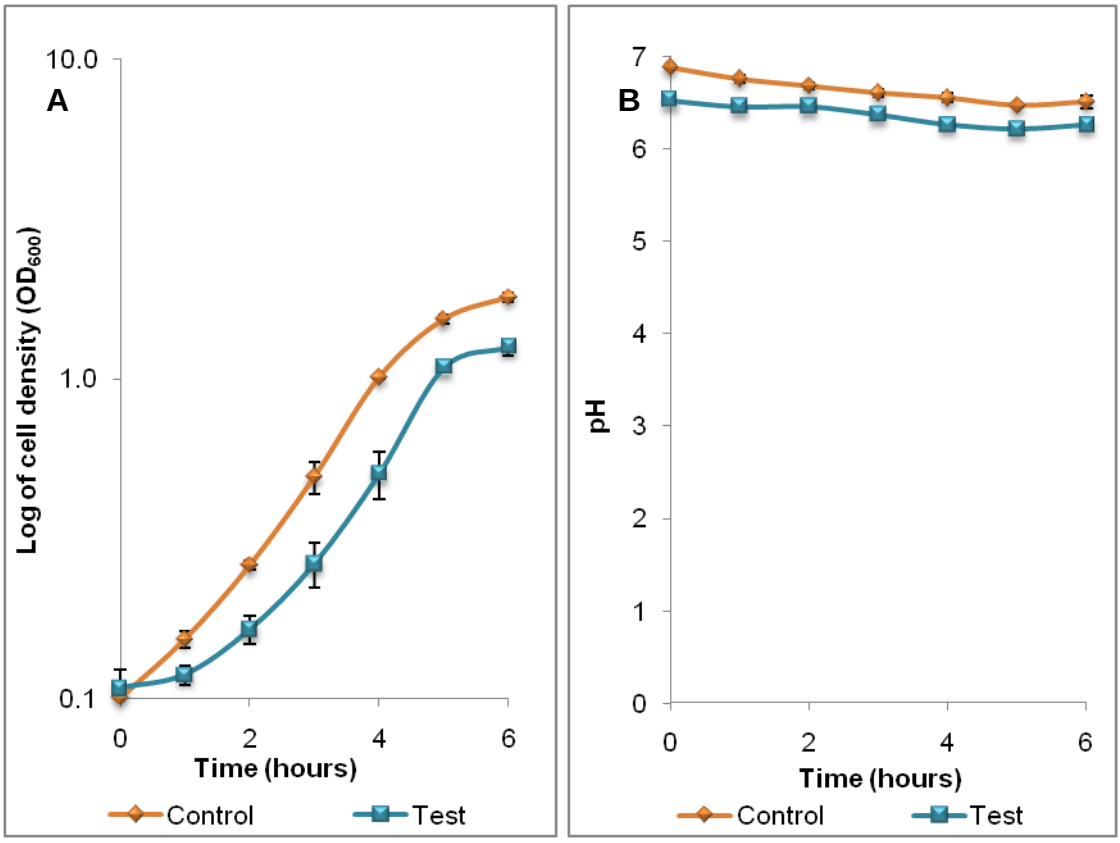


Figure 3.6: (A) Growth curve and (B) pH profile for *B. subtilis* JRL293 cultures grown in competence medium (Control) and in competence medium

supplemented with lyophilised spent medium (1.25% w/v) from *B. licheniformis* cultures at high cell density (Test). Samples were collected at 1 hour intervals for OD_{600} and pH determination. Experiments were performed in triplicates and the error bars represent the standard deviation.

3.1.2.2.2 Induction of β-galactosidase activity

β-galactosidase activity detected from samples collected throughout growth of *B. subtilis* JRL293 control and test cultures is illustrated in Figure 3.7. Addition of *B. licheniformis* lyophilised supernatant caused a drastic increase ($p<0.05$) in β-galactosidase activity in test cultures when compared to the control cultures. Enzyme activity in control cultures followed the same trend showed previously (Figure 3.5), reaching a peak of 400 MU L^{-1} after 6 hours of growth. Cultures supplemented with exogenous spent medium showed highest β-galactosidase activity at 1 hour after induction, corresponding to the highest 2.25-fold increase with respect to the control cultures.

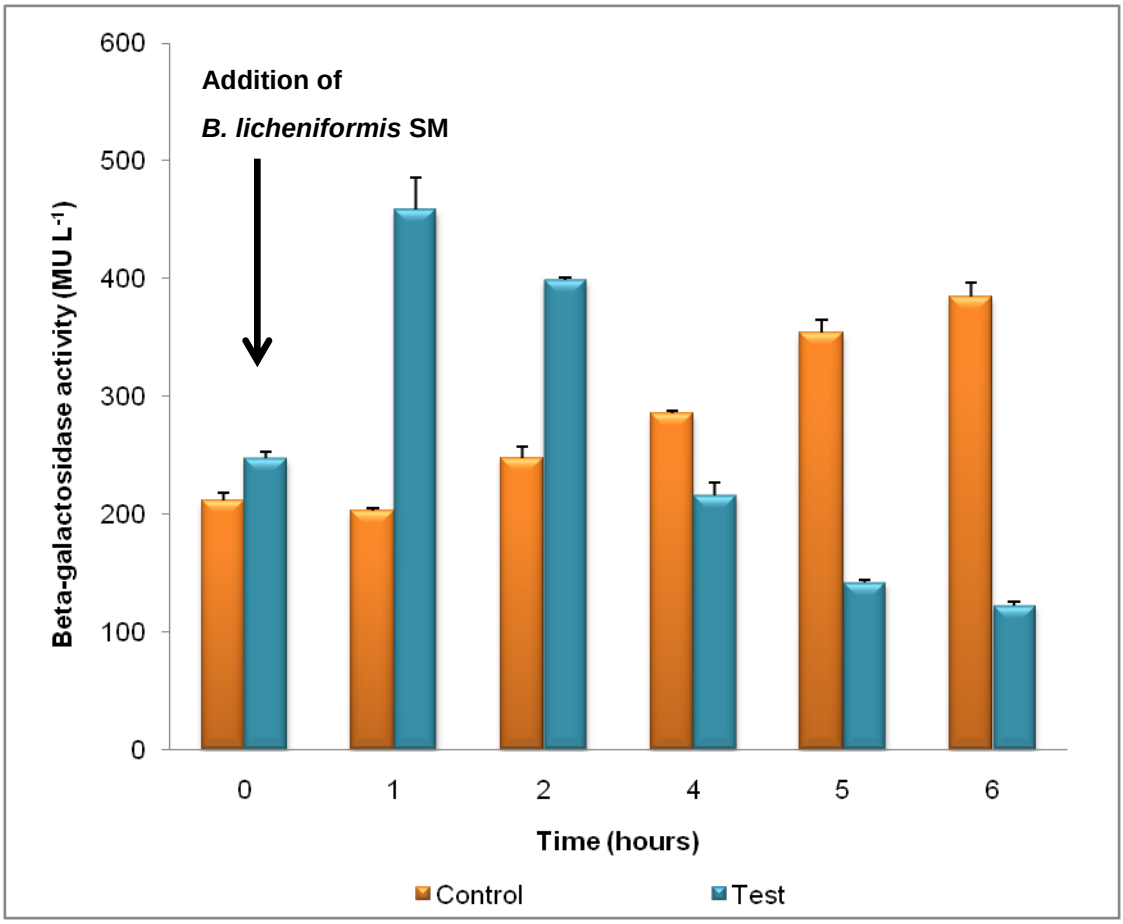


Figure 3.7: β-galactosidase activity in *B. subtilis* JRL293 cells grown in absence (Control) and in presence of 1.25% (w/v) spent medium from *B. licheniformis* cultures at high cell density (Test). Time of exogenous spent medium addition is indicated with a black arrow. Error bars indicate the standard deviation of the triplicate.

3.1.2.3 Effect of *B. licheniformis* cell-free supernatant on *B. subtilis* LS27

B. subtilis LS27 cells at a starting cell density of 0.16 OD_{600} were grown in competence medium supplemented with filter-sterilised spent medium collected from *B. licheniformis* cultures at high cell densities at 50% v/v (test cultures). Control cultures were prepared by growing *B. subtilis* LS27 cells in competence medium with no addition. Samples from both control and test cultures were

collected at 1 hour intervals during the course of fermentation for OD_{600} and pH measurements and for evaluation of β-galactosidase activity.

3.1.2.3.1 Growth curve and pH profile

Figure 3.8A depicts the growth curve for *B. subtilis* LS27 control and test cultures. Exogenous addition of *B. licheniformis* supernatant caused a statistically significant reduction ($p<0.05$) in the growth of *B. subtilis* LS27 test cultures as compared to control cultures in the first 3 hours of fermentation. However, at 4 hours after induction, cell growth resumed at a growth rate comparable to control cultures. The initial delay in the growth of *B. subtilis* LS27 test cultures by the addition of *B. licheniformis* spent medium resembles the effect previously described for *B. subtilis* JRL293 (Figure 3.4). The pH profile for *B. subtilis* LS27 test and control cultures is illustrated in Figure 3.8B. Test cultures, supplemented with *B. licheniformis* supernatant, showed no significant difference ($p>0.05$) in their pH pattern as compared with the control culture (no supernatant added).

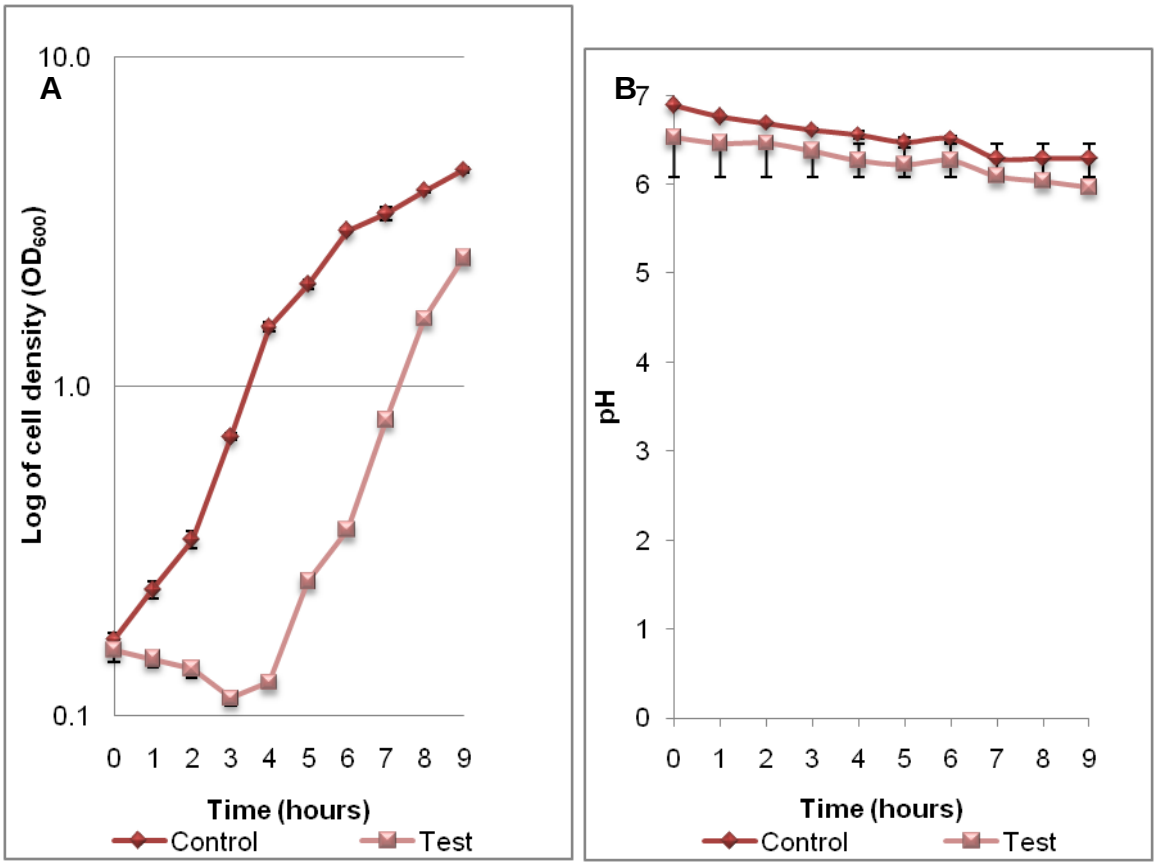


Figure 3.8: (A) Growth curve and (B) pH pattern for *B. subtilis* LS27 cultures cultivated in competence medium in the presence (Test) and absence of spent medium (50% v/v) collected from *B. licheniformis* cells at high cell density (Control) for 8 hours. Samples were collected at 1 hour intervals for OD_{600} and pH measurements. Experiments were performed in triplicates and the error bars represent the standard deviation.

3.1.2.3.2 Induction of β-galactosidase activity

Figure 3.9 shows the β-galactosidase activity measured in samples collected throughout growth of *B. subtilis* LS27 control and test cultures. When test cultures supplemented with 50% (v/v) *B. licheniformis* spent medium were compared to the control cultures, a statistically significant ($p<0.05$) increase in β-galactosidase activity was determined. Enhancement in β-galactosidase in test cultures started at 1 hour after induction, reaching highest β-galactosidase activity at 6 hours after

induction. The highest, 5.1-fold, increase in the enzyme activity with respect to control cultures was also registered after 5 hours of induction (Figure 3.9).

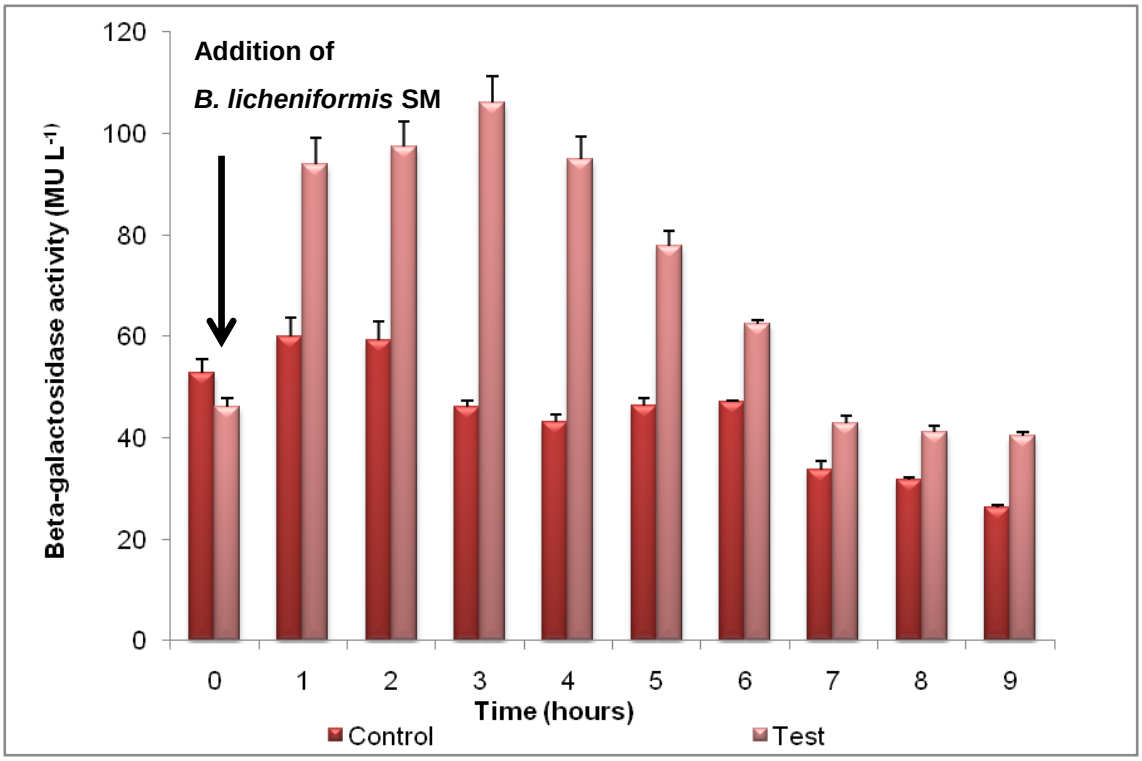


Figure 3.9: β-galactosidase activity in *B. subtilis* LS27 cells grown in absence (Control) and in presence of 50% (v/v) spent medium from *B. licheniformis* cultures at high cell density (Test). Time of exogenous spent medium addition is indicated with a black arrow. Error bars represent the standard deviation of the triplicate.

3.1.2.4 Effect of *B. licheniformis* lyophilised cell-free supernatant on *B. subtilis* LS27

Cell-free supernatant collected from *B. licheniformis* cells at high cell density was lyophilised and concentrated 25-fold prior to addition to *B. subtilis* LS27 cultures for investigation on the effects on growth, pH pattern and β-galactosidase activity. *B. subtilis* LS27 at a starting cell density of 0.15 OD_{600} were cultivated in competence medium in absence (Control) and presence of 1.25% (w/v) *B. licheniformis* lyophilised spent medium (Test). OD_{600}, pH and β-galactosidase

activity were measured by taking samples from both control and test cultures at 1 hour intervals during the course of 8 hours growth.

3.1.2.4.1 Growth curve and pH profile

The growth curves for *B. subtilis* LS27 control and test cultures is illustrated in Figure 3.1A. Exogenous addition of 1.25% (w/v) *B. licheniformis* lyophilised spent medium to the test cultures appears to have a negligible effect on *B. subtilis* LS27 growth ($p>0.05$), as compared to the control. Figure 3.10B shows the pH profile for *B. subtilis* LS27 test and control cultures. The difference in the pH pattern of test cultures, grown in presence of *B. licheniformis* lyophilised cell-free supernatant, and control culture (no supernatant added) was not significant ($p>0.05$).

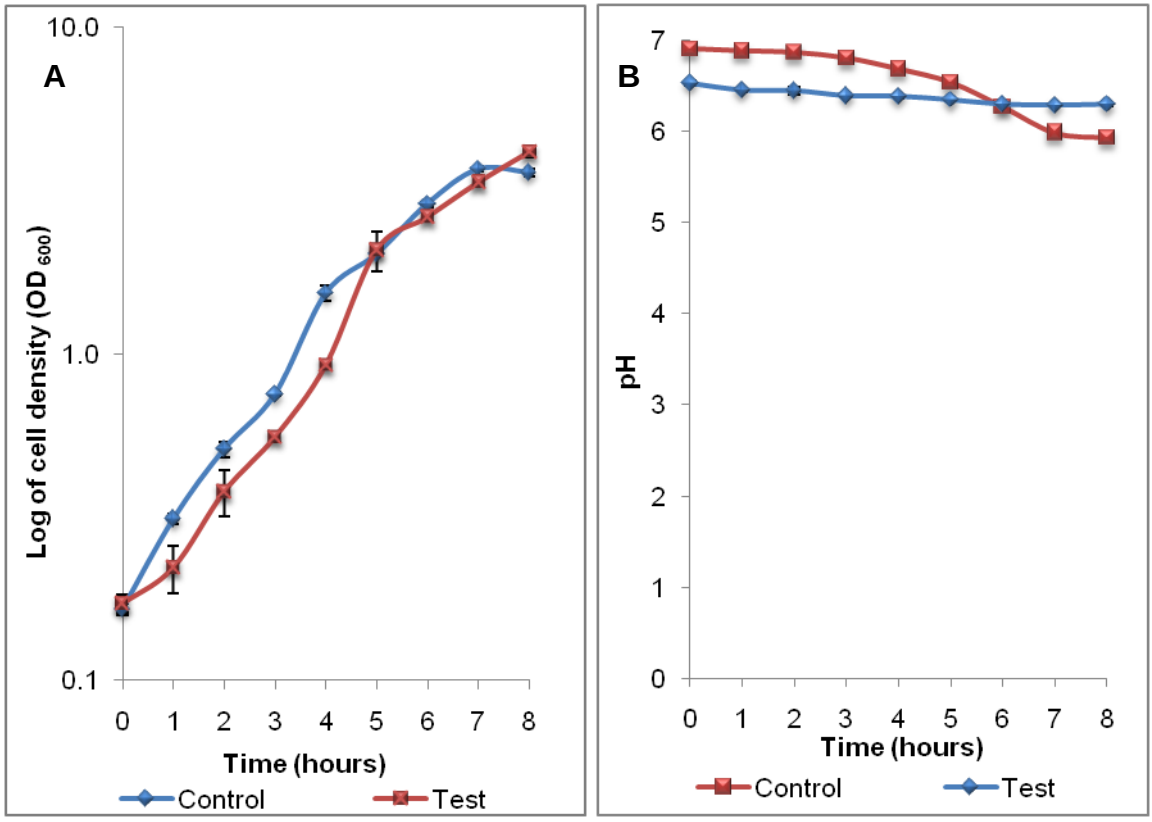


Figure 3.10: (A) Growth curve and (B) pH profile for *B. subtilis* LS27 cultures cultivated in competence medium in the presence (Test) and absence of spent medium (1.25% w/v) collected from *B. licheniformis* cells at high cell density (Control) for 8 hours. Samples were collected at 1 hour intervals for determination

of OD_{600} and pH. Experiments were performed in triplicates and the error bars represent the standard deviation.

3.1.2.4.2 Induction of β-galactosidase activity

The effect generated by the addition of *B. licheniformis* lyophilised spent medium on the β-galactosidase activity of *B. subtilis* LS27 test cultures in comparison with control cultures is illustrated in Figure 3.11. A statistically substantial ($p<0.05$) difference in β-galactosidase activity could be observed between test and control cultures. In cultures supplemented with *B. licheniformis* lyophilised supernatant the enzyme activity could be seen increasing from 1 hour after induction and peaking at 5 hours, when it registered the highest, 5.9-fold, increase as compared to control cultures (Figure 3.14). After 5 hours induction, β-galactosidase activity in test cultures started declining to reach values comparable to the control at the end of the growth (8 hours).

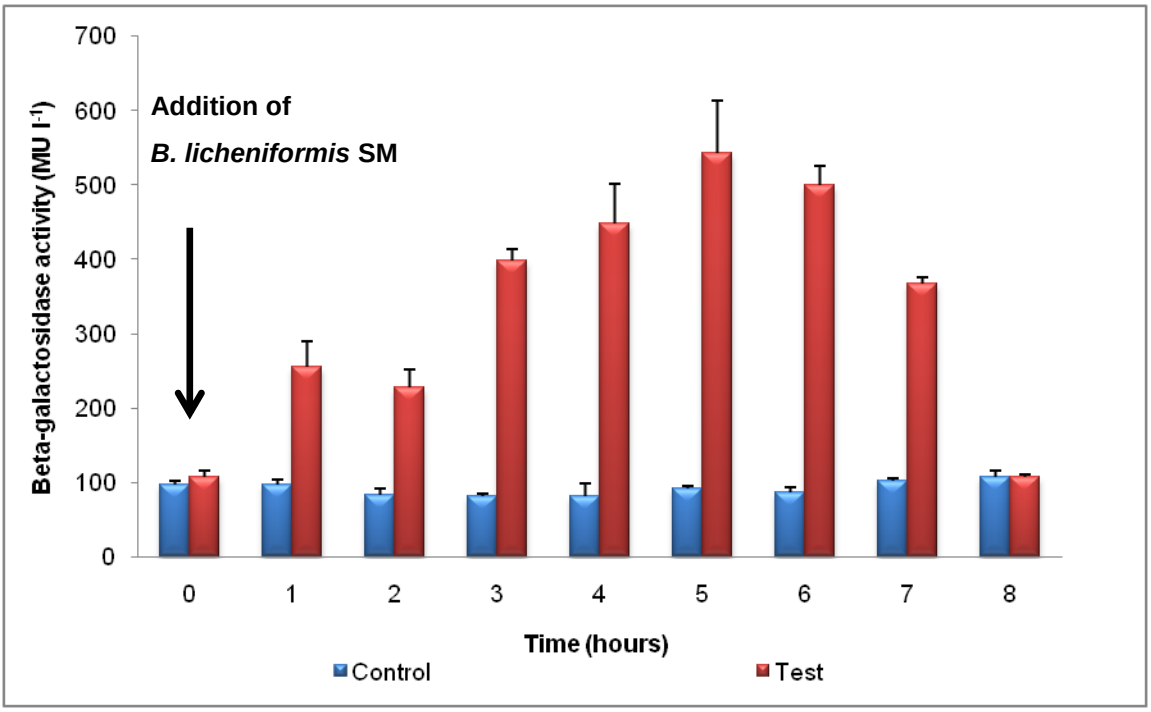


Figure 3.11: β-galactosidase activity in *B. subtilis* LS27 cells grown in the absence (Control) and in the presence of 1.25% (w/v) conditioned medium from *B. licheniformis* cultures at high cell density (Test). Time of exogenous spent medium addition is indicated with a black arrow. Error bars represent the standard deviation of triplicates.

3.1.3 Determination of production pattern for potential signalling molecules in *B. licheniformis*

To determine the production/secretion pattern of potential signalling molecules in *B. licheniformis* cultures, shaken flask fermentations (500 mL) in competence medium for competence development were set up. Single colonies of *B. licheniformis* and *B. subtilis* wild type strain were used to separately inoculate 100 mL competence medium, which was then incubated on a rotary shaker for 16 hours at 37 °C and 200 rpm. These pre-cultures, whose OD_{600} was determined to be ranging from 3-4, were diluted in 100 mL fresh medium to attain cultures with starting OD_{600} of 0.08-0.1. The cultures were then grown at 37 °C for 8-10 hours on a rotary shaker with a speed of 200 rpm. Samples were collected at 1 hour

intervals for OD_{600} measurement and centrifuged for collection of the cell-free supernatants. All the spent media collected were assayed for pheromone activity, by measuring the β-galactosidase activity induced in *B. subtilis* reporter strains JRL293 and LS27. In a 24-well plate, 0.25 mL of sample supernatant was mixed with 0.25 mL of reporter strain cells at low cell densities and incubated on a rotary shaker at 200 rpm for 70 minutes at 37 °C. The effect of added spent medium on *srfA* expression in *B. subtilis* reporter strain cells in one doubling time was then determined by β-galactosidase assay. Results are presented in Section 3.1.3.1 and 3.1.3.2.

3.1.3.1 Signalling molecule production pattern investigated with *B. subtilis* JRL293

Determination of pheromone production in *B. licheniformis* cultures was carried out using *B. subtilis* wild type strain as a positive control. For this study supernatants collected from *B. licheniformis* and *B. subtilis* cultures at different cell densities were used to induce β-galactosidase activity in *B. subtilis* strain JRL293. *B. subtilis* JLR293 cultures used for this assay were at 0.05-0.1 OD_{600}. Figure 3.12 shows the pattern of production of potential signalling molecules in *B. licheniformis* cultures compared to the pheromone activity profile of *B. subtilis*. In both cases production of signalling molecules follows the growth profile, thus confirming a cell density-dependent regulation. Moreover, the β-galactosidase activity detected when inducing *B. subtilis* JRL293 cells at low cell densities with cell-free supernatants from *B. licheniformis* and *B. subtilis* wild type cultures was in the same range.

