Bachelor thesis

"The Effect Of Some Hostile Environmental Conditions On Membrane Fatty Acids And Lipopeptide Production Of *Bacillus Subtilis*".

Arvanitidou Irene – Iro
email : iarvanit@hotmail.com
A.M.: 2233

University of Science and Technologies of Lille - Lamporatory ProBioGem

Technological Educational Institution of Thessaloniki - School of Food Technology and Nutrition

supervisors: professor Nour-Eddine Chihib
professor Maria Tsigga

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ABSTRACT

Bacillus Subtilis is a Gram positive bacterium, naturally found in soil and vegetation, rod shaped, which can form tough protective endospores. *Bacillus Subtilis ATCC6633* produces mycosubtilin, a non-ribosomally synthesized lipopeptide of the iturins family. Mycosubtilin is a cyclic peptide linked to one linear or branched fatty acid of length varying from C_{15} to C_{17}. The C_{17}*anteiso* homologue has been identified as a strong antifungal compound and offers many potential applications in agronomical, cosmetic or medical domains due to its biosurfactants character. Even within the natural production of mycosubtiline by the bacterium *Bacillus subtilis*, there are several isoform with activity that increases with the length of the fatty acid chain and the type of isomer.

In the following study, *Bacillus Subtilis ATCC6633* and its derivative *BBG132* have been used to examine the production of *isoC15, isoC17, anteisoC15* and *anteisoC17* under different temperatures (20^{o}C, 30^{o}C and 37^{o}C). The techniques that have been utilized among others were cultures for the previous temperatures, HPLC (High Performance Liquid Chromatography) and Gas Chromatography.

Even though the overproduction of anteisoC17 has been confirmed in this study, further research is considered necessary in order its properties to be used in industrial scale.

ΠΕΡΙΛΗΨΗ

Ο *Bacillus Subtilis* είναι ένα θετικό κατά Gram βακτήριο, το οποίο βρίσκεται στο χώμα και στη χλωρίδα, έχει ραβδοειδές σχήμα, και μπορεί να σχηματίζει ισχυρά προστατευτικά ενδοσπόρια. Ο *Bacillus Subtilis ATCC6633* παράγει τη *μυκοσουμπτιλίνη* (*mycosubtilin)*, η οποία είναι λιποπεπτίδιο που συντίθεται μη ριβοσωμικά και ανήκει στην οικογένεια των ιτουρινών. Η μυκοσουμπτιλίνη είναι κυκλικό πεπτίδιο που είναι συνδεδεμένο σε ευθείας ή σε διακλαδισμένης αλυσίδας λιπαρό οξύ με μήκος που ποικίλει από 15 ως 17 άνθρακες. Το ομόλογο C_{17}*anteiso* έχει χαρακτηρισθεί ως ισχυρή αντιμυκητιασική ένωση, με ποικίλες πιθανές εφαρμογές στην ιατρική, τη γεωπονία αλλά και την κοσμετική, λόγω του βιοεπιφανειοδραστικού (biosurfactant) χαρακτήρα της. Η μυκοσουμπτιλίνη, που παράγεται από τον *Bacillus Subtilis* έχει αρκετές ισομορφές που έχουν διαφορετική δραστικότητα η οποία εξαρτάται από το μήκος της αλυσίδας του λιπαρού οξέος και τον τύπο του ισομερούς.

Στην έρευνα που ακολουθεί ο *Bacillus Subtilis ATCC6633* και το παράγωγό του *BBG132,* χρησιμοποιήθηκαν για να εξεταστεί η παραγωγή των *isoC15, isoC17, anteisoC15* και *anteisoC17* υπό διαφορετικές θερμοκρασίες (20^{o}C, 30^{o}C and 37^{o}C). Οι τεχνικές που χρησιμοποιήθηκαν ήταν, ανάμεσα σε άλλες, οι καλλιέργειες στις παραπάνω θερμοκρασίες, HPLC (High Performance Liquid Chromatography) και Αέρια Χρωματογραφία.

Σε αυτή την εργασία, επιβεβαιώνεται η υπερέκκριση των anteisoC17. Παρόλα αυτά, περεταίρω επιστημονική έρευνα θεωρείται απαραίτητη προκειμένου οι σημαντικές του ιδιότητες να χρησιμοποιηθούν σε βιομηχανικό επίπεδο.

LIST OF ABBREVIATIONS

BACTERIA

B.subtilis :	Bacillus subtilis
B. cereus :	Bacillus cereus

CHEMICAL PRODUCTS

MOPS :	Acid 3 –[N-Morpholino]propanesulfonic

METHODS

HPLC :	High Performance Liquid Chromatography

DIVERS

A :	Adenylation
ACP :	Acyl carrier protein
AL :	Acyl ligase
AMT :	Amino-transferase
Asn:	Asparagine
ATP :	Adenosine triphosphate
C :	Condensation
DNA :	Deoxyribonucleic acid
Gln :	Glutamine
Iln :	Isoleucine
KS :	Ketide synthase-docking
Leu:	Leucine
m-RNA :	Ribonucleic acid-messenger
NRPS :	Non Ribosomal Peptide Synthetase
ORF :	Open Reading Frame
PCP :	Peptidyl carrier protein
PKS :	Polyketide Synthetase
Ppan :	Phosphopantetheinic
RNA :	Ribonucleic acid
Ser :	Serine
T :	Thiolation
TE :	Thioesterase
Thr :	Threonine
t-RNA :	Ribonucleic acid-transfer

UNITS

Da :	Dalton
g :	Gram
h :	Hour
Kb :	Kilobase
L :	Liter
M :	Molarity
mg :	Milligram
min :	Minute
mL :	Milliliter
mM :	Millimolar
nm :	Nanometer
OD :	Optical Density
pH :	Potential of Hydrogen
rpm :	Rounds per minute
µg :	Microgram
µL :	Microliter
µM :	Micromolar

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL FOR BACILLUS SUBTILIS

Bacillus subtilis is a Gram-positive, catalase-positive bacterium commonly found in soil (Madigan et al., 2005). A member of the genus *Bacillus, B. subtilis* is rod-shaped and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions (Nakano et al., 1998).

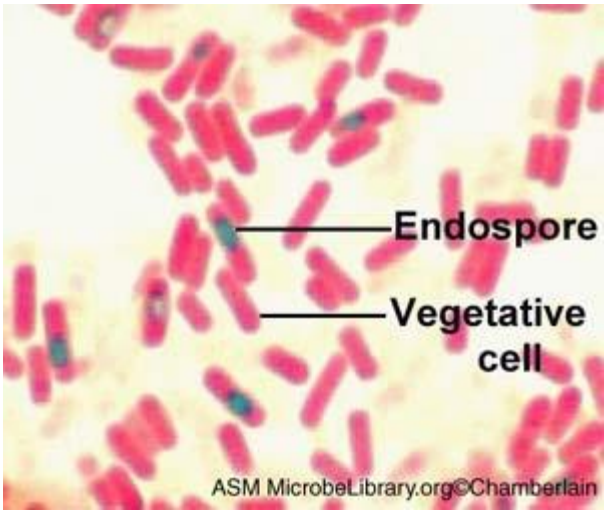

Figure1*Micrograph of bacillus subtilis, spore stain (http:/www.microbelibrary.org)*

Previous studies showed that *B. subtilis* grows anaerobically in the presence of nitrate. A more recent work has shown that the anaerobic growth with nitrate is truly nitrate respiration. Also is shown that *B. subtilis* is able to grow anaerobically by fermentation of glucose, which is stimulated by pyruvate (Michiko et al., 1997).
Bacillus subtilis lives in the upper layers of soil and is therefore subjected to frequent changes within the environment, especially in temperature, nutrient availability and osmolarity. Strategies and mechanisms to withstand these hostile conditions are crucial for survival. *Bacillus subtilis* is found mostly in a nongrowing or slow growing state due to these stress conditions (Klein 1999).

Bacillus subtilis is the best-characterized member of the Gram-positive bacteria. Its genome of 4.214.810 base pairs comprises 4,100 protein-coding genes. Of these protein-coding genes, 53% are represented once, while a quarter of the genome corresponds to several gene families that have been greatly expanded by gene

duplication, the largest family containing 77 putative ATP-binding transport proteins. In addition, a large proportion of the genetic capacity is devoted to the utilization of a variety of carbon sources, including many plant-derived molecules. The identification of five signal peptidase genes, as well as several genes for components of the secretion apparatus, is important given the capacity of *Bacillus* strains to secrete large amounts of industrially important enzymes. Many of the genes are involved in the synthesis of secondary metabolites, including antibiotics, that are more typically associated with Streptomyces species. The genome contains at least ten prophages or remnants of prophages, indicating that bacteriophage infection has played an important evolutionary role in horizontal gene transfer, in particular in the propagation of bacterial pathogenesis (Kunst et al., 1997).

