Impact of trehalose and mycolate biosynthesis on the cell envelope of a *Corynebacterium glutamicum* L-lysine production strain

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, Oktober 2005

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Tag der Disputation: 28. November 2005

Einfluß von Trehalose – und Mycolatbiosynthese auf die Zellwand eines *Corynebacterium glutamicum* L-Lysinproduktionsstamms

Corynebacterium glutamicum besitzt, im Gegensatz zu anderen Gram-positiven Bakterien, in der Zellwand eine äußere Lipiddoppelschicht, die Mycolatschicht, die eine Permeabilitätsbarriere darstellt. Trehalose ist ein wichtiger Bestandteil der Mycolatschicht und an der Biosynthese von Mycolat beteiligt. Es konnte gezeigt werden, dass der erste Schritt der Mycolatbiosynthese, die Kondensation zu Trehalosemonomycolat, in der Zellwand stattfindet. Um die Bedeutung von Trehalose für die Mycolatschicht zu untersuchen, wurde die Zusammensetzung der Mycolatschicht gezielt durch die Kultivierung eines trehalosedefizienten Stammes mit verschiedenen Kohlenstoffquellen, in An- und Abwesenheit von Trehalose manipuliert. Ein trehalosedefizienter Stamm, der auf einem *C. glutamicum* L-Lysinproduzenten basierte, wurde gewählt, um zu überprüfen, ob die Veränderung der Mycolatzusammensetzung die Exkretion von L-Lysin steigern könnte. Weiterhin sollte die Lysinexkretion mit der Permeabilität der Zellwand korreliert werden. Die Analysen zeigten, dass Trehalose essentiell für die Mycolatbiosynthese war, wenn Saccharose oder Fruktose als Kohlenstoffquelle dienten, während Glukose Trehalose als Akzeptor und Überträger von Mycolsäuren ersetzen konnte. Externe Trehalose konnte cytoplasmatische Trehalose nur teilweise für die Mycolatsynthese ersetzen, so dass die Supplementierung des Mediums mit Trehalose im trehalosedefizienten Stamm nicht vollständig die Eigenschaften der Mycolatschicht des Ausgangsstammes wiederherstellen konnte. Eine unvollständige Mycolatschicht erhöhte die Permeabilität der Zellwand, und gleichzeitig steigerte sie die Exkretion von Lysin und regte die Exkretion von Glutamat an. Die Permeabilitätsbarriere kann scheinbar nur dann aufgebaut werden, wenn alle Bestandteile in der Menge vorhanden sind, die eine korrekte Anordnung der Mycolatschicht ermöglicht.

Die physiologische Funktion von einem der drei Trehalosestoffwechselwege in *C. glutamicum*, dem OtsAB-Weg, war vor Beginn der Arbeit noch unbekannt. Die Analyse von Stämmen mit nur einem funktionellen Trehalosestoffwechselweg zeigte, dass der OtsAB-Weg der wichtigste Trehalosesyntheseweg unter Kohlenstofflimitierung war. Da Trehalose das wichtigste kompatible Solut unter Stickstofflimitierung ist, könnte der OtsAB-Weg für die Synthese von Trehalose als Schutzsubstanz gegen osmotischen Stress unter Kohlenstoff- und Stickstofflimitierung notwendig sein, eine Mangelsituation, die im Boden, dem natürlichen Lebensraum von *C. glutamicum,* häufig auftritt.

Impact of trehalose and mycolate biosynthesis on the cell envelope of a *Corynebacterium glutamicum* L-lysine production strain

In contrast to other Gram-positive bacteria all members of the suborder of *Corynebacterineae*, including *Corynebacterium glutamicum,* contain a cell envelope that comprises an outer lipid bilayer, the mycolate layer, which is considered as permeability barrier. Trehalose is an important component of the mycolate layer and involved in the biosynthesis of mycolate. The first step of mycolate biosynthesis, the condensation of trehalose monomycolate was proven to be located in the cell envelope. The composition of the mycolate layer was specifically manipulated by growing a trehalose deficient strain on different carbon sources in the absence and presence of trehalose to investigate the importance of trehalose for the corynebacterial mycolate layer. A strain deficient in trehalose biosynthesis deriving from a *C. glutamicum* L-lysine production strain was chosen to examine whether the alteration of the cell envelope could improve lysine production and to test whether lysine excretion was correlated with the permeability of the cell envelope. Trehalose was shown to be essential for mycolate synthesis, when sucrose or fructose were the carbon source, whereas glucose could replace trehalose as acceptor and translocator of mycolic acids. External trehalose substituted cytoplasmic trehalose only partially for mycolate synthesis so that supplementation of the medium with trehalose could not completely restore the properties of the mycolate layer of the trehalose deficient strain to those of the parental strain. An imperfect mycolate layer increased the permeability of the cell envelope, and at the same time enhanced the excretion of lysine and triggered the excretion of glutamate. Since synthesis alone of the native components of the mycolate layer was not sufficient to restore its native properties, the packing of the mycolate layer seemed to be crucial for its low permeability.

The physiological function of one of the three different trehalose metabolic pathways of *C. glutamicum*, of the OtsAB-pathway, was unknown. Analysis of trehalose synthesis of strains defective in individual trehalose synthesis pathways showed that the OtsAB-pathway was the predominant trehalose synthesis pathway under carbon limiting conditions. Since trehalose is the predominant compatible solute under nitrogen limitation, the OtsAB-pathway might be necessary to synthesise trehalose as protectant against osmotic stress, when *C. glutamicum* is exposed to the coincidental limitation of carbon and nitrogen, which occurs frequently in its natural soil habitat.

Abbreviations

AGM	Arabinogalactan mycolate
ATP	Adenosine 5'-triphosphate
BHI	Brain heart infusion
BSA	Bovine serum albumin
C	carbon
cdw	Cell dry weight
CTAB	N-cetyl-N,N,N-trimethylammoniumbromide
EDTA	Ethylenediamine-tetraacetic acid
FBP	Fibronectin binding protein
GC	Gas chromatography
GMM	Glucose monomycolate
HPLC	High performance liquid chromotography
kb	Kilo basepares
KPi	Potassium phosphate buffer
LB	Luria-Bertani
LDH	Lactate dehydrogenase
MIC	Minimum inhibitory concentration
MM	Minimal medium
MSTFA	N-methyl-N-trimethylsilyltrifluoracetamide
N	Nitrogen
OD	Optical density
ODHC	2-oxoglutarate dehydrogenase complex
OPA	Ortho-phthaldialdehyde
ORF	Open reading frame
osM	osmolal, dimension of osmolality, definied as number of osmotically active particles per kg solution
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PK	Pyruvate kinase
PPP	Pentose phosphate pathway
PTS	Phosphotransferase System
PTV	Programmed temperature vaporizer
TCA	Trichloro acetic acid
TDM	Trehalose dimycolate

THF	Tetrahydrofurane
TLC	Thin-layer chromatography
TMM	Trehalose monomycolate

1 Introduction

1.1 Functions of Trehalose

Trehalose (α-1,1-glucopyranosyl-glucopyranose) is a non-reducing disaccharide present in a large variety of both prokaryotes and eukaryotes, such as bacteria yeast, fungi, insects, invertebrates and plants, but not in mammals (Argüelles *et al.*, 2000; Elbein *et al.,* 2003). Its unique physical properties, including high hydrophilicity, chemical stability, non-hygroscopic glass formation and the absence of internal hydrogen bonding, make it an ideal protectant against different stresses such as heat, cold, dessication or osmotic stress. Furthermore, trehalose serves as carbon and energy source or as storage carbohydrate and controls metabolic pathways as a signalling molecule in yeast and in plants. In *Corynebacterineae,* a suborder within the *Actinomycetales*, trehalose is a crucial building block of the cell envelope. Exceptionally for Gram-positive bacteria, the cell envelope of *Corynebacterineae* comprises an outer lipid bilayer, named mycolate layer, distinct from the plasma membrane, which is composed of trehalose and mycolic acids. (Argüelles *et al.*, 2000; Reinders *et al.*, 1997; Hounsa *et al.,* 1998; Fillinger *et al.,* 2001; Elbein *et al.,* 2003).

In *Corynebacterium glutamicum* trehalose is utilized as protectant against osmotic stress, but it cannot serve as a carbon source. Since *C. glutamicum* belongs to the suborder of *Corynebacterineae,* its cell envelope comprises trehalose as a building block of the mycolate layer. The redundancy of three different pathways for trehalose synthesis emphasises the importance of trehalose for *C. glutamicum* (Wolf *et al.,* 2003; Wolf, 2002).

1.1.1 Trehalose as protectant against environmental stress

Unique chemical and physical properties make trehalose a good protectant against various stresses. Since trehalose is a non-reducing disaccharide which does not form internal hydrogen bonds, it is an inert substance which is stabile over a wide range of temperature and pH. Analogous to other compatible solutes, trehalose stabilizes the native configuration of proteins by preferential exclusion to protect them against osmotic stress (Arakawa & Timasheff, 1985). Furthermore, trehalose protects proteins during extreme drying in the complete absence of water. Trehalose forms hydrogen bonds between its hydroxyl groups and the polar residues of the protein to maintain the native conformation of the protein (Carpenter & Crowe, 1989).

One option for *C. glutamicum* to cope with osmotic changes is the *de novo* synthesis of trehalose (Wolf *et al.* 2003). The level of trehalose accumulation depends on the nutrient supply, especially the availability of nitrogen and the nature of the carbon source. Under

nitrogen limiting conditions trehalose is the predominant compatible solute, while in the presence of excess nitrogen trehalose synthesis is lower in favour of proline synthesis. *C. glutamicum* accumulates more trehalose grown on maltose than on sucrose.

1.1.2 Trehalose as carbon source

Microorganisms utilizing trehalose as carbon and energy source require an uptake system for trehalose and a possibility to degrade the disaccharide to glucose. *Escherichia coli* takes up trehalose *via* a phosphotransferase (PTS). The resulting trehalose-6-phosphate is cleaved by a phosphotrehalase into glucose and glucose-6-phosphate (Boos *et al.,* 1987; Boos *et al.,* 1990). At least two trehalose transport systems are present in *Saccharomyces cerevisiae*: the high-affinity H^+-trehalose symporter Agt1p and a low-affinity uptake system that could be a facilitated diffusion process (Han *et al.,* 1995; Stambuck *et al.,* 1996). Trehalose can be hydrolysed to glucose either by the neutral trehalase *Nth1* or by the acid trehalase *Ath1* (Jules *et al.,* 2004).

C. glutamicum cannot utilize trehalose as carbon source indicating that this bacterium has either no uptake system for trehalose or no possibility to degrade it into a consumable form. Analysis of the genome sequence provided no information which of the two systems might not function in *C. glutamicum* since similarities neither to genes encoding uptake systems for trehalose nor to genes encoding trehalases were identified (Wolf, 2002). Nevertheless, a degradation system for trehalose was detected in *C. glutamicum.* The trehalose synthase TreS which catalyses *in vitro* the transglycosylation of maltose to trehalose and the reverse reaction in equilibrium, was found to degrade rather than synthesise trehalose under physiological conditions (Wolf *et al.,* 2003). The presence of a degradation possibility indicates that the inability of *C. glutamicum* to utilize trehalose as carbon source might be caused by lack of an uptake system for trehalose.

1.1.3 Trehalose as component of the bacterial cell envelope

The cell envelope of Gram-negative bacteria contains a membrane composed of phospholipids and lipopoysaccharides which is distinct from the plasma membrane, whereas cell walls of most Gram-positive bacteria lack an outer membrane and are largely composed of peptidoglycan (Schlegel & Zaborosch, 1992). Although bacteria such as corynebacteria or mycobacteria which are members of the suborder of the *Corynebacterineae* are Gram-positive bacteria harbouring a peptidoglycan based cell envelope, their cell envelope comprises an outer membrane similar to the cell envelope of Gram-negative bacteria. This additional membrane, named mycolate layer, is composed of the glycolipids trehalose

monomyclolate (TMM) and trehalose dimycolate (TDM), which consist of a molecule trehalose esterified by one or two mycolic acids, and mycolic acids covalently linked to the cell wall compound arabinogalactan (Brennan & Nikaido, 1995). Trehalose is not only a component of the mycolate layer as building block of TMM and TDM, but also involved in the synthesis of arabinogalactan bound mycolate because TMM is believed to serve as mycolyl-donor for the synthesis of arabinogalactan mycolate (AGM) as well as of TDM (Shimakata & Minatogawa, 2000). Consequently, trehalose should be essential for the synthesis of TDM, TMM and arabinogalactan mycolate, the three main components of the mycolate layer.

In mycobacteria the mycolate layer is responsible for the extremely low permeability of the cell envelope and consequently for the resistance of the human pathogen *Mycobacterium tuberculosis* to most common antibiotics (Brennan & Nikaido, 1995). Furthermore, trehalose dimycolate, also known as cord factor, plays a role in the persistence of this pathogenic bacterium in the host cell, presumably by inhibiting the fusion between lysosomes and phagosomes containing the bacteria (Spargo *et al.*, 1991). The function of a variety of other trehalose containing glycolipids such as acetylated trehalose or sulphate containing glycolipids remains to be clarified.

In *C. glutamicum* trehalose seemed indeed to be essential for the synthesis of the mycolate layer since the trehalose deficient *C. glutamicum* ATCC 13032 strains Cgl*ΔotsAΔtreYΔtreS* and Cgl*ΔotsAΔtreY* were devoid of mycolate when they were cultured on sucrose as carbon source (Wolf *et al.*, 2003). However, when the trehalose deficient Cgl*ΔotsAΔtreY* was supplemented with maltose, this strain synthesised arabinogalactan mycolate and a glycolipid which was neither TMM nor TDM. This glycolipid was identified as maltose monomycolate (Wolf, 2002; M. Daffé, personal communication). The synthesis of maltose monomycolate suggests that apart from trehalose other sugars may be involved in mycolate synthesis. This suggestion was supported by the observation that glucose monomycolate was synthesised by a *C. glutamicum* strain deleted in the mycolyltransferase PS1 (Puech *et al.,* 2000). The type of carbon source could provide a tool to manipulate the mycolate composition in a trehalose deficient *C. glutamicum* strain to characterize the properties of the mycolate layer.