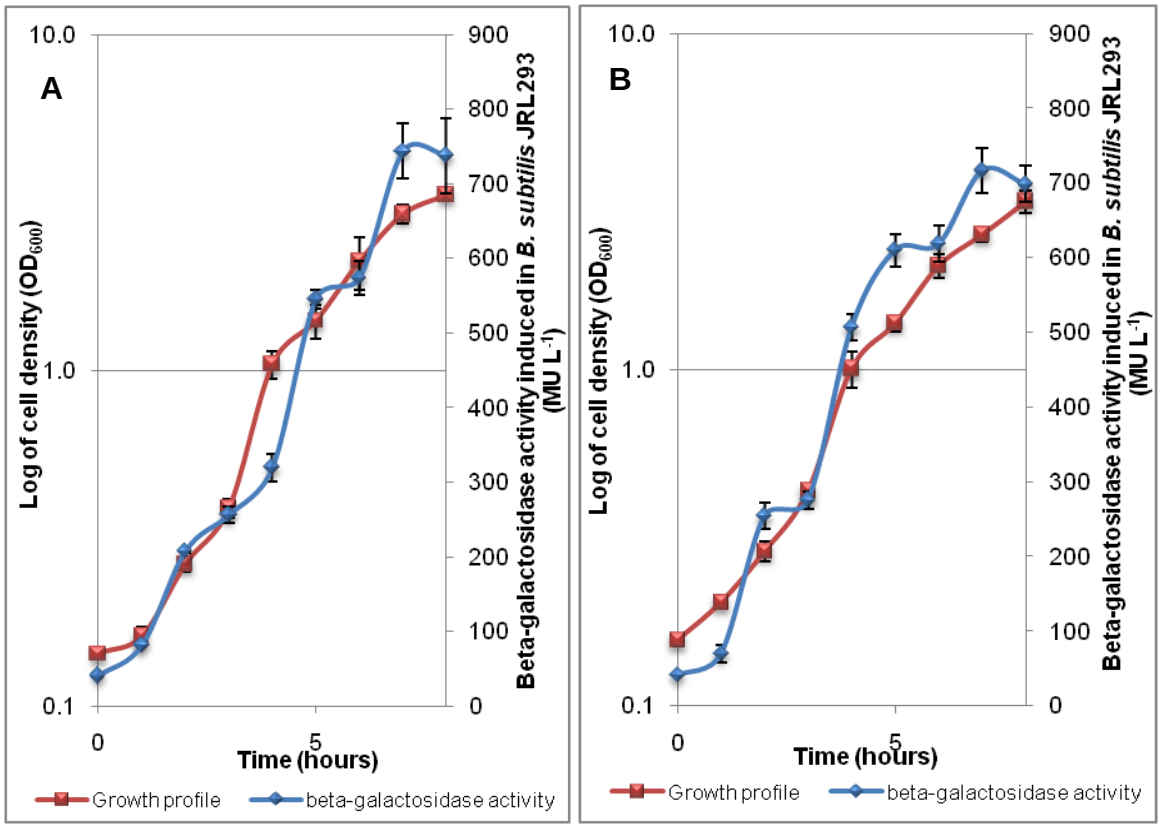


Figure 3.12: *B. licheniformis* (A) and *B. subtilis* wild type strain (B) growth curve (Red) and β-galactosidase activity (Blue). *B. licheniformis* and *B. subtilis* supernatants were collected at specific intervals throughout the growth and assayed for their ability to induce β-galactosidase activity in *B. subtilis* JRL293 cultures at low cell density. The experiments were performed in triplicates and the error bars represent the standard deviation.

3.1.3.2 Signalling molecule production pattern investigated with *B. subtilis* LS27

To confirm previous results and to rule out any possible interference due to ComX endogenous production from *B. subtilis* JRL293 cells, the experiment was performed using *B. subtilis* LS27, carrying a *comX* knock out, as a reporter. *B. subtilis* LS27 cultures used for this assay were at 0.1-0.2 OD_{600}. The pattern of production of potential signalling molecules in *B. licheniformis* cultures, as compared to *B. subtilis* wild type, is shown in Figure 3.13. The profile for β-galactosidase activity induced in *B. subtilis* LS27 cultures is consistent with the

one generated by *B. subtilis* JRL293, although the enzyme specific activity is ~10 times lower.

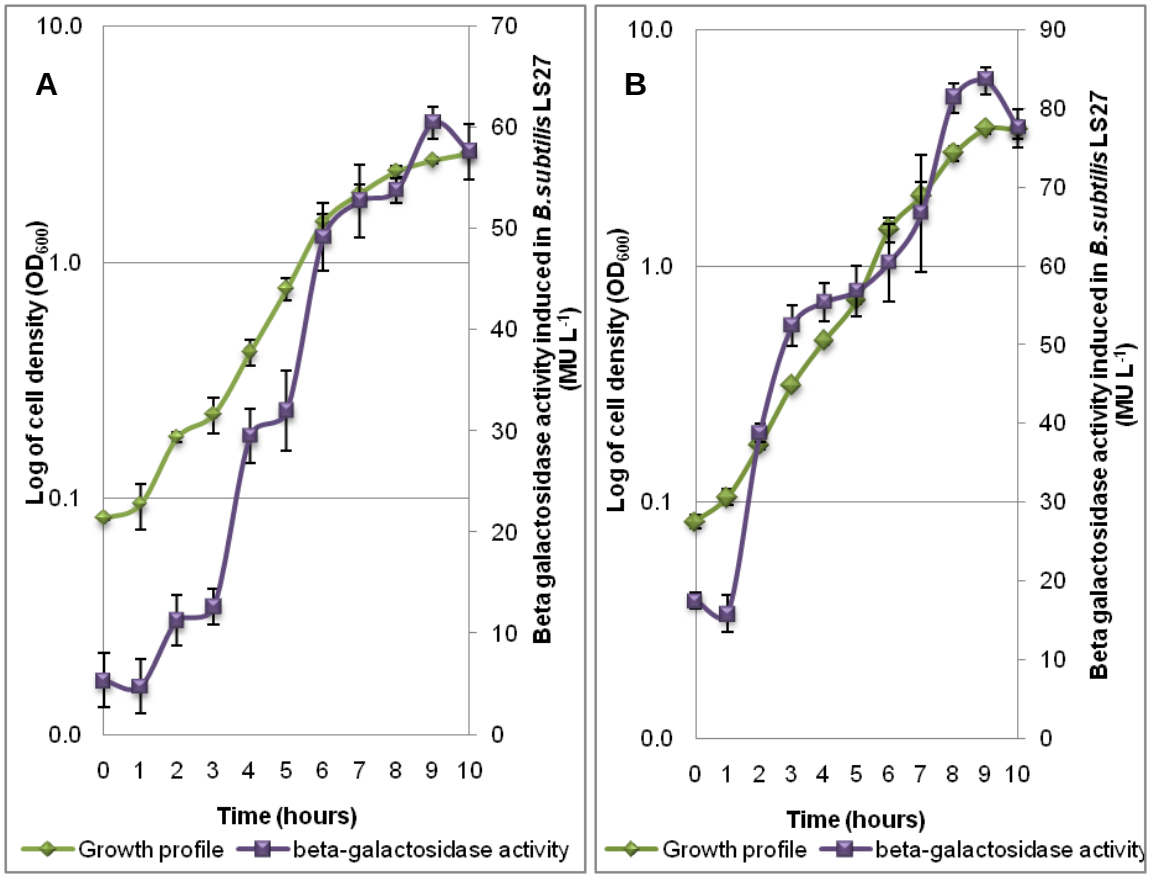


Figure 3.13: *B. licheniformis* (A) and *B. subtilis* wild type strain (B) growth curves (Green) and β-galactosidase activities (Purple). *B. licheniformis* and *B. subtilis* supernatants were collected at specific intervals throughout the growth and assayed for their ability to induce β-galactosidase activity in *B. subtilis* JRL293 cultures at low cell density. The experiments were carried out in triplicates and the error bars represent the standard deviation.

3.1.4 Isolation of putative signalling molecules from *B. licheniformis* cultures

Shaken flask fermentation (5 L) were carried out for isolation of potential signalling molecules from *B. licheniformis* cultures at high cell densities, using the ComX-producing *B. subtilis* wild type strain cultures as a control. In 5 L Erlenmeyer flasks, 900 mL of chemically defined medium were inoculated with 100 mL

cultures at 1 OD_{600} obtained by inoculating *B. licheniformis* and *B. subtilis* single colonies in chemically defined medium and incubating them at 37°C for 12 hours on a rotary shaker at 200 rpm. The resulting cultures were cultivated for 8 hours at 37°C on a rotary shaker with a speed of 150 rpm until they reached an OD_{600} of 3.5 and 4.5 for *B. licheniformis* and *B. subtilis* wild type strain, respectively. Supernatants harvested from these cultures were treated with 10% CH_3CN and 0.1% TFA prior to pheromone isolation by reverse-phase chromatography. The putative pheromone was eluted with a CH_3CN step gradient of 30, 60 and 90%. All the collected fractions were concentrated X1000 and assayed for pheromone activity by incubating them with *B. subtilis* LS27 cells at low cell densities. Amongst the fraction tested, only the 30% CH_3CN eluents from both *B. licheniformis* and *B. subtilis* wild type strain (Figure 3.14,) showed pheromone activity (ability to induce β-galactosidase activity in the reporter strain), whereas the fraction eluted with 60% CH_3CN, corresponding to the elution of the ComX pheromone, failed to induce β-galactosidase activity in the reporter strain.

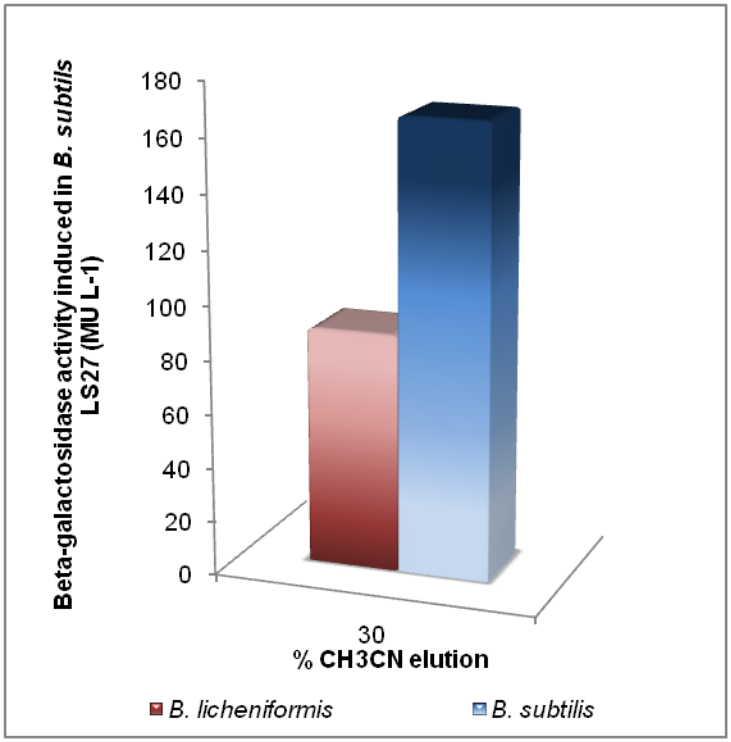


Figure 3.14: β-galactosidase activity in *B. subtilis* LS27 cultures at low cell densities induced by addition of chromatography fractions collected from *B. licheniformis* (Red) and *B. subtilis* (Blue).

3.2 Biosurfactants production in *B. licheniformis*

Studies on lichenysin production in *B. licheniformis* NCIMB 8874 were carried out in shaken flask fermentations (5 L) for biosurfactant extraction and determination of its microbial activity. Shaken flask fermentations (500 mL) were also set up to investigate the effect of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type strain supernatants on lichenysin production. The results are presented in Section 3.2.1.

3.2.1 Quantitative analysis of biosurfactants

Biosurfactants (lichenysin from *B. licheniformis* NCIMB 8874 and surfactin from *B. subtilis* wild type) production was investigated under two different growth conditions by cultivating the producing organisms in either complex or synthetic media (Sections 3.2.1.1 and 3.2.2.2). After biosurfactant extraction from culture supernatants, quantitative analysis was performed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) using commercially available surfactin as a standard. Surfactin chromatogram is characterised by six peaks, corresponding to different biosurfactant isoforms (Wei and Chu, 2002), as illustrated in Figure 3.15.

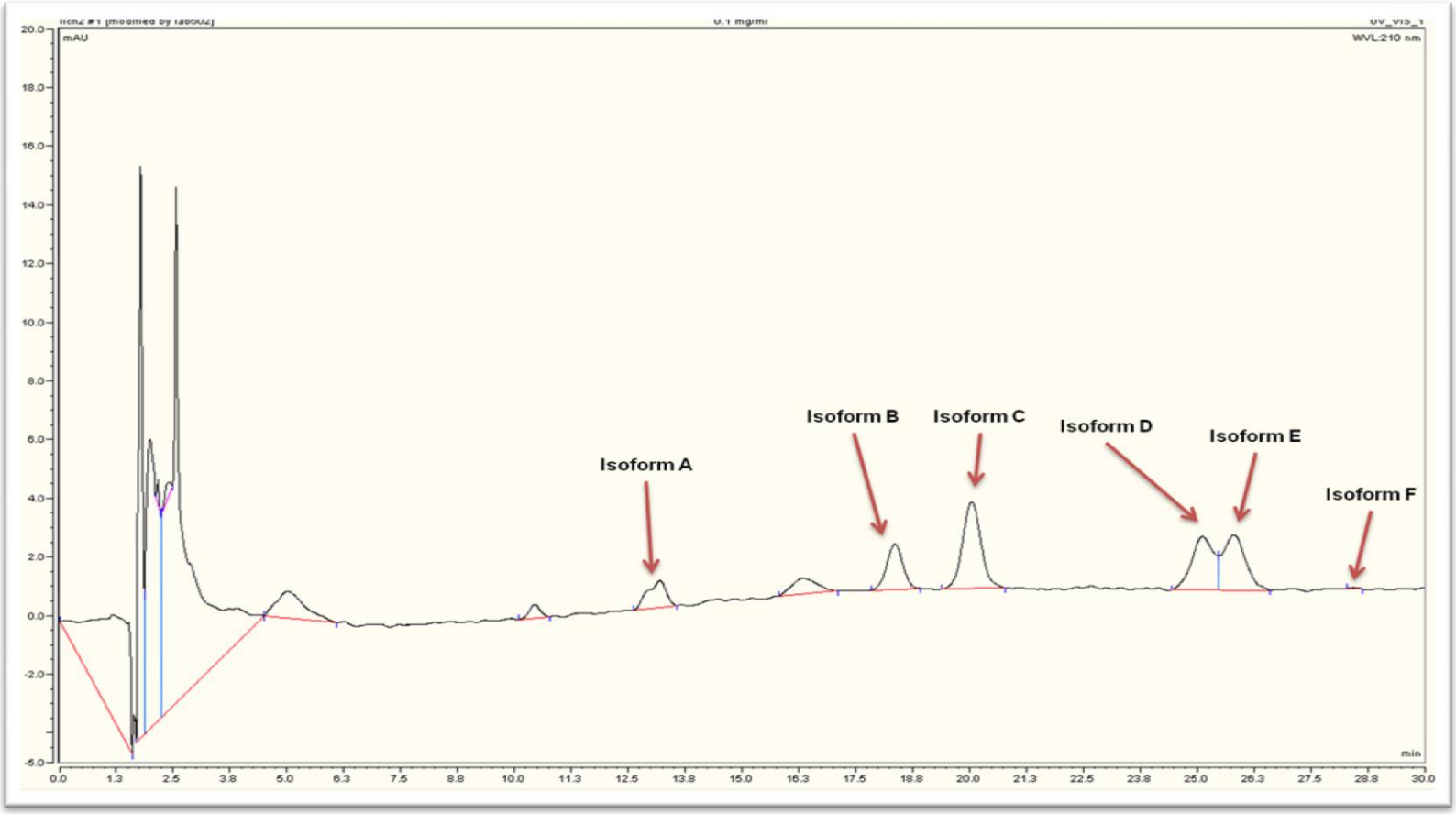


Figure 3.15: HPLC chromatogram of a 0.1 mg mL-1 surfactin standard sample. Red arrows indicate the chromatographic peaks representing the six isoforms of surfactin (A, B, C, D, E, F). The specific retention time for each peak is reported on the x-axis.

Single colonies of *B. licheniformis* NCIMB 8874 and the wild type *B. subtilis* were used to inoculate 100 mL YPD medium. The flasks were incubated at 37°C in an orbital shaker at a speed of 200 rpm with 2 cm throw for 16 hours. These cultures were used to inoculate 900 mL of YPD medium in 5 L flasks. The medium was then incubated at 37 °C at 150 rpm for 26 hours. At the end of the growth, supernatants were harvested by centrifugation and processed for biosurfactant extraction. HPLC analysis of both lichenysin and surfactin resulted in the identification of the isoforms: A; B; C; D and E (Figure 3.16). Isoform F could not be detected under the conditions analysed. Table 3.1 reports the concentrations calculated for each of the five isoforms detected for lichenysin and surfactin.

In *B. licheniformis* cultures cultivated in complex medium isoform A is the most abundant species, whereas *B. subtilis* produces surfactin mainly in the isoform D. Surfactin production showed to be significantly ($p<0.05$) higher than lichenysin, regardless of the isoform taken into consideration. In complex medium total surfactin concentration was 11.55-fold higher than lichenysin concentration.

Table 3.1: *Lichenysin and surfactin production in B. licheniformis NCIMB 8874 and B. subtilis wild type strain in YPD medium.*

	B. licheniformis	***B. subtilis***
	Lichenysin concentration (mg L^{-1})	**Surfactin concentration (mg L^{-1})**
Isoform A	63.32	177.81
Isoform B	29.28	469.63
Isoform C	7.77	169.06
Isoform D	37.52	672.71
Isoform E	16.59	296.23
Total	154.50	1785.45

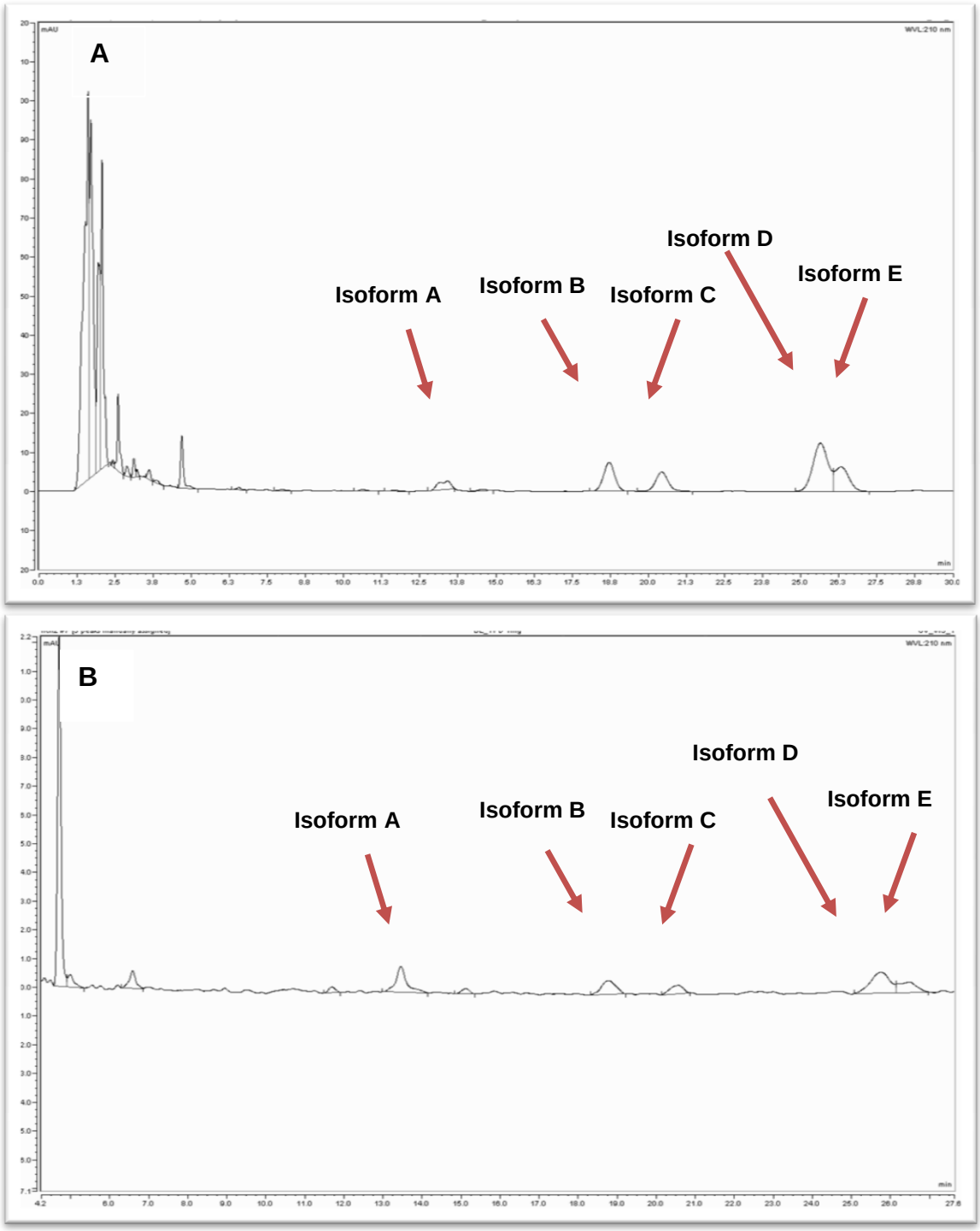


Figure 3.16: HPLC chromatogram of a 1 mg mL-1 surfactin (A) and lichenysin (B) samples exctracted from *B. subtilis* and *B. licheniformis* cultures, respectively, grown in YPD medium for 26 hours. Red arrows indicate the chromatographic

peaks representing the six isoforms of surfactin (A, B, C, D, E). The specific retention time for each peak is reported on the x-axis.

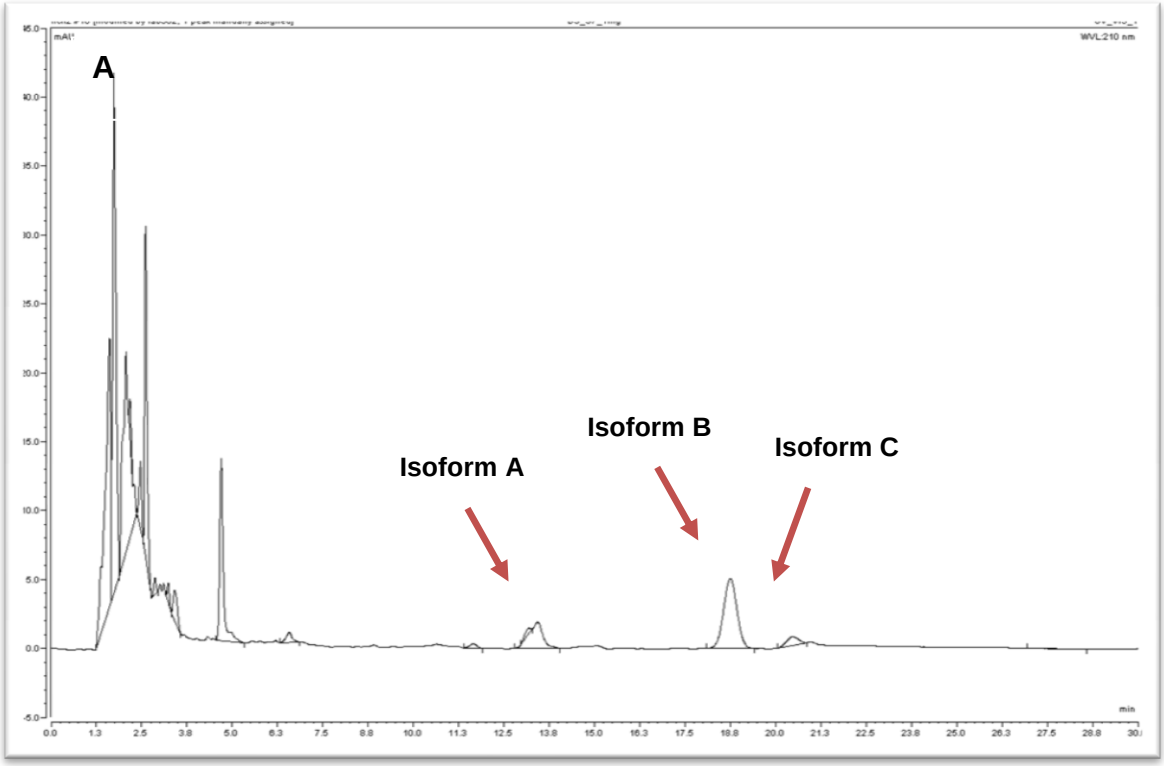
3.2.2 Synthetic medium

Single colonies of *B. licheniformis* and *B. subtilis* were used to inoculate 100 mL competence medium. The inoculated flasks were incubated at 37 °C in an orbital shaker at 200 rpm with 2 cm throw for 16 hours. These pre-cultures were used to inoculate 5 L shaken flasks containing 900 mL competence medium, subsequently incubated at 37 °C at 150 rpm for 13 hours. Biosurfactant extraction was carried out on cell-free supernatants collected after fermentation. Only three isoforms (A; B and C) were detected by HPLC analysis of the extracted biosurfactants (Figure 3,.17). The concentrations estimated for each of the three isoforms detected for lichenysin and surfactin are listed in Table 3.2.

Lichenysin was produced predominantly in the isoform A by *B. licheniformis* cultures grown in competence medium, whilst the isoform B was found to be the main component of surfactin in cultures of *B. subtilis*. Total surfactin concentration produced by *B. subtilis* wild type strain cultures in competence medium is 10-fold higher than the lichenysin detected in *B. licheniformis* supernatants.

Table 3.2: *Lichenysin and surfactin production in B. licheniformis* NCIMB 8874 *and B. subtilis wild type in competence medium.*

	B. licheniformis	***B. subtilis***
	Lichenysin concentration (mg L^{-1})	**Surfactin concentration (mg L^{-1})**
Isoform A	33.12	193.09
Isoform B	2.78	325.11
Isoform C	18.63	17.34
Total	54.53	535.54


A
Isoform A
Isoform B
Isoform C
mAU
WVL:210 nm
min

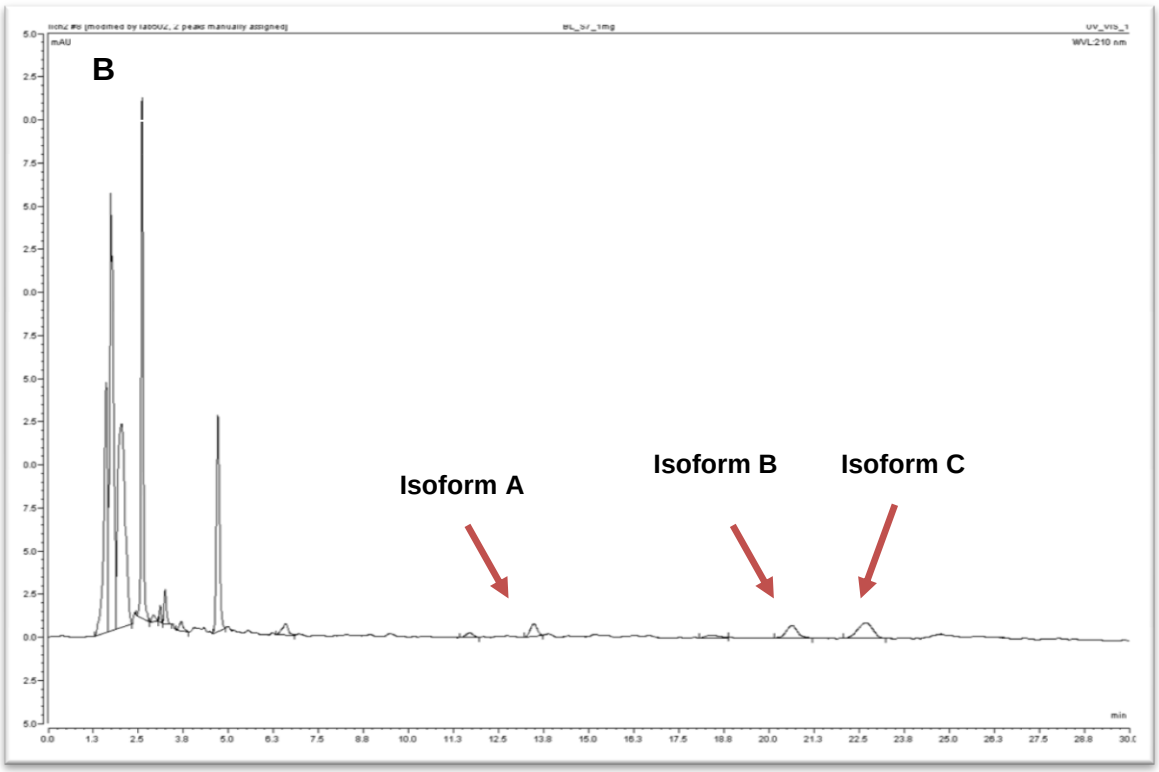

B
Isoform A
Isoform B
Isoform C
mAU
WVL:210 nm
min

Figure 3.17: HPLC chromatogram of a 1 mg mL-1 surfactin (A) and lichenysin (B) samples exctracted from *B. subtilis* and *B. licheniformis* cultures, respectively, grown in competence medium for 13 hours. Red arrows indicate the chromatographic peaks representing the six isoforms of surfactin (A, B, C). The specific retention time for each peak is reported on the x-axis.