1.1.1. *Bacillus Subtilis*: A Model Organism

The species *Bacillus subtilis* has quickly become a favorite tool for scientific research or industrial settings. *Bacillus subtilis* 168 was obtained in the laboratory after irradiation with X-rays, is as easily manipulated as its Gram-negative *Escherichia coli* homologue, and has great importance in the commercial production of enzymes such as amylases in breadmaking or proteases and cellulases in the manufacture of detergents.

Its biological significance in 1990 led to the development of a large consortium of 28 European laboratories, 7 Japanese laboratories, 2 Americans and one Korean, all gathered in the joint project to sequence the genome of *Bacillus subtilis*. Their work, completed and published in the journal Nature, at 20th November 1997, lists a set of 4.214.800 nucleotide bases, 4000 genes and 4000 putative proteins, which in 1500 still have unknown function (Kunst et al., 1997).

Many species assigned to *B. subtilis* group are associated with estuaries or marine habitats. The strains of *B. subtilis*, operating together in the soil, have the potential to exchange blocks of genes linked by a phenomenon of natural transformation. This leads to an extensive reorganization of the genotypic structure of the population. This practice, in relation to other species, is much rarer than the common plasmids transfer. In addition, examples of conjugation have been demonstrated between species *B. cereus* and *B. subtilis* (Priest, 1993).

In the laboratory, the bacterial colonies grow rapidly on agar medium in dendritic or by forming concentric circles slightly jagged. When the environment is appropriate, *Bacillus subtilis* has a propensity to spread. This expansion involves the differentiation of vegetative cells that migrate quickly to colonize the whole of a surface by taking on very specific style sheets (Leclere et al., 2006). This phenomenon is called swarming. Swarming is a special kind of bacterial spreading on a solid surface. All known swarming bacteria posses *peritrichous flagella* whose appearance in high numbers seem to be the only requirement known for swarming (Henrichsen, 1972).

Bacillus subtilis exhibits many interesting features, such as the synthesis and secretion of degradation enzymes, production of peptide antibiotics, sporulation and development of natural competence. The cells of *Bacillus subtilis*, rendered competent by treatment readily available, are able to take the exogenous DNA and integrate it in the future into their genome via homologous recombination (Duitman, 2003). This facilitates and improves the success rate of genetic manipulation. These include a major reason for its study as a procaryotic model.

1.2. NRPS PEPTIDE'S CHARACTERISTICS

In order to ensure its survival, each micro-organism produce naturally a number of molecules designed to function like an antibiotic, vitamin, toxin or other. These natural products may be of very different chemical compositions (Schwarzer et al., 2003).
The biosynthesis of peptides or proteins takes place ribosomally by transcription and translation of the genome sequence by the ribosome, or in a non-ribosomal way.

Most nonribosomally synthesized peptides produced by *B. subtilis* are cyclic peptides linked with a fatty acid, such as surfactin, fengycin, and the members of the iturin family, including mycosubtilin. *Bacillus subtilis* strain ATCC 6633 produces two lipopeptides, surfactin and mycosubtilin. Surfactin consists of a cyclic heptapeptide closed into a lactone ring by a β-hydroxy fatty acid. This lipopeptide exhibits strong antiviral and hemolytic activities but only a limited antibacterial activity. Surfactin seems to also be required for gliding motility. Mycosubtilin consists of a cyclic

heptapeptide closed into an amide ring by a β-amino fatty acid. Mycosubtilin exhibits a strong antifungal activity, especially against *filamentous fungi* (Erwin et al., 2007).

1.3. LIPOPEPTIDES PRODUCTION

B. subtilis ATCC 6633 produces subtilin, subtilosin, rhizocticin and two lipopeptides, surfactin and mycosubtilin, the last two are members of the iturin family. Mass spectrometry analyses of *B. subtilis* ATCC 6633 supernatant cultured at 30 °C revealed that the two main mycosubtilins produced belong to C_{16} and C_{17} homologues. The mycosubtilin gene cluster spans about 38 kb and consists of four ORFs (Open Reading Frame) designated fenF and mycosubtilin A, mycosubtilin B and mycosubtilin C, with all of them under control of the mycosubtilin promoter. The subunits encoded by the three mycosubtilin genes contain the seven modules necessary for synthesizing the peptide moiety of mycosubtilin. They show strong similarity to members of the peptide synthetase family and display the ordered assembly of conserved condensation, adenylation, and thiolation domains (Fickers, 2008).

1.3.1. Mycosubtilin

Mycosubtilin is a lipopeptide of the iturin family, isolated from the bacterium *Bacillus subtilis*. This molecule differs from iturin A by the inversion of two single amino acids, Ser (serin) and Asn (asparagin), in position six and seven.

The mycosubtilin's structure is a cyclic heptapeptide closed by a fatty acid β-amino, forming a ring amide (lactam). The amino acids present in position 2, 3 and 6 show a configuration D, accomplished during the synthesis by epimerisation areas. This structure is rigidified in the L-Pro5 and stabilized by a bend that allows the C7 carbonyl group of Ser6 interacting with its own hydroxyl group. By contrast, a large structural flexibility is observed in the region of the D-Tyr2 (Genest et al., 1987).

Mycosubtilin presents antagonistic activities toward various phytopathogens (Fickers, 2008).

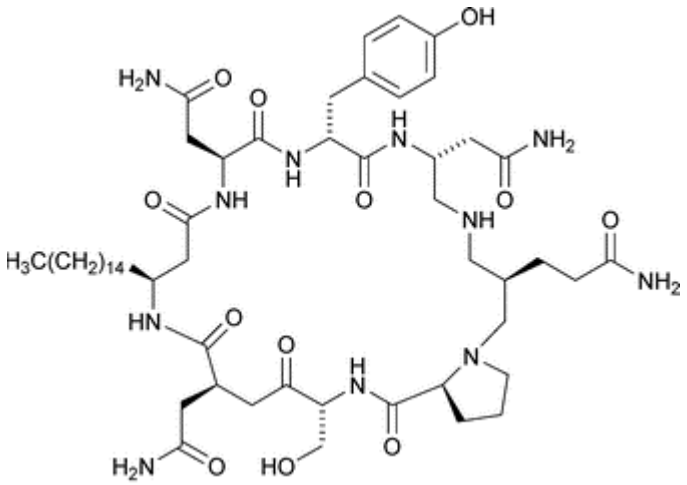

Figure 2: *Mycosubtilin*

This conformation of iturin facilitates their introduction into the lipid bilayer of cells and allows the formation of oligomers that disrupt, using phospholipases. The oligomers may aggregate lipopeptide, the lipopeptide-phospholipid complexes or lipopeptide-phospholipid-sterols forming channels within the bilayer and cause leakage of intracellular essential compounds. The loss of these molecules, such as potassium cations, nucleotides, proteins, lipids and polysaccharides (Maget-Dana and Ptak, 1990) therefore causes the death of the target cell.

1.3.2. Synthesis With Mycosubtilin Synthetase

Mycosubtilin is completely biosynthesised by a complex enzyme, called mycosubtilin synthetase. It is a hybrid enzyme PKS/NRPS that contains, therefore, areas of polyketide synthases as well as areas of non-ribosomal peptide synthetases. Mycosubtilin S encoding operon has been identified and sequenced in *B. subtilis* ATCC 6633 in 1999 Duitman and colleagues (Duitman et al., 1999).

This operon has a total length of 3.8 kb and comprises four ORFs (Open Reading Frame) designated by fenF, mycosubtilin A, Mycosubtilin B, mycosubtilin C and controlled by the same promoter P mycosubtilin. The intergenic regions between the four ORFs each contain a binding site (Figure 3).

The units are encoded by the three mycosubtilin genes contain the seven modules required for the synthesis of the peptide mycosubtilin.

Mycosubtilin A combines the functional areas of peptide synthetase aminosynthetase and fatty acid synthase. It is a natural hybrid of these different enzymes. This protein has a characteristic, since its field AL is similar to the acylCoA ligase and contains a binding site for ATP. Its function is to ensure the incorporation of fatty acid in the

molecule. The fatty acid is then β-amino via aminotransferase. Mycosubtilin B ensures, however, the adenylation of specific tyrosine and three following amino acids. Mycosubtilin C adds to the molecular structure of the amino acids serine and asparagine and causes the release and cyclization of the complete lipopeptide thanks to its thioesterase (Te) domain.