1.2 Trehalose synthesis pathways in *C. glutamicum*

At least five different trehalose synthesis pathways are identified in bacteria. Three pathways utilize activated glucose as substrate, while the others convert maltose or maltodextrin into trehalose. The most common synthesis route is the OtsAB-pathway. It catalyses the condensation of UDP-glucose and glucose-6-P to trehalose-6-P and the following

dephosphorylation to trehalose (Argüelles *et al.*, 2000). The trehalose glycosyltransferring synthase TreT in the hyperthermophilic Archaeon *Thermococcus litoralis* forms trehalose and ADP from ADP-glucose and glucose (Qu *et al*., 2004). *Pyrococcus horikoshii* possesses a similar glycosyltransferase utilizing UDP-glucose and glucose as substrate (Ryu *et al.*, 2005). In contrast, the bacterial species *Rhizobium* and *Arthrobacter*, the Archaeum *Sulfolobus* and *Brevibacterium helvolobum,* a close relative of *Corynebacterium glutamicum,* and mycobacteria utilize the storage carbohydrate maltodextrin as substrate for trehalose synthesis by the TreYZ-pathway (Maruta *et al.*, 1996a/b/c; Kim *et al.*, 2000; De Smet *et al.*, 2000). In the first step, the terminal maltosyl-residue of maltodextrin is transglycosylated by TreY to a trehalosyl-unit which is subsequently cleaved off by TreZ. A further way to synthesise trehalose is the transglycosylation of maltose to trehalose catalysed by the trehalose synthase TreS in a single step reaction. Not in all organisms is the synthesis of trehalose the prevalent direction of the reaction catalysed by TreS. Whereas in mycobacteria TreS catalyses predominantly the synthesis of trehalose (Pan *et al*., 2004), in *Rhodobacter sphaeroides f. sp. denitrificans* IL106 TreS catalyses the synthesis of maltose (Makihara *et al*., 2005).

In contrast to other bacteria, *C. glutamicum* possesses not only one trehalose synthesis pathway, but three of the described pathways - OtsAB, TreYZ and TreS - indicating an important function of trehalose (Fig. 1). Whereas an *in vitro* enzymatic assay showed that the trehalose synthase TreS of *C. glutamicum* catalysed the transglycosylation of trehalose to maltose and the reverse reaction in equilibrium, under *in vivo* conditions TreS catalyses in *C. glutamicum* the degradation rather than the synthesis of trehalose (Wolf *et al.*, 2003). Since no trehalases were identified in *C. glutamicum*, the TreS-pathway could be an alternative for the degradation of trehalose. The TreYZ-pathway seems to be the main trehalose synthesis pathway in *C. glutamicum.* It is responsible for the accumulation of trehalose as a compatible solute after a hyperosmotic shock. RNA-hybridisation experiments indicated that also the OtsAB-pathway is involved in the response of *C. glutamicum* to an osmotic shock, since the *otsA* gene was upregulated five fold after an osmotic upshift (Wolf *et al.*, 2003). In contrast, data of Shimakata and Minatogawa (2000) suggest a function of the OtsAB-pathway in the synthesis of the cell envelope component trehalose monomycolate. These authors concluded from an *in vitro* assay that trehalose-6-phosphate, the product of OtsA, the first enzyme of the OtsAB-pathway, is essential for the biosynthesis of trehalose monomycolate. This conclusion was contradicted by the fact that a *C. glutamicum* strain deleted in the *otsA* gene synthesised trehalose mycolate (Wolf *et al.*, 2003). Hence, the function of the OtsAB-pathway remained unclear. Only the simultaneous deletion of all three trehalose synthesis pathways in *C. glutamicum* resulted in a mutant devoid of mycolate suggesting that not OtsA alone, but both the enzymes of the OtsAB- and of the TreYZ-

pathway are involved in the synthesis of the components of the mycolate layer (Wolf *et al.*, 2003).

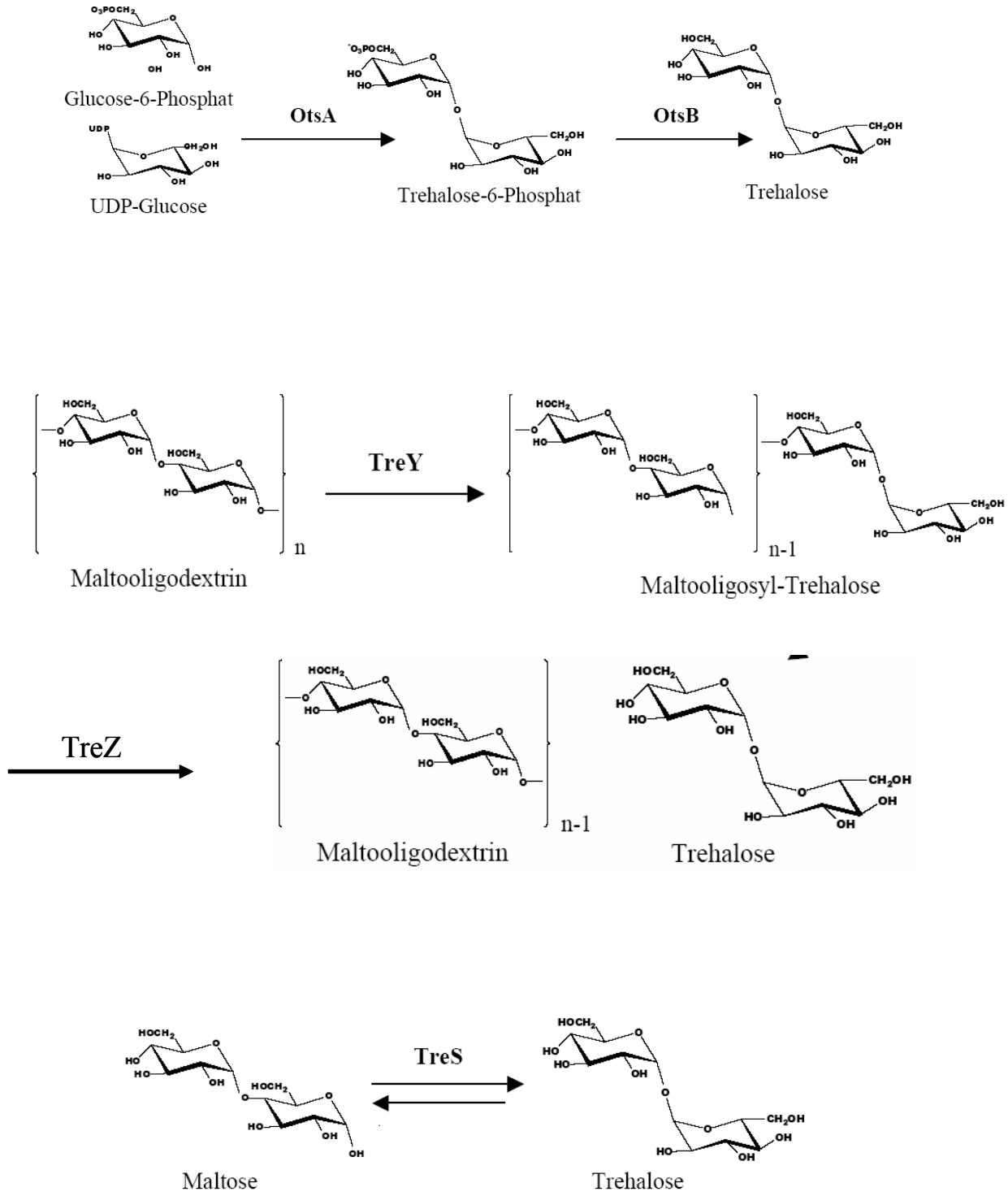


Fig. 1: Trehalose biosynthesis pathways in *C. glutamicum*

1.3 Corynebacterial cell envelope

C. glutamicum shares the structure of the cell envelope with all members of the suborder of *Corynebacterineae* which includes also nocardia, rhodococci and mycobacteria (CNM-group) (Fig. 2). The plasma membrane is the innermost layer of the cell envelope which is protected by the cell wall skeleton. Peptidoglycan covalently linked to arabinogalactan which in turn is esterified by mycolic acids forms the cell well skeleton. The mycolic acids pair with trehalose mycolates to a lipid bilayer called mycolate layer. This lipid bilayer in addition to the plasma membrane is a phylogenetic trait of the *Corynebacterineae* which distinguishes their cell envelope from a usual cell envelope of Gram-positive bacteria. The outer layer of the cell envelope consists to 90 % of polysaccharides. In some corynebacterial strains the S-layer protein is attached to the outermost surface (Puech *et al.*, 2001).

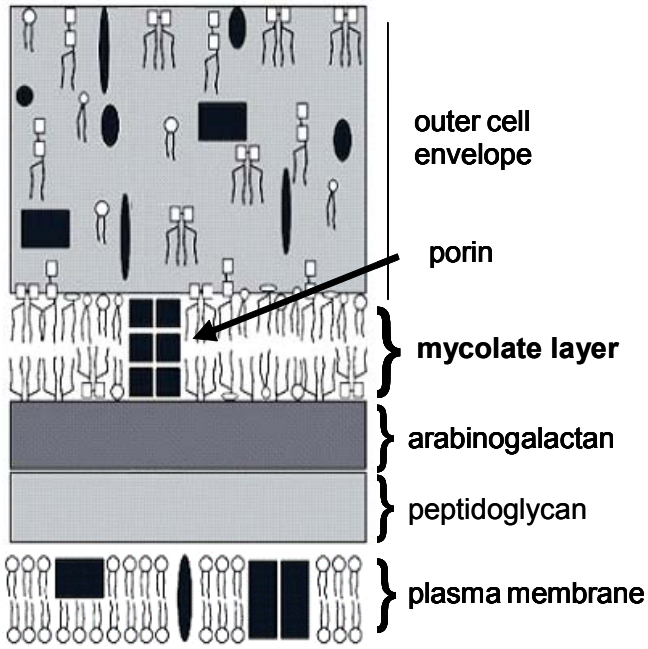


Fig. 2: Model of the cell envelope of *C. glutamicum* according to Puech *et al.* (2001), modified. From the cytoplasmic to the external side of the bacteria the cell envelope is composed of the plasma membrane, a cell wall skeleton and an outer layer. The plasma membrane is a lipid bilayer of phospholipids (empty oval symbols) and proteins (dark rectangles and elypses). The cell wall skeleton consists of peptidoglycan covalently linked to arabinogalactan which in turn is esterified by corynomycolic acids (thin parallel bars). The covalently bound mycolic acids arrange to the inner leaflet of a lipid layer with non-covalently linked lipids such as trehalose dimycolate (a pair of empty squares with two pairs of thin parallel bars) and trehalose monomycolate (a pair of empty squares with one pair of thin parallel bars). The outer leaflet of the lipid layer which is called mycolate layer is formed by non-covalently linked trehalose mycolate. Porins (dark squares) span the mycolate layer. The outer layer consists to 90% of polysaccharides, but contains also non-covalently linked lipids and proteins.

1.3.1 Plasma membrane

The plasma membrane of *C. glutamicum* is mainly composed of polar phospholipids possessing palmitic ($C_{16:0}$) and octadecenoic ($C_{18:1}$) acids as fatty acid chains. Phosphatidylglycerol is the main head-group of phospholipids found in *C. glutamicum* (80 %) complemented by diphosphatidylglycerol (cardiolipin), phosphatidylinositol and phosphatidylinositol dimannosides (PIM_2).

1.3.2 Cell wall skeleton

Three different layers build the cell wall skeleton in *C. glutamicum*. As common in all Gram-positive bacteria a thick peptidoglycan layer neighbours the plasma membrane. In *Corynebacterineae* as in most other bacteria the peptidoglycan layer is composed of β-1,4-linked N-acetylglucosamine and N-acetyl muramic acid residues linked to tri- or tetrapeptides such as L-Ala-D-Glu-*meso*-diaminopimelic acid or L-Ala-D-Glu-*meso*-diaminopimelic acid-D-Ala (Schleifer & Kandler, 1972).

The second layer consists of the heteropolysaccharide arabinogalactan which is composed of a linear alternating β-D-galactofuranosyl backbone connecting to a 3,5-branched α-D-arabinofuranosyl structure. The galactofuranosyl backbone is attached by a phosphodiester link to the peptidoglycan. The terminal β-arabinofuranosyl residues are esterifyed on position 5 by mycolic acids (Puech *et al*., 2001; Alderwick *et al*., 2005).