Table 3.3: *Comparison between lichenysin and surfactin yields (based on Cell Dry Weight, CDW) in both synthetic and complex media.*

	Lichenysin Yield	**Surfactin Yield**
Synthetic medium	0.05	0.38
Complex medium	0.13	1.19

When comparing lichenysin and surfactin yields (based on CDW) in both synthetic and complex media, the production of surfactin in *B. subtilis* wild type cultures at high cell densities was 7- and 9-fold higher than lichenysin produced by *B. licheniformis* in synthetic and complex medium, respectively.

3.2.3 Susceptibility assay

Lichenysin extracted from *B. licheniformis* cultures in complex and synthetic media was tested for antimicrobial activity against other bacterial species. *Bacillus subtilis* and *Bacillus smithii* were chosen as Gram positive targets, whereas antimicrobial activity against Gram negative bacteria was assayed using *Escherichia coli, Pseudomonas aeruginosa* and *Chromobacterium violaceum.* Lyophilised lichenysin was dissolved in distilled water to prepare solutions at different concentrations. Bacterial species to be tested were evenly streaked on DST agar plates to prepare a bacterial lawn and 10 μL of lichenysin solution was administered to disc diffusion discs (3 mm) placed on the inoculated agar. The inhibition zones were observed after overnight incubation at 37 °C. Results

obtained with lichenysin extracted from *B. licheniformis* cultures grown in complex and synthetic media are listed in Table 3.4 and 3.5, respectively.

Table 3.4: *Antimicrobial activity of lichenysin extracted from B. licheniformis cultures in complex medium assayed by disc diffusion test* [a].

Organism	Lichenysin concentration			
	0 mg mL^{-1}	1 mg mL^{-1}	2 mg mL^{-1}	4 mg mL^{-1}
B. subtilis	-	+	++	++
E. coli	-	-	+	++
P. aeruginosa	-	-	-	-
C. violaceum	-	-	-	-
B. smithii	-	+	++	++

[a] Maximum diameter of halos: -, < 5 mm; + > 6 to 7 mm; ++, > 8 to 9 mm.

Table 3.5: *Antimicrobial activity of lichenysin extracted from B. licheniformis cultures in chemically defined medium assayed by disc diffusion test* [b].

Organism	Lichenysin concentration			
	0 mg mL^{-1}	1 mg mL^{-1}	2 mg mL^{-1}	4 mg mL^{-1}
B. subtilis	-	-	-	-
E. coli	-	-	+	+
P. aeruginosa	-	+	++	+++
C. violaceum	-	-	-	-
B. smithii	-	-	-	-

[b] Maximum diameter of halos: -, < 5 mm; + > 6 to 7 mm; ++, > 8 to 9 mm; +++,> 10 to 11 mm.

3.2.4 Effect of endogenous and exogenous spent medium on lichenysin production

The regulation of surfactin production in *B. subtilis* is a well established quorum sensing-dependent phenomenon and exogenous addition of signalling-molecule-containing spent medium has been proven to increase expression of the *srfA* operon (Magnuson *et al.*, 1994). To determine whether lichenysin production might be similarly regulated in *B. licheniformis* NCIMB 8874 cells, a set of shaken fermentations was carried out by cultivating *B. licheniformis* NCIMB 8874 cultures in both complex and synthetic medium in the absence (control cultures) and presence of either *B. subtilis* wild type (Test 1) or *B. licheniformis* NCIMB 8874 (Test 2) cell-free supernatant. For preparation of the supernatants to be added *B. subtilis* wild type and *B. licheniformis* NCIMB 8874 cultures were grown in synthetic medium for induction of competence development until they reached the late exponential phase. Cells were pelleted by centrifugation and discarded and the filter-sterilised supernatants were lyophilised and subsequently added to *B. licheniformis* NCIMB 8874 cultures at low cell densities (0.1-0.2 OD_{600}) at 1.25% (w/v). At the end of *B. licheniformis* NCIMB 8874 fermentations using complex and synthetic medium (Section 2.7), lichenysin was extracted from both test and control cultures and quantified by RP-HPLC. No statistically significant differences ($p>0.05$) could be detected in lichenysin production between control and either test culture supplemented with *B. licheniformis* NCIMB 8874 or the wild type *B. subtilis* lyophilised supernatant (Figure 3.18).

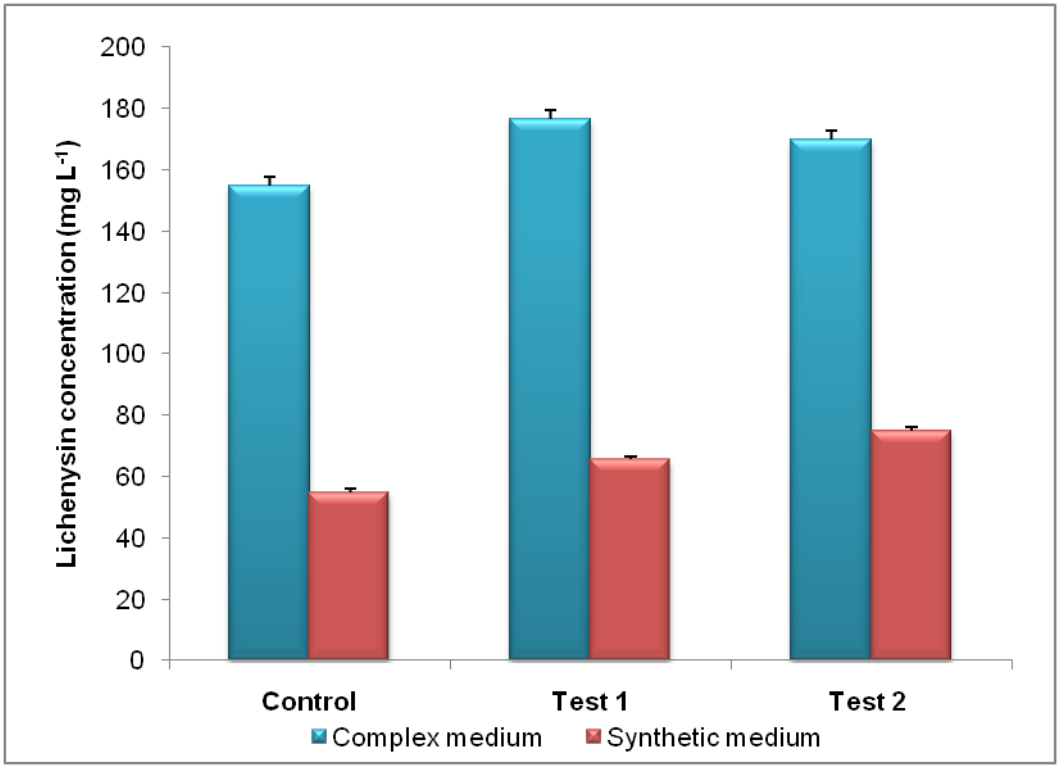


Figure 3.18: Lichenysin concentration detected in 1L cultures of *B. licheniformis* NCIMB 8874 grown in complex (Blue) and synthetic (Red) medium in the absence (control) and presence of 1.25% lyophilised spent medium obtained from either *B subtilis* wild type (Test 1) or *B. licheniformis* NCIMB 8874 (Test 2) at high cell densities. Experiments were carried out in triplicates and the error bars represent the standard error of the mean.

3.3 γ-PGA production

Studies on γ-polyglutamic acid production in *B. licheniformis* NCIMB 8874 were carried out in shaken flask fermentations (500 mL) for γ- PGA extraction. Shaken flask fermentations (500 mL) were also set up to investigate the effect of the addition of *B. licheniformis* NCIMB 8874 and the wild type *B. subtilis* strain supernatants on γ-PGA production in *B. licheniformis* NCIMB 8874. The results are presented in Section 3.3.1.

3.3.1 γ-PGA production in *B. licheniformis* NCIMB 8874

For determination of γ-PGA production in *B. licheniformis*, single colonies were inoculated into 100 mL of LB medium and incubated overnight at 200 rpm and 37°C. These cultures were then diluted in 100 mL PGA production medium to 0.1-0.2 OD_{600} and cultivated at 37°C for 45 hours on a rotary shaker at 200 rpm. The cultures were checked at regular intervals for biomass and γ-PGA production.

In order to determine the effect of potential signalling molecules on the biopolymer production, the cultures were treated at 0 hours, separately, with 1.25% (w/v) lyophilised supernatants collected from the wild type *B. subtilis* (Test 1) and *B. licheniformis* NCIMB 8874 (Test 2) cultures at high cell density. Cultures with no addition were used as controls. Prior to the additions, the supernatants were ultrafiltered through a membrane with a 3kDa cut-off and the filtrate was collected and lyophilised.

3.3.1.1 Growth curve

B. licheniformis NCIMB 8874 cultures grown in γ–PGA production medium in the absence (Control) and presence of 1.25% (w/v) lyophilised wild type *B. subtilis* (Test 1) and *B. licheniformis* NCIMB 8874 (Test 2) spent medium, were sampled

at regular intervals (0; 5; 20; 25;30 and 45 hours) for determination of OD_{600} and γ–PGA production.

The growth curves of *B. licheniformis* NCIMB 8874 control and test cultures are illustrated in Figure 3.19. Exogenous addition of 1.25% (w/v) lyophilised spent medium from either *B. licheniformis* NCIMB 8874 or *B. subtilis* wild type to *B. licheniformis* NCIMB 8874 test cultures has a negligible effect on cell growth ($p>0.05$), as compared to the control.

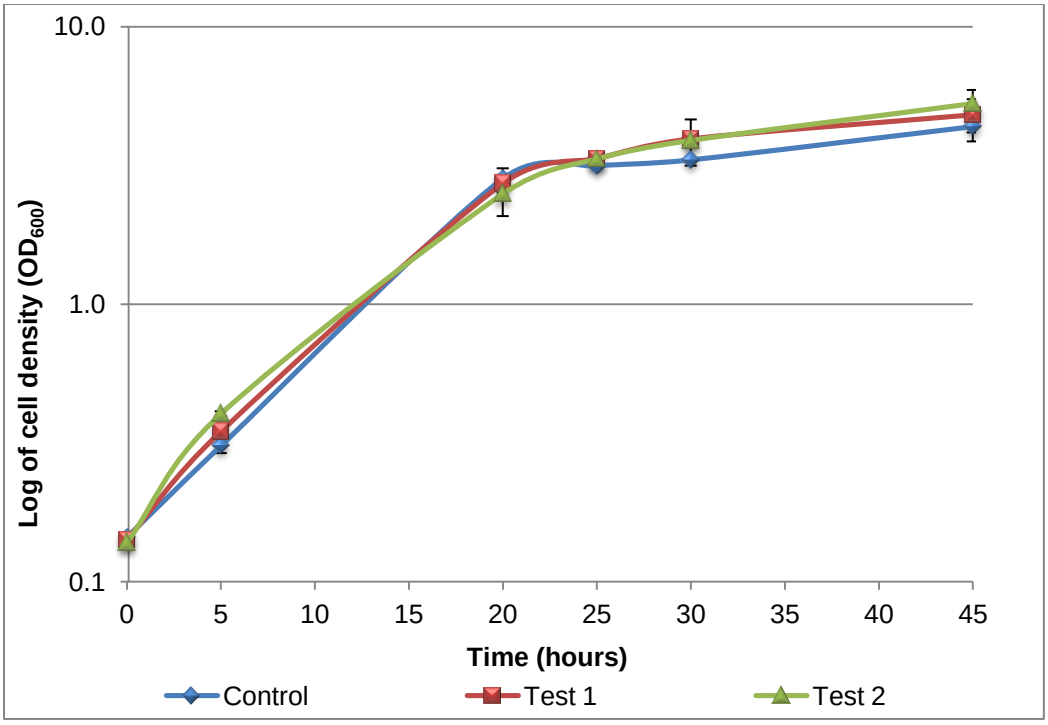


Figure 3.19: *B. licheniformis* NCIMB 8874 growth curve in PGA production medium in presence of *B. subtilis* (Test 1) and *B. licheniformis* NCIMB 8874 (Test 2) lyophilised spent media at 1.25% (w/v). The spent media were passed through a 3 kDa membrane prior to lyophilisation. Cultures grown without any addition were used as control. Samples were collected at specific times for OD_{600} determination. The experiments were performed in triplicates and the error bars represent the standard deviation.

3.3.1.2 Effect of cell-free supernatant on γ-PGA production

To evaluate γ–PGA production in the course of fermentation of *B. licheniformis* NCIMB 8874 control and test cultures supplemented with 1.25% (w/v) lyophilised spent media from *B. subtilis* wild type (Test 1) and *B. licheniformis* NCIMB 8874 (Test 2), crude γ-PGA was extracted from 1 mL supernatants collected at regular intervals (0; 5; 20; 25; 30 and 45 hours) throughout the growth. γ-PGA was also extracted from control cultures grown without any addition.

γ–PGA production pattern for *B. licheniformis* NCIMB 8874 control and test cultures is shown in Figure 3.20. γ–PGA production in the control cultures increases during the initial 20 hours of growth, reaching highest concentration of 19 g L^{-1} at 25 hours. This is followed by a steady decrease until the end of fermentation at 45 hours. No significant differences ($p>0.05$) could be detected in γ-PGA production from the cultures supplemented with 1.25% (w/v) lyophilised spent medium from *B. licheniformis* NCIMB 8874 cultures at high cell density (Test 2). However, an enhancement in γ–PGA production was detected as a peak after 20 hours growth, with γ-PGA concentration reaching 18.6 g L^{-1}, 1.5-fold higher than the control (13 g L^{-1}). Similarly, cultures grown in presence of 1.25% (w/v) lyophilised spent medium from *B. subtilis* wild type (Test 2) show an 2.3-fold increase corresponding to 27.5 g L^{-1} γ–PGA, in the production of biopolymer at 20 hours after induction, as compared to control cultures. However, the effect of exogenous addition of spent medium appears to be transient in both Test 1 and Test 2 cultures, as γ–PGA production after 25 hours does not show significant change ($p>0.05$) when compared to the control.

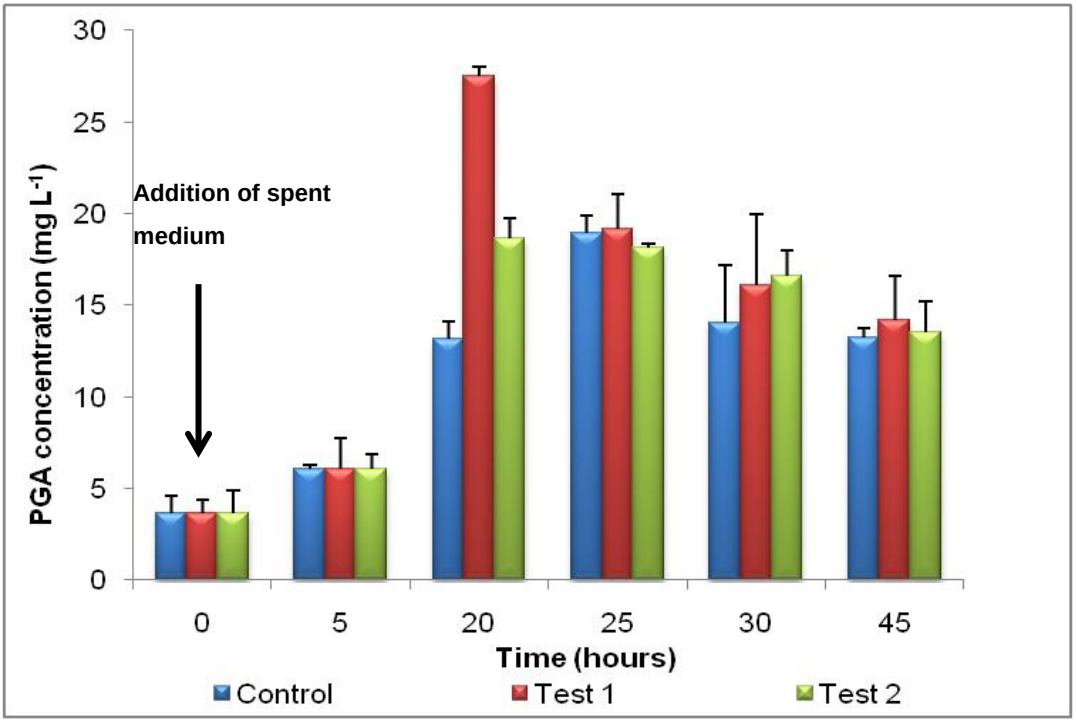


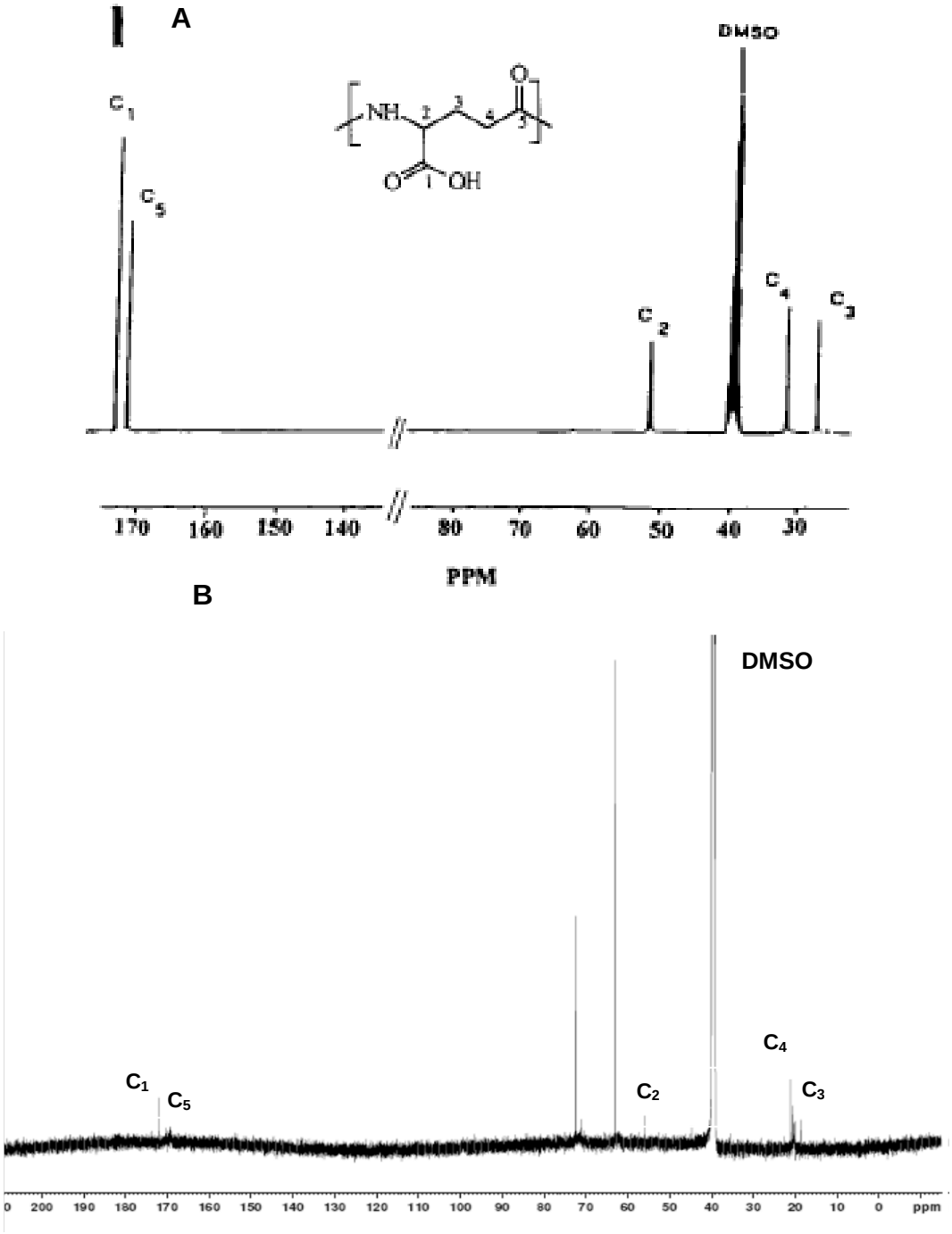
Figure 3.20: γ–PGA production in *B. licheniformis* NCIMB 8874 cultures grown in absence (Control) and presence of the wild type *B. subtilis* (Test 1) and *B. licheniformis* NCIMB 8874 (Test 2) lyophilised spent media at 1.25% (w/v). Crude γ–PGA was extracted from 1 mL supernatants collected at regular intervals throughout the growth. The experiments were performed in triplicates and the error bars represent the standard deviation.

3.3.1.3 NMR analysis of γ–PGA extracted from *B. licheniformis* NCIMB 8874 and *B. subtilis* LS27

In order to confirm the chemical composition of the crude polymer extracted from *B. licheniformis* NCIMB 8874 supernatants, NMR spectroscopy was performed on methanol-precipitated γ-PGA obtained from 100 mL supernatants collected after 45 hour fermentation. The extract obtained from supernatants of *B. licheniformis* test cultures at 45 hours growth in PGA production medium is illustrated in Figure 3.21.

Figure 3.21: γ-PGA extracted from *B. licheniformis* NCIMB 8874 cultures after 45 hours cultivation in PGA production medium.

Figure 3.22 shows the ^{13}C spectrum of γ–PGA extracted from *B. licheniformis* NCIMB 8874 supernatants. The spectrum shown corresponds to biopolymer extracted from the control cultures, as no difference in the number or composition of the peaks could be detected between the control and test cultures. As pure γ–PGA was not available to use as a standard for NMR analysis, assignation of the chemical shifts for each carbon atom was attempted by comparing with the NMR spectrum presented by Birrer and co-workers (1994).



Figures 3.22: ^{13}C spectrum of A) HCl-precipitated γ-PGA from *B. licheniformis* 9945A (Birrer at al., 1994) and B) MetOH-precipitated γ-PGA from *B. licheniformis* NCIMB 8874 analysed with NMR spectroscopy. Samples (20 mg) were dissolved in d_6-DMSO. The chemical shift of the peaks is indicated in parts per million (ppm).

3.3.1.4 Effect of supernatant addition on culture morphology

Figure 3.23 shows the effect of supernatant additions (as mentioned earlier) on *B. licheniformis* NCIMB 8874 culture morphology after 20 hours in the cultures grown in PGA production medium.

Both test cultures (1 and 2) showed an increase in the red pigmentation following the addition of the supernatants as compared to the control cultures. The increase in the production of the red pigment caused by exogenous addition of lyophilised supernatant from the wild type *B. subtilis* cultures appears to be greater than the one observed for cultures supplemented with *B. licheniformis* NCIMB 8874 supernatant. The increase in pigmentation was observed until the end of the growth at 45 hours.

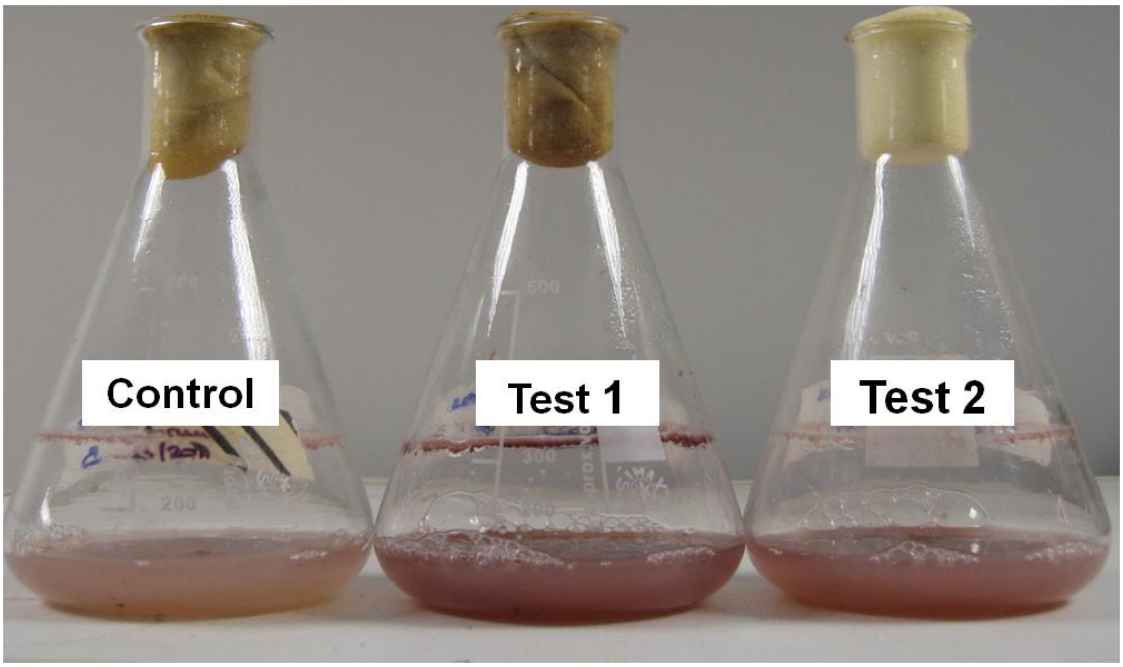


Figure 3.23: Effect of the addition of 1.25% (w/v) lyophilised supernatants collected from *B. licheniformis* NCIMB 8874 (Test 2) and *B. subtilis* wild type (Test 1) on the morphology of *B. licheniformis* NCIMB 8874 cultures in PGA production medium after 20 hours growth. Cultures without any addition were used as a control.

3.4 Effect of exogenous addition of spent medium on exoprotease production

Proteolytic activity in *B. licheniformis* NCIMB 8874 culture supernatants was qualitatively and sub-quantitatively evaluated by performing disc diffusion assay on agar plates containing skimmed milk (10%). The effect of exogenous addition of 1.25% (w/v) lyophilised supernatants collected from *B. licheniformis* NCIMB 8874 (Test 2) and *B. subtilis* wild type (Test 1) cultures on protease production in cultures of *B. licheniformis* NCIMB 8874 was also evaluated. Single colonies of *B. licheniformis* NCIMB 8874 were inoculated into 100 ml of LB medium and incubated overnight at 200 rpm and 37°C. These pre-cultures were then used to inoculate 100 mL LB and cultivated at 37°C for 12 hours on a rotary shaker at 200 rpm. Cultures at low cell densities (0.1 OD_{600}) at 0 hours were supplemented with 1.25% (w/v) lyophilised supernatants collected from *B. licheniformis* NCIMB 8874 (Test 2) and *B. subtilis* wild type (Test 1) cultures at high cell densities at 0 hours. Non treated cultures were used as a control. Samples (1 mL) were collected at regular intervals (2 hours) throughout the growth for supernatants collection. An aliquot (10 μL) of pre-filter-sterilised cell-free supernatants from each sample was dropped on paper discs positioned on M9 agar plates containing skimmed milk. After 25 hours incubation at 37 °C the halos generated by the supernatant proteolytic activities were measured and compared. The exogenous addition of 1.25% (w/v) lyophilised supernatants collected from *B. licheniformis* NCIMB 8874 (Test 2) and *B. subtilis* wild type (Test 1) cultures at high cell densities to lag phase cultures of *B. licheniformis* NCIMB 8874 did not result in any significant change in exoproteolytic activity as compared to the controls (data not shown).