The number of modules in the synthetase and the position areas of epimerisation reflect perfectly the amino acid sequence of the mycosubtilin. Epimerisation generally takes place at the end but in the case of mycosubtilin synthetase the epimerisation is integrated within the modules.

In addition, ORFs Mycosubtilin B and mycosubtilin C begin with an area of adenylation instead of a field of condensation, which gives a very high genetic flexibility. It is therefore an exceedingly complex biosynthesis, in which the interruption of a single gene completely blocked the production of lipopeptide (Duitman et al., 1999). The synthesis itself takes place following a number of well-ordered steps (Figure 3).

FenF is the first ORF of the operon responsible for synthesis of mycosubtilin and encodes a protein of 45.2 kDa similar to malonyl-CoA transacylase. This molecule catalyzes the binding of the cofactor malonylCoA with 4-phosphopantetheine of the second field ACP2 necessary for elongation of lipopetide. ACP is small protein, highly acidic with a prosthetic group phosphopantetheine, allowing them to set acyl groups (Mendoza et al., 1993).

The other ORFs, mycosubtilin A, Myc B and myc C encode proteins which have molecular weight respectively 449.3 kDa, 612.3 kDa and 297.9 kDa (Duitman et al., 1999).

The acylCoA ligase, domain AL of MycA, is grouping CoenzymeA in a long chain fatty acid by an ATP dependent reaction. The fatty acid activated, is transferred to the cofactor 4-phosphopantetheine of the first ACP domain (Hansen et al., 2007). The condensation of malonyl thioester with the acyl thioester is catalyzed by the KS domain, with a β-ketoacyl synthase, and leads to the formation of a β-keto acyl thioester covered by the field ACP2.

Unlike the normal synthesis of fatty acids by reduction and hydration, the β-keto acyl thioester is converted into a β-amino fatty acid. This reaction of transamination is carried out by the AMT. The fatty acid needs to be activated by a thioester on the field of PCP1, and is coupled to asparagine thioester by the condensation domain which preceding the NRPS module of Mycosubtilin A (Duitman et al., 1999).

Finally, the different reactions of condensation of modules Mycosubtilin B and Mycosubtilin C can incorporate the following amino acids and lead, after cyclisation

by a thioesterase domain, Te, to the formation of the complete skeleton of mycosubtilin.

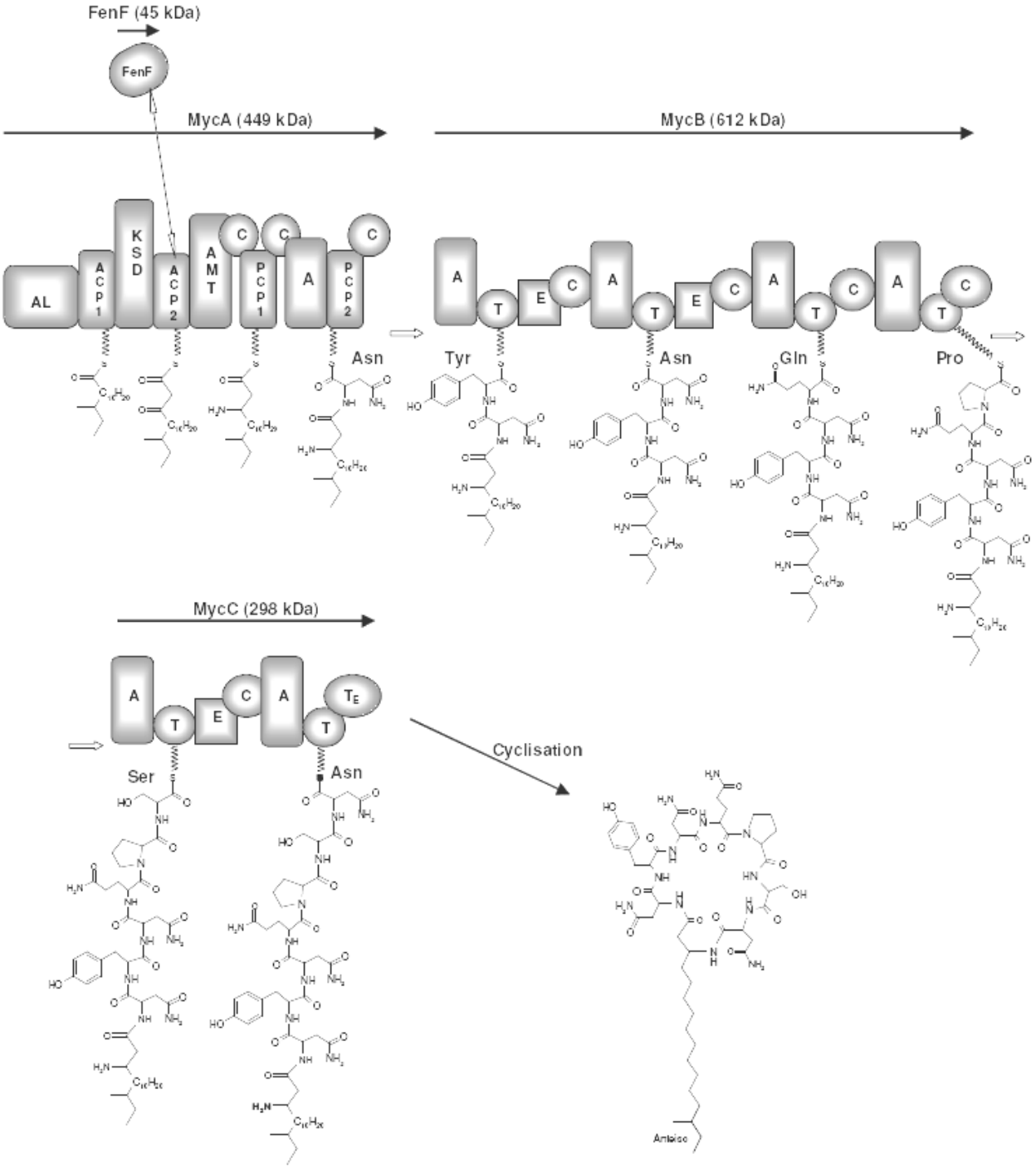

***Figure 3**: Mycosubtilin biosynthesis anteisoC_{17} from mycosubtilin synthetase Mycosubtilin S. The domain FenF is an acyltransferase. The 11 domains of Mycosubtilin A are symboled as AL (acyl ligase), ACP (acyl carrier protein), KS (ketide synthase-docking), AMT (amino-transferase), C (condensation), PCP*

(peptidyl carrier protein) et A (adenylation). The other domains NRPS of Mycosubtilin B and Mycosubtilin C are symboled as A (adenylation), T (thiolation), C (condensation) et TE (thioesterase). The peptide under elongation is linked to domain T of mycosubtilin synthetase by the effect of phosphopantetheinic (Ppan) (Jacques et al., 2007).

Members of the *Bacillus subtilis* family produce a wide variety of antibacterial and antifungal antibiotics (T. Stein, 2005). Some of them, such as bacilysin, chlorotetain, mycobacillin, difficidin and lipopeptides, are formed by non-ribosomal peptide synthetases and/or polyketide synthetases. The lipopeptides belonging to the surfactin, iturin and fengycin families are amphiphilic cyclic peptides composed of seven α-amino acids (surfactins and iturins) or ten α-amino acids (fengycins) linked to a single β-amino fatty acid (iturins) or β-hydroxy fatty acids (surfactins and fengycins). The length of the fatty acid moiety may vary from C_{13} to C_{16} for surfactins, from C_{14} to C_{17} for iturins and from C_{14} to C_{18} in the case of fengycins. Different homologous compounds with a linear or branched fatty acid moiety are usually co-produced for each lipopeptide family (M. Ongena 2008).

1.4. ITURINS

Iturins are amphiphilic compounds from the connection of hydrophilic peptides and hydrophobic lipids. This leads to an organization of micelles in these molecules, which confers biological properties, physico-chemical and interfacial particular.
Iturins present strong fungitoxic activity against different phytopathogens such as *Botrytis cinerea, Fusarium oxysporum* and *Pythium aphanidernatum*. Biological activities of iturins have been shown to increase with the number of carbon atoms in the fatty acid chain. Indeed, C_{17} homologues are 20-fold more active against pathogens than the C_{14} isoform (Fickers et al., 2008).

1.4.1 Surface Properties

Surfactants are compounds capable to reduce surface tension between liquids,

solids or gases. They lead to the formation of micellar structures enabling the mixing and dispersion of compounds in a liquid form an emulsion.
The biosurfactants have areas of very extensive application in various industrial processes. They are used in agriculture, cosmetics, pharmaceuticals, detergent manufacturing, food industries, textile industries, as well as in bioremediation of soils contaminated with heavy metals (Banat et al., 2000).