Mycolic acids are long chain α-alkyl-β-hydroxy fatty acids and characteristic components of the mycolate layer, the third layer of the cell wall skeleton. In mycobacteria mycolic acids possess a very long chain (C_{60-90}) and may be oxygenized or hydroxylated, whereas in nocardia and corynebacteria the alkyl-chain is shorter and the mixture of saturated and unsaturated fatty acids homologous (C_{40-50} nocardomycolic acids; C_{22-36} corynomycolic acids) (Qureshi *et al*., 1984). Mycolic acids linked to arabinogalactan constitute the arabinogalactan mycolate (AGM) or cell wall-bound mycolate. The esterification of one or two mycolic acids with a molecule trehalose yields trehalose monomycolate (TMM) and trehalose dimycolate (TDM), respectively, which are part of the extractable mycolate. TMM is the mycolyl-donor for the synthesis of arabinogalactan mycolate and TDM (Shimakata & Minatogawa, 2000). Electron microscopy of freeze-fractured preparations of whole cells of *C. glutamicum,* a method revealing lipid bilayers by characteristic fracture planes, indicated that the cell envelope of the *C. glutamicum* wild type contained, additionally to the plasma membrane, a second lipid bilayer closer to the cell surface. Since this lipid bilayer was missing in *Corynebacterium amycolatum,* a naturally mycolate deficient strain, it was considered to be composed of mycolate (Puech *et al*., 2001). Whereas the inner lipid layer of the mycolate layer is formed mainly by arabinogalactan mycolate and minor amounts of extractable mycolate, the outer leaflet consists exclusively of TDM and TMM (Daffé & Draper, 1998;

Puech *et al.,* 2001). Thus, the cell envelope of the Gram-positive *Corynebacterineae* possesses an outer membrane similar to the cell envelope of Gram-negative bacteria. In Gram-negative bacteria phospholipids form the inner and lipopolysaccharides the outer leaflet of the outer membrane. This outer membrane exhibits a permeability barrier for hydrophilic as well as hydrophobic solutes. The low permeability of the outer membrane explains why Gram-negative bacteria are more resistant to some antibiotics than most Gram-positive bacteria (Schlegel & Zarborosch, 1992). In contrast, Jalier and Nikaido (1994) showed that the permeability of the cell envelope to some hydrophilic antibiotics of the Gram-positive *Mycobacterium chelonea,* harbouring a mycolate layer, was 3 times lower than that of Gram-negative *E. coli* and 10 times lower than that of Gram-negative *Pseudomonas aeruginosa*. Similar to the outer membrane in Gram-negative bacteria, in mycobacteria the mycolate layer is considered to be responsible for the low permeability of the cell envelope and consequently it is supposed to be crucial for the low susceptibility to most antibiotics (Brennan & Nikaido, 1995). A correlation of mycolate layer and permeability of the cell envelope in *C. glutamicum* was revealed by the inactivation of a mycolyltransferase in *C. glutamicum.* The inactivation decreased the cell wall linked-mycolate of the mutant to 50 % and caused increased uptake rates for glycerol and acetate (Puech *et al*., 2000). These results indicated that the nature of the mycolate layer may influence also the permeability of the cell envelope in *C. glutamicum.*

1.3.3 Porins

Similar to the outer membrane in Gram-negative bacteria, the mycolate layer of the Gram-positive *Corynebacterineae* may constitute a permeability barrier impeding the uptake of hydrophilic nutrients. The outer membrane of Gram-negative bacteria contains channel-forming proteins named porins facilitating the permeation of small hydrophilic molecules across the outer membrane. These porins are trimers of identical subunits. Each monomer contains one channel of 4 nm length (Cowan *et al*., 1992). Also the mycolate layer of *Corynebacterineae* comprises porins which passage hydrophilic solutes through the lipid bilayer, but the structure of these porins differs from that of the Gram-negative porins. In *Corynebacterineae* a porin is an oligomeric protein forming a single channel (Kartmann *et al*., 1999; Lichtinger *et al*., 1998; Riess *et al*., 1998). The best studied porin of this type is MspA, the main porin of *Mycobacterium smegmatis.* It is a tetrameric-protein forming a cone-like structure with a single pore of 10 nm length. A further difference between *Corynebacterineae* and Gram-negative bacteria is the number of porins. *M. smegmatis* contains 15-fold less porins per μm^2 cell wall than a Gram-negative bacterium which results in 45 less pores per μm^2 since one porin of a Gram-negative bacterium contains three pores. Longer channels

and lower pore numbers may cause lower permeability of the cell envelope of *M. smegmatis* compared to Gram-negative bacteria (Niederweis, 2003). Expression of MspA in the pathogenic and slow growing *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG accelerated the uptake of glucose and enhanced the susceptibility to β-lactam antibiotics and to isoniazid, ethambutol and streptomycin, indicating that porins are responsible for nutrient supply as well as the uptake of drugs currently used in tuberculosis chemotherapy (Mailaender *et al.*, 2004). Furthermore, deletion of the porin gene *mspA* in the non-pathogenic *M. smegmatis* rendered it multidrug resistant (Stefan *et al*., 2004) and enhanced its intracellular persistence in macrophages, demonstrating that persistence depends on the permeability of the mycolate layer (Sharbati-Tehrani *et al*., 2005).

In *C. glutamicum* three different porins are identified which exhibit an oligomeric structure similar to MspA. The major porin is PorA, a cation-selective cell wall channel formed by an oligomer of a 45-amino-acid polypeptide (Lichtinger *et al*., 1998 and 2001). The characterisation of a *porA* deletion mutant indicated the presence of an alternative anion-selective cell wall channel with low channel conductivity. An anion-selective channel protein was identified with a conductance of 700 pS in 1M KCL and called PorB. Analysis of the genome sequence of *C. glutamicum* suggested the presence of a further anion-selective porin similar to PorB which was named PorC (Costa-Riu *et al*., 2003a/b). Additionally to PorA, the cation-selective channel PorH was identified (Hunten *et al*., 2005). Since the pore diameter of PorA is broader and the channel conductivity higher than that of the other porins, PorA was considered as the predominant hydrophilic channel in *C. glutamicum.* This assumption was confirmed by the fact that the deletion of the *porA* gene in the *C. glutamicum* wild type ATCC 13032 rendered the mutant less susceptible to the antibiotics ampicillin, kanamycin, streptomycin and tetracycline (Costa-Riu *et al*., 2003a). Furthermore, higher resistance to the antibiotics of the *porA* deletion mutant indicated that similar to mycobacteria, also the mycolate layer in *C. glutamicum* constitutes a permeability barrier for the uptake of antibiotics. Whereas in mycobacteria the impact of the mycolate layer on the uptake of antibiotics is of special interest, in *C. glutamicum* the influence of the cell envelope on the efflux of solutes is a crucial issue*,* since it is one of the most important industrial producers of amino acids.

1.3.4 Outer layer

The outer layer of the corynebacterial cell envelope consists to 90% of polysaccharides which are composed of glucose, mannose and arabinose (Puech *et al*., 2001). Some corynebacterial strains, but not the strains used in this study, have a protein attached to the cell surface – the S-layer protein – PS2 (Chami *et al*., 1997). Furthermore, the outer layer

contains various lipids, mostly TDM and TMM, but also phospholipids like PIM_2 and phosphatidyl glycerol (Puech *et al*., 2001).

1.4 Biosynthesis of mycolic acids and of mycolate

Mycolic acids are α-alkyl-β-hydroxy fatty acids and a phylogenetic trait of the *Corynebacterineae* suborder within the *Actinomycetales*. As the α-alkyl-β-hydroxy structure, the so called mycolic motif, is common to the whole suborder, the enzymatic set for mycolic acid biosynthesis is supposed to be shared by the *Corynebacterineae*, but the mechanism of mycolic acid biosynthesis is not known in detail. Use of cell-free extracts showed that two palmitic acids serve as precursors for the condensation to a C_{32} mycolic acid (Shimakata *et al*., 1984; Walker *et al*., 1973). Recently, the enzyme catalysing the condensation of two fatty acids to a mycolic acid was identified as polyketide synthase Pks13 in *C. glutamicum* (Portevin *et al.,* 2004).

Also the following mycolate synthesis is still a matter of discussion. The first step is the transport of a newly synthesised mycolic acid and its condensation with trehalose to yield trehalose monomycolate (TMM). Based on results of an *in vitro* assay in *Corynebacterium matruchotii* Shimakata and Minatogawa (2000) suggest a role of trehalose-6-phosphate as an intermediate acceptor of mycolic acids. Contradicting these results, Wolf *et al.* (2003) demonstrated that trehalose-6-phosphate is not essential for mycolate syntheses in *C. glutamicum in vivo.* A mutant devoid of trehalose-6-phosphate due to deletion of *otsA,* the gene encoding trehalose-6-phosphate synthase, synthesised trehalose mycolate. In contrast, in Cgl Δ*otsA*Δ*treS*Δ*treY,* which is devoid of both trehalose and trehalose-6-P due to inactivation of all trehalose synthesis pathways, no mycolate was detected. These results emphasize the essentiality of trehalose rather than the importance of trehalose-6-phosphate for mycolate synthesis. Regardless of the acceptor, a mycolyltransferase is predicted for synthesis of TMM. The identity of this mycolyltransferase is unclear.

The last steps of mycolate synthesis are the least controversial. Mycolyltransferases transfer the mycolyl-residue from TMM either to the free position 6 of the trehalose-residue of another TMM to yield trehalose dimycolate or to a terminal arabinofuranosyl of the arabinogalactan layer to yield arabinogalactan mycolate. In *Mycobacterium tuberculosis* the three antigen 85 proteins were identified as mycolyltransferases by site directed mutagenesis and *in vitro* enzymatic assays. The antigen 85 proteins, which are also known as fibronectin binding enzymes (FBP), comprise a fibronectin-binding site which enables interaction with macrophages of the host immune system. The fibronectin-binding-site demonstrates that these mycolyltransferases are exported from the cell. The presence of a signal peptide which facilitates export through the plasma membrane is a further proof of the extracellular activity

of these enzymes. Since the mycolyltransferases are exported from the cytoplasm, the synthesis of TDM and AGM is supposed to be localised in the cell envelope (Belisle *et al*., 1997; Ronning *et al*., 2000; Ronning *et al*., 2004).

In *C. glutamicum* the first mycolyltransferase identified due to sequence similarity to the antigen 85 proteins was encoded by the *csp-1* or *cop-1* gene and called PS1 (Joliff *et al.,* 1992). A *csp-1* mutant accumulated TMM and synthesised less TDM than the wild type proving the mycolyltransferase activity of PS1 and indicating the presence of further mycolyltransferases in *C. glutamicum.* In the genome of *C. glutamicum* six putative mycolyltransferase genes were identified. One gene was the already known, *cop-1.* The other five were named *cmt1-5* (Brand *et al*., 2003) or *cmytA-E* (Sousa-D'Auria *et al*., 2003). Since a *C. glutamicum* ATCC 13032 mutant deleted in *cop1*, *cmt1* and *cmt2* could not synthesise TDM, but the single and double mutants produced TDM, Brand *et al*. (2003) concluded that *cop1*, *cmt1* and *cmt2* code for mycolyltransferases synthesising TDM. Cross-complementation experiments in *C. glutamicum* CGL 2005 suggested two different classes of mycolyltransferases. CMytA (PS1) and cMytB (Cmt2) could replace each other in TDM as well as in arabinoglactan-mycolate synthesis, whereas a *cmytA* deletion mutant was only complemented in TDM production by the overexpression of *cmytC* (*cmt1*) or *cmytD* (*cmt5*). Sousa-D'Auria *et al*. (2003) concluded that the mycolyltransferases cMytA (PS1) and cMytB (Cmt2) transfer a mycolyl-residue to TMM as a well as to arabinogalactan, whereas cMytC (Cmt1) and cMytD (Cmt5) synthesise TDM, but no arabinogalactan mycolate.

1.5 Amino acid production with *C. glutamicum*

1.5.1 L-lysine production with *C. glutamicum*

L-lysine is an essential amino acid for mammals which has to be provided in sufficient amounts in animal feed to meet the nutritional requirements. Since plant based feedstuff like corn, wheat or barley is poor in lysine, supplementation with lysine provides a possibility to increase the nutrient value. *C. glutamicum* is the sole organism utilized for industrial production of L-lysine which exceeds 600,000 tons per year of lysine. Lysine belongs to the aspartate familiy of amino acids as well as homoserine, methionine, threonine and leucin, which are all synthesised starting from oxalacetate, a component of the tricarboxylic acid cycle (Fig. 3).

Lysine biosynthesis is regulated by the aspartate-kinase which is feedback inhibited by lysine and threonine. Since this feedback inhibition limits lysine synthesis, an aspartate-kinase resistant to feedback inhibition provides a tool to optimize lysine production. A further option

to increase lysine synthesis is the restriction of the synthesis of the by-products homoserine, methionine and threonine. Conventional lysine production strains are based on the mutagenesis of aspartate-kinase and of enzymes of by-product synthesis, since they were obtained by random mutagenesis followed by screening for resistance to lysine or threonine analogues and/or nutritional requirement for by-products (Nakayama *et al.,* 1973).

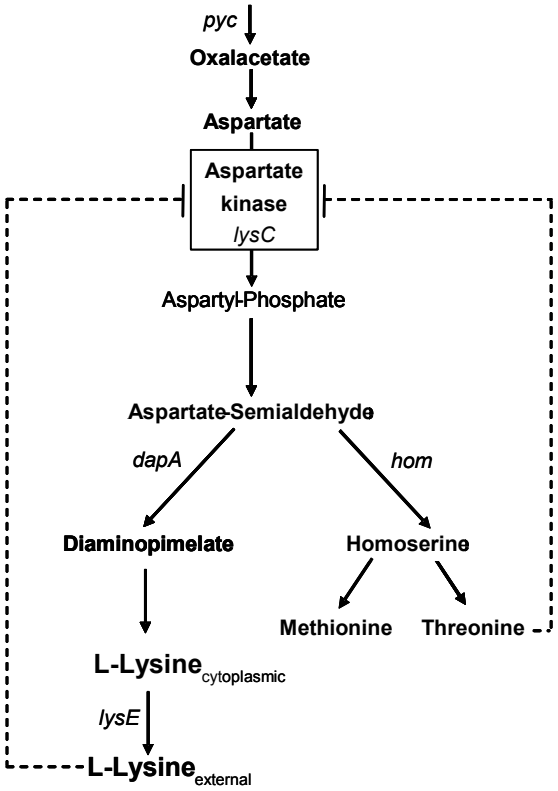


Fig. 3: Biosynthesis of the amino acids of the aspartate-family in *C. glutamicum.* Dashed arrows, regulation at enzyme level (feedback inhibition).