3.5 Genetic analysis

Molecular studies for the investigation of potential quorum sensing processes in *B. licheniformis NCIMB 8874* were mainly focused on two genes of the

competence regulating *comQXPA* locus: i) *comP*, encoding the histidine kinase responsible for recognition of the ComX pheromone and downstream transmission of the signal, and ii) *comX*, coding for a polypeptide that undergoes maturation to become the ComX pheromone. Primers for PCR amplification were designed for both the genes by using *B. licheniformis* ATCC 14580 genome sequence as a template. Results are presented in section 3.5.1 and 3.5.2

3.5.1 PCR amplification of overlapping *comP* regions

Sequence alignment performed between the *comQXPA* cluster of *B. licheniformis* ATCC 14580 and *B. subtilis* 168 have highlighted that *comP* gene sequence is interrupted by a 1288-bp insertion sequence, named as *IS3Bli* (Lapidus *et al.*, 2002). As the genome sequence of *B. licheniformis* NCIMB 8874 was not available earlier, PCR analysis was carried out in order to determine whether the gene *comP* of this organism is disrupted by the same transposon insertion. To this aim, five sets of primers were designed and used in alternate combinations to amplify the gene sequence. The primers were designed to span the *IS3Bli* transposon insertion sequence in order to give products of different sizes in accordance with the presence or absence of the insertion into the gene. The localisation of the different sets of primers on *comP* gene sequence and the expected product sizes are illustrated in Figure 3.24.

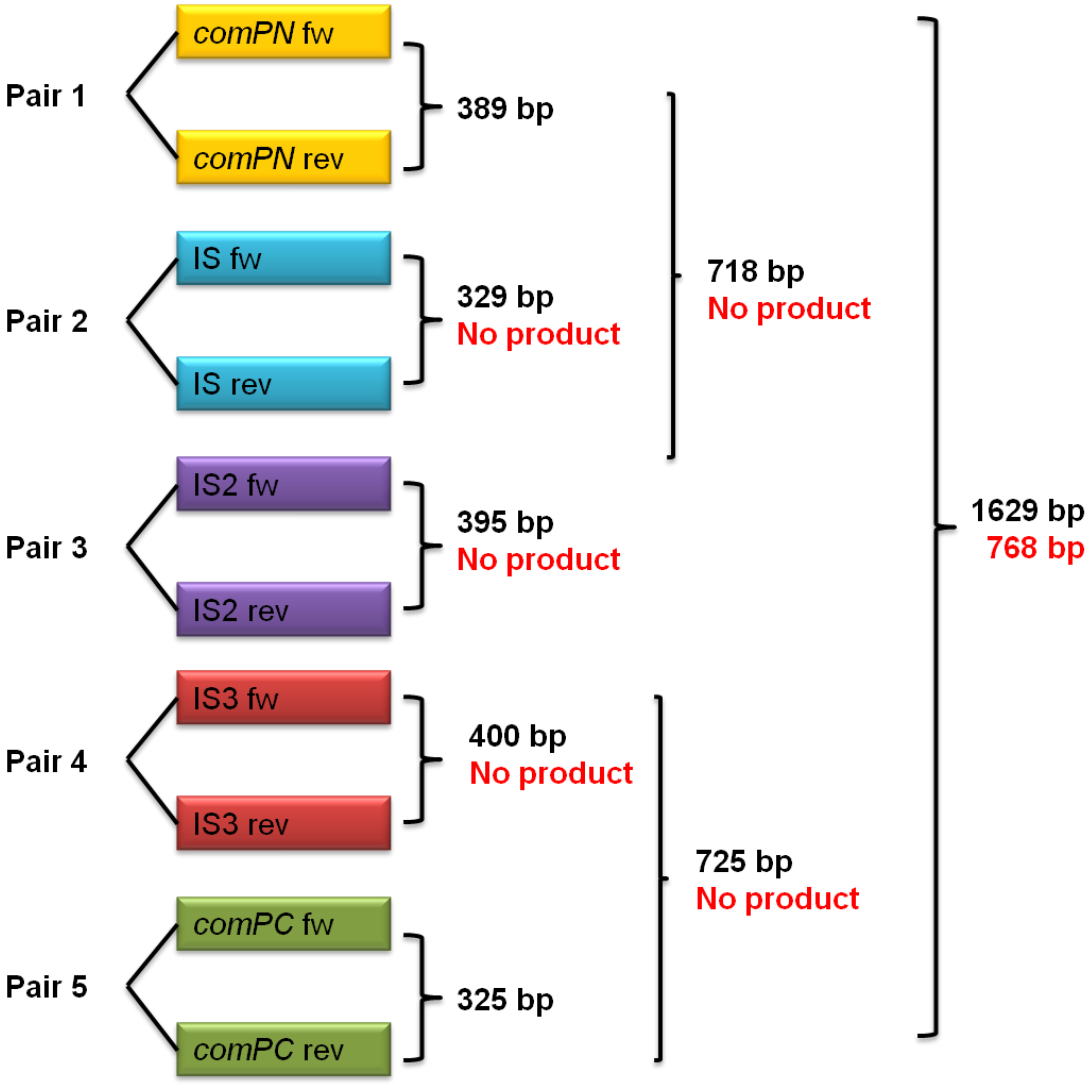


Figure 3.24: *comP* gene sequence from *B. licheniformis* strain ATCC 14580. The five sets of primers used for PCR amplification are highlighted (Pair 1: Yellow; Pair 2: Blue; Pair 3: Purple; Pair 4: Red; Pair 5: Green). Expected product sizes for *comP* amplification are also illustrated. The primers were designed to span the *IS3Bli* transposon insertion sequence in order to give different products depending on the presence (Black) or absence (Red) of the insertion into the gene.

The primer combination *comPN fw/ comPC rev* resulted in a band of approximately 700 bp, consistent with the expected product size (data not shown). This PCR product was purified and re-amplified using the purified PCR product as the DNA template. The product obtained from re-amplification was extracted from the gel, purified and sequenced. Sequence alignment with *B. licheniformis* ATCC

14580 genome revealed that the band was derived from an unspecific PCR product. No products were obtained with other sets of primers, although the primer pair *bacA* fw/*bacA* rev, used as a positive control, generated a band of the expected size (150 bp).

3.5.2 PCR amplification of *comX*

Primers were designed for PCR amplification of *comX* in order to confirm its sequence in *B. licheniformis* NCIMB 8874 genome. Given the rather small size of *comX* gene (only 165 bp), one pair of primers was designed from the extreme 5' and 3' ends of the gene. Figure 3.25 depicts the localisation of the primers for *comX* PCR amplification and the expected product size.

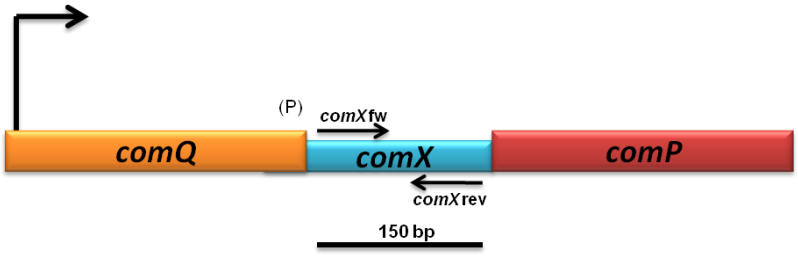


Figure 3.25: Schematic representation of primer design for *comX* amplification and expected product sizes.

No band was obtained from PCR amplification of *comX* with the designed primers (data not shown), whereas the primer pair *bacA* fw/*bacA* rev, used as in internal control, generated a band of the expected size (150 bp).

3.6 Bioinformatics analysis of quorum sensing-related genes on *B. licheniformis* NCIMB 8874 genome

To confirm the presence and the organisation of genes involved in cell-cell communication in *B. licheniformis* NCIMB 8874, the genomic DNA of this bacterium was isolated and sequenced for bioinformatics studies. Quorum sensing

related genes previously identified and annotated in other Bacilli were used to search for homologues on *B. licheniformis* NCIMB 8874 genome and to perform comparative analysis.

The genome sequence of *B. licheniformis* strain NCIMB 8874 used for this study was obtained from the alignment of output sequences from two different sequencers, 454 GS Junior and Illumina GAII. The assembly resulted in a single nucleotide sequence consisting of 304 contigs. This sequence is to be considered preliminary as it may contain erroneously assembled contigs and further analysis is necessary to reduce the gaps between the contigs.

Bioinformatics studies on *B. licheniformis* NCIMB 8874 genome sequence were carried out to investigate the *comQXPA* cluster of this organism and to confirm the presence of quorum sensing- regulated genes. Results are presented under Sections 3.5.1, 3.5.2 and 3.5.3.

3.6.1 The *comQXPA* gene cluster

Initial studies were performed for the identification of the competence regulating locus, *comQXPA* in *B. licheniformis* NCIMB 8874 genome. The *comQXPA* gene cluster comprises the genes coding for the isoprenyl transferase ComQ, dedicated to the maturation of the competence pheromone; the precursor protein of the ComX pheromone; and the two-component signal transduction system ComPA. The Basic Local Alignment Search Tool (BLAST) was used to carry out pairwise alignment between the *comQXPA* cluster of *B. licheniformis* ATCC 14580 and the whole genome sequence of *B. licheniformis* NCIMB 8874. The alignment allowed the identification of all the genes of the cluster with 95% identity for *comQ*; 94% for *comX*; 89% for *comP;* and 98% for *comA*.

The localisation of the *comQXPA* cluster on *B. licheniformis* NCIMB 8874 genome was investigated by searching for the genes flanking the *comQXPA* locus on *B. licheniformis* ATCC 14580 genome and carrying out alignment with strain NCIMB 8874 whole genome sequence. The locus was found to be located

between *degQ,* the gene immediately upstream of *comQ and yuxO,* the coding sequence immediately downstream of *comA*. To determine the conservation of *comQXPA* genetic organisation, the position of the cluster was investigated in other Bacilli. The organisation and localisation of the *comQXPA* locus on *B. licheniformis* NCIMB 8874 and other *Bacillus* species are presented in Figure 3.26. However, the current nucleotide sequence for *B. licheniformis* NCIMB 8874 does not allow the determination of the exact position of the locus on the genome as this sequence has yet to be annotated.

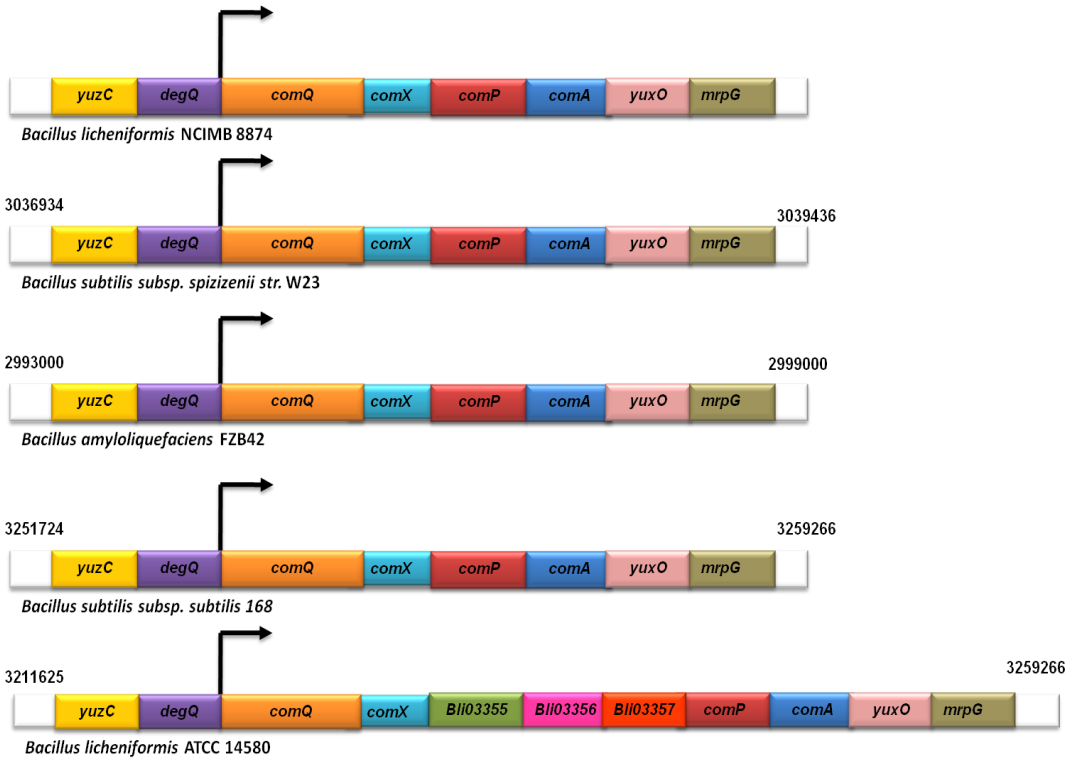


Figure 3.26: Genetic organisation and localisation of the *comQXPA* cluster in different *Bacillus* species.

The *comQXPA* gene cluster of *B. licheniformis* NCIMB 8874 appears to share the same organisation found in *B. subtilis subsp. subtilis* 168, *B. subtilis subsp. spizizenii* W23 and *B. amyloliquefaciens* FZB42 , thus indicating that the locus is conserved amongst Bacilli. *B. licheniformis* ATCC 14580 is an exception within the

group, having three unidentified genes, namely *Bli03355*, *Bli03356 and Bli03357*, located between *comX* and *comP.*

The whole *comQXPA* cluster of *B. licheniformis* NCIMB 8874 was aligned with the recently annotated counterparts of strains 9945A and F11 (Hoffmann *et al.*, 2010), resulting in 100 and 97% identity, respectively. Nucleotide sequence alignment of the *comQXPA* cluster of *B. licheniformis* NCIMB 8874 could not be carried out with more genetically distant Bacilli as the BLAST search tool was not able to identify any significant similarity between the gene sequences submitted for the analysis. Therefore, further investigation of the *comQXPA* locus of *B. licheniformis* NCIMB 8874 was performed at the protein level.

3.6.2.1 Conservation of proteins encoded by the *comQXPA* locus

Following the discovery of quorum sensing in *B. subtilis subsp. subtilis* 168 (Magnuson *et al.*, 1994), each protein encoded by the *comQXPA* locus has been thoroughly investigated and compared with homologues from related species. These studies highlighted a certain polymorphism in the proteins directly engaged in the cell-cell signalling production (ComQ and the ComX precursor) and reception (the N-terminal portion of ComP). Contrastingly, the C-terminus of ComP and the response regulator ComA appear to be the only conserved regions in all the Bacilli studied.

For investigation of the proteins encoded by the quorum sensing cluster of *B. licheniformis* NCIMB 8874 and determination of their evolution in relation with other Bacilli, the nucleotide sequence of each gene located on *comQXPA* cluster was translated into amino acids with the aid of Expasy DNA translation tool. Alignment of the amino acid sequences was then carried out with homologues from other Bacilli, listed in Table 3.6, using ClustalW. The colour code used to indicate amino acids with similar characteristics in the protein alignments is as follows: Red (small and hydrophobic); Blue (acidic); Magenta (basic); Green (hydroxyl and amine) and Gray (others).

***Table 3.6**: Bacillus species used for comparative analysis of quorum sensing-related genes of B. licheniformis NCIMB 8874.*

NCBI Accession number	Organism	Competence	Genome annotation
NC_000964	*Bacillus subtilis subsp. 168*	competent	Annotated (Kobayashi *et al.*, 2003)
NC_006270	*Bacillus licheniformis* ATCC 14580	non competent	Annotated (Rey *et al.*, 2004)
GQ505081.1	*Bacillus licheniformis* 9945A	competent	comQXPA, comS and mecA annotated (Hoffmann *et al.*, 2010)
GQ505080.1	*Bacillus licheniformis* F11	non competent	comQXPA, comS and mecA annotated (Hoffmann *et al.*, 2010)
NC_009725	*Bacillus amyloliquefaciens* FZB42	competent	Annotated (Chen *et al.*, 2007)
AF456135.1	*Bacillus mojavensis* R-O-B2	not identified	comQXP annotated (Ansaldi *et al.*, 2002)
NZ_ACWC00000000	*Bacillus sp*. BT1B_CT2	not identified	Annotated (unpublished)
NC_014479	*Bacillus subtilis subsp. spizizenii* W23	not identified	Annotated (unpublished)

The choice of the *Bacillus* species to use for alignment studies was mainly based on the presence of annotated *comQXPA* clusters on their genomes. As the ComX pheromone of *Bacillus mojavensis* R-O-B2 has been isolated (Ansaldi *et al.*, 2002) this bacterium was selected despite the partial annotation of its competence cluster.

3.6.2.1.1 The isoprene synthases ComQ

ComQ of *B. licheniformis* NCIMB 8874 was identified as a 303 amino acid protein, whose comparison with homologues from genetically related Bacilli selected from the Uniprot database confirmed as a member of the FPP/GGPP synthetase family engaged in isoprene biosynthesis. The % identities between ComQ of *B. licheniformis* NCIMB 8874 and other *Bacillus* species are listed in Table 3.7. The alignment of the different ComQ amino acid sequences is illustrated in Figure 3.27. Figure 3.28 provides a phylogenetic tree for determination of protein conservation amongst selected *Bacillus* species.

Table 3.7: *Percentage identities obtained from the alignment of ComQ protein sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
D9YRK9	*Bacillus licheniformis* 9945A	303	100
Q65FH4	*Bacillus licheniformis* ATCC 14580	289	97
GQ499198	*Bacillus licheniformis* F11	289	97
E5W7I2	*Bacillus sp*. BT1B_CT2	293	95
E0TZW4	*Bacillus subtilis subsp. spizizenii* W23	286	52
AF456135	*Bacillus mojavensis* R-O-B2	286	51
NC_000964	*Bacillus subtilis subsp. subtilis* 168	239	46
NC_009725	*Bacillus amyloliquefaciens* FZB42	286	40

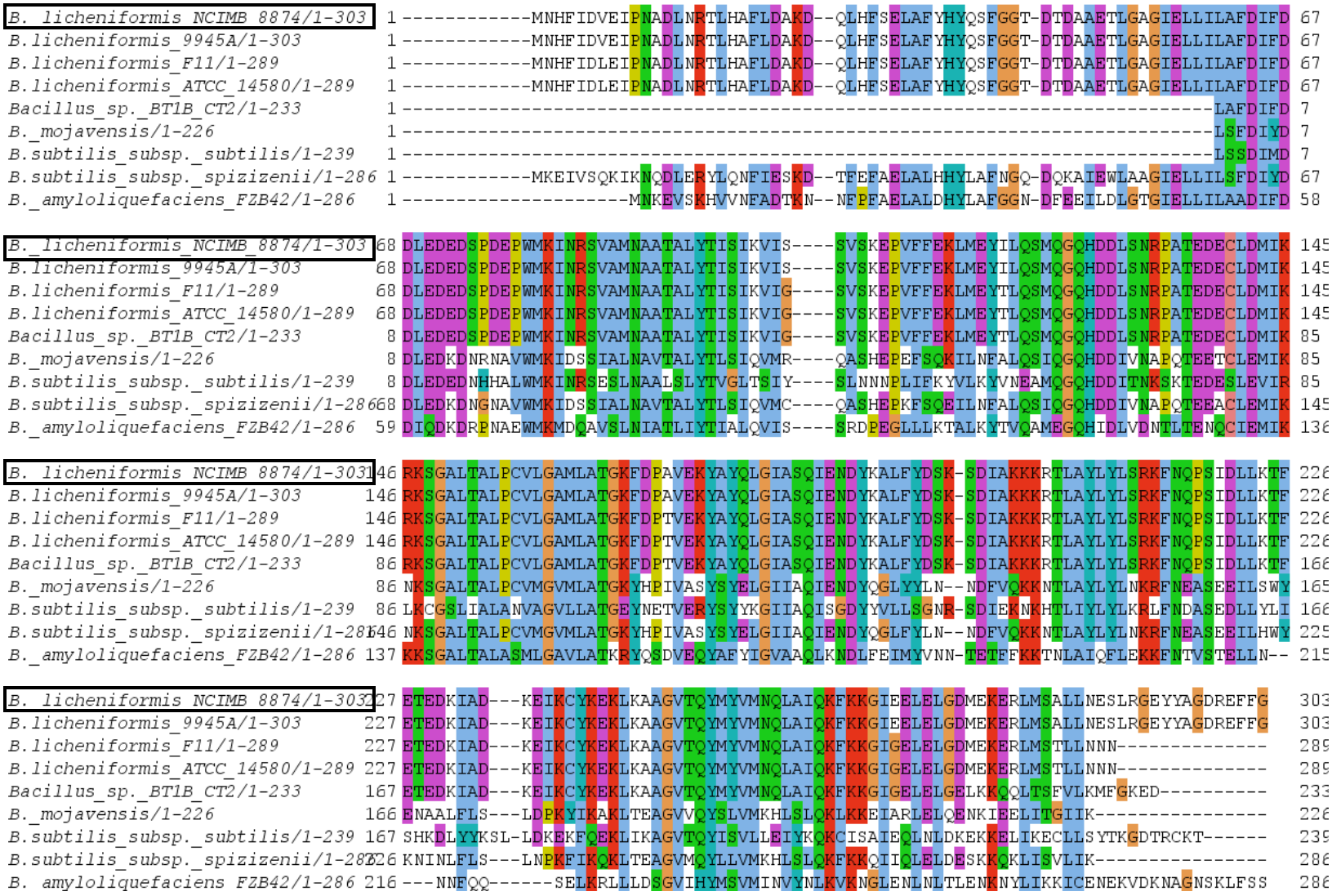


Figure 3.27: ClustalW alignments of ComQ coloured with JalView. Conserved amino acids are indicated with the same colours in all rows. The black box denotes *B. licheniformis* NCIMB 8874.

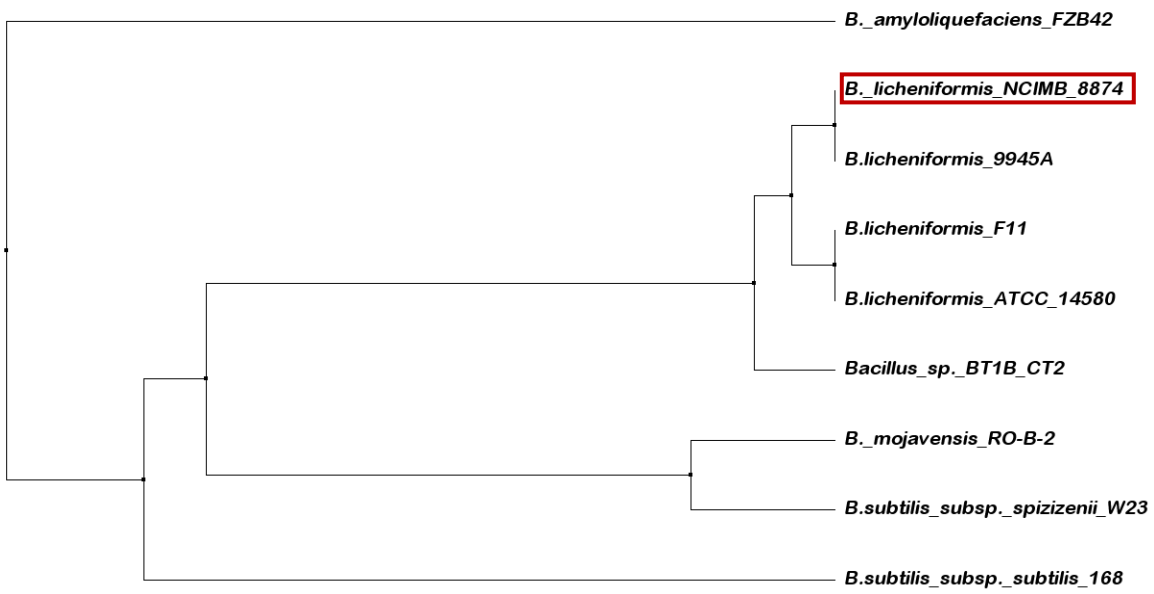


Figure 3.28: ComQ phylogenetic tree based on protein sequences aligned using Clustal W. The evolutionary tree was generated using Jalview software. The red box denotes *B. licheniformis* NCIMB 8874.

The results from *B. licheniformis* NCIMB 8874 ComQ alignment with other homologues revealed that the highest degree of identity was obtained with homologues from other *B. licheniformis* strains. In particular, the ComQ sequences from strain NCIMB 8874 and 9945A are 100% identical, whist 97% identity was detected with counterparts from strain F11 and ATCC 14580. ComQ from *B. amyloliquefaciens* FZB42 appeared to be the most divergent, with only 40% identity.

Although the alignment of ComQ sequences (Figure 3.27) highlights the polymorphism of the protein at the amino acid level, the phylogenetic tree derived by the alignment (Figure 3.28) shows that the *Bacillus* species analysed can be clustered in four distinct groups based on ComQ relative conservation.

3.6.3.1.2 The ComX pheromone precursor

The translated *comX* nucleotide sequence of *B. licheniformis* NCIMB 8874 resulted in a 56 amino acid protein, identified as the precursor of the ComX pheromone after alignment with homologues from other *Bacillus* species. Table 3.8 presents the results of the alignment between the pre-ComX of *B. licheniformis* NCIMB 8874 with selected homologues. The ClustalW alignment of the amino acid sequences of ComX precursor is depicted in Figure 3.29. Figure 3.30 provides a phylogenetic tree for determination of conservation of the protein amongst different Bacillus species.

Table 3.8: *Percentage identities obtained from the alignment of ComX precursor amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
D9YRL0	*Bacillus licheniformis* 9945A	56	100
E5W7I1	*Bacillus sp.* BT1B_CT2	57	65
E0TZW3	*Bacillus subtilis subsp. spizizenii* W23	54	53
AY003901	*Bacillus mojavensis* RO-H-1	53	53
AF456135	*Bacillus mojavensis* R-O-B2	54	52
AF456130	*Bacillus subtilis* RO-FF-1	57	49
Q65FH5	*Bacillus licheniformis* ATCC 14580	54	41
A7Z883	*Bacillus amyloliquefaciens* FZB42	57	39
GQ499198	*Bacillus licheniformis* F11	47	34
AF456134	*Bacillus mojavensis* RO-C-2	56	34
AF456138	Bacillus mojavensis RO-E-2	58	34
AF456137	*Bacillus subtilis* RO-F-3	73	29
NC_000964	*Bacillus subtilis subsp. subtilis* 168	55	27

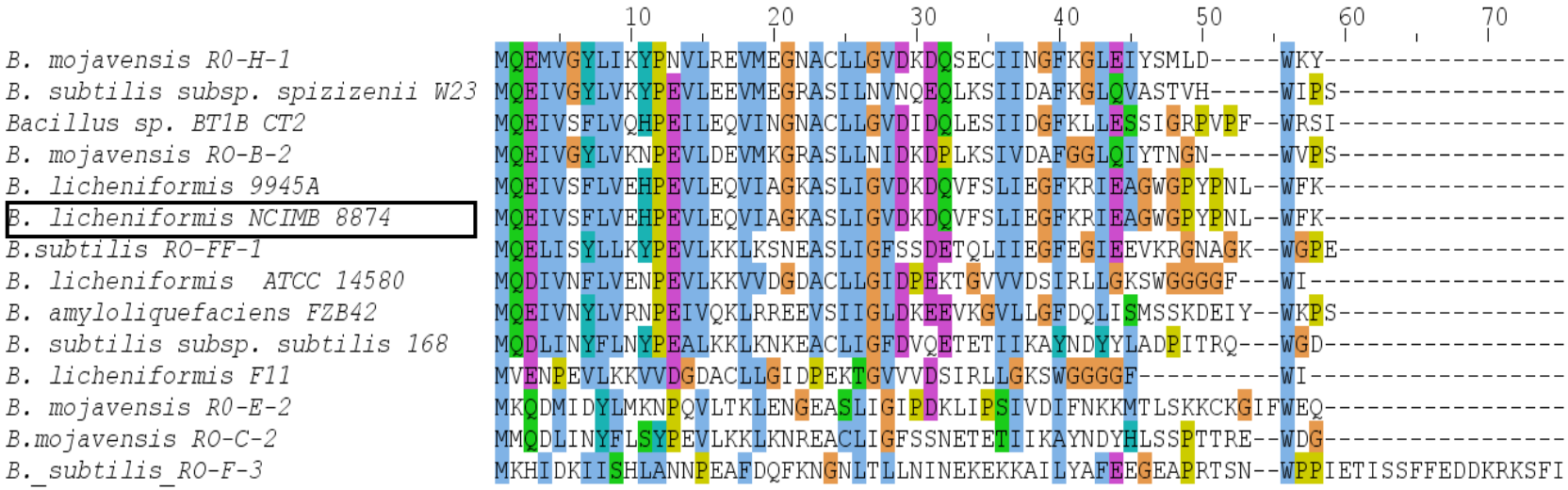


Figure 3.29: ClustalW alignments of *B. licheniformis* NCIMB 8874 pre-ComX with other homologues**.** The aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows. The black box denotes *B. licheniformis* NCIMB 8874. The sequences of pre-ComX from *B. mojavenis* isolates RO-H-1, RO-B-2, RO-E-2 and RO-C-2 and *B. subtilis* isolates RO-FF-1 and RO-F-3 were taken from Ansaldi and Dubnau, 2004.