1.4.2 Biological properties

1.4.2.1 Antifungal

Mycosubtilin is the lipopeptide that has the highest antifungal capacity (Besson et al., 1979). Different dilutions of lipopeptide that comes from mutant BBG100 which overproduce mycosubtilin on Saccharomyces cerevisiae indicate that a minimum inhibitory concentration of only 8 µM was required for its toxic activity (Leclere et al., 2005).

1.4.2.2 Antibacterial

Secondary metabolites with antibiotic activity are often produced during stress conditions or during the stationary phase to maintain the competitiveness of their nutrients for bacteria.

There are two types, the lantibiotics that are synthesized ribosomal and those from the thiotemplate multienzymatic mechanism, such as iturin by *Bacillus subtilis* (Zuber et al., 1993).

1.4.2.3 Haemolytic

The iturin show a fairly significant haemolytic activity. This effect is especially marked for mycosubtilin. It is also interesting to compare the effect of the length of the fatty acid in the different isoforms of iturin A. Indeed, the hemolytic activity also increases with the length of the aliphatic chain and isoform anteisoC17 presents the best results (Guez, 2007).

1.5 POTENTIAL APPLICATIONS

1.5.1 Food

At the moment, the science of food focuses again on the use of beneficial organisms for the treatment of plant diseases. In their constant nutritional competition, bacterial strains in the soil play a range of antifungal and lytic enzymes. Specifically, they enable plants to defend themselves, by stimulating the onset of systemic resistance. This feature allows increasing the protection of plants against the phytopathogenic (Priest, 1993).

The use of active lipopeptide is a promising solution to consider in the treatment of seeds and fields to minimize many infections and thus increase crop yields. It has been shown that the strain BBG100 converted by replacing Pmycosubtilin by a strong promoter from *Staphyloccocus aureus*, can treat and reduce significantly the infection of tomato seeds (Leclere et al., 2005).

1.5.2 Cosmetics

The faculties of lipopeptide biosurfactants from B. subtilis are interesting in the field of cosmetology, as regards their poor wetting properties and their compatibility with the skin (Banat et al., 1999). In this context, the mycosubtilin, is also a potent antifungal, could be very advantageous as composed of different emulsions, creams or cosmetics. It should nevertheless remain vigilant as to its hemolytic activity.

1.5.3 Medical

The endospore-forming soil bacterium *Bacillus subtilis* is able to produce more than two dozen antibiotics with an amazing variety of structures. Most of these components show antimicrobial or antiviral activity (Stein, et al., 2005). *Bacillus subtilis* is amenable towards genetic manipulations, thanks to its ability to become naturally genetically competent. Unfortunately, natural *Bacillus subtilis* isolates that to make antibiotics appear to be difficult to transform, due to a much reduced (natural) level of competence (Erwin et al., 2007). The importance of the nature of antifungal iturin *B. subtilis* is felt not only on *filamentous fungi*, but also on different types of yeast. The fungicidal activity of mycosubtilins has already shown significant results

on *Saccharomyces cerevisiae*, as well as *Candida albicans*. If *Saccharomyces* does virtually no health hazard, *candidia* is, however, a disease that affects a very large number of people and can cause serious damage. It is extremely encouraging to consider the use of these powerful lipopeptide in the hospital domain to treat this type of pathology.

1.6. FATTY ACIDS

Fatty acids are one of the most important building blocks of cellular materials. In bacterial cells, fatty acids occur mainly in the cell membranes as the acyl constituents of phospholipids. Membrane fatty acids can be divided into two major families on the basis of their biosynthetic relationships.

One is the straight-chain fatty acid family, which includes palmitic, stearic, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic, and 2- or 3-hydroxyl fatty acids. These fatty acids occur most commonly in bacteria. They are synthesized from acetyl coenzyme A (acetyl-CoA) as the primer and malonyl-CoA as the chain extender, followed, in some cases, by a modification of the fatty acid products.

The other is the branched-chain fatty acid family, which includes iso-, anteiso- and ω-alicyclic fatty acids with or without a substitution (unsaturation and hydroxylation). The occurrence of these fatty acids in bacteria is not nearly as common as that of the straight-chain fatty acid family, but is still very significant. These fatty acids are synthesized in certain bacteria from iso, anteiso, or cyclic primer and malonyl-CoA with or without a subsequent modification.

The clear difference between these two families of cell membranes exists in the mechanism that controls their fluidity. The fluidity of membranes composed of straight-chain fatty acids is adjusted to the proper level by the inclusion of monounsaturated fatty acids, whereas that of membranes with branched-chain fatty acids is controlled mainly by 12- and 13-methyltetradecanoic acids. Thus, bacteria with the straight-chain membrane system usually require unsaturated fatty acids for growth, but these fatty acids are nonessential for bacteria with the branched-chain membrane system.

The occurrence of branched-chain fatty acids as major constituents in bacteria was first reported for *Bacillus subtilis*. The genus *Bacillus* includes bacteria with a wide variety of physiological and biochemical properties, such as psychrophiles, mesophiles, thermophiles, insect pathogens, animal pathogens, antibiotic producers, and industrial enzyme producers. This genus has been the most extensively studied with respect to branched-chain fatty acids (Kaneda, 1991).

Membranes of several gram-positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis* contain under normal growth conditions at 37°C more than 70% methyl-branched acyl residues and up to 30% straight chain acyl residues.

The physicochemical effect of a methyl branch in a long chain fatty acid is similar to that of a cis double bond in the same hydrocarbon molecule. Both alterations of the

hydrocarbon chain can cause a decrease in the melting point and an increase of the surface area in monolayer films formed from the corresponding free fatty acid or phosphoglycerides. These similarities led several authors to assume that branched chain fatty acids might fulfil the same role as unsaturated fatty acids for the structure and function of biological membranes.

The methyl-branched portions of fatty acids in *B. subtilis* are derived from carbon skeletons of the three branched chain amino acids valine, isoleucine, and leucine. Oxidative decarboxylation of the corresponding 2-keto acids leads to the branched chain C_4- and C_5-coenzyme A derivatives. These are the “primer” molecules of branched chain fatty acid biosynthesis which are lengthened by successive addition of C_2 units derived from malonyl-CoA. Therefore, this pathway produces three different types of long branched chain fatty acids, even numbered iso fatty acids (from valine), odd numbered anteiso fatty acids (from isoleucine), and odd numbered iso fatty acids (from isoleucine).

B. subtilis cells use branched chain fatty acids to maintain a certain mobility and spacing of lipid hydrocarbon chains in the membrane. It is now established that fatty acid chains of lipids in biomembranes are in a liquid-like state. Bacteria are apparently not able to survive when the temperature drops below a transition point at which the hydrocarbon chains of lipids lose their mobility by assuming a semicrystalline state. Long chain iso fatty acids have similar melting points as their straight chain isomers, but their cross-sectional area in monolayer films is at least 1.5 times larger. Anteiso fatty acids have significantly lower melting points than the corresponding straight chain fatty acids and occupy a still larger cross section area than the corresponding iso fatty acids in monolayer films (Willecket and Pardee 1971).

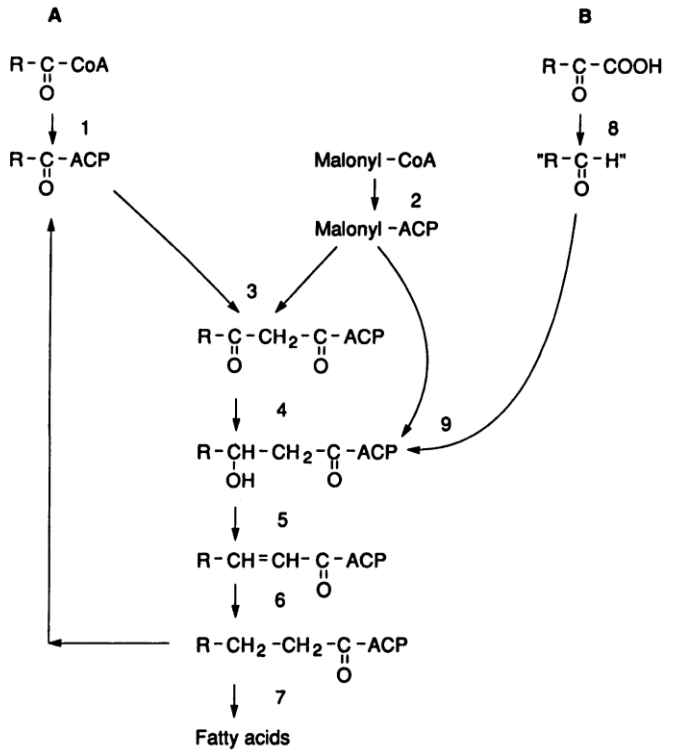

Figure 4 *Pathways of branched-chain fatty acids synthesis in B. Subtilis and other organisms, which possess branched-chain fatty acids as major cellular fatty acids. A, pathway of the synthesis from branched-chain acyl-CoA ester as a primer; B, the other pathway of the synthesis from branched-chain a-keto acid as a primer (Kaneda T., 1991).*

Temperature has played an important part in membrane research for two reasons: It has a marked effect on the physical properties and functions of cell membranes and most organisms, particularly poikilotherms, alter their membrane lipid composition in response to a change in environmental temperature. These alterations are most often in the fatty acyl constituents of phospholipids and glycolipids and much less often in the head group. This is because membrane fluidity is altered much more effectively by changes in fatty acid composition than in head group.