A further approach to improve lysine production uses genetic engineering. Defined mutagenesis avoids secondary mutations which affect growth or stability. A crucial target also of genetic engineering is the feedback inhibition of the aspartate-kinase which is encoded by *lysC.* Overexpression of *lysC* alleles encoding aspartate-kinases, which were not feedback inhibited, increased lysine synthesis (Thierbach *et al.,* 1990). A genetic approach to reduce the formation of by-products is the inactivation of the homoserine dehydrogenase (*hom*) the first enzyme of the metabolic branch leading to homoserine, methionine and threonine synthesis (Eikmanns *et al.,* 1991). Consequently, overexpression of *dapA* encoding dihydrodipicolinate synthase, an enzyme of the lysine biosynthesis pathway competing with the homoserine dehydrogenase for their common substrate L-aspartate semialdehyde, increased lysine synthesis (Eggeling *et al.,* 1998). A further bottleneck for lysine production are anaplerotic reactions as shown by overexpression of *pyc* encoding

pyruvate carboxylase which synthesises oxalacetate, the precursor of all amino acids of the aspartate familiy (Peters-Wendisch *et al.,* 2001).

A further obstacle for the production of lysine is the export of the amino acid. The first permeability barrier for lysine is the plasma membrane. Transport across the plasma membrane is mediated by the exporter LysE (Broer *et al.,* 1991a/b; Vrljic *et al.,* 1995; Vrljic *et al.,* 1996; Bellmann *et al.,* 2001). Product excretion seems to constitute a further bottleneck for lysine production, since overexpression of *lysE* increased lysine excretion (Pfefferle *et al.,* 2003). Apart from the plasma membrane the cell envelope of *C. glutamicum* comprises a second lipid bilayer, the mycolate layer, which is supposed to impede the permeation of solutes. However, the exact impact of the mycolate layer on the excretion of amino acids is unknown (Eggeling & Sahm, 2001).

1.5.2 L-glutamate production with *C. glutamicum*

Although more than 1,000,000 tons per year of L-glutamate are produced by *C. glutamicum*, little is known about the mechanism causing efflux of glutamate from the cell. Special treatment of the cell is always required such as biotin limitation, temperature upshift or addition of penicillin (Kimura, 2003). Some of these treatments interfere with the fatty acid composition of the plasma membrane e. g. the biotin containing acyl-CoA carboxylase, the first enzyme of fatty acid biosynthesis, might be the target of biotin limitation. The “leak model” suggested that increased permeability of the plasma membrane facilitated the passive diffusion of glutamate, but evidence for an L-glutamate exporter ruled out this model (Hoischen & Krämer, 1990; Gutmann *et al.,* 1992).

Currently, two different models for glutamate efflux are discussed. Eggeling & Sahm (2001) propose that the excretion of glutamate is determined by the structure of the corynebacterial cell envelope. Treatments inducing glutamate excretion may facilitate the passage through the mycolate layer by increasing its permeability. Since the components of the cell wall skeleton peptidoglycan, arabinogalactan and mycolic acids are linked by covalent bonding, alteration of any of these components influences the mycolate layer. Thus, treatment with penicillin, which inhibits the synthesis of peptidoglycan, as well as addition of ethambutol, which interferes with the synthesis of arabinogalactan, may increase the permeability of the mycolate layer (Nunheimer *et al.,* 1970; Radmacher *et al.,* 2005b).

Treatments which manipulate fatty acid synthesis, e. g. biotin limitation, influence the fatty acid composition of the plasma membrane and they may similarly alter the composition of mycolate layer since fatty acids are essential for mycolate synthesis. This assumption was supported by the inactivation of the two fatty acid synthases FAS-IA and FAS-IB in *C. glutamicum.* The inactivation mutants *fasA* and *fasB* excreted glutamate without special

treatment and they exhibited an altered plasma membrane as well as a reduced amount of mycolic acids (Radmacher *et al.,* 2005a).

Systematic genetic engineering of fatty acid biosynthesis indicated that also the alteration of the plasma membrane may influence glutamate excretion since the appropriate lipid composition and the suitable membrane tension activate the L-glutamate carrier (Nampoothiri *et al.,* 2002).

Kimura (2003) explains glutamate overproduction by the “metabolic flux change model”. Already 30 years ago the central role of the activity of the 2-oxoglutarate dehydrogenase complex (ODHC) in glutamate production had been discovered (Shingu & Terui, 1971). ODHC belongs to the tricarboxylic acid cycle (TCA cycle) and catalyses the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA. Thus, ODHC competes with glutamate dehydrogenase, the first enzyme of the glutamate synthesis pathway, for their common substrate 2-oxoglutarate. Low ODHC activity may shift metabolic fluxes from the tricarboxylic acid cycle to glutamate production. The significance of ODHC for glutamate excretion was confirmed by the observation that activity of ODHC was reduced under conditions which cause glutamate overproduction such as biotin limitation or treatment with Tween 40 or penicillin (Kawahara *et al.,* 1997), and that inactivation of ODHC by disrupting the *odhA* gene triggered glutamate excretion (Kimura, 2005). Analysis of metabolic fluxes proved that decreased ODHC activity directed carbon fluxes towards glutamate synthesis (Shirai *et al.,* 2005).

How the special treatments triggering glutamate efflux influence the change of metabolic fluxes is unknown. Kimura (2005) suggests that fatty acid synthesis, the target of most treatments, and metabolic fluxes in TCA cycle and glutamate synthesis are controlled on the transcriptional level by the same global metabolic regulator.

1.6 Objectives of the PhD-Thesis

The first part of this project assessed the role of trehalose as protectant against osmotic stress. Since an involvement of the OtsAB trehalose synthesis pathway in mycolate synthesis, as suggested by Shimakata and Minatogawa (2000) could be ruled out (Wolf *et al.*, 2003), the function of the OtsAB pathway remained unknown. RNA-hybridisation experiments indicated a role of this pathway in the response to osmotic stress in *C. glutamicum* (Wolf *et al.,* 2003). Since the TreYZ-pathway is regarded as the predominant trehalose synthesis pathway after a hyperosmotic shock, conditions were searched which required the OtsAB-pathway for the synthesis of trehalose under osmotic stress.

The major part of this project aimed to investigate the function of trehalose as an important component of the corynebacterial cell envelope. In contrast to other Gram-positive bacteria all members of the suborder of *Corynebacterineae*, including *C. glutamicum,* contain a cell envelope that comprises a second lipid bilayer apart from the plasma membrane, the mycolate layer, which is considered as permeability barrier (Puech *et al.,* 2001). An important building block for the biosynthesis of the mycolate layer is trehalose, since trehalose monomycolate (TMM) serves as a precursor for trehalose dimycolate (TDM) and arabinogalactan mycolate (AGM), which are, together with TMM, the main components of the mycolate layer. Whereas TDM and AGM are known to be synthesised in the cell envelope, it is unclear where the condensation to TMM is located. One objective of this project was to find out whether TMM is synthesised in the cell envelope like TDM and AGM or in the cytoplasm, where its building blocks trehalose and mycolic acids are synthesised.

Since the trehalose deficient *C. glutamicum* strains Cgl*ΔotsAΔtreYΔtreS* and Cgl*ΔotsAΔtreY* were devoid of mycolate when they were cultured on sucrose, trehalose seemed to be essential for mycolate synthesis (Wolf *et al.,* 2003). However, Cgl*ΔotsAΔtreY* cultivated in medium supplemented with maltose synthesised AGM and a glycolipid identified as maltose monomycolate (Wolf, 2002; M. Daffé, personal communication) indicating that trehalose was not the sole sugar acceptor for mycolic acids. This assumption was supported by the detection of glucose monomyolcate in a *C. glutamicum* strain deleted in the mycolyltransferase PS1 (Puech *et al.,* 2000). A further objective of this project was to analyse how the carbon source specifically influences the composition of the mycolate layer of a *C. glutamicum* strain inactivated in trehalose synthesis. Supplementation of the medium with trehalose was tested as an additional tool to influence the synthesis of the mycolate layer (Tzvetkov *et al.,* 2003). The comparison of strains harbouring differently composed mycolate layers should reveal how the composition determines the properties of the mycolate layer, especially its permeability. A *C. glutamicum* L-lysine production strain inactivated in trehalose biosynthesis was chosen for these experiments to examine whether excreted lysine as a measure for the efflux of solutes could be correlated to the permeability of the cell envelope. Moreover, analysis of this strain should reveal whether the alteration of the cell envelope improves lysine production.

2 Materials and Methods

2.1 Strains, plasmids and oligonucleotides

Tab. 1: Bacterial strains used in this study

Strain	Description	Reference
C. glutamicum		
ATCC 13032	Wild type	Abe *et al*. (1967)
Cgl∆*otsA*∆*treS*	Wild type carrying chromosomal deletions in the *otsA* and *treS* ORFs	Wolf *et al*. (2003)
Cgl∆*treY*∆*treS*	Wild type carrying chromosomal deletions in the *treY* and *treS* ORFs	Wolf *et al*. (2003)
Cgl∆*otsA*∆*treS*∆*treY*	Wild type carrying chromosomal deletions in the *otsA*, *treY* and *treS* ORFs	Wolf *et al*. (2003)
Cgl∆*otsA*∆*treS*∆*glgC*	Wild type carrying chromosomal deletions in the *otsA*, *treS* and *glgC* ORFs	this study
Cgl∆*treY*∆*treS*∆*glgC*	Wild type carrying chromosomal deletions in the *treY, treS* and *glgC* ORFs	this study
Cgl∆*glgC*	Wild type carrying chromosomal deletions in the *glgC* ORF	this study
ATCC 21527	L-Lysine production strain, *lysC*^{FBR}, *leu*CD, *hse*CD	Nakayama *et al.* (1972)
LP∆*treS*	L-Lysine production strain carrying a chromosomal deletion in the *treS* ORF	this study
LP∆*treS*∆*otsA*	L-Lysine production strain carrying chromosomal deletions in the *otsA* and *treS* ORFs	this study
LP∆*treS*∆*otsA*∆*treY*	L-Lysine production strain carrying chromosomal deletions in the *otsA*, *treY* and *treS* ORFs	this study

Tab. 2: Oligonucleotides used in this study.

Designation	Oligonucleotide sequence (5'-3')	Size of amplified fragment (kb)
otsAs	5'-tct gcc agt gga tat gac tgt cc-3'	1.808
otsAa	5'-cgt tga cgt cgt ggg tat aga cc-3'	
treYs	5'-gca cgt cca att tcc gca ac-3'	2.739
treYa	5'-tca aaa ctc act atc ggg tac-3'	
treSgs2	5'-atg act gat acc tct ccg ttg-3'	1.800
treSGa	5'-tca ttc cat atc gtc ctt ttc-3'	

Tab. 3: Plasmids used in this study

Plasmids	Description	Reference
pUC18/19	*plac*, Ap^R	Yanisch-Perron *et al.* (1985)
pUC18*otsA*	pUC18 carrying a 1.8 kb fragment containing the *otsA* ORF	Wolf, 2002
pUC18*treY*	pUC18 carrying a 2.7 kb fragment containing the *treY* ORF	Wolf, 2002
pUC19*treS*	pUC19 carrying a 2.5 kb fragment containing the *treS* ORF	Wolf, 2002
pK19*mobsacB*	*ori* pUC, Km^R, *mob sacB*	Schäfer *et al.* (1994)
pK19*mobsacB* Δ*otsA*	pK19*mobsacB* carrying a 0.21 kb deletion in the *otsA* ORF	Wolf *et al*. (2003)
pK19*mobsacB* Δ*treY*	pK19*mobsacB* carrying a 0.34 kb deletion in the *treY* ORF	Wolf *et al*. (2003)
pK19*mobsacB* Δ*treS*	pK19*mobsacB* carrying a 0.71 kb deletion in the *treS* ORF	Wolf *et al*. (2003)
pK19IMC	pK19*mobsacB* carrying Δ*glgC*	Eikmanns, Ulm

2.2 Media and culture conditions

All cultivations were performed in shake flasks under aerobic conditions. *Escherichia coli* strains were kept at 37 °C and *C. glutamicum* strains at 30 °C. In both cases, LB medium was used as complex medium. CgXII medium (Keilhauer *et al.,* 1993) was used as minimal medium for *C. glutamicum*. As carbon source 4 % sucrose, 4 % glucose or 4 % fructose were used. When indicated 2 % or 0.5 % of trehalose were added to the medium. The lysine producer strain ATCC 21527 and its derivatives were supplemented with 0.2 g/L L-leucin and 0.4-0.8 g/L D/L-homoserine. High osmolality was adjusted by the addition of 750 mM NaCl (equivalent to an osmotic upshift of approx. 1.5 osM). For all strain characterizations, cells from LB precultures were washed once in CgXII medium and used to inoculate CgXII precultures, which were grown to exponential or early stationary phase. Subsequently, from those cultures, the CgXII main cultures were inoculated to an initial optical density (OD_{600}) of 1. If the main culture differed from the preculture in any parameter, e.g. carbon source or NaCl content, a washing step between pre- and main culture was included.

2.3 Molecular biology methods

2.3.1 DNA digestion, ligation and purification

Standard techniques like DNA digestion (enzymes purchased from New England Biolabs; Schwalbach), ligation using T4 ligase (New England Biolabs, Schwalbach) and plasmid DNA purification (NucleoSpin Plasmid; Macherey Nagel, Düren) were performed according to the manufacturer's protocol.

2.3.2 Competent cells and transformation

Competent *E. coli* cells were prepared and transformed according to Inoue *et al.* (1990). Competent *C. glutamicum* cells were prepared as described by van der Rest *et al.* (1999) and transformed by electroporation (2.5 kV, 600 Ω, 2.5 µF) with a Gene-Pulser (Bio-Rad, München).