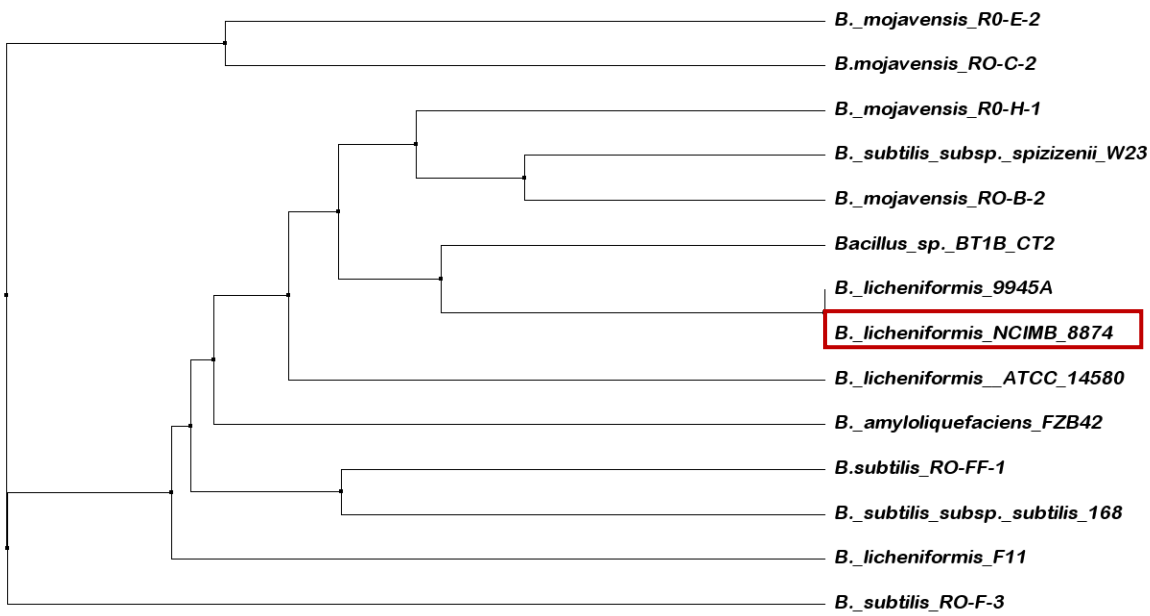


Figure 3.30: Pre-ComX phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. *B. licheniformis* NCIMB 8874 is indicated with a red box.

As Table 3.8 and Figure 3.29 show, the precursor of the ComX pheromone is highly polymorphic and, with the exception of *B. licheniformis* strains NCIMB 8874 and 9945A which share 100% identity, the alignment of the amino acid sequences resulted in percentage identities ranging from 65 to 27. However, similarly to what has been shown for ComQ, the pre-ComX sequences of the *Bacillus* species analysed can be classified into four main phylogenetic groups (Figure 3.30).

3.6.3.1.3 The sensor kinase ComP

Translation of *comP* nucleotide sequence from *B. licheniformis* NCIMB 8874 resulted in a 771 amino acid protein, confirmed as the sensor histidine kinase of the ComPA two component system. *B. licheniformis* NCIMB 8874 ComP was aligned with its counterpart of selected Bacilli. As the gene coding for ComP of *B. licheniformis* ATCC 14580 is interrupted by a transposon insertion, the amino acid

sequence used for this study was translated from the first 1130 bp of *comP* gene until the start of the *IS3Bli* insertion sequence, resulting in a 376 aa truncated protein. The amino acid sequence found in the NCBI database corresponds to the C-terminus of the protein, which cannot be encoded due to the presence of the transposon insertion. Results of this alignment are listed in Table 3.9 and illustrated in Figure 3.31. Figure 3.32 shows the phylogenetic tree derived from amino acid sequence alignments.

Table 3.9: *Percentage identities obtained from the alignment of ComP protein sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
D9YRL1	*Bacillus licheniformis* 9945A	771	100
E5W7I0	*Bacillus sp.* BT1B_CT2	766	71
GQ499198	*Bacillus licheniformis* F11	773	68
A7Z882	*Bacillus amyloliquefaciens* FZB42	766	55
NC_000964	*Bacillus subtilis subsp. subtilis* 168	769	53
E0TZW2	*Bacillus subtilis subsp. spizizenii* W23	774	51
Q65FH9	*Bacillus licheniformis* ATCC 14580	376	45
AF456135	*Bacillus mojavensis* R-O-B2	577	48

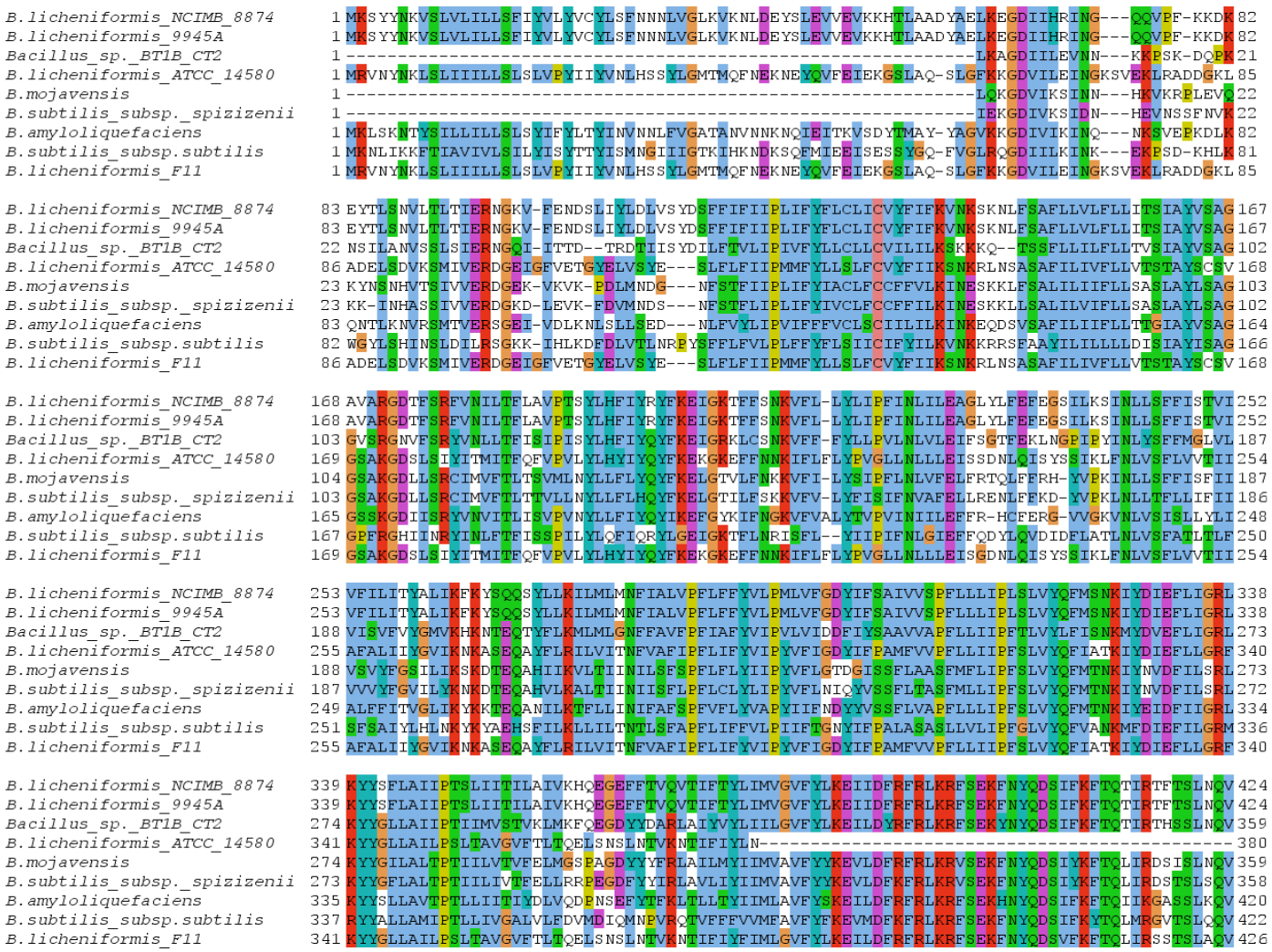
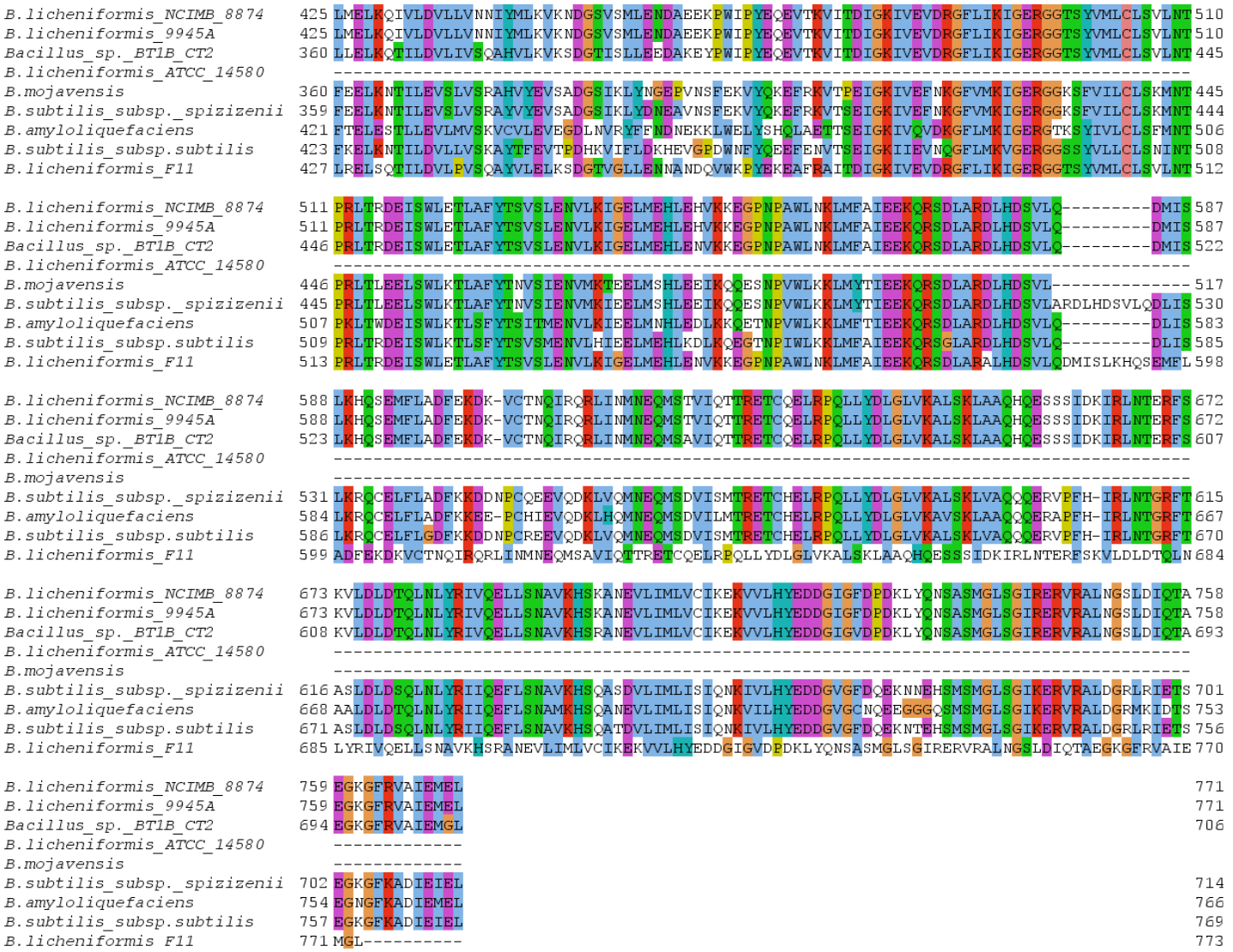


Figure 3.31: ClustalW alignments of *B. licheniformis* NCIMB 8874 ComP with selected homologues. The amino acids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows. The black boxes denote *B. licheniformis* NCIMB 8874. The sequence continues on the next page.


B.licheniformis_NCIMB_8874 425 LMELKQIVLDVLLVNNIYMLKVKNDGSVSMLENDAEEKPWIPYEQEVTKVITDIGKIVEVDRGFLIKIGERGGTSYVMLCLSVLNT 510
B.licheniformis_9945A 425 LMELKQIVLDVLLVNNIYMLKVKNDGSVSMLENDAEEKPWIPYEQEVTKVITDIGKIVEVDRGFLIKIGERGGTSYVMLCLSVLNT 510
Bacillus_sp._BT1B_CT2 360 LLELKQTILDVLIVSQAHVLKVKSDGTISLLEEDAKEYPWIPYEQEVTKVITDIGKIVEVDRGFLIKIGERGGTSYVMLCLSVLNT 445
B.licheniformis_ATCC_14580 ----------
B.mojavensis 360 FEELKNTILEVSLVSRAHVYEVSADGSIKLYNGEPVNSFEKVYQKEFRKVTPEIGKIVEFNKGFVMKIGERGGKSFVILCLSKMNT 445
B.subtilis_subsp._spizizenii 359 FEELKNTILEVSLVSRAYVYEVSADGSIKLYDNEAVNSFEKVYQKEFRKVTSEIGKIVEFNKGFVMKIGERGGKSFVILCLSKMNT 444
B.amyloliquefaciens 421 FTELESTLLEVLMVSKVCVLEVEGDLNVRYFFNDNEKKLWELYSHQLAETTSEIGKIVQVDKGFLMKIGERGTKSYIVLCLSFMNT 506
B.subtilis_subsp.subtilis 423 FKELKNTILDVLLVSKAYTFEVTPDHKVIFLDKHEVGPDWNFYQEEFENVTSEIGKIIEVNQGFLMKVGERGGSSYVLLCLSNINT 508
B.licheniformis_F11 427 LRELSQTILDVLPVSQAYVLELKSDGTVGLLENNANDQVWKPYEKEAFRAITDIGKIVEVDRGFLIKIGERGGTSYVMLCLSVLNT 512
B.licheniformis_NCIMB_8874 511 PRLTRDEISWLETLAFYTSVSLENVLKIGELMEHLEHVKKEGPNPAWLNKLMFAIEEKQRSDLARDLHDSVLQ---------DMIS 587
B.licheniformis_9945A 511 PRLTRDEISWLETLAFYTSVSLENVLKIGELMEHLEHVKKEGPNPAWLNKLMFAIEEKQRSDLARDLHDSVLQ---------DMIS 587
Bacillus_sp._BT1B_CT2 446 PRLTRDEISWLETLAFYTSVSLENVLKIGELMEHLENVKKEGPNPAWLNKLMFAIEEKQRSDLARDLHDSVLQ---------DMIS 522
B.licheniformis_ATCC_14580 ----------
B.mojavensis 446 PRLTLEELSWLKTLAFYTNVSIENVMKTEELMSHLEEIKQQESNPVWLKKLMYTIEEKQRSDLARDLHDSVL-------------- 517
B.subtilis_subsp._spizizenii 445 PRLTLEELSWLKTLAFYTNVSIENVMKIEELMSHLEEIKQQESNPVWLKKLMYTIEEKQRSDLARDLHDSVLARDLHDSVLQDLIS 530
B.amyloliquefaciens 507 PKLTWDEISWLKTLSFYTSITMENVLKIEELMNHLEDLKKQETNPVWLKKLMFTIEEKQRSDLARDLHDSVLQ---------DLIS 583
B.subtilis_subsp.subtilis 509 PRLTRDEISWLKTLSFYTSVSMENVLHIEELMEHLKDLKQEGTNPIWLKKLMFAIEEKQRSGLARDLHDSVLQ---------DLIS 585
B.licheniformis_F11 513 PRLTRDEISWLETLAFYTSVSLENVLKIGELMEHLENVKKEGPNPAWLNKLMFAIEEKQRSDLARALHDSVLQDMISLKHQSEMFL 598
B.licheniformis_NCIMB_8874 588 LKHQSEMFLADFEKDK-VCTNQIRQRLINMNEQMSTVIQTTRETCQELRPQLLYDLGLVKALSKLAAQHQESSSIDKIRLNTERFS 672
B.licheniformis_9945A 588 LKHQSEMFLADFEKDK-VCTNQIRQRLINMNEQMSTVIQTTRETCQELRPQLLYDLGLVKALSKLAAQHQESSSIDKIRLNTERFS 672
Bacillus_sp._BT1B_CT2 523 LKHQSEMFLADFEKDK-VCTNQIRQRLINMNEQMSAVIQTTRETCQELRPQLLYDLGLVKALSKLAAQHQESSSIDKIRLNTERFS 607
B.licheniformis_ATCC_14580 ----------
B.mojavensis ----------
B.subtilis_subsp._spizizenii 531 LKRQCELFLADFKKDDNPCQEEVQDKLVQMNEQMSDVISMTRETCHELRPQLLYDLGLVKALSKLVAQQQERVPFH-IRLNTGRFT 615
B.amyloliquefaciens 584 LKRQCELFLADFKKEE-PCHIEVQDKLHQMNEQMSDVILMTRETCHELRPQLLYDLGLVKAVSKLAAQQQERAPFH-IRLNTGRFT 667
B.subtilis_subsp.subtilis 586 LKRQCELFLGDFKKDDNPCREEVQDKLVQMNEQMSDVISMTRETCHELRPQLLYDLGLVKALSKLVAQQQERVPFH-IRLNTGRFT 670
B.licheniformis_F11 599 ADFEKDKVCTNQIRQRLINMNEQMSAVIQTTRETCQELRPQLLYDLGLVKALSKLAAQHQESSSIDKIRLNTERFSKVLDLDTQLN 684
B.licheniformis_NCIMB_8874 673 KVLDLDTQLNLYRIVQELLSNAVKHSKANEVLIMLVCIKEKVVLHYEDDGIGFDPDKLYQNSASMGLSGIRERVRALNGSLDIQTA 758
B.licheniformis_9945A 673 KVLDLDTQLNLYRIVQELLSNAVKHSKANEVLIMLVCIKEKVVLHYEDDGIGFDPDKLYQNSASMGLSGIRERVRALNGSLDIQTA 758
Bacillus_sp._BT1B_CT2 608 KVLDLDTQLNLYRIVQELLSNAVKHSRANEVLIMLVCIKEKVVLHYEDDGIGVDPDKLYQNSASMGLSGIRERVRALNGSLDIQTA 693
B.licheniformis_ATCC_14580 ----------
B.mojavensis ----------
B.subtilis_subsp._spizizenii 616 ASLDLDSQLNLYRIIQEFLSNAVKHSQASDVLIMLISIQNKIVLHYEDDGVGFDQEKNNEHSMSMGLSGIKERVRALDGRLRIETS 701
B.amyloliquefaciens 668 AALDLDTQLNLYRIIQEFLSNAMKHSQANEVLIMLISIQNKVILHYEDDGVGCNQEEGGGQSMSMGLSGIKERVRALDGRMKIDTS 753
B.subtilis_subsp.subtilis 671 ASLDLDSQLNLYRIIQEFLSNAVKHSQATDVLIMLISIQNKIVLHYEDDGVGFDQEKNTEHSMSMGLSGIKERVRALDGRLRIETS 756
B.licheniformis_F11 685 LYRIVQELLSNAVKHSRANEVLIMLVCIKEKVVLHYEDDGIGVDPDKLYQNSASMGLSGIRERVRALNGSLDIQTAEGKGFRVAIE 770
B.licheniformis_NCIMB_8874 759 EGKGFRVAIEMEL 771
B.licheniformis_9945A 759 EGKGFRVAIEMEL 771
Bacillus_sp._BT1B_CT2 694 EGKGFRVAIEMGL 706
B.licheniformis_ATCC_14580 ----------
B.mojavensis ----------
B.subtilis_subsp._spizizenii 702 EGKGFKADIEIEL 714
B.amyloliquefaciens 754 EGNGFKADIEMEL 766
B.subtilis_subsp.subtilis 757 EGKGFKADIEIEL 769
B.licheniformis_F11 771 MGL---------- 773

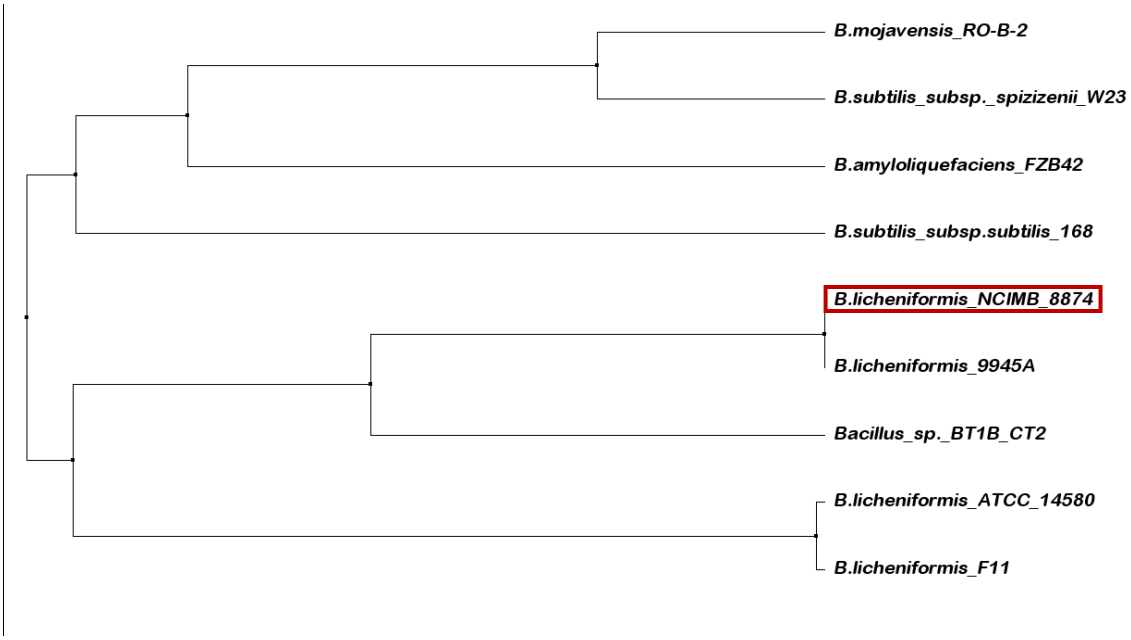


Figure 3.32: ComP phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. *B. licheniformis* NCIMB 8874 is indicated with a red box.

The sequence alignment between ComP of *B. licheniformis* NCMB 8874 and selected homologues from other species resulted in a reasonably variable distribution of identities, which ranged from 100% (strain 9945A) to 48% (*Bacillus mojavensis* R-O-B2). This variability is a result of ComP polymorphism, which, as Figure 3.31 shows, was found to characterise only the N-terminal portion of the protein, whereas the C-terminus appears to be conserved in all the species analysed. Figure 3.32 shows the phylogenetic congruence of ComP in selected Bacilli. On the tree, derived from the alignment of amino acid sequences, ComP homologues are grouped in four distinct clusters.

3.6.1.4.4 The response regulator ComA

The 212 amino acid-long sequence of *B. licheniformis* NCIMB 8874 ComA was aligned with counterparts to study its conservation in relation with other Bacilli. Results from the alignment are shown in Table 3.10. Comparison between

selected ComA amino acid sequences is illustrated in Figure 3.33, whist the phylogenetic distribution of the response regulator in different Bacillus species in shown in Figure 3.34.

Table 3.10: *Percentage identities obtained from the alignment of ComA amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
Q65FI0	*Bacillus licheniformis* ATCC 14580	212	100
E5W7H9	*Bacillus sp*. BT1B_CT2	212	100
GQ499198	*Bacillus licheniformis* F11	212	100
D9YRL2	*Bacillus licheniformis* 9945A	212	100
NC_000964	*Bacillus subtilis subsp. subtilis* 168	214	77
E0TZW1	*Bacillus subtilis subsp. spizizenii* W23	214	77
A7Z881	*Bacillus amyloliquefaciens* FZB42	214	75

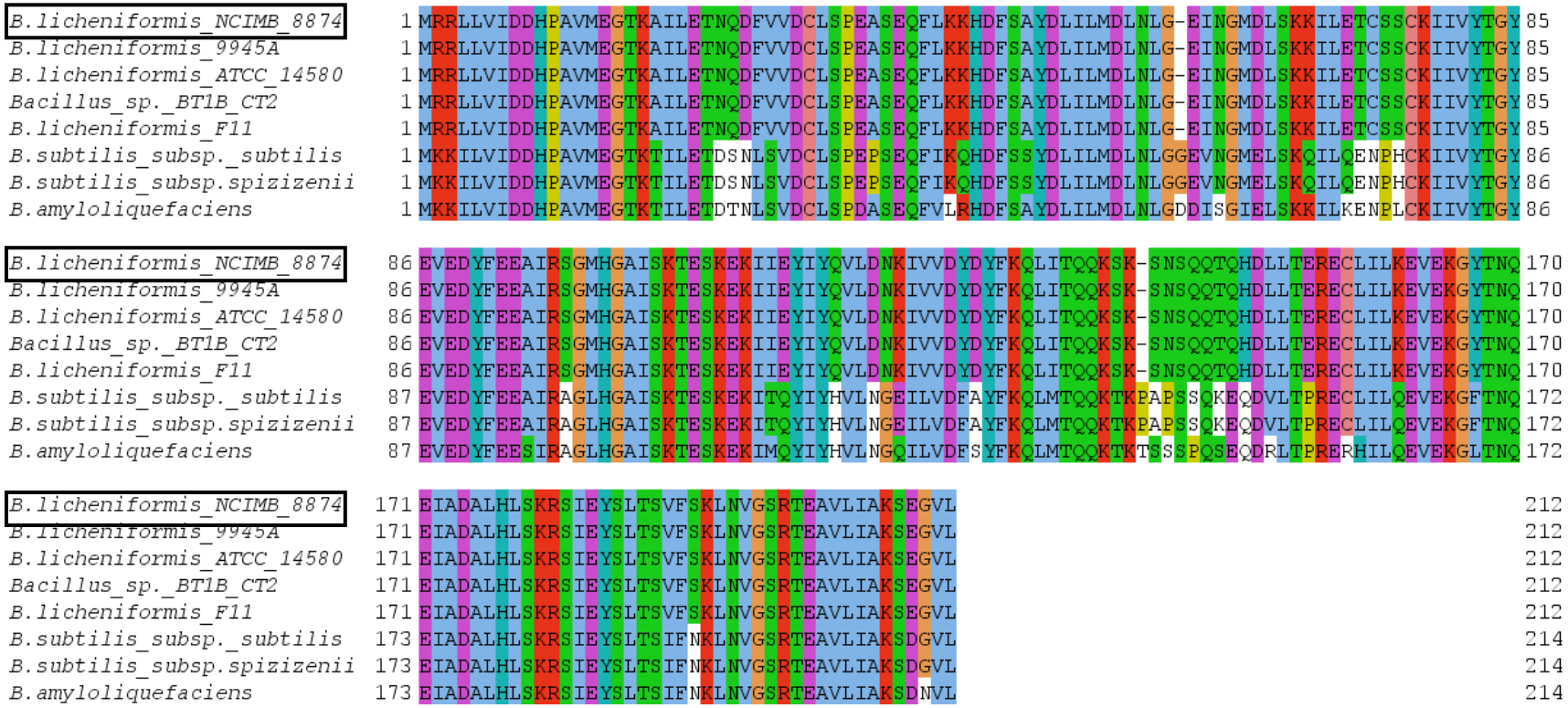


Figure 3.33: Sequence alignment of *B. licheniformis* NCIMB 8874 ComA with homologues from other *Bacillus* species. The alignment was performed with ClustalW and the aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows.