Genotypic adaptations of fatty acid composition can be sought by comparing psychrophilic, mesophilic and thermophilic bacteria. If this is done for species of the same genus there may be a correlation between, for example, lipid unsaturation and growth temperature. But different types of bacteria, even within the same genus, may have involved quite different ways of creating a fluid membrane – their “lipid genotype”. When the growth temperature of a bacterium is lowered, a number of changes in the pattern of fatty acids can occur, depending on the species. The most frequently and widely recognized change is in the unsaturation of lipid acyl chains. Also the average fatty acyl chain length may be shortened, the amount of branched

fatty acid may increase and this can sometimes involve an alteration in the kind of branches, or there may be a decrease in the proportion of cyclic fatty acids. All these changes produce lipids with a lower gel-to-liquid-crystalline transition temperature, thereby maintaining fluidity and compensating for the decreased growth temperature. This phenomenon has been called homeoviscous adaptation but in some bacteria the compensation is incomplete. In others it enables the growth temperature range to be extended.

The way in which the unsaturation of lipids in a bacterial membrane changes depends on the mechanism used to synthesize fatty acids. Two mechanisms are found, but only one type exists in a particular species. All anaerobic and some facultatively (an)aerobic bacteria use the “anaerobic pathway” of fatty acid biosynthesis. The enzyme b-ketoacyl-ACP synthase, which catalyses the condensation reaction in which 2C units are added, exists in two forms synthase I and II.

The second way in which bacteria make unsaturated fatty acids is by aerobic desaturation of saturated fatty acids. Such species contain a fatty acid synthetase that synthesizes saturated fatty acids only.

The most common fatty acids in bacteria are iso- and anteiso- branched. The methyl branch originates in the primer molecule used by fatty acid synthetase and is usually derived from an amino acid, leucine giving rise to an iso-branched fatty acid and isoleucine to an anteiso-branched fatty acid; similarly valine leads to a straight-chain, odd-numbered fatty acid product.

Changes in branching involve de novo synthesis of fatty acids (and phospholipids) and again there is no evidence that phospholipid turnover contributes to altered lipid composition after a shift in growth temperature. There are a few species which can methylate fatty acids directly, either as acyl-CoA or in a phospholipid, but in no case this appears to be involved in adaptation to temperature changes.

Fatty acids are elongated by the addition of 2C units at the carboxyl terminus and therefore the chain length of phospholipid acyl chains cannot be altered in situ. Acyl chain shortening as well as lengthening is possible and both are achieved by new membrane synthesis during growth after a temperature shift.

Bacillus provides a real example in which both types of adaptation are used by the same cell. Following a sudden decrease in temperature, bacillus first increase membrane lipid unsaturation by modification synthesis involving the hyperinduction of desaturase; the levels of desaturase then decline but by this time the addition

synthesis mechanism involving the altered pattern of fatty acid branching is contributing to the change in membrane lipid composition (Russell, 1984).

In its natural environment, the surface layer of soil, *B. subtilis* is exposed to temperature fluctuations which induce modifications in its physiology and metabolism. At lower temperature, cells are confronted with several problems, including low membrane fluidity, reduced enzyme activities and decreased initiation of translation due to stabilized secondary structures of mRNAs or slower protein folding. Fatty acids are one of the most important building blocks of cellular materials. In *B. subtilis*, membrane composition is characterized by a fatty acid profile dominated to a large extent by odd-numbered branched-chain fatty acids, with the major C_{15} and C_{17} species. The latter were shown to play a major role in the correct physical state of membrane lipids, which is required for optimal membrane structure and function. At lower temperatures, membrane fluidity must increase to avoid transition from a liquid crystalline into a gel-like phase state of the lipid bilayer. *Bacillus* cells respond to a decrease in the growth temperature by desaturating the fatty acids of their membrane lipids though activation of the Des pathway and by increasing the proportion of ante-iso branched fatty acids which present a lower melting point.

However, it was shown that deletion of des genes does not lead to a detectable phenotype after cold shock, indicating, rather, that *B. subtilis* adapts to low temperature by modifying its iso- and anteiso-fatty acid membrane composition. These modifications must involve de novo fatty acid synthesis. Anteiso-methyl branches cannot be added by methylation of existing fatty acids, but are introduced as part of the primer molecule during initiation of fatty acid synthesis. Anteiso-branched C_{15} and C_{17} fatty acids are formed from α-keto-β-methylvalerate and 2-methyl-butyryl-CoA, both of which are derived from isoleucine. The modification of the membrane composition at low temperature was demonstrated in *B. subtilis* JH642. These results suggested de novo synthesis of anteiso C15:0 and C17:0 fatty acids from isoleucine or threonine present in the culture medium as a response to cold stress (Patrick Fickers 2008).

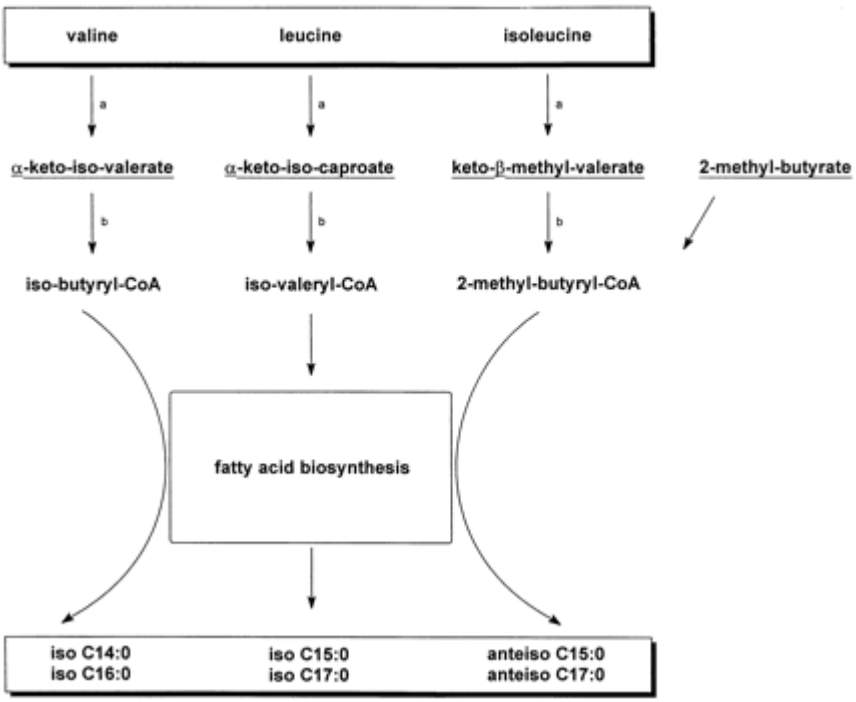

Figure 5 *: Schematic representation of branched-chain fatty acid biosynthesis with leucine, isoleucine, and valine leading to iso- and anteiso-branched fatty acid products, respectively. a, a soluble branched-chain amino acid amino transferase; b, NAD- and CoA-dependent branched-chain α-keto-acid decarboxylase. The underlined metabolites were supplied externally, as indicated in the text. This scheme is adapted from the one presented by Kaneda (20).*

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. CULTURE MEDIAS

2.1.1. E medium

The E medium (Clark et al., 1981) that is used in this work, is modified, and comes from the mix of stock solutions which are:

- Solution base: 5.62g/L KH_2PO_4, 39.37 g/L K2HPO4, 1g/L yeast extract
- Solution 1: 200g/L glucose
- Solution 2: 0.5g/L EDTA, 6.14 g/L $MgSO_4$, 0.56 g/L $MnSO_4$, 1 g/L NaCl, 0.12 g/L $CaCl_2$, 0.18 g/L $ZnSO_4$, 0.18 g/L $FeSO_4$, 0.02 g/L $CuSO_4$, 0.01 g/L Na_2MoO_4, 0.01 g/L H_3BO_3, 0.01 g/L Na_2SO_3, 0.037 g/L $NiCl_2$
- Solution 3: 200g/L NH_4NO_3
- Solution 4: 102.3 g/L $MgSO_4$

All those solutions, are autoclaved at 120^{o}C for 20min.