2.3.3 Polymerase chain reaction

The knock-out of genes in the genome in *C. glutamicum* mutants was examined by the amplification of DNA fragments containing internal deletions in the case of allelic replacement. Amplification of DNA fragments was carried out using *Taq* PCR Master Mix Kit (Qiagen, Hilden) according to the manufacturer's protocol. Per reaction 2.5 mM $MgCl_2$ were added. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). Sequence information on the *C. glutamicum* genome was generously provided by the Degussa AG (Hanau, Germany).

2.3.4 Agarose gel electrophoresis

To test *C. glutamicum* mutants for allelic replacement DNA fragments were amplified by PCR and analyzed by agarose gel electrophoresis (1 x TAE: 0.04 M Tris, 0.5 mM EDTA, pH adjusted to 7.5 with acetic acid) according to Sambrook *et al.* (1989). DNA extraction from the agarose gels was done with the NucleoSpin Extract II-Kit (Macherey Nagel, Düren) according to the manufacturer's protocol.

2.3.5 Construction of a strain defective in trehalose synthesis

The knock-out of genes in the genome of *C. glutamicum* ATCC 21527 was performed by the method of allelic replacement described by Schäfer *et al*. (1994) to construct a *C. glutamicum* lysine producer strain defective in all three trehalose synthesis pathways. Derivatives of the vector pK19*mobsacB* containing the coding regions of *otsA*, *treY* and *treS* with internal deletions were kindly provided by A. Wolf and described recently (Wolf *et al.*, 2003; Tab. 3). For the allelic replacement, competent *C. glutamicum* cells were transformed

with pK19*mobsacB* derivatives by means of electroporation (van der Rest *et al*., 1999). Plasmid integration into the genome was verified by selecting kanamycin-resistant and sucrose-sensitive colonies (the expression of the *sacB* gene encoding the levan sucrase is toxic in sucrose-containing media). To promote re-excision of the plasmid DNA, positive clones were grown overnight in LB broth containing 25 µg/ml kanamycin, washed and cultivated in CgXII without phosphate and ammonium source containing 0.5 % glucose for 6 h. Since the knock-out mutants were supposed to have a slower growth rate than the wild type, the starvation step should prevent that the knock-out mutants were overgrown by clones comprising the wild type allele. The starved cells were plated on LB agar supplemented with 15 % sucrose in different dilutions, usually between 10^{-3} and 10^{-5}. Positive colonies obtained from the subsequent selection (Km^S, Suc^R) were tested for allelic replacement by PCR (oligonucleotides are listed in Tab. 2). Single or double deletion strains were used instead of *C. glutamicum* ATCC 21527 to generate strains carrying multiple gene deletions. LPΔ*treS*Δ*otsA* was constructed from LPΔ*treS* and LPΔ*treS*Δ*otsA*Δ*treY* from LPΔ*treS*Δ*otsA* (Tab. 1).

2.3.6 Construction of strains inactivated in glycogen synthesis

Glycogen biosynthesis was inactivated by inserting the vector pK19IMC (kindly provided by B. Eikmanns) *via* homologous recombination into the *glgC* gene in the genomes of *C. glutamicum* ATCC 13032, CglΔ*otsA*Δ*treS* and CglΔ*treY*Δ*treS* (Schäfer *et al*. 1994). In short, competent wild type cells were transformed with pK19IMC by means of electroporation (van der Rest *et al*., 1999). Plasmid integration into the genome was verified by selecting kanamycin-resistant colonies. The Δ*glgC* insertion strains CglΔ*glgC* CglΔ*otsA*Δ*treS*Δ*glgC* and CglΔ*treY*Δ*treS*Δ*glgC* were constructed (Tab. 1). These insertion strains were examined for cytoplasmic glycogen accumulation.

2.4 Biochemical methods

2.4.1 Determination of protein concentration

Protein concentration was determined according to the method of Bradford (1976). The solution was calibrated using bovine serum albumin.

2.4.2 Cell disruption

2.4.2.1 Cell disruption by permeabilisation with CTAB

For the analysis of cytoplasmic amino acids by HPLC, 1 ml cell culture was separated from the medium by rapid filtration using 0.45 µm glass fibre filters (GF, Schleicher & Schuell GmbH, Dassel, Germany). The cells were washed twice with 2 ml of CgXII medium and incubated in 1 ml 0.1 % N-Cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) for 10 min to release the cytoplasmic solutes. After a centrifugation step (20,000 *g*; 7 min, 4 °C) the supernatant was collected and stored at -20 °C until use.

2.4.2.2 Cell disruption by methanolysis

Cells were disrupted by methanolysis for the quantitative analysis of cytoplasmic trehalose. In short, 2 ml samples were taken from cell cultures. Cells were separated from the growth medium by centrifugation. The cells were washed once with 1 ml of fresh, isoosmotic CgXII medium and frozen with liquid nitrogen. Cells were permeabilized by incubation in methanol at 70 °C. Cell debris was removed by centrifugation and the supernatant was used for the preparation of GC-samples.

2.4.2.3 Mechanical cell disruption

2 ml samples were taken from cell cultures. The cells were washed with 50 mM Tris, 50 mM NaCl, pH 6.3 and 300 mg glass beads were added. The cells were broken up mechanically by means of a *FastPrep* FP120 (QBiogene, Heidelberg, D) (2 x 4.5 s, 6.5 m/s). The cell debris was removed by centrifugation (20,000 *g*; 10 min, 4 °C).

2.4.3 Quantification of amino acids by HPLC

For the quantitative analysis of external amino acids, especially lysine and glutamate, 1 ml samples were taken from cell cultures. Cells were separated from the growth medium by centrifugation and analysis was performed from the diluted supernatant. Cytoplasmic fractions were analysed for the quantification of cytoplasmic amino acids (cf. 1.4.2.1). The concentration of the amino acids was determined using a reversed phase high-performance liquid chromatography (HPLC) system (HP 1090 Liquid chromatograph, HP1046A fluorescence detector; Hewlett Packard) with automated fluorescent precolumn derivatization by ortho-phthaldialdehyd/mercaptopropionic acid (OPA). Solutes were separated by means of a reversed phase column (Multospher, CS Chromatographie Service) at 40 °C with a flow rate of 0.5 ml/min. The mobile phase was a mixture of solvent A (20 mM sodium actetate, pH 7.2, 0.3 % tetrahydrofurane, 0.04 % triethylamine) and 100% methanol (solvent B). A

hydrophilic-to-hydrophobic solvent gradient was used ranging from 35 % solvent B in the beginning to 100 % solvent B at the end of a run.

2.4.4 Quantification of trehalose by GC

Cytoplasmic and external trehalose were analysed quantitatively by means of gas chromatography (GC). Either 1 ml of the aqueous phase obtained after methanolysis of the cells (cf. 1.4.2.2) or 0.1 ml of the cultural supernatant was dried under N_2 at 60 °C. The resulting extract was methoxymized by incubation with 35 µl of a methoxylamine/pyridine mixture (20 mg /ml pyridine) at 30 °C for 90 min. Subsequently, 65 µl MSTFA was added and the samples were derivatised at 65 °C for 60 min (Sweely *et al.,* 1963). GC was performed using a Finnigan Trace-GC equipped with a MEGA fused-silica capillary column (10 m length, 0.1 mm internal diameter, 0.25 mm film thickness) obtained from MEGA (Legnano, Italy). The samples were applied by split-injection (1:25) / PTV. Separation was achieved by a linear temperature gradient from 60 °C to 280 °C with a heating rate of 13 °C/min, starting at 2 min after injection. The final temperature was kept constant for 3 min for complete elution. Myo-inositol was used as internal and trehalose as external standard.

2.4.5 Determination of the cytoplasmic glycogen concentration

For the determination of the cytoplasmic glycogen concentration in *C. glutamicum* cells the protocol of Parrou *et al.* (1997) was modified. Pellets of 2 ml bacterial culture were suspended in 40 mM potassium acetate and incubated for 5 min at 100 °C. Cells were broken up mechanically (cf. 1.4.2.3) and the cell debris was removed by centrifugation. To degrade glycogen to glucose, 100 µl of the supernatant were incubated for 2 h at 57 °C with 70 U/ mg amyloglucosidase (Fluka, Buchs, Schweiz). The liberated glucose was determined by an enzymatic colorimetric test for glucose (Glucose liquicolor, Human, Wiesbaden).

2.4.6 Quantification of carbon sources in bacterial cultures

For the quantification of the carbon source in bacterial cultures, cells were removed by centrifugation. Lactate concentrations in the supernatant were determined by an UV-test (R-Biopharm, Darmstadt) based on the following principle. In the presence of lactate dehydrogenase (LDH), lactate is oxidized to pyruvate by NAD. The equilibrium of this reaction lies on the side of lactate. By trapping pyruvate in a subsequent reaction catalyzed by the enzyme glutamatepyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favour of pyruvate and NADH. The amount of NADH formed in the above reactions is stoichiometric to the amount of lactate. The increase in NADH was determined by means of its light absorbance at 340 nm.

Sucrose concentrations were determined by measuring the D-glucose concentration before and after enzymatic hydrolysis of sucrose (R-Biopharm, Darmstadt). Glucose concentrations were determined by an enzymatic colorimetric test (Glucose liquicolor, Human, Wiesbaden).

2.4.7 Isolation, fractionation and analysis of lipids

2.4.7.1 Extraction of glycolipids

For the qualitative analysis of trehalose mycolate and glucose monomycolate, lipids were extracted from wet cells of 100 ml cultures for 16 h with $CHCl_3/CH_3OH$ [1:2, (v/v)] at room temperature. This procedure was repeated with $CHCl_3/CH_3OH$ [1:1, (v/v)] and $CHCl_3/CH_3OH$ [2:1, (v/v)]. The organic phases were pooled and dried. The crude lipid extracts were re-extracted in $CHCl_3/H_2O$ [8:2, (v/v)]. The lower organic phases were collected, evaporated to dryness to yield the crude lipid extracts from each strain and comparatively examined using thin layer chromatography (TLC) on silica gel-coated plates (Durasil-25, 0.25 mm thickness, Macherey-Nagel) developed with $CHCl_3/CH_3OH/H_2O$ [65:25:4, (v/v/v)]. Glycolipids were visualized by spraying plates with 0.2 % anthrone in concentrated H_2SO_4, followed by heating to 110 °C. Blue spots indicate the presence of glycolipids.

2.4.7.2 Extraction of arabinogalactan bound mycolic acids

The cell residues obtained after the extraction of glycolipids (cf. 1.4.7.1) were dried for the qualitative analysis of arabinogalactan bound mycolic acids. Fatty acids were separated from the bacterial residues by saponification with 40 % KOH and subsequently neutralized with 20 % H_2SO_4. They were extracted with diethylether, converted to methyl esters with diazomethane, dried under vacuum and weighed. Lipid extracts resolved in ether (10 mg/ml) were examined using TLC on silica gel-coated plates (Durasil-25, 0.25 mm thickness, Macherey-Nagel) and developed with $CHCl_2$. Detection of mycolate was carried out by spraying the TLC plates with 0.01 % rhodamine B in 0.25 M NaH_2PO_4.

2.4.7.3 Quantification of mycolic acids by GC

The quantification of corynomycolic acids was performed as follows: extracts of glycolipids (10 mg/ml in $CHCl_3/CH_3OH$ [1:1, (v/v)]) were dried under vacuum, saponified with 40 % KOH and neutralized with 20 % H_2SO_4. The fatty acids were extracted with diethyl ether, converted to methyl esters with diazomethane, dried under vacuum and weighed. Portions of fatty acid methyl esters from extractable lipids (1 - 2 mg) were treated with trimethylsilyl reagents to derivatise hydroxylated components of the mixtures, i.e. corynomycolates, and analyzed by GC. Fatty acid methyl esters of arabinogalactan bound mycolic acids (cf. 1.4.7.2) were similarly derivatised with trimethylsilyl reagents for the analysis by means of GC. GC of fatty

acid methyl esters was performed using a Hewlett Packard HP4890A equipped with a fused silica capillary column (25 m length x 0.22 mm i.d.) containing WCOT OV-1 (0.3 mm film thickness, spiral). A temperature gradient of 100 - 300 °C at 5 °C / min was used, followed by a 5 - 9 min isotherm plateau at 300 °C.

2.4.8 Determination of the uptake of glucose, trehalose or betaine into *C. glutamicum* cells

Uptake rates for [^{14}C]labeled solutes were measured to determine whether trehalose is taken up into *C. glutamicum* cells. Cultures of the wild type and Cgl*ΔotsAΔtreSΔtreY* were grown overnight in CgXII medium with 4 % sucrose as carbon source. Cells were harvested and washed once in KP$_i$ buffer (50 mM KP$_i$ pH 7.5, 20 mM NaCl). Subsequently, they were suspended in the same buffer and kept on ice. Cells with an optical density of 3 were preheated for 3 min at 30 ºC before the uptake measurement was started by the addition of either 500 µM [^{14}C]glucose (Hartmann Analytic, Braunschweig, Germany, 100 µCi/ml) or 50 µM [^{14}C]trehalose (Trenzyme GmbH, Konstanz, Germany, 1 µCi/ml) i. e. final concentrations of 0.025 µCi/ml or 0.05 µCi/ml of labelled glucose or trehalose, respectively, in the assays. In the case of the uptake of betaine, an osmotic shock was performed by the addition of 600 mM NaCl to activate the betaine uptake carriers of *C. glutamicum*. Cells were preheated for 3 min before the uptake reaction was started by the addition of 250 µM [^{14}C]betaine. At different time intervals 100 µl samples were taken and filtered rapidly through 0.45 µm glass fibre filters (GF, Schleicher & Schuell GmbH, Dassel, Germany). The filters were washed twice with 2.5 ml of 100 mM LiCl solution and the radioactivity was determined by liquid scintillation counting (Beckmann).