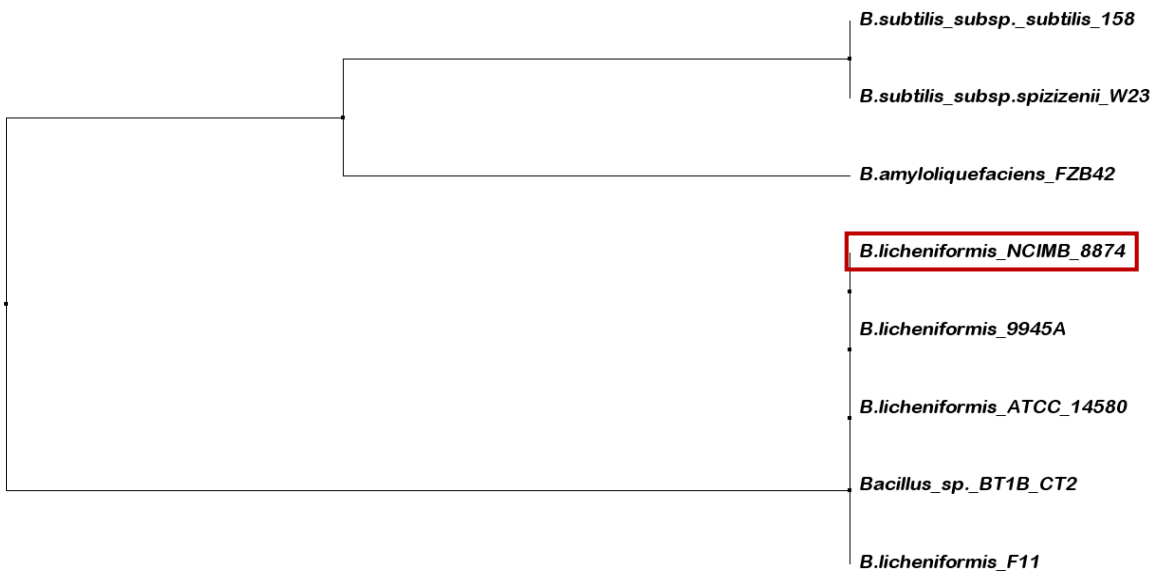


Figure 3.34: ComA phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. The red box denotes *B. licheniformis* NCIMB 8874.

The alignment results shown in Figure 3.33 confirmed that ComA is the most conserved protein encoded by the quorum sensing-regulating cluster. The amino acid sequence of ComA of *B. licheniformis* NCIMB 8874 showed 100% identity with homologues from all the other *B. licheniformis* strains and from *Bacillus sp.* BT1B_CT2. Alignment with homologues from more distant Bacilli revealed the lowest identity of 75% with ComA of *B. amyloliquefaciens* A7Z881 (Table 3.10).

The conservation of ComA can be observed in the phylogenetic tree drawn from the alignment (Figure 3.33), where the Bacilli analysed in relation to the conservation of the competence response regulator are distributed in only two groups.

3.6.3.2 Identification of essential proteins for quorum sensing in *B. licheniformis* NCIMB 8874

To determine whether the quorum sensing system of *B. licheniformis* NCIMB 8874 might be functional the presence of essential genes/proteins involved in the development of competence and other quorum sensing related processes, such as exoproteases, lichenysin and γ-polyglutamate production, needed to be established in this bacterium.

Quorum sensing in Bacilli is mainly regulated by the competence transcription factor ComK, whose expression in strictly regulated by the small protein ComS which releases ComK from the inhibition operated by the MecA-ClpCP proteasome-like complex. For this reason the presence of *comK*, *comS* and *mecA* genes was investigated in *B. licheniformis* NCIMB 8874 genome and their translated amino acid sequences were compared with homologues from selected Bacilli.

3.6.3.2.1 The competence transcription factor ComK

The gene encoding the ComK transcription factor was identified on *B. licheniformis* NCIMB 8874 genome by performing whole genome alignment with *comK* from *B. licheniformis* ATCC 14580 using BLAST. The alignment resulted in the identification of comK in *B. licheniformis* NCIMB 8874 with 97% identity with its counterpart in strain ATCC 14580.

The position of the gene coding for the transcription factor was established by performing alignment of the nucleotide sequence comprising *yhzC*, the gene located upstream of *comK*, *comK* and *yhjD*, the gene downstream of *comK,* of *B. licheniformis* ATCC 14580 with the whole genome sequence of strain NCIMB 8874. Search of the NCBI database determined that *comK* position is conserved, as the same genetic organization was found in more distant Bacilli, such as

B. subtilis subsp. subtilis 168 and *B. subtilis subsp spizizenii* W23. However, in *B. amyloliquefaciens FZB42 yhjD* is replaced by an unidentified gene. The gene encoding ComK in *B. licheniformis* strains 9945A and F11 has not been annotated in the NCBI database. The *comK* nucleotide sequence of *B. licheniformis* NCIMB 8874 was translated in its correspondent 192 amino acid protein using Expasy DNA translation tool and aligned with homologues from other Bacilli. Results of the alignment are listed in Table 3.11 and illustrated in Figure 3.35. Figure 3.36 depicts ComK phylogenetic tree based on the alignment.

Table 3.11: *Percentage identities obtained from the alignment of ComK amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
Q65LN7	*Bacillus licheniformis* ATCC 14580	192	98
E5W578	*Bacillus sp.* BT1B_CT2	192	98
CAA74548	*Bacillus subtilis subsp. subtilis 168*	192	97
E0TYQ8	*Bacillus subtilis subsp. spizizenii* W23	192	68
A7Z348	*Bacillus amyloliquefaciens* FZB42	192	66

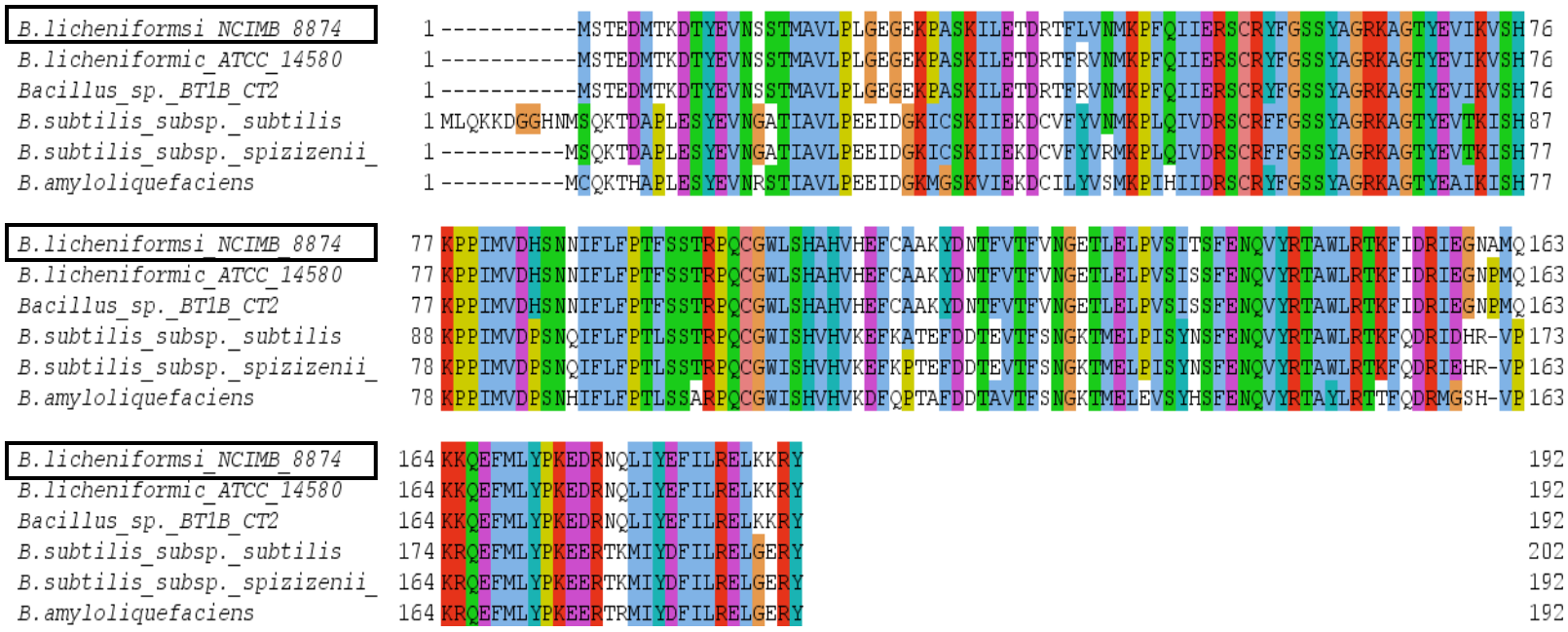


Figure 3.35: Sequence alignment of *B. licheniformis* NCIMB 8874 ComK with homologues from other *Bacillus* species. The alignment was performed with ClustalW and the aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows.

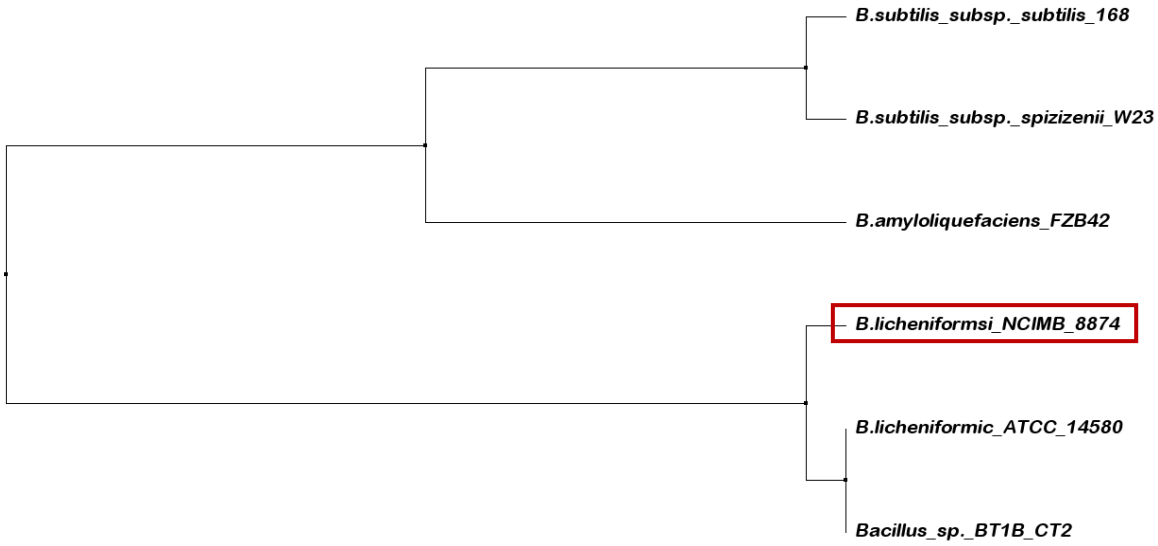


Figure 3.36: ComK phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. *B. licheniformis* NCIMB 8874 is denoted with a red box.

ComK of *B. licheniformis* NCIMB 8874 was found to share the highest identity (98%) with its counterpart in *B. licheniformis* ATCC 14580 and *Bacillus sp.* BT1B_CT2, whist only 68% identity was detected with ComK sequences from *B. subtilis subsp. spizizenii* and *B. amyloliquefaciens* (Table 3.11). These results suggest that the transcription factor ComK is characterised by a certain degree of conservation within two distinct groups of homologues, as confirmed by ComK phylogenetic tree (Figure 3.36).

3.6.3.2.2 ComS

The gene coding for ComS in *B. licheniformis* strain ATCC 14580 is located on the lichenysin biosynthetic cluster. However, the *comS* coding sequence of *B. licheniformis* ATCC 14580 has not been annotated in the NBCI database. Therefore search for *comS* of *B. licheniformis* NCIMB 8874 was carried out by

aligning the whole genome sequence of this organism with *comS* nucleotide sequence of strain 9945A, where *comS* was originally identified. The alignment resulted in a 99% identity between the nucleotide sequences of the two strain. Alignment with *comS* of *B. licheniformis* F11 revealed 94% identity. The genetic organisation of *comS* in *B. licheniformis* NCIMB 8874 was confirmed by alignment of its genome sequence with the lichenysin biosynthetic operon of strains 9945A and F11. The putative *comS* was translated into its amino acid sequence (61 amino acids) and compared with selected *Bacillus* species for investigation of protein conservation. For comparative analysis with *B. licheniformis* ATCC 14580 the nucleotide sequence of the lichenysin biosynthetic operon of this strain was aligned with *comS* sequence from *B. licheniformis* NCIMB 8874. The alignment led to identification of a 201 bp sequence, located on the lichenysin synthase B, with 94% identity with *comS* of *B. licheniformis* NCIMB 8874.

Table 3.12 shows the list of identity percentages obtained from the alignment of *B. licheniformis* NCIMB 8874 ComS with homologues from different Bacilli. ClustalW alignment is illustrated in Figure 3.37 and Figure 3.38 depicts the ComS phylogenetic tree.

Table 3.12: *Percentage identities obtained from the alignment of ComS amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
ADK89160	*Bacillus licheniformis* 9945A	66	100
E0U2C1	*Bacillus subtilis subsp. spizizenii* W23	46	97
GQ505079	*Bacillus licheniformis* F11	78	89
N/A	*Bacillus licheniformis* ATCC 14580	67	88
A7Z190	*Bacillus amyloliquefaciens* FZB42	54	61
AAA61567	*Bacillus subtilis subsp. subtilis* 168	46	39

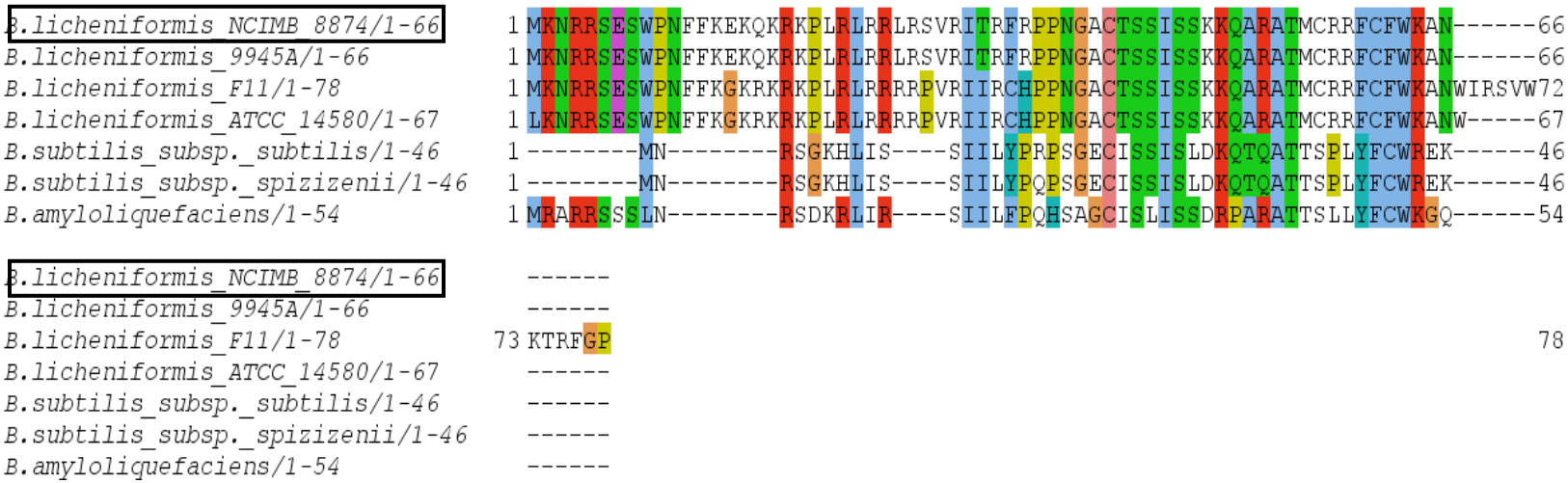


Figure 3.37: Sequence alignment of *B. licheniformis* NCIMB 8874 ComS with homologues from other *Bacillus* species. The alignment was performed with ClustalW and the aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows.

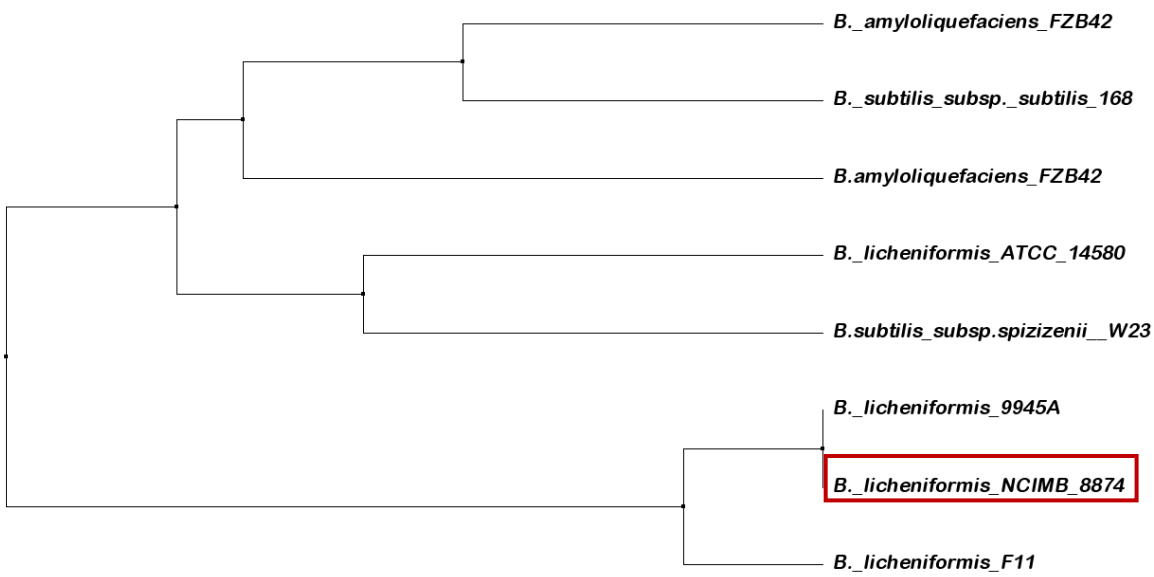


Figure 3.38: ComS phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. *B. licheniformis* NCIMB 8874 is denoted with a red box.

B. licheniformis NCIMB 8874 ComS showed 100% identity with its counterpart from strain 9945A, whilst 89% and 88% identity was revealed in the alignment with homologues from strains F11 and ATCC 14580 (Table 3.12). Intriguingly, identity of 97% emerged from the alignment between ComS sequences of *B. licheniformis* NCIMB 8874 and *B. subtilis subsp. spizizenii*.

Figure 3.38 shows that ComS phylogenetic tree can be divided in two main groups, with one of the clusters comprising all the *B. licheniformis* strains, thus implying that ComS is conserved in this species. *Bacillus subtilis subsp. subtilis* 168 ComS was found to be the most divergent, with only 39% identity with its counterparts from *B. licheniformis* strains NCIMB 8874 and 9945A and was therefore clustered in a different phylogenetic group (Figure 3.38).

3.6.3.2.3 The negative regulator of competence MecA

The adaptor protein MecA, engaged in the negative regulation of ComK during the development of competence in *B. subtilis*, was recently discovered and annotated in *B. licheniformis* strains 9945A and F11. To determine whether this protein might be produced *B. licheniformis* NCIMB 8874, the gene encoding MecA (*mecA*) was identified in the genome of the bacterium by performing nucleotide alignment with the coding sequence of strain 9945A. A nucleotide sequence sharing 100% identity with *mecA* of *B. licheniformis* 9945A was identified. The position of *mecA* on the genome is not strictly conserved among Bacilli as determined by research into NCBI database. MecA nucleotide sequence was translated in the corresponding 212 amino acid-long protein and aligned with annotated homologues for phylogenetic analysis. Table 3.13 includes the results of the alignment between MecA of *B. licheniformis* NCIMB 8874 and its counterparts from other Bacillus species. The amino acid sequence alignment is shown in Figure 3.39, whereas Figure 3.40 depicts the phylogenetic tree derived from the alignment.

Table 3.13: *Percentage identities obtained from the alignment of MecA amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
GQ505082.1	*Bacillus licheniformis* 9945A	212	100
GQ505080	*Bacillus licheniformis* F11	212	98
Q65LB7	*Bacillus licheniformis* ATCC 14580	212	98
E5W4U9	*Bacillus sp.* BT1B_CT2	212	98
E0U152	*Bacillus subtilis subsp spizizenii* W23	218	88
A7Z3E0	*Bacillus amyloliquefaciens*	249	82
NC_00094	*Bacillus subtilis subsp subtilis 168*	218	82

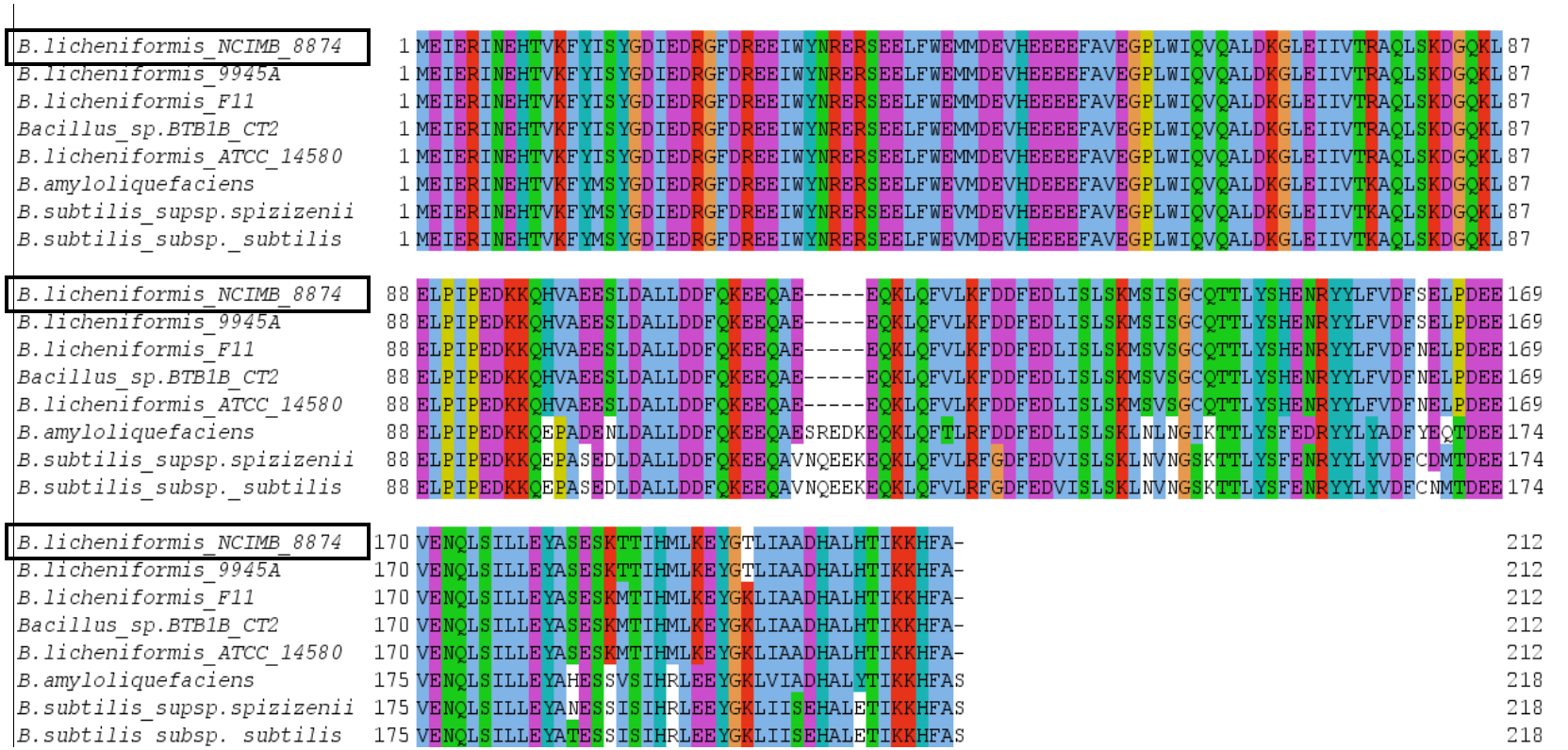


Figure 3.39: Sequence alignment of *B. licheniformis* NCIMB 8874 MecA with homologues from other *Bacillus* species. The alignment was performed with ClustalW and the aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows. MecA sequence of *B. licheniformis* NCIMB 8874 is indicated by a black box.

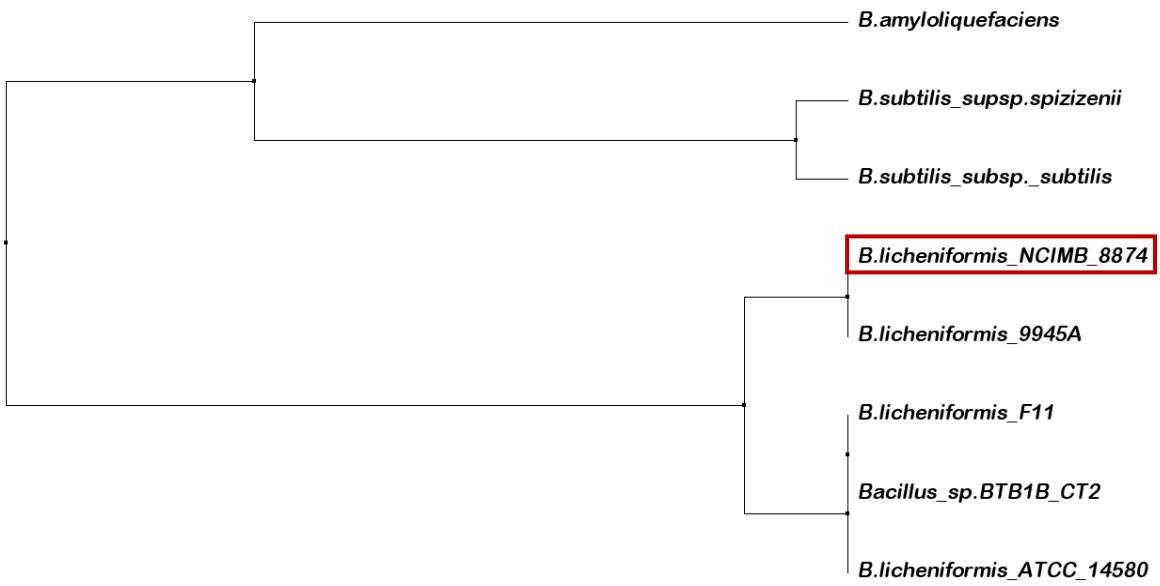


Figure 3.40: MecA phylogenetic tree generated using Jalview software. The tree was based on protein alignments with Clustal W. *B. licheniformis* NCIMB 8874 is denoted with a red box.

MecA sequences of *B. licheniformis* strains NCIMB 8874 and 9945A were found to be 100% identical at the amino acid level, whilst 98% identity was detected with homologues from strains F11 and ATCC 14580 (Table 3.13), thus suggesting that this protein is conserved within the species. The conservation was found to be extended to *Bacillus sp.* BT1B_CT2, as shown in Figure 3.40.

MecA phylogenetic tree (Figure 3.40) revealed that homologues of this protein can be classified in two groups, although results from alignment analysis listed in Table 3.13 suggest that members of the two clusters are not highly divergent.

3.6.3.3 The Rap-Phr system

Lapidus and co-workers (2002) reported that the genes coding for the Competence and Sporulation factor (CSF), *phrC*, and the co-transcribed RapC phosphatase were not identified on the genome of *B. licheniformis* ATCC 14580.

RapC belongs to the family of Rap phosphatases, comprising 11 members which have been identified in *B. subtilis*. Rap phosphatases are regulated by pentapeptides, whose precursors are coded by *phr* genes.

In order to determine whether *B. licheniformis* NCIMB 8874 possesses *rapC* and *phrC* and which members of this superfamily might be produced by this bacterium, its whole genome sequence was analysed by alignment with *phr* and *rap* genes from other Bacilli annotated in the NCBI database.