One liter of modified E medium can be prepared by mixing 480ml of base solution, 100ml of solution 1, 10ml of solution 2, 20ml of solution 3 and 10ml of solution 4 and fill with distilled sterilized water up to the volume of 1000ml.
The PH is corrected with HCl 10%.

2.1.2. Landy medium

The Landy medium (Landy et al. 1948) is modified and its composition is 20g/L glucose, 2g/L glutamic acid , 1g/L yeast extract, 2.3g/L $(NH_4)_2SO_4$, 1g/L K_2HPO_4, 0.5g/L $MgSO_4$, 0.5g/L KCl, 1.6mg/L $CuSO_4$, 1.2mg $Fe_2(SO_4)_3$ and 0.4mg/L $MnSO_4$. The solution's PH is adjusted at 7 $\pm$ 0,5 by KOH 5M and the buffer solution is MOPS 100mM.
The medium is prepared under sterilized conditions at PSM from stock solutions. Those solutions are:

- Solution Glucose 200g/L, autoclaved at 121^{o}C for 20min
- Solution glutamic acid 20g/L, filtrated with filters 0.2μm

- Solution yeast extract 20g/L, autoclaved at 121°C for 20min
- Solution $(NH_4)_2SO_4$ 23g/L, autoclaved at 121°C for 20min
- Solution 1 : 40g/L K_2HPO_4, 20g/L $MgSO_4$, 20g/L KCl, filtrated with filters 0.2µm
- Solution 2 : 64mg/L $CuSO_4$, 48mg $Fe_2(SO_4)_3$ 16mg/L $MnSO_4$, filtrated with filters 0.2µm
- Buffer solution concentrated 20x : 42g of MOPS (acid 3 –[N-Morpholino]propanesulfonic) in 100ml of water, filtrated with filters 0.2µm

One liter of modified Landy medium, comes up from the mixture of 100ml of glucose solution, 100ml of solution glutamic acid, 50ml solution yeast extract, 100ml of solution $(NH_4)_2SO_4$, 25ml of solution 1, 25ml of solution 2 and 50ml MOPS. The total volume of 1000ml is reached with distilled sterilized water, after the PH correction with KOH 5M.

2.1.3. Luria-Bertani (LB) medium

The composition of LB medium is 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl and 950ml deionized water, adjust the pH at 7.0 with 5N NaOH and then fill with deionized water up to 1L. Autoclave for 20min at 120°C.

2.1.4 Mossel medium agar

The composition of Mossel agar is: 1 g/L meat extract10 g/L peptone, 10 g/L D-mannitol, 10 g/L NaCl, 0.025 phenol red, 12 g/L agar, 10ml/L egg yolk, 0.01 g/L polymixine and the PH is 7.1.

2.2. ANALYTICAL CHEMISTRY TECHNIQUES

2.2.1. Lipopeptides purification

The protocol for the extraction of lipopeptides is the above. The cartridges are C18 maxiclean, Altech (1 g of gel). The first injection is 20ml of methanol 100% and then 8ml of water mQ for the preparation of the cartridge. Afterwards 1ml of culture's supernatant. The column is rinsed with 8ml of water mQ and then with 8 ml of methanol/water mQ (50/50). After that, 20ml of air pass, to dry the column and then 5ml of methanol 100% to collect the lipopeptides. Those 5 ml that have the lipopeptides, must be dried in Speed-Vac to evaporate the methanol for the HPLC analysis.

2.2.2. High performance liquid chromatography (HPLC)

The lipopeptides samples, can be analysed, with HPLC. The HPLC's label is Waters (Online Degaser, 717 Autosampler, 660S Controller, 626 Pump, 2996 PhotoDiodeArray) with a column C18 (5μm, 250 x 2,5 mm, VYDAC 218 TP).
During the HPLC, 10μl from the lipopeptides sample are injecting and comparing to a standard, which is Iturine 500mg/L (Sigma) at 0,6ml/min. The elution was performed in isocratic mode with solvent of water/acetonitrile/trifluoroacetic acid 60/40/0,1 (v/v/v).The retensional time and the derive second of the spectrum at 214 nm of every pic (barette diode, PDA 2996, Waters) are analysed automatically with a software named as Millenium to identify the molecules.

2.2.3. Extraction of fatty acids

The protocol for the extraction of fatty acids has four steps. The first one is *Saponification*, to the pellet that has the fatty acids and comes from a culture, with 1ml of NaOH-Methanol, homogenizing in Vortex, bath at 100^{o}C for 5 min, homogenizing in Vortex once again and then bath at 100^{o}C for 25 min.
The second step is *Methylation*, with 2ml of HCl-Methanol, then homogenizing at Vortex, bath at 80^{o}C $\pm$ 1 for 10 min $\pm$ 1 and then set the tubes in the ice for 10 min.

The third step is the *Extraction*, with 1,5ml of TBME/HEXAN and set the tubes in the machine that is spinning around, for 30 min. After this step there are two phases in the tubes and the upper phase is the one that has the fatty acids.
The fourth step is *Washing*, at the upper phase from the third step which is placed in clean heated tubes, with 3ml of NaOH-water-UHQ and once again set of the tubes in the machine that is spinning around, for 5 min. At this step there are also two phases. The upper phase is the one that has the fatty acids and it is removed and placed in clean heated tubes for the analyzing at gas chromatography.

2.2.4. Gas chromatography

The gas chromatography needs a 10μl sample (from the extraction of fatty acids) that being vaporised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid.

2.3 ANALYSIS

2.3.1. Optical density (O.D.) measurements

The optical density is measured at 600nm to quantify the cell density. Indeed, at this wavelength there is a measure of the distribution of the light beam, proportional to the number of cells present and not the absorbance.
The spectrometer used is the model Prim SECOCAM .

2.3.2. pH measurements

The pH-meter that was used for the Ph measurements of the cultures is the Inolab pH ION+sentix61 MANO39210012/001.

2.4. CULTURES

2.4.1. Preparation of the inoculum

In 5ml of modified E medium which is adjusted at pH 7, is inoculated one drop of each bacterium (ATCC6633 and BBG132) under sterilized conditions. The incubation is for 24h in an agitator at 30ºC at 250 rpm. Afterwards the tubes are homogenized in Vortex and follow the optical density measurement at 600nm.
The next step is to prepare a first preculture with 50ml of E medium in a 500ml flask by taking from the tube a volume that will give a final optical density, equals to 3. To find the exact volume we use the above mathematical types.

- $O.D._f = O.D._{in} * e^{(\mu * t)} \Rightarrow O.D._{in} = \frac{O.D.f}{e(\mu * t)}$

$O.D._f$: final optical density =3
$O.D._{in}$: initial optical density
μ : specific growth yield = 0,4
t : inoculation time in hours

- $O.D._{in} * 50ml_{(preculture)} = x\ ml$

$O.D._{in}$: initial optical density
50ml of E medium for the preculture
X ml : the quantity 50ml

- $X\ ml = O.D._{real} * V$

X ml : the quantity of 50ml
$O.D._{real}$: the real optical density at the end of the inoculation in tubes
V : the volume that will give optical density = 3

The incubation is in an agitator at 30ºC at 250 rpm. At the end of the first preculture the optical density must be 3 to 5. With the optical density's value and the same mathematical types we can find the volume we have to take to prepare a second

preculture. The second preculture is in 100ml of E medium in an agitator at 30°C at 250 rpm for 15h.

2.4.2. Cultures in flasks

To prepare a culture, we have to rinse twice with distilled sterilized water and centrifuge for 10 min at 2500 rpm at 30^0C a volume from a second preculture, that corresponds to the biomass necessary to reach an optical density comprised between 0,2 and 0,3. The cells that we have after the centrifugations are inoculated in 500ml flasks that have 100ml of modified Landy medium.

The culture last for 48h, at 20°C, 30°C and 37°C in agitators at 250 rpm. At 24h samples from each flask are taken for extraction of lipopeptides and also for measurements of optical density and pH.

At 48h to stop the culture, we centrifuge the entire volume of the flasks twice at 4°C at 10000rpm for 10min. The supernatant from the first centrifugation is kept in -20°C for the extraction of lipopeptides and the pellet at the end of the centrifugations also kept in -20°C for the extraction of fatty acids.

At the end of every culture a purity test is done in LB medium and Mossel medium, to verify that the samples have not contaminated during the manipulations.