To determine whether the label was located in the cell wall fraction or the cytosol, the assay conditions were changed as follows. Cells were prepared and used in the transport assay as described above. After an incubation time of 10 min with 50 µM [^{14}C]trehalose, 500 µM [^{14}C]glucose or 250 µM [^{14}C]betaine 100µl cells were filtered on glass fibre filters. They were partly permeabilized by the addition of 1 ml of 50 mM KP$_i$ buffer containing 0.1 % N-Cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) in order to release the cytosol without destroying the cell envelope. After 1 min the CTAB solution was filtered and the remaining cell envelopes were washed twice with 2.5 ml of 100 mM LiCl solution. In a parallel approach the filtered cells were incubated for 1 min in 50 mM KPi buffer instead of the CTAB solution before being washed with LiCl. The radioactivity determined in these control cells represented the total amount of the accumulated substrate present after 10 min of incubation with [^{14}C]trehalose, [^{14}C]glucose or [^{14}C]betaine. The radioactivity was determined by liquid scintillation counting.

2.4.9 Determination of the permeability of the cell envelope

2.4.9.1 Determination of glycerol uptake rates

Uptake of glycerol was used as a measure for the permeability of the cell envelope. Concentrations from 20 - 100 µM [^{14}C]glycerol (PerkinElmer, 100 µCi/ml) were added to 1 ml of 50 mM KPi, 20 mM NaCl containing 1 mg wet cells i. e. the final concentration in the assay was 2 µCi/ml of labelled glycerol. Aliquots (0.1 ml) were taken after 6, 12, 18, 24, 30, 60 min and cells were separated from the accumulation medium by filtration through glass fiber filters (GF, Schleicher & Schuell GmbH, Dassel, Germany). The filters were washed twice with 2.5 ml of 100 mM LiCl solution and the radioactivity was determined by liquid scintillation counting (Beckmann).

2.4.9.2 Assay for glycerol kinase activity

To determine the activity of the glycerol kinase in ATCC 21527 and in LP∆*treS*∆*otsA*∆*treY,* cells of both strains were grown in CgXII with 4 % sucrose to stationary phase, harvested, washed twice in 50 mM potassium phosphate (pH 7.5) and suspended in 2 ml of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$ and disrupted by glass beads. Cell debris was removed by centrifugation. Mixtures for the determination of glycerol kinase activity, based on the method described by Kwakman *et al*. (1994), contained 100 µl cell extract, 50 mM Tris HCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM phosphoenolpyruvate, 1.2 U pyruvate-kinase and 1.1 U L-lactate-dehydrogenase. The mixture was allowed to equilibrate for 90s prior to activity measurement. The reaction was started by adding 0.3 mM NADH and 1 mM glycerol. The conversion of NADH was monitored at 340 nm. Protein concentrations were determined by the method of Bradford (1976). The protein content in the assay was in the range between 2 - 5 mg/ml. The specific activities were expressed as nmoles of NAD formed per minute per mg protein.

2.4.9.3 Determination of the susceptibility of *C. glutamicum* strains to antibiotics

The susceptibility of ATCC 21527 and LP*∆treS ∆otsA ∆treY* to the antibiotics penicillin, erythromycin or ethambutol was determined as an indicator for the permeability of the cell envelope. The minimum inhibitory concentrations (MIC) which inhibit the growth of the strains were determined by means of Etest® strips (AB Biodisk, Solna, Sweden). This test consists of an inert plastic strip with a MIC scale on one side and an immobilized exponential grade of antibiotic on the other side. When an Etest strip is applied to an inoculated agar surface the antibiotic gradient diffuses into the agar matrix. After incubation, whereby bacterial growth becomes visible, a symmetrical inhibition ellipse centred along the strip is seen. The MIC value is read from the scale where the ellipse intersects the strip. For inoculation, a CgXII overnight culture diluted to OD_{600} = 1 was spread with a cotton swab over the CgXII agar

plates. The plates inoculated with ATCC 21527 were incubated at 30 °C for 2 days, inoculated with LP*ΔtreS ΔotsA ΔtreY* for 4 days.

2.4.9.4 Measurement of the outer membrane permeability by using β-lactamases

The permeation of cephalothin through the mycolic acid layer of *C. glutamicum* was measured spectrophotometrically using the method of Zimmermann and Rosselet (1977) as modified by Jarlier and Nikaido (1990). *Mycobacterium smegmatis* and *C. glutamicum* cells were grown to an OD_{600} of 1, harvested by centrifugation for 5 min at 3000 *g* at room temperature, washed with PBS and resuspended in 2.5 mM PIPES (pH 6.5) to a concentration of 80 mg of cells (wet weight) ml^{-1}. This cell suspension (100 µl) was mixed with 400 µl of a 2.5 mM PIPES (pH 6.5) buffer containing 1 mM cephalothin. A sample of 300 µl of this mixture was quickly transferred to a cuvette with 1 mm light path, and the OD at 260 nm was recorded for 40 min at 25 °C. In order to determine the activity of the periplasmic β-lactamases, 160 mg of cells (wet weight) resuspended in 1 ml of 2.5 mM PIPES (pH 6.5) were broken mechanically, and the hydrolysis of cephalothin by the supernatant, corresponding to 80 mg (wet weight) of broken cells, was measured as described above.

3 Results

3.1 Function of the trehalose synthesis pathway OtsAB

As *C. glutamicum* is an immotile soil bacterium it has to cope with changes of osmolality in its natural habitat. The bacterium adapts to altering osmolality by accumulation or synthesis of compatible solutes e. g. trehalose. *C. glutamicum* harbours three different trehalose synthesis pathways: OtsAB, TreYZ and TreS. Within the scenario of redundant biosynthesis pathways, TreS catalyses under physiological conditions rather the degradation of trehalose to maltose than the synthesis of trehalose. The TreYZ-pathway seems to be the main trehalose synthesis pathway in *C. glutamicum.* It is responsible for the accumulation of trehalose as a compatible solute after hyperosmotic shock (Wolf *et al*., 2003). The function of the OtsAB-pathway in *C. glutamicum* is unknown, but transcriptional regulation of the *otsA* gene indicated a function of the OtsAB-pathway in the osmostress response of *C. glutamicum.* After hyperosmotic shock the *otsA* gene was upregulated fivefold in carbon rich medium, although no osmostress dependent trehalose synthesis by OtsA and OtsB was detected (Wolf *et al*., 2003). We suggest that the OtsAB-pathway is necessary to synthesise trehalose after hyperosmotic shock under conditions of carbon limitation when glycogen, the substrate for the TreYZ-pathway, becomes limiting. Carbon limitation should not inhibit the OtsAB-pathway because its substrates are glucose-6-P and UDP-glucose which are essential for the central metabolism.

3.1.1 Carbon source triggered glycogen limitation

Since the accumulation of cytoplasmic glycogen depends on the type of carbon source (B. Eikmanns, personal communication), nature and quantity of the carbon source were varied to manipulate intracellular glycogen concentrations to analyse the influence of glycogen limitation on the trehalose biosynthesis pathways TreYZ and OtsAB. For this purpose, *C. glutamicum* ATCC 13032 was cultivated with 4 % glucose, 1 % glucose, 2 % acetate or 2 % lactate and intracellular glycogen concentrations were determined.

Whereas biomass production of *C. glutamicum* ATCC 13032 was twice as high with 4 % glucose (13 g/L) than with 1 % glucose or 2 % acetate (6 g/L), the growth rate of *C. glutamicum* ATCC 13032 depended on the nature of the carbon source rather than on the quantity (Fig. 4). The growth rate in the exponential phase was equal with 1 % or 4 %

glucose (μ = 0.4 h^{-1}), whereas with 2 % acetate (μ = 0.2 h^{-1}) it was only half as high. Also the glycogen concentration was influenced by the nature of the carbon source. *C. glutamicum* ATCC 13032 accumulated up to 2 μg/mg dcw glycogen after 24 h when it was cultured on 1 % or 4 % glucose, whereas only 0.5 μg/mg dcw were detected in cells cultured on acetate. Since even with acetate cells contained glycogen, it was investigated whether lactate was more suitable to induce glycogen deficiency in *C. glutamicum.*

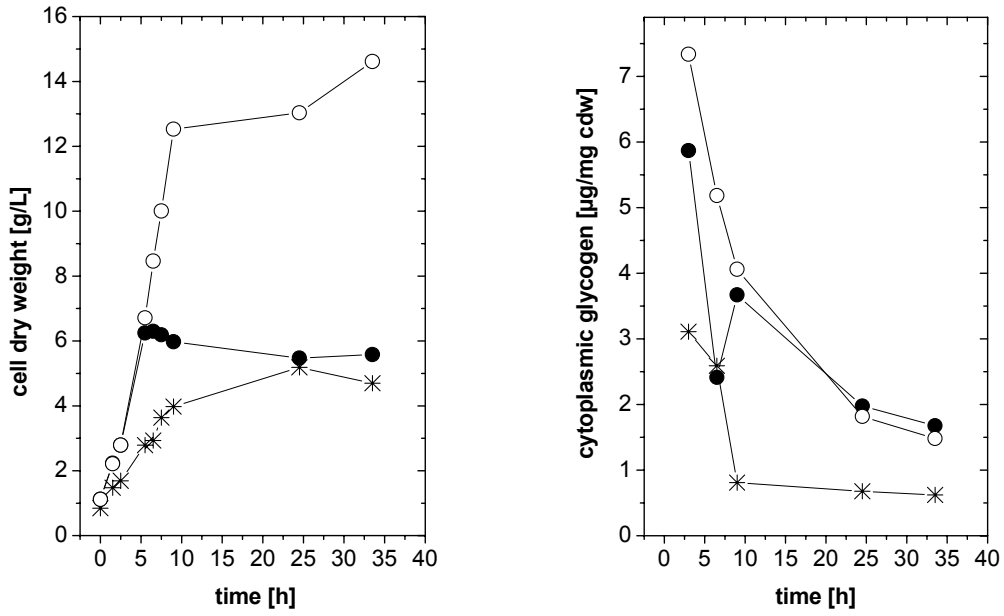


Fig. 4: Effect of the carbon source on bacterial growth and cytoplasmic glycogen synthesis of *C. glutamicum* ATCC 13032. ● 1 % glucose; O 4 % glucose, ∗ 2 % acetate

When ATCC 13032 was cultured on 2 % lactate, growth rate and biomass production was similar to ATCC 13032 cultured on 2 % acetate (Fig. 5). In contrast to cultivation on acetate, no glycogen was detected after 50 h cultivation on 2 % lactate. Thus, cultivation of *C. glutamicum* strains in medium supplemented with 2 % lactate could be used to reach the glycogen deficiency required for the elucidation of the function of the OtsAB-pathway.

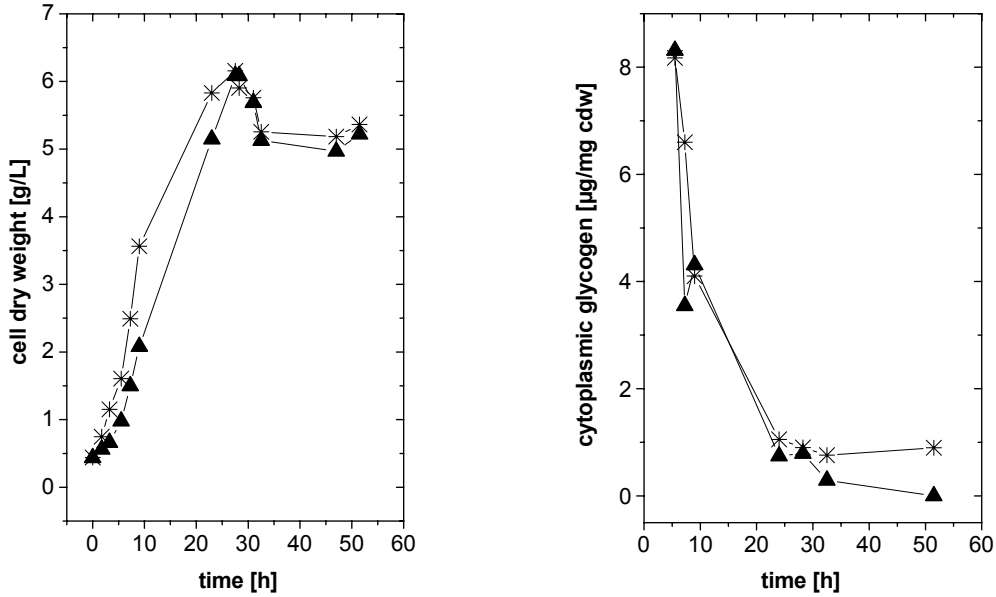


Fig. 5: Effect of the carbon source on bacterial growth and cytoplasmic glycogen synthesis of *C. glutamicum* ATCC 13032. ✳ 2 % acetate; ▲ 2 % lactate.

3.1.1.1 Significance of single trehalose synthesis pathways under carbon limitation

Two mutants of *C. glutamicum* ATCC 13032 were selected to quantify the contribution of the TreYZ-pathway and of the OtsAB-pathway to trehalose biosynthesis. The mutant Cgl*ΔtreYΔtreS* depended solely on the OtsAB trehalose synthesis pathway whereas Cgl*ΔotsAΔtreS* had only a functional TreYZ trehalose synthesis pathway. These strains and the wild type as a control were grown on 2 % lactate to reach glycogen limiting conditions. Cytoplasmic trehalose concentrations were analysed by means of GC.