Confirming results obtained with *B. licheniformis* ATCC 14580, neither *phrC* nor *rapC* could be identified in the genome of strain NCIMB 8874. However, the analysis led to the identification of *rapA* and *rapB* genes, coding for RapA and RapB phosphatases. However, no *phr* gene could be detected downstream of these genes, or in any other region of *B. licheniformis* NCIMB 8874 genome.

3.6.3.3.1 RapG and PhrG

Further research resulted in the identification of *rapG* and *phrG* on the genome sequence of *B. licheniformis* NCIMB 8874. The nucleotide sequences of both genes were translated into amino acids and aligned with homologues found in the NCBI database. Results from RapG alignment are presented in Table 3.14 and Figure 3.41.

Table 3.14: *Percentage identities obtained from the alignment of RapG amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
Q65LL9	*Bacillus licheniformis* ATCC 14580	366	90
E5W560	*Bacillus sp*. BT1B_CT2	366	90
E0TZA4	*Bacillus subtilis subsp. spizizenii* W23	365	52
NP_391910	*Bacillus subtilis subsp. subtilis* 168	365	52

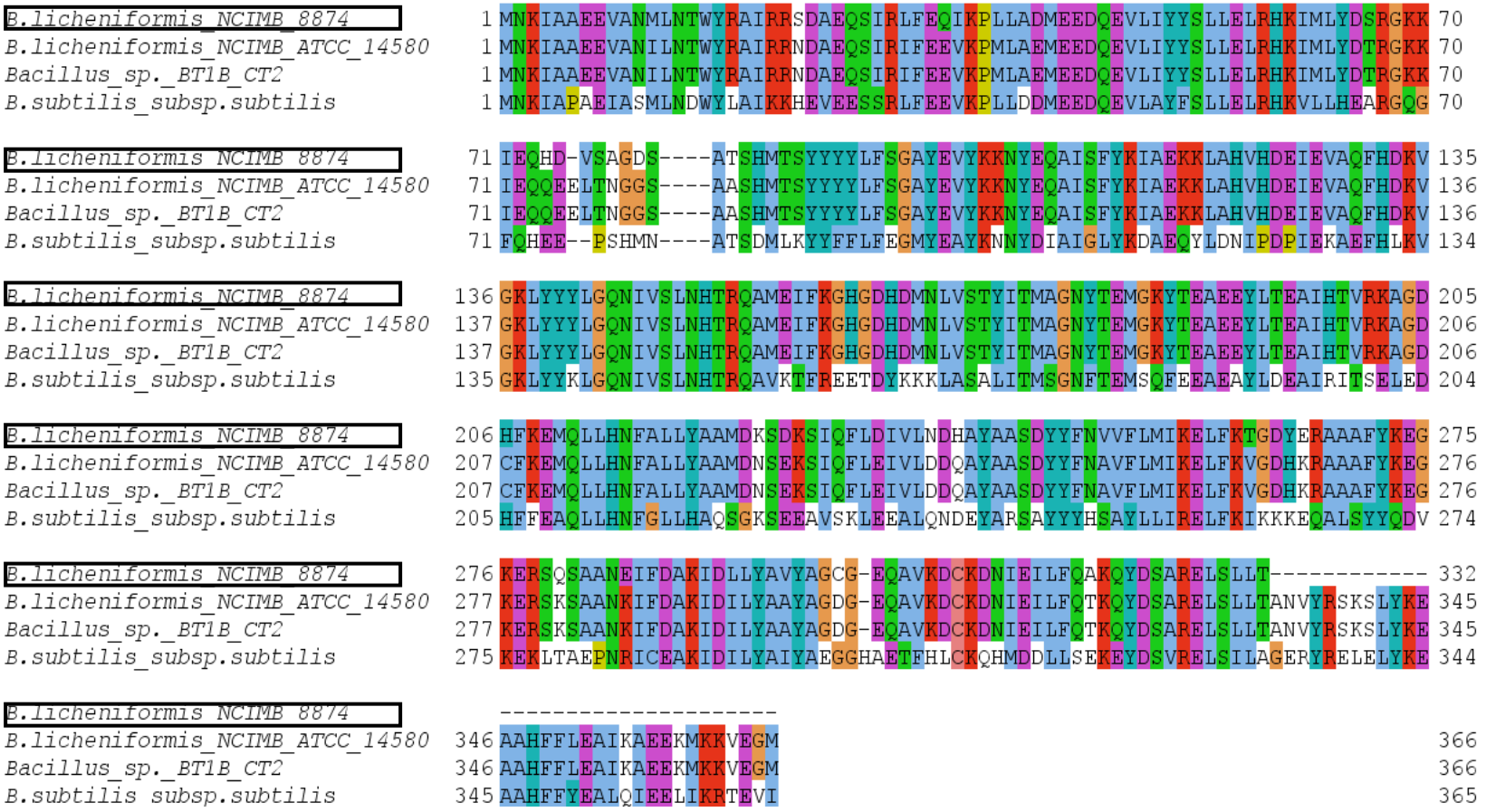


Figure 3.41: Sequence alignment of *B. licheniformis* NCIMB 8874 RapG with homologues from other *Bacillus* species**.** The alignment was performed with ClustalW and the aminoacids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows. RapG of B. licheniformis NCIMB 8874 is indicated with a black box.

RapG appears to be conserved in *B. licheniformis*, as homologues from strains NCIMB 8874 and ATCC 14580 share 90% identity. However, this conservation does not extend to *B. subtilis*, as only 52% identity was detected between RapG sequence of *B. licheniformis* NCIMB 8874 and both the *B. subtilis* strains analysed (Table 3.14).

PhrG was identified in the same Bacillus species where *rapG* was previously detected, with the exception of *Bacillus sp.* BT1B_CT2. The PhrG amino acid sequences were aligned to their *B. licheniformis* NCIMB 8874 counterpart. Results of the alignment are shown in Table 3.15 and Figure 3.42.

Table 3.15: *Percentage identities obtained from the alignment of PhrG amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
Q65MM8	*Bacillus licheniformis* ATCC 14580	38	92
E0TZA4	*Bacillus subtilis subsp. spizizenii* W23	38	53
NP_389772.1	*Bacillus subtilis subsp. subtilis* 168	38	50

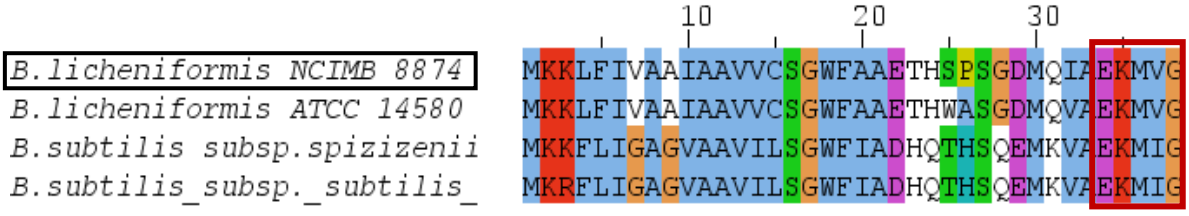


Figure 3.42: ClustaW alignment of PhrG homologues from different Bacilli. Amino acids were coloured using JalView software. Conserved amino acids are indicated with the same colours in all rows. PhrG amino acid sequence of *B. licheniformis* NCIMB 8874 is indicated by a black box. The red box denotes the putative pentapeptides generated after maturation.

PhrG congruence appears to follow similar pattern of RapG, with 92% identity between homologues from *B. licheniformis* strains. The conservation does not extend to members of *B. subtilis* species, as highest 53% identity was detected between PhrG of strain 168 and its *B. licheniformis* NCIMB 8874 homologue.

The C-terminus of the protein, which putatively gives rise to signalling pentapeptides is almost identical in both the *Bacillus* species (Figure 3.42). Interestingly, the pentapeptides of both *B. licheniformis* strains are characterised by a valine residue in position 4, whereas in their counterparts from both *B. subtilis* strains this amino acid is substituted with an isoleucine residue.

3.6.3.3.2 RapK and PhrK

The presence of another member of the Rap family, *rapK*, was established on the genome sequence of *B. licheniformis* NCIMB 8874. A gene coding for a putative signalling peptide precursor, *phrK*, was found immediately downstream of *rapG*. The nucleotide sequences of both genes were translated into amino acids and aligned with homologues found in the NCBI database. Results from RapK alignment are presented in Table 3.15 and Figure 3.43. Figure 3.44 illustrates the outcome of comparative analysis performed on PhrK homologues.

Table 3.16: *Percentage identities obtained from the alignment of RapK amino acid sequence of B. licheniformis NCIMB 8874 with homologues from other Bacilli.*

Accession	Organism	Length	% Identity
Q65MM8	*Bacillus licheniformis* ATCC 14580	370	96
E5W5Z1	*Bacillus sp.* BT1B_CT2	370	96
NP_389772.1	*Bacillus subtilis subsp. subtilis* 168	371	46

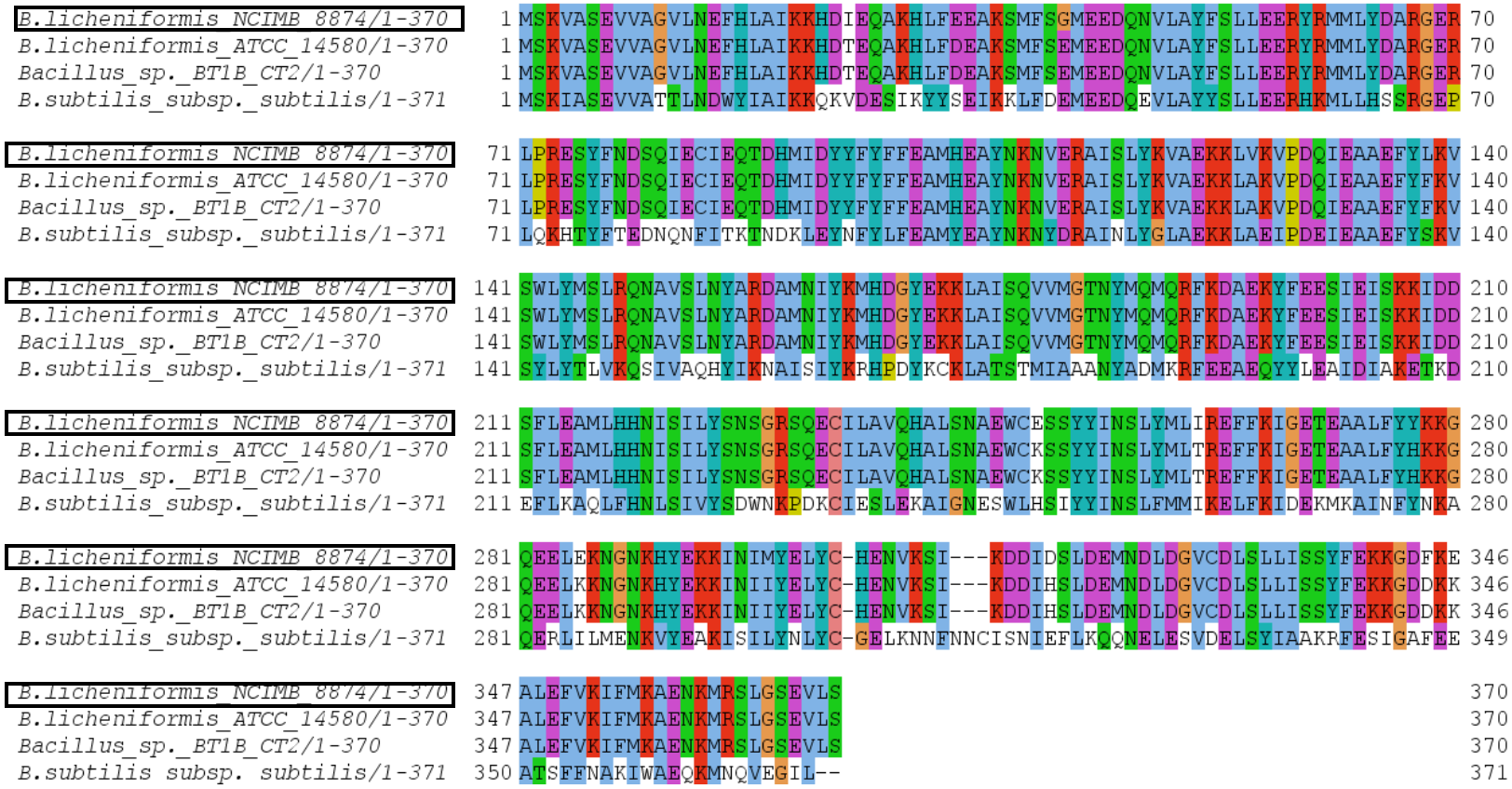


Figure 3.43: ClustaW alignment of RapK homologues from different Bacilli. Amino acids were coloured using JalView software to indicate conserved residues. RapK amino acid sequence of *B. licheniformis* NCIMB 8874 is indicated by a black box. The red box denotes the putative pentapeptides generated after maturation.

RapK sequences of *B. licheniformis* strains NCIMB 8874 and ATCC 14580, and Bacillus sp. BT1B_CT2 were found to be 96% identical at the amino acid level, whilst only 46% identity was detected with their *B. subtilis* homologue.

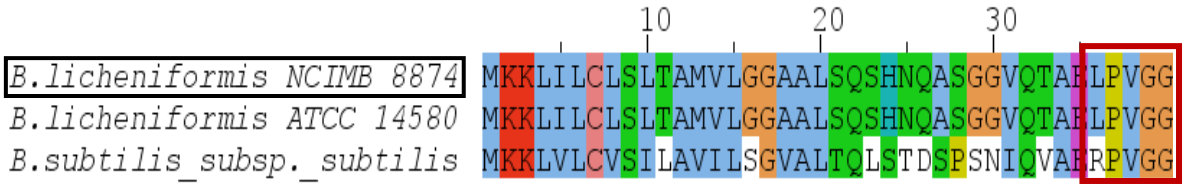


Figure 3.44: ClustaW alignment of PhrK homologues from different Bacilli**.** Amino acids were coloured using JalView software to indicate conserved residues. PhrK amino acid sequence of *B. licheniformis* NCIMB 8874 is indicated by a black box. The red box denotes the putative pentapeptides generated after maturation.

Rap phosphatases and their associated Phr proteins appear to evolve together. PhrK sequences of *B. licheniformis* strains NCIMB 8874 and ATCC 14580 were found to be 100% identical at the amino acid level, whilst only 50% identity was detected with their *B. subtilis* homologue, similar to the results shown for RapG alignment. The polymorphism of PhrK reaches the putative signals generated by the two species, as the leucine residue at position 1 of the *B. licheniformis* pentapeptide is substituted with an asparagine in *B. subtilis* (Figure 3.44).

Chapter IV

Discussion

DISCUSSION

4.1 Quorum sensing in *B. licheniformis*

Competence development in *B. licheniformis* cells has been extensively studied in the past and the transformation system of *B. licheniformis* has been shown to be similar to that of *B. subtilis* in numerous molecular aspects. In another line of research, the quorum sensing mediated development of competence has been widely investigated in *B. subtilis*. Although a *comQXPA* cluster and other genes involved in competence development, such as *comS*, *comK* and *mecA*, have been identified in the genome sequence of *B. subtilis* and different *B. licheniformis* strains, the production of peptide signalling molecules in *B. licheniformis has* not been investigated so far.

Taking into account the above background and given the importance of *B. licheniformis* as an industrial workhorse, this work was aimed to establish production of putative signalling molecule/s in *B. licheniformis.* To this end *B. licheniformis* strain NCIMB 8874 was chosen as a natural isolate, bacitracin producer, with potential industrial applications. Investigation was carried out on the production of signalling molecules able to generate a quorum sensing response in *B. subtilis* reporter strains. Production of secondary metabolites with industrial potential, such as the biosurfactant lichenysin, the biopolymer γ-PGA and extracellular proteases, whose regulation was proven to be controlled in a cell density-dependent fashion, was also established. Finally, following the acquisition of *B. licheniformis* NCIMB 8874 genome sequence the quorum sensing regulating *comQXPA* gene cluster and other genes involved in cell-cell communication were identified and analysed in comparison with other Bacilli.

4.1.1 Effect of *B. licheniformis* supernatant on *B. subtilis* reporter strains

Initially, due to the unavailability of *B. licheniformis* NCIMB 8874 genome sequence and the absence of previous reports on quorum sensing in this organism, this study was based on the use of *B. subtilis* reporter strains carrying a *PsrfA-lacZ* fusion in their α–amylase coding gene. Shaken flask fermentations for investigation of the production of potential peptide signalling molecules in *B. licheniformis* were performed by growing the cells in competence medium containing glucose as the only carbon source. This medium was originally developed by Vasantha and Freese (1980) to study the turnover in enzyme production at the onset of sporulation in *B. subtilis* and since then it has also been used to investigate the development of competence in the same organism, as its composition has been proven to induce the natural process of DNA uptake (Dubnau *et al.*, 1994). Moreover, the use of glucose in the medium as the only carbon source represents an additional advantage for the study, as this medium does not comprise other substrates that could induce β-galactosidase production.

B. subtilis reporter strain JRL293 was grown in the presence of cell-free supernatants taken from *B. licheniformis* cultures at late exponential phase, when the production of potential signalling molecules should reach its threshold. A significant increase in β-galactosidase activity, 8-fold (Figures 3.5), was measured in the test cultures of *B. subtilis* JRL293 supplemented with 50% (v/v) as compared to control cultures. Furthermore, the addition of *B. licheniformis* supernatant resulted in the growth impairment of *B. subtilis* JRL293 (Figures 3.4), thus suggesting the presence of compound/s (i.e. antimicrobials) accumulating in the extracellular milieu of *B. licheniformis* cultures, toxic to *B. subtilis*. This toxic effect might have been exacerbated by the dilution of the nutrient content of the medium (50% was replaced with spent medium) and the low cell density of the reporter strain at the beginning of the experiment.

Despite the impaired cell growth, *B. subtilis* JRL293 cultures supplemented with *B. licheniformis* supernatant showed significant increase in β-galactosidase activity.

This may be attributed to a quorum sensing response of the cells to the addition of *B. licheniformis* spent medium. Moreover, enhancement of β-galactosidase activity (2.25-fold) was detected again when *B. subtilis* JRL293 cultures were supplemented with the lyophilised supernatants of *B. licheniformis*. The amount of lyophilised supernatant to be added was calculated so that its composition would correspond to 50% (v/v) fresh spent medium. However, the details of the effect of the lyophilisation on the composition of the spent medium are not known.

However, since *B. subtilis* JRL293 cultures showed a decrease in their growth rate, it may be speculated that either the higher nutrient content counteracted the toxic effect of the added supernatant or the lyophilisation process diminished the effect of the factor that inhibited the growth of *B. subtilis*.

Further studies on the production of potential signalling molecules by *B. licheniformis* were carried out using *B. subtilis* LS27, a *comX* knock-out, as a reporter strain. Two, and potentially more, signalling molecule are engaged in the regulation of cell-density-dependent phenomena in *B. subtilis*: the ComX pheromone and the competence and sporulation factor (CSF). This implies that the inducing effect of *B. licheniformis* spent medium on *B. subtilis* JRL293 might be due to one or more molecules. However, the gene coding for CSF, *phrC*, could not be identified in the genome sequence of *B. licheniformis* ATCC 14580, suggesting that the *B. licheniformis* strain is not a CSF-producer. Therefore, the ComX pheromone was suggested as the cause of induction of *srfA* expression in *B. subtilis* JRL293. An essential criterium for the identification of a quorum sensing molecule is its ability to restore a quorum sensing response in a null mutant (Bainton *et al.*, 1992c, Winzer *et al.*, 2002b). So, to establish whether ComX was the molecule responsible for the quorum sensing response in *B. subtilis* cells, *B. licheniformis* supernatant was tested against the *comX* null mutant strain, *B. subtilis* LS27.

B. subtilis LS27 test cultures grown in the presence of either fresh or lyophilised spent medium collected from *B. licheniformis* cultures at high cell density showed a significant increase, 5.1- and 5.9- fold (Figures 3.9 and 3.11), respectively, in their β-galactosidase activity. These results are comparable with the previously shown

effect for *B. subtilis* JRL293, although the activity of the enzyme in strain LS27 was significantly lower. This difference in the activity depends on the fusion harboured by the reporter strain, as the exact position of the junction between the end of *PsrfA* and *lacZ,* and the nature of the ribosome binding site greatly affect the absolute activity of the strain (Prof. Grossman, personal communication). Although the *B. subtilis* LS27 does not produce ComX a basal level of β-galactosidase activity which might be attributed by the presence of CSF in the extracellular milieu, could still be detected throughout the growth of control cultures (Figures 3.9 and 3.11). Addition of *B. licheniformis* supernatant also effected a delay in the growth of *B. subtilis* LS27, although the culture was able to return to a normal growth rate after 4 hours post-addition of the exogenous spent medium. It can be suggested that this growth delay might have been due to the same factor causing growth inhibition of *B. subtilis* JRL293 culture. In this case, however, the higher initial cell density allowed a subpopulation of cells to sense and respond to the exogenous ComX addition, restoring cell-cell communication, thus reinstating a normal growth rate. Results obtained from both *B. subtilis* reporter strains confirmed that a signalling molecule, presumably the ComX pheromone, is produced by *B. licheniformis* cultures in competence-inducing medium.

4.1.2 ComX production pattern in *B. licheniformis*

The “pheromone assay” originally developed by Magnuson and co-workers (1994) for identification of the ComX during the purification process, was adapted to the present studies to monitor the pattern of production of putative ComX pheromone in *B. licheniformis* cultures using both *B. subtilis* JRL293 and LS27.

The profiles of β-galactosidase activity generated by *B. licheniformis* supernatants collected throughout the cell growth using either of the reporter strains are very similar and both reproduce *B. licheniformis* growth curve, thus indicating that the signalling molecule is produced in a cell-density dependent fashion (Figures 3.12 and

3.13). Moreover, comparison with β-galactosidase activity generated by supernatants from the ComX-producer wild type *B. subtilis* revealed that the two bacteria share the same pattern of pheromone production and are able to induce *srfA* expression at the same level (Figures 3.12 and 3.13) in both the reporter strains. This agrees with the reports on ComX discovery in *B. subtilis*, where investigation on *srfA* expression using a *srfA-lacZ* fusion determined that β-galactosidase activity increased as cells grew to high density, before the onset of stationary phase (Magnuson *et al.*, 1994).

Quorum sensing mediated communication in *B. subtilis* is species-specific and induction of the signalling cascade that leads to the activation of *srfA* expression by signalling molecules other than ComX and CSF has not been reported so far. In the case of the ComX pheromone this process involves signal recognition by the histidine kinase ComP, whose extracellular sensor domain specifically interacts with its cognate ComX (Ansaldi *et al.*, 2002). ComX activity depends on the isoprenylation of the conserved tryptophan residue, as synthetic analogues lacking the modification have been shown no pheromone activity (Magnuson *et al.*, 1994). So far, ComX pheromones isolated from different Bacilli have been classified in four pherotype groups which diverge in the sequence of the mature peptides and their modifications (Okada *et al.*, 2005, Okada *et al.*, 2004).

4.2 ComX pheromone purification

Attempts at isolation of ComX from cultures of *B. licheniformis* and *B. subtilis* wild type at high cell densities yielded 60% CH_3CN elution fractions which were unable to induce a quorum sensing response in *B. subtilis* reporter strain LS27, despite previous evidence indicating that supernatants from these cultures have pheromone activity. As the same results were obtained with both *B. licheniformis*, whose ComX production is yet to be demonstrated, and the established pheromone-producer *B. subtilis*, it can be suggested that the concentration of ComX present in cultures of Bacilli which are not genetically manipulated is not sufficient for the isolation process in the 1L fermentation used in this study. The first ComX pheromone was isolated

from high cell density cultures of *B. subtilis* ROM183, a sporulation deficient strain harbouring a double mutation in the sporulation-regulating gene *spoOA* and in *abrB*¸ encoding a DNA-binding protein required for the expression of late competence genes (Albano *et al.*, 1987, Perego *et al.*, 1988). This double mutation results in a ~1.7-fold increase in ComX production with respect to the wild type (Solomon *et al.*, 1995b). Since the discovery of ComX, the competence pheromone has been isolated from many *Bacillus* species by cloning *comQ* and *comX* genes of selected strains into *E. coli* cells, under the control of IPTG- inducible promoters (Tortosa *et al.*, 2001, Ansaldi *et al.*, 2002, Bacon Schneider *et al.*, 2002). This procedure could not be used in the present study as until the very late stage of the project the genome sequence of *B. licheniformis* NCIMB 8874 was unavailable. Higher amount of the starting material (i.e. cell free supernatant from the strains under examination) would have probably led to successful isolation of the pheromone. However, this would have involved the use of large pilot-scale fermentations and several steps of downstream processing.

4.3 Production of *comQXPA* regulated secondary metabolites in *B. licheniformis*

In *B. subtilis* the *comQXPA* gene cluster has been involved in the regulation of a number of secondary metabolites at the onset of stationary phase, concomitantly with the control of competence development. Examples of these metabolites are the antimicrobial biosurfactant surfactin, the capsule constituent biopolymer γ-poly glutamic acid and extracellular degradative enzymes. Biosynthesis of surfactin is directly under the control of the ComPA two-component signal transduction system with ComA activating transcription of the *srfA* operon. In *B. licheniformis*, the biosurfactant lichenysin has been suggested to be regulated in a similar fashion. γ-PGA and exoproteases production, on the other hand, are indirectly regulated by ComPA through a second two-component system composed of DegS and DegU (Msadek *et al.*, 1991). In *B. subtilis natto* NAF5, the insertion of an IS element in the *comP* coding sequence was established to be detrimental for the production of both

γ-PGA and extracellular proteases (Nagai *et al.*, 2000b). A similar sequence interrupts the *comP* gene sequence of *B. licheniformis* ATCC 14580, the only *B. licheniformis* strain whose genome has been entirely annotated so far.

In this study, due to the lack of genetic information on *B. licheniformis* NCIMB 8874 until the later stage, lichenysin and γ-PGA production as well as exoproteolytic activity were investigated in order to determine the functionality of the *comQXPA* locus.

4.3.1 Lichenysin

The lichenysin biosynthetic operon, comprising three large ORFs encoding peptide synthetases and designated *licA*, *licB* and *licC*, followed by a gene, *licTE*, coding for a thioesterase-like protein, has been identified in *B. licheniformis* NCIMB 8874 (ATCC 10716). The lichenysin biosynthetic genes are characterised by a genetic organisation resembling the *srfA* surfactin operon of *B. subtilis* and are predicted to generate a lichenysin variant with the primary amino acid sequence: L-Gln–L-Leu–D-Leu–L-Val–L-Asp–D-Leu–L-Ile (Konz *et al.*, 1999). However, isolation of this lipopeptide from *B. licheniformis* NCIMB 8874 has not been reported. Therefore, 1 L shaken flask fermentations of *B. licheniformis* NCIMB 8874 and *B. subtilis* wild type (used as a control) were set up in both complex and synthetic media for production of biosurfactants. As shown in Tables 3.1 and 3.2 lichenysin and surfactin were successfully isolated and quantified, using a surfactin standard as a reference. Regardless of the medium used for the production, total surfactin concentration (calculated as the sum of different isoforms) was always found to be approximately 10 times higher than lichenysin. These results are in accord with previous reports showing that lichenysin is produced in much lower amounts (at least one order of magnitude) than surfactin (Yakimov *et al.*, 1996). However, this difference appeared to be slightly reduced when comparing lichenysin and surfactin yield (based on CDW) in the two organisms, thus revealing that the yields of *B. subtilis* biosurfactant were 9- and 7-fold higher with respect to its counterpart of *B. licheniformis* in complex

and competence medium, respectively. Results presented in Figures 3.16 and 3.17 also highlighted that the quantity and the isoform composition of the biosurfactant produced are significantly affected by the nutrients present in the media. Nutrient rich medium resulted in increased biosurfactant production, as well as a higher number of isoforms, compared to the synthetic medium. Both *B. licheniformis* and *B. subtilis* cultures produced five isoforms of biosurfactant in complex medium, whereas only three were detected in supernatants from cultures grown in synthetic medium. The effect of media composition on biosurfactant production has been established to be influenced by a number of factors, including the nature and concentration of the carbon and nitrogen sources, the amount of phosphorus, magnesium, iron, and manganese ions in the medium as well as other culture conditions, such as pH, temperature and agitation (Karanth *et al.*, 2000). A direct correlation has been established between biosurfactant yields and sugar concentrations, whereas the number of biosurfactant isoforms produced appears to be related to the nature of the nitrogen source used (Grangemard *et al.*, 2001, Rodrigues *et al.*, 2006). The nitrogen sources used in the complex medium were yeast extract and bactopeptone whilst glutamic acid was added to the synthetic medium to provide nitrogen; therefore a change in number of isoforms produced is not surprising.