CHAPTER 3

RESULTS

3. RESULTS

3.1. GROWTH KINETICS OF *B. SUBTILIS ATCC6633* AND *BBG132* AT 30ºC

The growth kinetics of the strains ATCC6633 and BBG132 at 30ºC came from the value of the strain's optical density, to time. The strains ATCC 6633 and BBG132 were cultured at 30ºC in modified Landy medium adjusted at 7.0 pH. All the manipulations were performed under sterile conditions in a laminar flow hood.

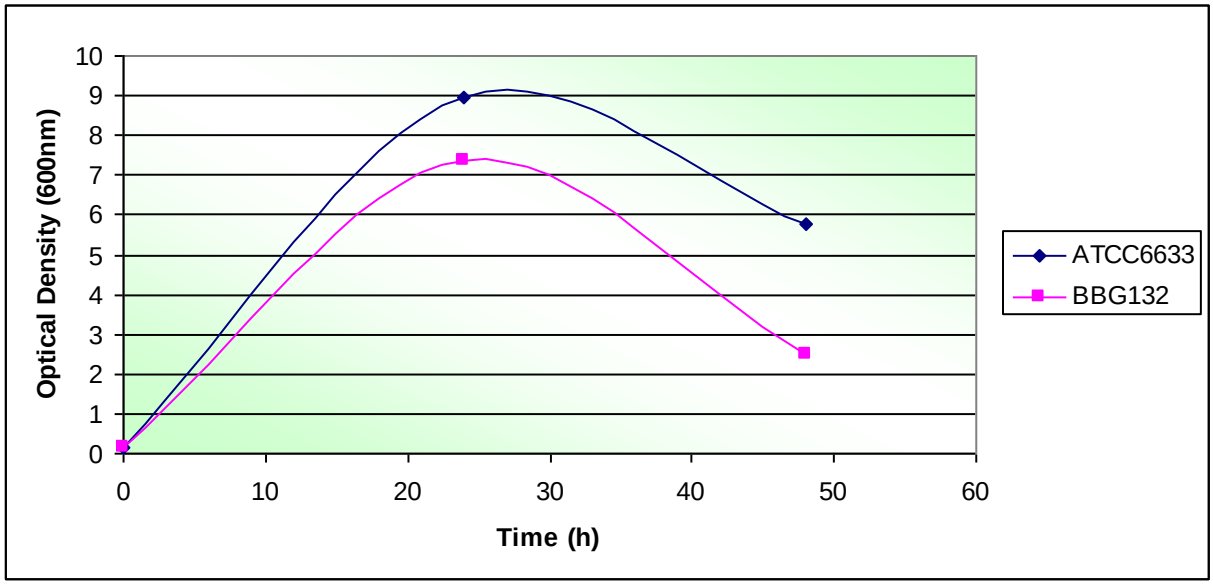

***Figure 6** : The Optical Density's evolution at 600nm to time for cultures at 30ºC for the strains ATCC6633 and BBG132, in modified Landy medium adjusted at 7.0 pH. The data represent mean value of three independent experiments.*

According to the growth kinetics of *B. subtilis ATCC6633* and *B. subtilis BBG132* we observed that the higher cell concentration was at 24 h for both strains which is correlated with the highest Optical Density at 600nm, which were 8.94 and 7.35 respectively. At 0h the Optical Density was 0.147 and 0.138 and at 48h were 5.75 and 2.48 respectively.

3.2. EFFECT OF GROWTH TEMPERATURE ON MEMBRANE FATTY ACIDS PROFILE OF *B. SUBTILIS ATCC6633* AND THE MUTANT STRAIN *B.SUBTILIS CspB*

The extraction of fatty acids, carried out, as described in 2.2.3. After the extraction follows the gas chromatography. Each peak represents a different fatty acid. The overexpression of the gene CspB is carried out in B. subtilis by the substitution of its native promoter by the strong constitutive promoter P_{repU} of the replica's gene of *Staphylococcus aureus* to characterize the effect of this genomic modification on the level of production of the isoform mycosubtiline anteisoC17.

Analytically this happened by the interference on the metabolic pathway synthesis of branched chain fatty acids by improving the stability of *mRNAs* encoding for peptides and enzymes involved. The molecules having the capacity to prevent the degradation of *mRNA*, chaperones proteins, are encoded by the gene CspB. These CspB proteins are essential for bacterial survival, especially in heat stress conditions. The overexpression of the gene *CspB* could therefore increase the proportion of cold shock proteins within the cell and promote the formation of branched chain fatty acids.Thus, it is extremely probable that the constitutive expression of the gene cspB at BBG132 increases the chaperonnes protein concentration and promotes this fact, the pathway of synthesis of the branched fatty-acids anteiso type.

Growth conditions	**aC_{15}/aC_{17}**		**iC_{15}/iC_{17}**	
	B. subtilis 6633	*B. subtilis* CspB	*B. subtilis* 6633	*B. subtilis* CspB
20°C	4.1	2.3	1.7	0.7
30°C	3.5	2.5	1.0	1.3
37°C	4.3	4.0	3.8	2.2

***Table** 1 Evolution of ai15:0 to ai17:0 ratio and i15:0 to i17:0 ratio in Bacillus subtilis 6633 and the mutant strain B. subtilis CspB, depending on the growth temperature.*

The result of table 1 showed that the changes of growth temperature affect the *ai*15:0 to *ai*17:0 ratios in the wild type and the mutant strain of *B. subtilis*. Thus, the *ai*15:0 to *ai*17:0 ratio increase from 3.5 to 4.3 and from 2.5 to 4.0 when the growth temperatures decreased from 37 to 30°C for the wild type and the mutant strain, respectively. Moreover, the *ai*15:0 to *ai*17:0 ratios are similar for the wild type strain at 20 and 37°C. However, this ratio is of *ca.* 1.6 to1.7-fold higher, in the *B. subtilis* CspB strain at 37°C, than the *ai*15:0 to *ai*17:0 ratios calculated at both 30 and 20°C. In addition, the *ai*15:0 to *ai*17:0 ratio in *B. subtilis* CspB strain are similar at both 20 and 30°C.

The *i*15:0 to *i*17:0 ratio decrease with the decrease of growth temperature in both the wild type and *B. subtilis* CspB strains. This ratio decrease 3-fold and 2-fold in the mutant and the wild type strains when the growth temperature decreases from 37 to 20°C, respectively.

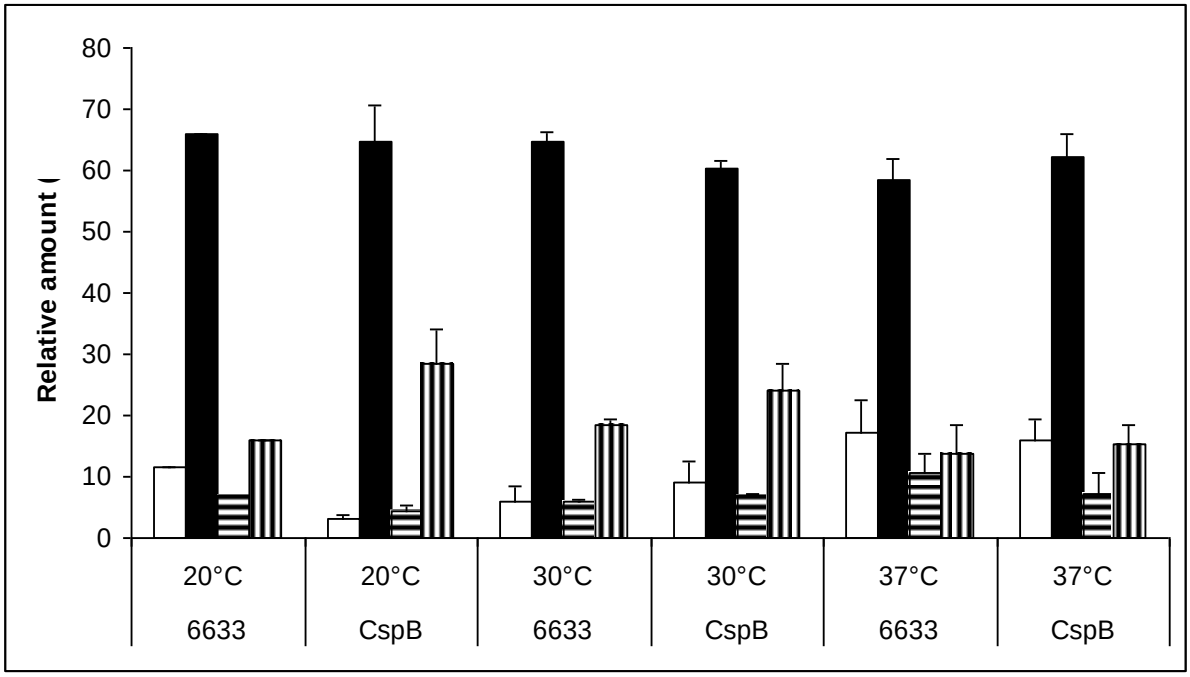

***Figure 7**: Effect of growth temperature on fatty acids composition of Bacillus subtilis 6633 and the mutant strain Bacillus subtilis CspB. The cells were grown in Landy medium at 20°C, 30°C and 37°C. ai15:0 solid bars; ai17:0 vertically hatched bars; i15:0 open bars; i17:0 horizontally hatched bars. The data represent mean ±S.D of three independent experiments.*

The results of figure 6 show that the wild type strain and the mutant have similar fatty acids profiles at 37°C. In contrast, the two strains have different fatty acids profiles at 20°C and at 30°C. Under these conditions, *B. subtilis* CspB cells showed an amount of *ai*17:0 of *ca.*1.7 and 1.3 higher at 20 and 30°C respectively when compared with the wild type strain. However, the relative amount of *ai*15:0 was similar at both 20 and 30°C for both strains.