The strain harbouring only the TreYZ pathway (Cgl*ΔotsAΔtreS)* accumulated 0 - 5 µmoles/g dcw trehalose, whereas the strain depending only on the OtsAB pathway (Cgl*ΔtreYΔtreS)* synthesised nearly wild type levels (Fig. 6). Cultivating these three strains under carbon surplus (4 % glucose) the opposite was found. Under these conditions, deletion of *otsA* did not influence trehalose synthesis at all, whereas a defect TreYZ-pathway reduced trehalose accumulation (Wolf *et al*., 2003). These results indicate that the cytoplasmic pools of precursors of the two pathways determine which biosynthetic route is used by *C. glutamicum*.

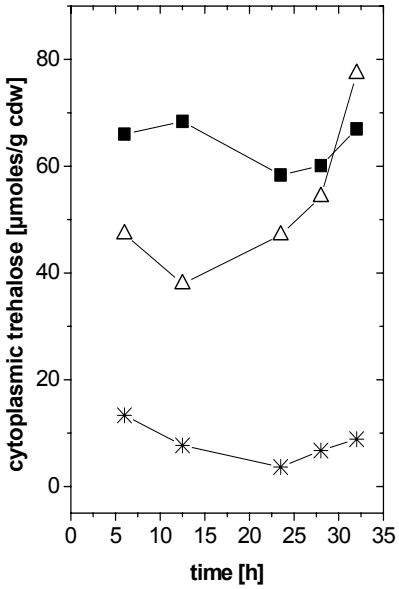


Fig. 6: Accumulation of cytoplasmic trehalose under carbon limitation in *C. glutamicum* strains with one functional trehalose synthesis pathway. Cultivation in minimal medium supplemented with 2 % lactate. ∗ Cgl*ΔotsAΔtreS*; △ Cgl*ΔtreYΔtreS;* ■ ATCC 13032

3.1.1.2 Significance of single trehalose synthesis pathways under carbon limitation after hyperosmotic shock

As the transcription of the *otsA* gene was significantly induced after a hyperosmotic upshift (Wolf *et al.,* 2003), it was investigated whether the OtsAB-pathway may become the predominant trehalose synthesis pathway under carbon limiting conditions. *C. glutamicum* strains Cgl*ΔtreYΔtreS,* Cgl*ΔotsAΔtreS* and ATCC 13032 were cultivated in 2 % lactate for 24 h to reach glycogen limitation. Then a hyperosmotic shock was applied by addition of NaCl to the growth medium. The osmolality of the basis medium CgC (0.9 osM), was increased by salt addition to 2.4 osM. Cytoplasmic glycogen and trehalose concentrations were analysed by an enzymatic assay or by means of GC, respectively.

Immediately before and after hyperosmotic shock, Cgl*ΔotsAΔtreS* (TreYZ) accumulated only 2 - 3 µmoles/g dcw of trehalose whereas Cgl*ΔtreYΔtreS* (OtsAB) accumulated trehalose at wild type level (Fig. 7A). These data confirmed that the OtsAB-pathway was the predominant trehalose synthesis pathway under carbon limitation in the absence of osmotic stress.

In spite of glycogen limitation, Cgl*ΔotsAΔtreS* (TreYZ) increased the cytoplasmic trehalose concentration from 2 - 180 µmoles/g dcw between 15 - 180 min after hyperosmotic shock, thus reaching wild type level, whereas Cgl*ΔtreYΔtreS* accumulated only 1/3 of the wild type level in the same time frame. Analysis of the cytoplasmic glycogen level showed that these

strains did not synthesise glycogen shortly before and directly after hyperosmotic shock, but surprisingly, they started to accumulate glycogen 15 min after hyperosmotic shock (Fig. 7B). Thus, the substrate of the TreYZ-pathway was available 15 min after hyperosmotic shock facilitating trehalose synthesis by the TreYZ-pathway. Analysis of the lactate concentration in the supernatant of Cgl*ΔotsAΔtreS,* Cgl*ΔtreYΔtreS* and ATCC 13032 cultures revealed that after 24 h of cultivation the lactate level (4-7 g/L) was still sufficient to facilitate glycogen synthesis after hyperosmotic shock (data not shown). Thus, cultivation of *C. glutamicum* strains in medium supplemented with 2 % lactate was not the right tool to obtain glycogen deficiency under hyperosmotic conditions.

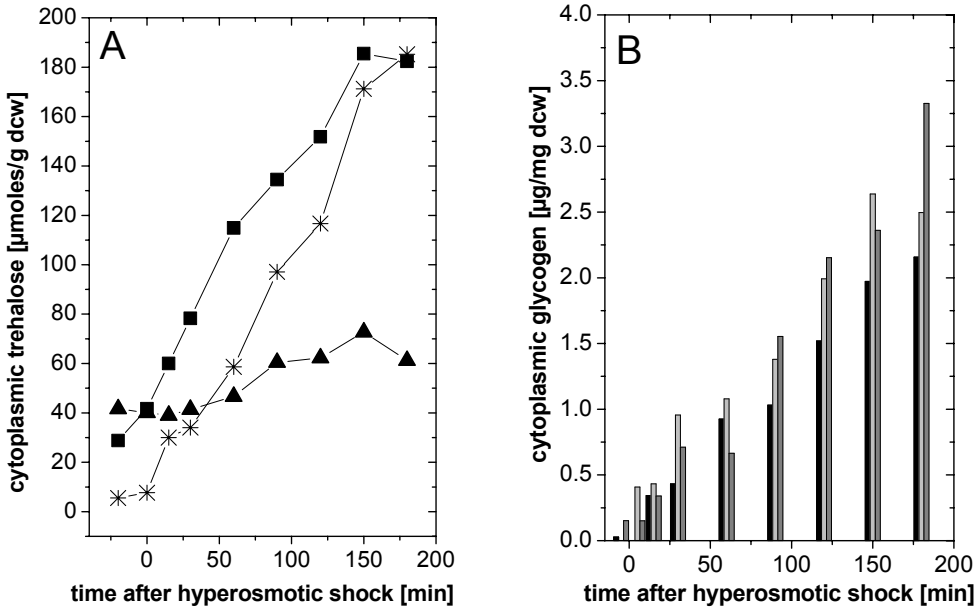


Fig. 7: A, Relevance of single trehalose synthesis pathways under carbon limitation (2 % lactate) after hyperosmotic shock. Results from one out of three experiments are shown. ∗ Cgl*ΔotsAΔtreS*; △ Cgl*ΔtreYΔtreS;* ■ ATCC13032. B, Glycogen accumulation by *C glutamicum* strains harbouring only one trehalose synthesis pathway under carbon limitation after hyperosmotic shock. Light grey bars, Cgl*ΔotsAΔtreS* (TreYZ), dark grey, Cgl*ΔtreYΔtreS* (OtsAB); black bars, ATCC 13032.

3.1.2 Inactivation of glycogen biosynthesis

3.1.2.1 Construction of *C. glutamicum* strains inactivated in glycogen synthesis

Glycogen biosynthesis was inactivated in *C. glutamicum* strains to avoid glycogen accumulation. The *glgC* gene encoding ADP-glucose-pyrophosphorylase, the first enzyme of the glycogen synthesis pathway, was inactivated in ATCC 13032 and in Cgl*ΔotsAΔtreS* and Cgl*ΔtreYΔtreS* by inserting the plasmid pK19IMC (kindly provided by B. Eickmanns) *via* homologous recombination. The resulting insertion strains Cgl*ΔglgC*, Cgl*ΔotsAΔtreSΔglgC* and Cgl*ΔtreYΔtreSΔglgC* were glycogen deficient when cultivated in CgC supplemented with 1 % sucrose whereas exponentially growing wild type cells accumulated 10 µg/mg of glycogen under the same conditions (data not shown). Lower growth rate and decreased biomass accumulation of the *ΔglgC* insertion strains compared to the wild type signalled that inactivation of glycogen biosynthesis weakened the general metabolism of the cell. Nevertheless, the glycogen deficient strains provided a tool to prevent trehalose synthesis by the TreYZ-pathway enabling the analysis of the function of the OtsAB-pathway.

3.1.2.2 Significance of single trehalose synthesis pathways in *C. glutamicum* mutants inactivated in glycogen biosynthesis after hyperosmotic shock

Growth and accumulation of internal solutes were analysed in the glycogen deficient *C. glutamicum ΔglgC* insertion mutants Cgl*ΔglgC*, Cgl*ΔotsAΔtreSΔglgC* and Cgl*ΔtreYΔtreSΔglgC* to find out whether the OtsAB-pathway replaces the TreYZ-pathway under carbon limitation after hyperosmotic shock. These strains were cultivated in CgC with 1 % sucrose and a hyperosmotic shock was applied during exponential growth. The concentration of the compatible solute trehalose was determined, but also the concentration of the amino acid proline because under nitrogen surplus proline is the predominant *do novo* synthesised compatible solute in the wild type (Rönsch *et al.,* 2003; Ley, 2005). Glycogen concentrations were measured to prove the carbon limiting conditions.

All strains started to accumulate trehalose after hyperosmotic shock, but trehalose synthesis in the three *ΔglgC* insertion strains was 10 fold lower than in wild type cells (Fig. 8A). Cgl*ΔtreYΔtreSΔglgC,* the strain harbouring the OtsAB-pathway, synthesised up to 10 µmol/g dcw trehalose, whereas Cgl*ΔotsAΔtreSΔglgC* synthesised only 1 µmol/g dcw trehalose. Thus, the OtsAB-pathway was the predominant trehalose synthesis pathway after hyperosmotic shock in the *ΔglgC*-background.

Surprisingly, Cgl*ΔotsAΔtreSΔglgC,* harbouring only the TreYZ-pathway, synthesised a small, but significant amount of trehalose. In spite of the inactivation of *ΔglgC,* all three insertion strains accumulated 0.5 µg/mg cdw cytoplasmic glycogen after hyperosmotic shock, a low amount compared to 10 µg/mg cdw glycogen accumulated by the wild type, but obviously sufficient for the TreYZ-pathway to synthesise trehalose.

Growth of Cgl*ΔtreYΔtreSΔglgC* recovered faster than that of Cgl*ΔotsAΔtreSΔglgC* after hyperosmotic shock emphasizing the importance of the OtsAB-pathway at the first glance (Fig. 8C). Since Cgl*ΔtreYΔtreSΔglgC* grew also faster than the type strain ATCC 13032, trehalose could not be responsible for faster recovery of the mutant because cytoplasmic trehalose concentrations were 10 fold lower in the mutant compared to the wild type.

Under conditions of nitrogen surplus, proline is the predominant *de novo* synthesised protectant against osmotic stress, therefore cytoplasmic proline concentrations were analysed in the glycogen deficient *C. glutamicum* strains Cgl*ΔglgC,* Cgl*ΔtreYΔtreSΔglgC,* Cgl*ΔotsAΔtreSΔglgC* and in the wild type. Cgl*ΔtreYΔtreSΔglgC* and Cgl*ΔotsAΔtreSΔglgC* accumulated slightly more proline than the wild type (Fig. 8B). Thus under the conditions tested, proline rather than trehalose seemed to be responsible for the adaptation to osmotic stress also in the glycogen deficient *C. glutamicum* strains.

The importance of proline was supported furthermore by the surprising phenotype of Cgl*ΔglgC.* Growth of this strain hardly recovered after hyperosmotic shock. As the proline concentration of Cgl*ΔglgC* was a fourth of that of the other *ΔglgC* insertion strains, it was probably not sufficient to protect this strain against osmotic stress. These results were reproduced with two Cgl*ΔglgC* clones which derived from two independent inactivation experiments confirming that low proline accumulation was not due to accidental inactivation of proline biosynthesis genes. The reason for the special phenotype of Cgl*ΔglgC* is unknown.

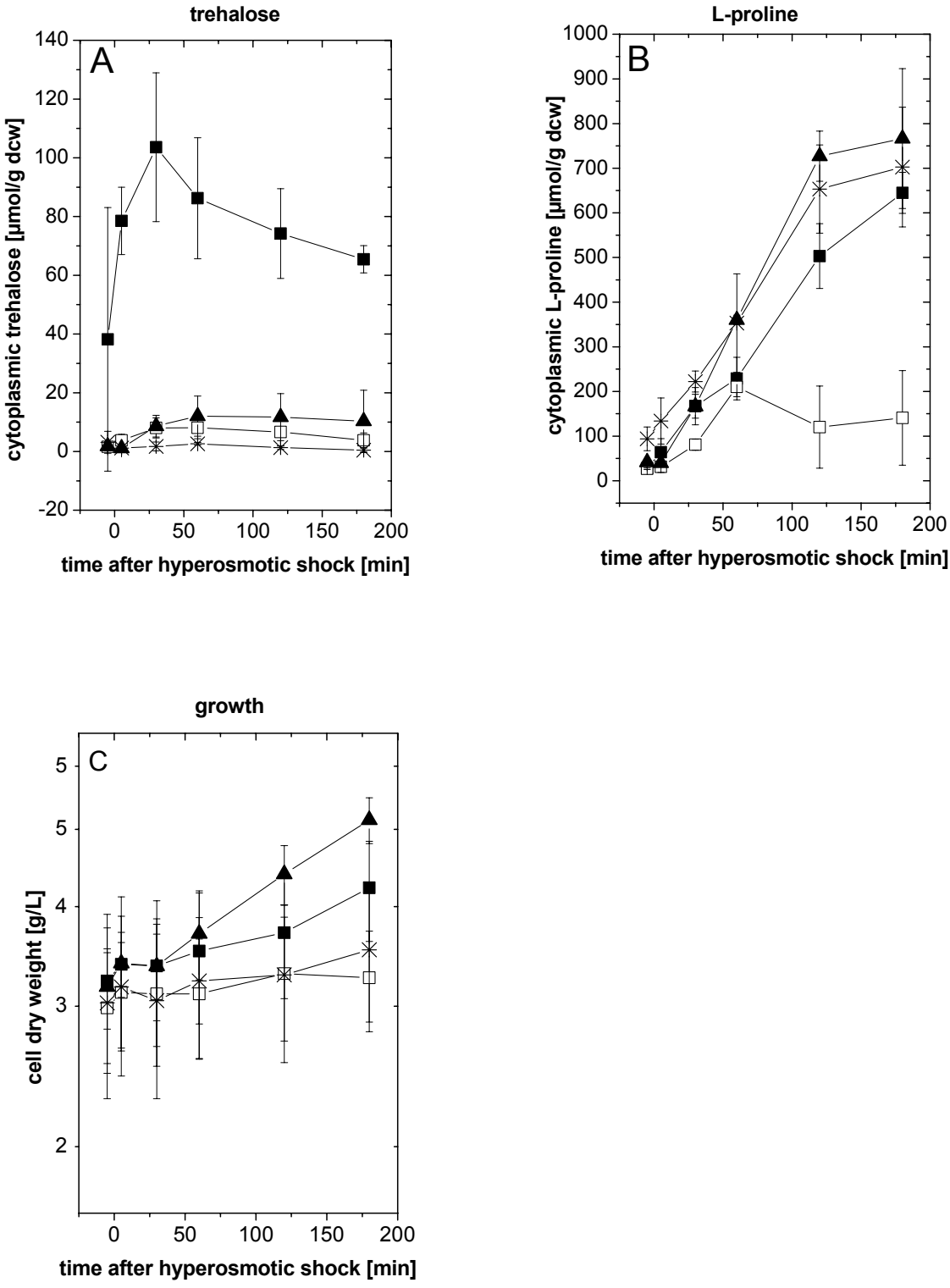


Fig. 8: Cytoplasmic accumulation of trehalose (A) and proline (B) in *C. glutamicum ΔglgC* insertion mutants after hyperosmotic shock, (C) growth. ■ ATCC 13032; □ Cgl*ΔglgC*, ∗ Cgl*ΔotsAΔtreSΔglgC*; ▲ Cgl*ΔtreYΔtreSΔglgC*.