Although in this study the number of isoforms produced was determined to be the same for the two biosurfactants, the isoforms were identified by using commercially available surfactin as a standard, thus resulting only in the identification of isoforms which are common between the two species. Other peaks were in fact identified on lichenysin chromatogram (data not shown) which might be specific for this biosurfactant.

4.3.1.1 Lichenysin antimicrobial activity

Antimicrobial activity of biosurfactants, including lichenysin, has been correlated to their amphipathic nature which resembles the phospholipids forming the cell membrane (Grangemard *et al.*, 1999a). Insertion of biosurfactant lipid moiety into the cell membrane causes significant structural changes together with alteration of

membrane permeability, thus leading to the disruption of the cell membrane and consequent loss of the internal cytoplasmic contents (Carrillo *et al.*, 2003).

Lichenysin extract from both complex and competence medium was tested against different bacteria in order to determine the antimicrobial activity of the biosurfactant against both Gram negative and Gram positive bacteria, and especially against *B. subtilis*, whose growth was found to be impaired when exposed to supernatants from *B. licheniformis*. Of all the strain tested, only *Chromobacterium violaceum* was not inhibited by any of the used concentrations of lichenysin extract from either complex or synthetic medium. In the case of *C. violaceum*, it is possible to infer that either the Minimum Inhibitory Concentration (MIC) for this bacterium is higher than 4 mg mL^{-1} or the microorganism is not affected by lichenysin. On the other hand, inhibition by lichenysin was observed in *Escherichia coli* culture under all the conditions tested confirming previous reports (Yakimov *et al.*, 1995). The results for *Pseudomonas aeruginosa* are ambiguous as the growth of this bacterium appears to be inhibited by lichenysin extract obtained from synthetic medium but not by the one extracted from complex medium. It may be speculated that the inhibition observed with the ichenysin extracted from cells grown in the competence medium is due to the unidentified lichenysin isoforms (data not shown). Opposite results were obtained against Gram positive *Bacillus* species, where a 1 mg mL^{-1} of lichenysin extract from culture grown in complex medium was lethal for the tested organism, whereas no effect was detected using biosurfactant extracted from the synthetic medium. These differences in antimicrobial activities between lichenysin extracts obtained from cultures grown in two different media might be attributed to the different amount of active compound present in the extract.

Due to the strain specificity of biosurfactant production and the variability of their composition depending upon environmental conditions, the studies on lichenysin antimicrobial activity were inconclusive. However, the majority of studies show that lichenysin has a higher antimicrobial potential against Gram positive rather than Gram negative bacteria (Fernandes *et al.*, 2007, Quadros *et al.*, 2011, Yakimov *et al.*, 1995). Lichenysin antimicrobial activity also appears to be especially directed

towards other *Bacillus* species (Yakimov *et al.*, 1995, Quadros *et al.*, 2011). These findings could not be confirmed in the present study, as the number of bacteria tested was not sufficient to draw definite conclusions. However, this study succeeded in establishing *B. licheniformis* NCIMB 8874 as a lichenysin producer and the antimicrobial activity of this biosurfactant was confirmed against other Bacilli. As biosurfactant production in Bacilli is under the control of quorum sensing and as some species have evolved to recognise each other's signals (same pherotype) it could be speculated that biosurfactant biosynthesis in these bacteria has developed as a defence mechanism against evolutionary closely related microorganisms. However, this study concluded that lichenysin is not the agent responsible for the growth inhibition in liquid cultures of *B. subtilis* reporter strains, as lichenysin extracted from cultures grown in the competence medium was ineffective against *B. subtilis*.

4.3.2 γ-PGA production and exoproteolytic activity

B. licheniformis ATCC 14580 genome shows a transposon insertion, named as *IS3Bli*, into the gene sequence coding for the histidine sensor kinase ComP. In *B. subtilis natto* NAF4, a similar element frequently integrates into *comP* (Nagai *et al.*, 2000b, Tran *et al.*, 2000), thus causing a decrease in natural competence and extracellular protease secretion as well as the complete loss in γ-PGA formation (Nagai *et al.*, 2000b) (Takahashi *et al.*, 2007, Weinrauch *et al.*, 1990). Recent studies have shown that *B. licheniformis* ATCC 14580 is characterised by a similar phenotype (Hoffmann *et al.*, 2010).

In order to determine whether *B. licheniformis* NCIMB 8874 possesses a functional competence sensor kinase, the production of γ-PGA in this organism was investigated. The results (Figure 3.20) confirmed production of γ-PGA in *B. licheniformis* NCIMB 8874 cultures in PGA production medium for 45 hours. The production of γ-PGA in *B. licheniformis* NCIMB 8874 continued over the initial 20

hours of growth, reaching highest concentration (19 g L^{-1}) at 25 hours and then steadily decreased until the end of fermentation. These results are in accordance with previous studies on γ-PGA production in *B. subtilis* using the same medium composition (Goto and Kunioka, 1992). However, NMR spectroscopy could not identify γ-PGA unambiguously (Figure 3.22) as the purity of the biopolymer was not at the standard required for the analysis. Moreover, the different positions of the peaks corresponding to each carbon residue on the spectrum from Birrer and co-workers (1994) and the one presented in this study might be due to the different methods used for the isolation of the biopolymer, resulting in a shift of the peaks. Even though the NMR spectrum did not confirm *B. licheniformis* NCIMB 8874 as a γ-PGA producer, collective evidence exist that this bacterium does produce the biopolymer, as confirmed by the extract shown in Figure 3.21. Also, *B. licheniformis* NCIMB 8874 colonies are characterised by a mucoid nature, which recent studies correlated it with biofilm formation and exopolymers production (Stanley and Lazzazzera. 2005). Moreover, γ-PGA production in *B. licheniformis* NCIMB 8874 was previously established by Fourier transform infrared spectroscopy (FTIR) (Dr Vydyanath, personal communication).

Exoproteolytic activity present in the supernatants of *B. licheniformis* NCIMB 8874 was investigated to attain a complete picture of quorum sensing dependent secondary metabolism in this organism. Exoproteases production in *B. licheniformis* NCIMB 8874 was found to be analogous to the results obtained with other *B. licheniformis* strains carrying a functional *comP* gene on their genome sequence (Hoffmann *et al.*, 2010). *B. licheniformis* NCIMB 8874 was proven to produce γ-PGA and showed extracellular proteolytic activity expected from a strain possessing a functional *comQXPA* quorum sensing system.

4.4 Effect of exogenous addition of spent medium on secondary metabolites production

Secondary metabolites production in microbes includes antibiotics, pigments, toxins, pheromones, biosurfactants, receptor antagonists and agonists, and antitumor agents. Their regulation is influenced by specific low molecular mass compounds, whose biosynthetic genes are often clustered on the chromosomal DNA, produced in the late exponential stage of cell growth at the onset of stationary phase (Demain, 1998). The products of secondary metabolism often carry industrial importance; therefore it is not surprising that extensive research has been dedicated to the investigation of factors, such as nutrients, growth rate, and enzyme inhibition or induction, which might increase the product yields. Expression of many genes controlling secondary metabolites production is regulated by quorum sensing mechanism. This process has been studied in a number of bacteria and more recently in fungi, thus providing potential industrial applications for signalling molecules. Examples of these processes are the acyl homoserine lactones-induced productions of the antibiotic carbapenem in *Erwinia carotovora* (Bainton *et al.*, 1992c, Welch *et al.*, 2005) and the violet colour pigment in *Chromobacterium violaceum* (McClean *et al.*, 1997), the self-induced production of bacteriocins in *Lactobacillus acidophilus* (Barefoot *et al.*, 1994) and *Lactobacillus plantarum* (Diep *et al.*, 1995). More recently, oxylipins have been reported to act as signaling molecule in the filamentous fungus *Aspergillus terreus* for the regulation of lovastatin production. Moreover, exogenous addition of linoleic acid (oxylipins precursors) to *A. terreus* cultures was found to enhance lovastatin production as well as the level of transcription of lovastatin biosynthetic genes (Sorrentino *et al.*, 2010).

In this study the effect of signalling molecule-containing spent medium from *B. licheniformis* and *B. subtilis* wild type cultures on secondary metabolites, i.e. lichenysin, γ-PGA and exoproteases production in *B. licheniformis* NCIMB 8874 was investigated to determine whether any enhancement could be detected. Figure 3.18 shows that addition of 1.25% lyophilised supernatant from either *B. licheniformis* or *B. subtilis* cultures to *B. licheniformis* test cultures had no significant effect on lichenysin production as compared to test cultures. The addition of signalling molecule-containing supernatants should result in induction of lichenysin production

in early stages of growth in a way that resembles the effect observed on the expression of *sfrA-lacZ* fusion. However, detection of lichenysin in cultures at such low cell densities will necessitate a sensitive assay, such as the construction of a fusion comprising the promoter of the lichenysin operon connected to a reporter gene. In this study a similar construct could not be designed due to the lack of genetic information and lichenysin production had to be analysed from the extracts obtained from the stationary phase cultures of *B. licheniformis.*

Similar results were obtained when investigating the effect of lyophilised spent medium addition on the production of extracellular proteases (data not shown). However, this evidence cannot exclude possible ComX-driven induction of extracellular proteases production, as recent reports have revealed that the decrease in exoproteolytic activity generated by disruption of ComP coding gene could be reinstated to its standard levels when the defective ComP of *B. licheniformis* ATCC 14580 was replaced with its functional counterpart from strain F11 (Hoffmann *et al.*, 2010), thus suggesting that a direct correlation exists between ComX signalling and production of exoproteases. A more detailed investigation into the pattern of extracellular proteases production in *B. licheniformis* NCIMB 8874 will be necessary in order to prove this correlation.

Interestingly, addition of 1.25% lyophilised spent media from *B. licheniformis* and *B. subtilis* cultures to test cultures of *B. licheniformis* at low cell densities resulted in a transient increase in γ-PGA production after 25 hours growth (Figure 3.20). Surprisingly, this effect was accompanied by an increase in red pigmentation in both the test cultures (Figure 3.23). It is interesting to note that the addition of *B. subtilis* spent medium had a greater effect on both γ-PGA and red pigment production than *B. licheniformis*, thus not only confirming that the two species are capable to cross-induction, but that this phenomenon can be stronger than autoinduction. The spent media added to the test cultures were filtered through a 3kDa membrane, thus excluding all molecules of higher sizes, including potential γ-PGA produced by growing *B. licheniformis* and *B. subtilis* wild type cells in competence medium. The increase of γ-PGA production in test cultures of *B. licheniformis* does not appear to

be related to any unutilised nutrient remained in the spent medium, as the effect caused by *B. subtilis* and *B. licheniformis* was different, albeit not significantly, whereas the two bacteria were grown in the same medium. Also, the concomitant increase in red pigmentation appears to be a further indication of a quorum sensing response. This red pigment was detected previously in cultures of a different *B. licheniformis* strain, known as EI-34-6. Although the study could not identify the red pigment, it has been found that the production of this compound, which requires $FeCl_3$ and glycerol, is related to biofilm formation and is induced by small molecules accumulating in the extracellular milieu in a cell density dependent fashion (Yan *et al.*, 2003). Also, production of a similar red pigment, named as pulcherrimin, has been reported in *B. subtilis* (Uffen and Canale-Parola, 1972).

4.5 Genetic and bioinformatics analysis

4.5.1 The competence locus

Cell-cell communication in *Bacillus subtilis* is under the control of the *comQXPA* locus, whose products, the ComX pheromone and the two-component transduction system ComP and ComA, regulate the occurrence of natural competence in this organism (Weinrauch *et al.*, 1990, Dubnau *et al.*, 1994). The activation of the regulatory cascade that ultimately leads to the development of competence, and related secondary metabolites production, is driven by accumulation of the pheromone in the extracellular milieu (Magnuson *et al.*, 1994). In this picture, where each component plays an essential role for ensuring a correct communication, the key factors are undoubtedly ComX and ComP, the signal and the sensor of the system, respectively. Therefore, preliminary genetic analysis carried out in the present study focussed on the genes coding for the ComX pheromone precursor protein and the histidine kinase, and primers were designed for amplification of the corresponding coding sequences annotated in the genome of *B. licheniformis* ATCC

14580. However, none of the amplifications gave rise to the desired products, thus suggesting that genetic organisation of the *comQXPA* clusters of *B. licheniformis* strains ATCC 14580 and NCIMB 8874 might be different. Previous studies investigating *Bacillus* natural isolates have shown that the competence regulating locus is highly polymorphic, with the polymorphism stretching through *comQ*, *comX* and the region of *comP* encoding the N-terminal part of the protein, whilst the C-terminus of *comP* and *comA* were proved to be highly conserved (Tran *et al.*, 2000, Tortosa *et al.*, 2001). Bioinformatics analysis performed on the recently acquired genome sequence of *B. licheniformis* NCIMB 8874 confirmed this hypothesis, as the ComP coding regions of strains ATCC 14580 and NCIMB 8874 were found to share only 89% identity at the nucleotide level, whereas the identity for *comX* was 94%.

Bioinformatics analysis was extended to the localisation of the *comQXPA* cluster on *B. licheniformis* NCIMB 8874 genome (Figure 3.26), revealing a high degree of conservation within the group of Bacilli analysed, with the exception of *B. licheniformis* ATCC 14580, whose competence regulating gene cluster is interrupted by three unidentified coding sequences.

When the *comQXPA* gene cluster of *B. licheniformis* NCIMB 8874 was aligned with the recently annotated locus from strain 9945A, which has been shown generate a functional quorum sensing system (Hoffmann *et al.*, 2010), the two loci were found to be identical at the nucleotide level. The alignment also revealed that the *comP* gene sequence of *B. licheniformis* NCIMB 8874 is not interrupted by a transposon insertion, thus confirming thus confirming experimental evidence of a functional cell-cell communication mechanism.

Previous research has shown that the *Bacillus* pheromones can be classified in four pherotypes groups depending on their amino acid sequences and the nature of the modification (operated by ComQ) on their conserved tryptophan residues; only pheromones belonging to the same group are able to generate a cross-induction phenomenon (Ansaldi *et al.*, 2002). According to these findings, the polymorphism characterising the *comQXP* locus suggests that a given pheromone specifically

interacts with its receptor protein ComP and its processing protein ComQ, thus determining a specific pattern of activation of the cell density- dependent response (Tortosa *et al.*, 2001, Tran *et al.*, 2000).

As the present study showed that the ComX pheromone produced by *B. licheniformis* NCIMB 8874 was able to activated a quorum sensing response in *B. subtilis* reporters strains, comparative analysis was performed between the *comQXPA* loci of these bacteria, as well as other selected Bacilli, in order to investigate the relationship between the polymorphism of the competence regulating gene cluster and the specificity of activation of the quorum-sensing system.

To this end, each product of the *comQXP* locus was aligned with homologues from selected Bacilli and a phylogenetic tree was drawn for each protein, as illustrated in Figure 3.28 for ComQ, Figure 3.30 for ComX, and Figure 3.32 for ComP. Taken together, the phylogenetic relationships between these proteins appeared to confirm previous results, as each protein could be classified in four different groups. *B. licheniformis* strains were usually found in the same cluster, as well as a not identified species, namely *Bacillus sp*. BT1B_CT2. Interestingly, the ComX precursor proteins of *B. licheniformis* strains ATCC 14580, which has been proven to possess a non functional quorum sensing system, and F11, which has been shown to be unable to develop natural competence, were classified under a distinct group. ComA congruence, shown in Figure 3.34, indicated that, whilst this protein is conserved in bacteria belonging to the same species, the conservation does not extend to the genus, as two different clusters could be identified in the phylogenetic tree, corresponding to as many analysed species. *B. subtilis subsp. subtilis* and *B. licheniformis* NCIMB 8874 were found to be classified under different phylogenetic groups for all the products of the *comQXPA* cluster. *B. licheniformis* NCIMB 8874 and *B. subtilis subsp. subtilis* positions on pre-ComX evolutionary tree are not too distant, thus confirming the possibility of cross induction between the two species. The sequence alignment between the two precursor proteins, however, highlighted that their conservation is only restricted to the N-terminal regions, whereas high variability characterises the pheromone-forming C-terminus, where the conserved tryptophan

residue is located. Classification of a given pheromone under a particular pherotype is not possible merely on the basis of its amino acid sequence and, since little is known about the mechanism of the modification/maturation catalysed by ComQ, a prediction on the specific isoprenoid added by ComQ cannot be made. As experimental evidence suggests that the ComX pheromone of *B. licheniformis* NCIMB 8874 is able to induce a quorum sensing response in *B. subtilis* reporter strains derived from *B. subtilis subsp. subtilis*, it may be inferred that the two molecules are characterised by a similar modification.

4.5.2 The competence transcription factor ComK and its regulatory proteins ComS and MecA

Investigation of quorum sensing-related proteins was extended to ComK, the competence transcription factor, MecA, a modulator protein involved in ComK inhibition prior to quorum sensing induction, and ComS, a small protein responsible for ComK relief from inhibition when the quorum sensing response is initiated. The role of these proteins is essential in the regulatory cascade generated by ComX signalling; therefore the corroboration of their presence on *B. licheniformis* NCIMB 8874 genome was necessary to confirm that this organism possesses a functional cell-cell communication mechanism. Alignment of the coding sequences of these proteins with their counterparts from *B. licheniformis* 9945A resulted in 100% identity and their position in the genome was found to be conserved with respect to other Bacilli. In particular, the localisation of ComS coding sequence on the lichenysin biosynthetic operon, previously reported in *B. licheniformis* strains 9945A and F11, reproduces the same genetic organisation found in *B. subtilis*, with comS located on *srfA* operon, thus confirming previous reports (Hoffmann *et al.*, 2010). Moreover, ComK, MecA and ComS phylogenetic relationships revealed that, whilst these proteins are highly conserved within a particular species, they show variability throughout the genus. This indicates that each quorum sensing system, albeit generated from a common progenitor, has evolved in each species to ensure

specificity in the communication. As quorum sensing signalling is usually initiated at the onset of the stationary phase, when the environmental conditions become limiting for a cell population, it is not surprising that these systems have evolved independently to generate specific messages that cannot be decoded by their close neighbours and possible competitors.

4.5.3 The Rap and Phr families

Previous studies on *B. licheniformis* ATCC 14580 reported no coding sequence could be identified for PhrC, the precursor protein of the Competence and Sporulation factor (CSF), and the co-transcribed gene encoding RapC phosphatase (Lapidus *et al.*, 2002). PhrC belongs to a family of eight Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK), whereas RapC is one of the 11 members of the Rap phosphatases family (RapA to RapK) of *B. subtilis*.

Neither phrC nor rapC could be detected on B. licheniformis NCIMB 8874, thus confirming that this species does not produce the signalling molecule CSF. However, two other members of the Rap family were identified on B. licheniformis NCIMB 8874 genome, namely RapA and RapB. Whereas the gene coding for RapB is not reported to be co-transcribed with any *phr*, *rapA* is co-transcribed with *phrA* in other *Bacillus* species. However, bioinformatic search failed to identify a *phrA* gene downstream of *rapA B. licheniformis* NCIMB 8874 genome.

Indeed, no *phr* gene could be detected downstream of either *rapA* or *rapB* genes. The presence of RapB coding gene on the genome of an organism that does not produce CSF is quite surprising, as the PhrC peptide was proven to be responsible of the inhibition of the unpaired phosphatase RapB (Perego, 1997).

Further analysis led to the discovery of the *rapG-phrG* and *rapK-phrK* loci in *B. licheniformis* NCIMB 8874. PhrK was recently shown to regulate ComA activity via inhibition of its cognate phosphatase RapK in *B. subtilis* cells (Bongiorni *et al.*, 2005, Auchtung *et al.*, 2006). According to one of these studies (Auchtung *et al.*, 2006) PhrC, PhrF, and PhrK act jointly, although to a different extent, to stimulate the

response regulator ComA, whose regulation of target gene expression requires all three peptides. As regarding RapG and PhrG, their mechanism of action has not been fully understood, though RapG regulatory activity has been shown to be directed towards DegU (Ogura *et al.*, 2003).

These findings are in accordance with experimental results obtained in the course of ComX purification. Although the pheromone could not be isolated, induction of *srfA* in *B. subtilis* LS27 cultures was observed with the fraction collected at 30% CH_3CN elution (Figure 3.14), which corresponds to the elution of CSF. As it has been confirmed that *B. licheniformis* NCIMB 8874 does not produce the competence and sporulation factor, *srfA* induction might have been caused by another Phr peptide, possibly PhrK, which regulates ComA activity.

Chapter V

Conclusions

CONCLUSION

The aim of this study was to investigate cell-cell communication in *B. licheniformis* NCIMB 8874 and establish the role of *comQXPA* gene cluster of this bacterium in secondary metabolite production. The study provided evidence for the presence of signalling molecule/s in *B. licheniformis* NCIMB 8874 supernatant, able to induce quorum sensing response in two *B. subtilis* reporter strains, one of which harboured a *comX* null mutation.

Bioinformatics analysis of *B. licheniformis* NCIMB 8874 genome sequence confirmed the existence of an intact *comQXPA* gene cluster identical to its counterpart from *B. licheniformis* strain 9945A, a naturally competent bacterium. Studies on the production of quorum sensing-regulated secondary metabolite, such as lichenysin, γ-γ-PGA and extracellular proteases confirmed that the *comQXPA* cluster gives rise to a functional communication system.

However, exogenous addition of spent medium from high cell density cultures of either *B. licheniformis* NCIMB 8874 or the wild type *B. subtilis* showed no significant effect on lichenysin production and exoproteolytic activity in *B. licheniformis* NCIMB 8874.

A potential quorum sensing effect was observed in *B. licheniformis* NCIMB 8874 cultures supplemented with supernatants of *B. licheniformis* NCIMB 8874 and the wild type *B. subtilis*, resulting in a transient increase in γ-PGA production and red pigmentation.

This study has established, for the first time, the production of industrially valuable secondary metabolites, such as the antimicrobial biosurfactant lichenysin and the capsular biopolymer γ-PGA, in *B. licheniformis* NCIMB 8874 under the control of the quorum sensing cluster *comQXPA*, with potential industrial exploitation.

The ComX pheromone was established as the signalling molecule in *B. licheniformis* NCIMB 8874 and a second potential inducer was also identified as the product of the *phrK* gene.

It can be concluded from these findings that the ubiquitous quorum sensing process covers *B. licheniformis* NCIMB 8874 as well. This initial characterization of cell-cell communication mechanism in *B. licheniformis* NCIMB 8874 opens up further possibilities for improved production of industrially desirable products through the adoption of strategies based on microbial signalling processes.

Chapter VI

Future Work

FUTURE WORK

Taking into account the results of this study and the derived conclusions, the following suggestions for future work may be addressed.

6.1 Annotation of *B. licheniformis* NCIMB 8874 genome

The present work highlighted the importance of genetic information for the investigation of biological processes in microbes. Bioinformatics analysis was carried out using a preliminary sequence of *B. licheniformis* NCIMB 8874 genome which was obtained at a later stage of the project. Although the annotation of the selected genes of *B. licheniformis* NCIMB 8874 was carried out in this study, the present genome sequence only comprised partially assembled contigs. Hence further work is necessary to reduce the gaps between all the contigs and to annotate individual coding sequence(s).

6.2 Cloning and overexpression of *comQX* for pheromone purification

In order to further investigate cell-cell communication processes in *B. licheniformis* NCIMB 8874, the isolation and purification of the ComX pheromone is of utmost importance. To this end, the best strategy would be the cloning of the *comQX* locus into a shuttle vector under the control of an inducible promoter. The genes *comQ* and *comX* are sufficient for the process leading to the production and the maturation of the peptide in host cells. This technique will result in pheromone production at high concentrations for subsequent harvesting and purification using reverse phase chromatography and HPLC.

Following the purification of ComX pheromone of *B. licheniformis* NCIMB 8874, Edman degradation might be used to determine the exact amino acid sequence of the peptide, whereas techniques such as MALDI-TOF and LC-MS, and NMR will aid the identification of the modification on the tryptophan residue. The isolation of the

ComX pheromone will allow further insight into the regulation of the secondary metabolite production controlled by quorum sensing.

6.3 Microarray analysis

Whereas cell-cell communication in *B. subtilis* has been extensively investigated, very little is known about this process in *B. licheniformis*. However, it has been established that quorum sensing in the two bacteria are different, as demonstrated by the absence, in *B. licheniformis* NCIMB 8874, of the competence and sporulation factor, the second signalling molecule of *B. subtilis*,. Therefore, further investigation will be needed to elucidate the regulation of cell density dependent phenomena in this bacterium.

The recently acquired genomic sequence could be used to design probes for gene expression profiling analysis by using microarray technology. This would allow comparison of the transcription levels in *B. licheniformis* cultures grown in the absence and presence of the ComX pheromone. This would generate a comprehensive profile of genome wide changes in gene expression in response to the addition of the signalling molecule.

6.4 Construction of *B. licheniformis* reporter strains

The use of bioassays is a well established practise in the investigation of cell-cell communication in microbial cells. Bioassays have been developed to detect various aspects of autoinducer-mediated quorum sensing by exploiting bacteria characterised by an easily observed phenotype, such as pigmentation or bioluminescence. In bacteria which do not possess an easy-to-detect phenotype, this is created by introducing a reporter gene.

Reporter genes, such as the β-galactosidase encoding gene *lacZ*, could be used to investigate the expression of the lichenysin biosynthetic operon, or other genes regulated by quorum sensing, in *B. licheniformis* NCIMB 8874, by constructing a reporter strain carrying a fusion of the promoter of this operon and the *lacZ* gene (*licA-lacZ* fusion). This type of construct would allow the investigation of cell density dependent production of lichenysin, or other secondary metabolites regulated by quorum sensing, through β-galactosidase assay.

Moreover, these reporter strains could be used to investigate the effect of exogenous addition of ComX on the production of secondary metabolites. Different concentrations of pheromone could be added at different times to the cultures of *B. licheniformis* reporter strains to establish the best conditions for optimisation of the production of quorum sensing-regulated secondary metabolites.

6.5 Production of secondary metabolites in continuous culture systems

Quorum sensing is generally considered as a cell-density-dependent cell-to-cell signalling process. There is, however, increasing evidence that cell-to-cell signalling mediated by quorum sensing can be affected strongly by environmental factors other than the cell density; hence a novel approach is needed to investigate quorum sensing processes under defined, stable and controllable set of physico-chemical conditions. For this purpose, an ideal experimental system for quorum sensing studies would be the chemostat.

In order to establish whether the environmental parameters might affect the ComX-regulated cell-cell communication in *B. licheniformis* NCIMB 8874, cell population density could be studied under the steady state in a continuous culture system, where the production of secondary metabolites could be investigated in the course of parallel fermentations with and without the addition of the signalling molecule(s).

6.6 Investigation of other potential quorum sensing molecules in *B. licheniformis* NCIMB 8874

Quorum sensing processes in *B. subtilis* are under the control of two convergent signalling pathways, each regulated by a specific quorum sensing molecule. One of these molecules, the ComX pheromone, has been identified in *B. licheniformis* NCIMB 8874, whereas the coding sequence of the second molecule, *phrC*, could not be located in the genome of this organism. However, a second potential signalling molecule has been identified in *B. licheniformis* NCIMB 8874, possibly the product of *phrK* gene.

The isolation and identification of this potential signalling molecule would necessitate further studies. Reverse phase chromatography and HPLC would be the favourable techniques for the isolation of this peptide, whilst Edman degradation would provide information on its amino acid sequence.

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