Under 20°C *B. subtilis* CspB strain showed the lowest relative amounts for both *i*15:0 and *i*17:0, while the wild type strain showed the highest relative amounts for the fatty acid under 37°C.

3.2. EFFECT OF GROWTH TEMPERATURE ON LIPOPEPTIDES PRODUCTION OF *B.SUBTILIS ATCC6633* AND THE MUTANT STRAIN *B.SUBTILIS CspB*

The lipopeptides extraction for ATCC 6633 and BBG132 from culture's supernatant caried out according to 2.2.1. To perform the HPLC analysis we have to recapture the dried sample from the tubes with 500 µl of methanol (100%). Each peak corresponds to a mycosubtiline's isoform.

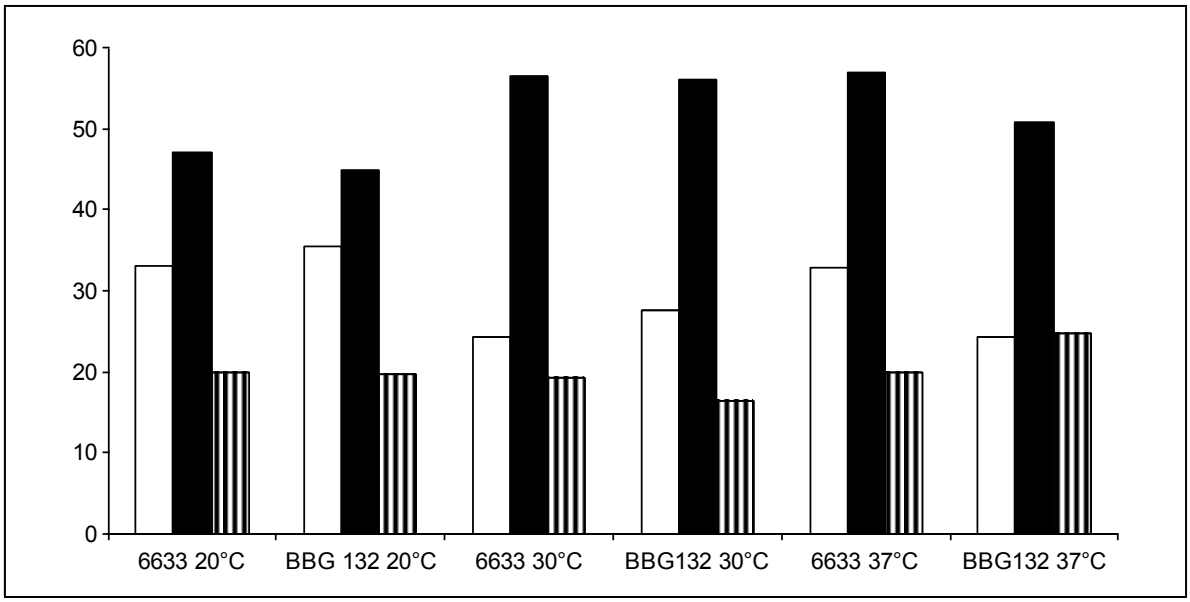

***Figure 8**: Effect of growth temperature on lipopeptides mycosubtiline isoform biosynthesis by the wild type strain Bacillus subtilis 6633 and the mutant strain BBG132. The bacteria were grown in Landy medium at 20°C, 30°C and 37°C. The open bars represent the iso16:0, the solid bars the ai17:0 and the vertically hatched bars represent the i17:0. The data represent mean QS.D. of three independent experiments.*

At 30°C, a decrease of isoform isoC17 is visible in BBG132 while obtaining a higher production of mycosubtiline anteisoC17 (Figure 8). This difference, compared to the wild type strain, is probably due to the effect of the cold shock proteins which, present in stronger concentration and support the isoleucine synthesis to form branched chain fatty-acids of anteiso type.

CHAPTER 4

DISCUSSION

4. DISCUSSION – CONCLUSIONS

Many organisms of different species have evolved sophisticated mechanisms to overcome the life-endangering influence of low temperatures*. *Bacillus subtilis* has become a model organism for studies of the bacterial CSR (Cold Shock Response) representing the Gram positive branch of mesophilic soil bacteria. This extraordinarily versatile, rod-shaped micro-organism is genetically easily accessible and has been accepted as one of the best-studied bacteria even before its genome was entirely sequenced a few years ago (Kunst et al., 1997). *B. subtilis* is broadly distributed all over the world and lives in the upper layers of soil, where it is subject to every imaginable stress situation over the entire year.

Regarding the kinetics growth at 30 ° C, the strain BBG132 grows significantly worse than the natural strain ATCC 6633. Moreover, after 24 h of culture, the cell concentration drops very intensely and the culture medium turns to a brownish colour from a creamy one. This colour can be explained by the production of pigment in response to stress, or by the sporulation of the strain as a result of higher mortality rate.

The overexpression of the gene CspB does not seem beneficial to the development of BBG132 at the optimum temperature. This result could possibly come because of an energy waste from the transformed strain which should produce constitutively the cold shock proteins sufficiently at 30° C even in lower concentrations. This overproduction would probably use many cellular resources, which significantly disadvantage BBG132 strain at optimal conditions of growth. Therefore, it is a metabolic imbalance caused by global deregulation of gene CspB, following its constitutive expression. They are involved in several cellular processes and may also have a direct effect on growth and adaptation of cells in stationary phase. However, it is necessary to investigate these hypotheses in future studies and experiments on the multiple effects engendered by the overproduction of these proteins.

* Over 80% of the earth's biosphere is governed by permanent cold, i.e. temperatures below 5^{o}C.

During the data processing of membrane's fatty acids, we observed that the production of anteisoC17 at 20ºC is the highest (Figure 7). This result corresponds with the literature. Graumann and Marahiel (1998) proved that the cold shock proteins, CspB, are naturally produced in large quantities in *B.subtilis* under stress conditions and act as a chaperone protein for RNA. Specifically at 20 ºC and 30ºC *B. subtilis* CspB cells showed an amount of *ai*17:0 of *ca.*1.7 and 1.3 higher at 20 and 30°C respectively when compared with the wild type strain.

For all organisms, maintenance of functional cellular membranes is a limiting factor for survival. Numerous investigations in the past have shown that biomembranes are highly complex structures that exist in several physically different states.

The mycosubtiline anteisoC17 at 20 ºC and 30ºC is higher, while the proportion of the iso type isoform is shorter. This result is probably explained by the preferential metabolic pathway which is initiated by the enzymatic reaction of the threonine dehydratase. This is actually the first enzyme in the pathway synthesis of branched chain amino acids which directly leads to the formation of isoleucine (Fink, 1993). The cellular isoleucine concentration was not analyzed, but it would seem normal to be higher. An overproduction of fatty acids anteiso type follows, so isoleucine is the precursor (Klein et al., 1999). A more important proportion of anteiso fatty-acids within the cell directly affects the selectivity of these precursors by the mycosubtiline synthetase and leads to the formation of mycosubtilin anteiso counterparts.

The lipopeptides production of *B. subtilis ATCC6633* and *BBG132* also have higher production of the isoform anteisoC17 comparing to the isoforms isoC17 and isoC16, under 20ºC, 30 ºC and 37 ºC (figure 8). The difference in the lipopeptides production between the two strains is minimal. This result is contrary to previous studies between the two strains. In particular *BBG132* should produce more anteisoC17 than ATCC6633.

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