In summary, the enzymes of the OtsAB-pathway synthesised 10 times more trehalose than the enzymes of the TreYZ-pathway in a glycogen deficient background after hyperosmotic shock confirming its importance under these conditions. Nevertheless, the amount of trehalose accumulated was not sufficient for protection against osmotic stress as in the wild type 100 µmoles/g cdw of trehalose were necessary for recovery (Wolf *et al.*, 2003). Slower growth and lower biomass accumulation of *⊿glgC* insertion strains compared to the wild type indicated that the energy metabolism might have been changed by the deletion of glycogen biosynthesis. In turn, an altered energy metabolism might explain the extremely low trehalose synthesis of the *⊿glgC* insertion strains.

In contrast to trehalose, the compatible solute proline was synthesised in wild type amounts in the glycogen deficient background after hyperosmotic shock indicating that, similar to the situation in the wild type, proline might be the most important compatible solute in the *⊿glgC* insertion strains under conditions of N-surplus.

3.2 Localisation of the synthesis of trehalose monomycolate

Although the mycolate layer is a phylogenetic trait of the *Corynebacterineae,* little is known about its biosynthesis. The mycolyltransferase catalysing the first step, i. e. the condensation a molecule of trehalose with a mycolyl-residue to trehalose monomycolate (TMM), is not identified. The following reactions, the transfer of the mycolyl-residue from TMM to arabinogalactan or to another TMM to form arabinogalactan mycolate or TDM, respectively, are catalysed by fibronectin binding proteins (FBP) or their analogues in *C. glutamicum.* Since these proteins are secreted, these reactions take place in the cell envelope. Whether synthesis of TMM is also localized in the cell envelope or in the cytoplasm where its building blocks are synthesised, is not known.

The combination of the two following observations suggests that TMM synthesis is localized in the cytoplasm. The *C. glutamicum* wild type strain ATCC 13032 could not grow on trehalose as carbon source indicating that the disaccharide cannot be taken up by *C. glutamicum* (Wolf, 2002), and the addition of external trehalose to the medium of a trehalose deficient mutant of *C. glutamicum* facilitated synthesis of TMM (Tzvetkov *et al.,* 2003).

Uptake rates for [^{14}C]trehalose were determined for the wild type ATCC 13032 to prove the assumption that trehalose is not taken up into the cytoplasm by *C. glutamicum.* These uptake rates were compared to uptake into Cgl*ΔtreYΔtreSΔotsA,* which was mycolate deficient under the chosen cultivation conditions, to differentiate between transport into the cytoplasm and incorporation into the mycolate layer. Uptake rates of [^{14}C]glucose were measured to verify whether the energy state of Cgl*ΔtreYΔtreSΔotsA* was high enough to trigger sugar uptake in spite of trehalose and mycolate deficiency. Both strains were cultivated in minimal medium with 4 % sucrose as carbon source.

Nearly identical glucose uptake rates for both strains indicated a similar energy metabolism excluding that different uptake rates for trehalose resulted from lower energy status of the mycolate deficient strain compared to the wild type (Tab. 4).

The wild type accumulated trehalose, but the uptake rate was 100 times lower than that of glucose. In contrast, no uptake of trehalose was detected in Cgl*ΔtreYΔtreSΔotsA* which lacked the mycolate layer under the chosen cultivation conditions. Thus, trehalose accumulation in the wild type cells could reflect integration of trehalose into the cell envelope and more precisely into the mycolate layer rather than uptake into the cytoplasm.

Tab. 4: Uptake rates of [^{14}C]trehalose and of [^{14}C]glucose of ATCC 13032 and Cgl*ΔtreYΔtreSΔotsA* after 10 min (trehalose) or 5 min (glucose) incubation

Strain	Trehalose uptake	Glucose uptake
	*nmol/(min*mg dcw)*	*nmol/(min*mg dcw)*
ATCC 13032	0.13 +/- 0.07	9.0 +/- 0.7
Cgl*ΔtreYΔtreSΔotsA*	no uptake	9.0 +/- 0.6

Trehalose accumulation in isolated cell envelopes was compared to accumulation in whole cells to test the hypothesis that trehalose is integrated into the cell envelope by external enzyme activity rather than taken up into the cytoplasm. Therefore, cells were permeabilized with 0.1 % CTAB and washed thoroughly to separate the cytoplasmic solutes from of the remaining cell envelope. The compatible solute betaine served as a control for permeabilisation and washing conditions because it is avidly taken up into the cytoplasm after hyperosmotic shock and not metabolized. As a further control, the localisation of labelled glucose was determined because *C. glutamicum* takes up glucose into the cytoplasm and metabolizes it into cell envelope components. Accumulation of [^{14}C]trehalose was determined after 10 min, while accumulation of [^{14}C]glucose was already determined after 5 min as the uptake was much faster. In the case of the uptake of betaine, an osmotic shock was performed to activate the betaine uptake carriers of *C. glutamicum* and accumulation of [^{14}C]betaine was measured 10 min after the osmotic shock.

Accumulation of trehalose in ATCC 13032 was similar in isolated cell walls and in intact cells (Tab. 5). Thus, accumulation of trehalose detected in the whole cells represented accumulation only in the cell envelope. This proved that trehalose was not taken up into the cytoplasm, but that it was only integrated into the cell envelope. Permeabilisation and washing conditions were suitable to separate the cell wall from the cytoplasm and to prevent adhesion of the labelled substance to the cell wall as shown by the controls. Whereas 10 min after hyperosmotic shock 85.7 nmol/mg cdw betaine were taken up into the whole cells, no labelled betaine was detected in the cell wall, confirming that washing conditions were sufficient. After incubation with [^{14}C]glucose 2/3 of the label was detected in the intact cells whereas 1/3 accumulated in the isolated cell envelopes. Labelling of the cell wall might result from [^{14}C]glucose integrated into cell wall components, confirming that incorporation of [^{14}C]labelled substances into the cell wall can be detected.

In contrast to wild type cells, no label was detected in the intact cells or in the cell wall when Cgl*ΔtreYΔtreSΔotsA* was incubated with [^{14}C]trehalose proving the uptake measurements. Cgl*ΔtreYΔtreSΔotsA* accumulated the control substances betaine and glucose similar to the

wild type. 60.2 ± 6.6 nmol/mg cdw of betaine was detected in the intact cells, but nothing in the isolated cell walls confirming that the washing conditions were sufficient also for the mycolate deficient strain. After 5 min incubation with [^{14}C]glucose, 2/3 of the label were in the intact cells whereas 1/3 was in the cell walls demonstrating that also in the strain lacking the mycolate layer incorporation of [^{14}C]labelled substances into the cell wall could be detected. Presumably, the label detected in the cell wall corresponded to glucose metabolised into components of the peptidoglycan or the arabinogalactan layer.

Tab. 5: Accumulation of [^{14}C]trehalose, [^{14}C]betaine and [^{14}C]glucose in ATCC 13032 and Cgl*ΔtreYΔtreSΔotsA* after 10 min (trehalose, betaine) or 5 min (glucose) incubation

Strain	Trehalose accumulation		Betaine accumulation		Glucose accumulation	
	in whole cells	in cell walls	in whole cells	in cell walls	in whole cells	in cell walls
	nmol/mg cdw	*nmol/mg cdw*	*nmol/mg cdw*	*nmol/mg cdw*	*nmol/mg cdw*	*nmol/mg cdw*
ATCC 13032	1.6 ± 0.5	1.5 ± 0.3	85.7 ± 10	0.5* ± 0.05	7.5 ± 0.2	3.2 ± 0.0
Cgl*ΔtreYΔtreSΔotsA*	0.1* ± 0.4	0.2* ± 0.1	60.2 ± 6.6	0.3* ± 0.06	5.4 ± 0.7	1.8 ± 0.3

* in these measurements the detected radioactivity was below 70 counts per minute, which is the level of background activity

Whereas Cgl*ΔtreYΔtreSΔotsA,* which lacked the mycolate layer, did not accumulate trehalose during this short incubation time, neither in cell envelopes nor in intact cells, the wild type accumulated trehalose exclusively in the cell envelope. These data show that the wild type incorporated trehalose into the cell envelope, more precisely into the mycolate layer and furthermore, that the wild type could not take up trehalose into the cytoplasm. Thus, the assumption was substantiated that TMM is synthesised in the cell envelope and not in the cytoplasm.

3.3 Importance of trehalose for the cell envelope of a *C. glutamicum* L-lysine production strain

Exceptionally for Gram-positive bacteria, the cell envelope of *Corynebacterineae* comprises a second lipid bilayer apart from the plasma membrane, the mycolate layer (Puech *et al.,* 2001). The mayor building blocks of the mycolate layer are mycolic acids and trehalose. Since *C. glutamicum* as well as the human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae* belong to the suborder of the *Corynebacterineae* they share the mycolate layer as a characteristic trait. In mycobacteria the mycolate layer renders the cell envelope impermeable to most of the common antibiotics (Brennan & Nikaido, 1995). As recombinant *C. glutamicum* strains with reduced mycolate content showed increased uptake rates for glycerol and acetate, the mycolate layer might determine the permeability of the cell envelope also in corynebacteria (Puech *et al.* 2000). Trehalose seemed to be essential for the synthesis of the mycolate layer since the trehalose deficient *C. glutamicum* ATCC 13032 strains Cgl*ΔotsAΔtreYΔtreS* and Cgl*ΔotsAΔtreY* were devoid of mycolate when they were grown on sucrose as carbon source (Wolf *et al.*, 2003). However, when the trehalose deficient Cgl*ΔotsAΔtreY* was supplemented with maltose, this strain synthesised arabinogalactan mycolate and maltose monomycolate (Wolf, 2002; M. Daffé, personal communication) indicating that trehalose was not the sole sugar acceptor for mycolic acids. This assumption is supported by the detection of glucose monomycolate in a *C. glutamicum* strain deleted in the mycolyltransferase PS1 (Puech *et al.,* 2000). This project addressed the question how the carbon source and the availability of trehalose influence the synthesis of mycolate and how in turn an altered mycolate layer may change the properties of the cell envelope, especially the permeability.

Whereas in mycobacteria the influence of the cell envelope on the uptake of antibiotics is important, in *C. glutamicum* the influence of the cell envelope on the efflux of substances is more relevant as it is the sole industrial producer of the amino acids L-lysine and L-glutamate. Whereas excretion of glutamate requires specific conditions e. g. biotin limitation or treatment with detergents (Eggeling and Sahm, 2001), the production of lysine is carried out with especially mutated *C. glutamicum* strains. The lysine biosynthesis pathway or pathways leading to side-products are manipulated, either by undirected mutagenesis and screening or by genetic engineering, to obtain *C. glutamicum* lysine producer strains (Pfefferle *et al*., 2003). The discovery of the lysine exporter LysE in *C. glutamicum* revealed that efficient lysine efflux is a further prerequisite for lysine production (Broer *et al*., 1991 a/b; Vrljic *et al*., 1995; Vrljic *et al*., 1996; Bellmann *et al*., 2001). LysE exports lysine across the plasma membrane into the cell envelope where lysine has to cross a second lipid bilayer -

the mycolate layer. As the deletion of trehalose synthesis pathways influenced the composition of the mycolate layer, trehalose deficiency may provide a further tool to facilitate the efflux of amino acids. However, it has to be considered that amino acids may not cross the mycolate layer directly, but that porins may facilitate the crossing.
Consequently, the question whether trehalose deficiency could enhance amino acid excretion by a lysine producer strain was a further focus of this project. Moreover, it was examined whether excreted lysine as a measure of the efflux of solutes could be correlated to the permeability of the cell envelope.

3.3.1 Characterisation of *C. glutamicum* L-lysine production strain ATCC 21527

The L-lysine production strain ATCC 21527 was chosen to address the question how the availability of trehalose influences the properties of the mycolate layer and the excretion of amino acids, and moreover, whether trehalose deficiency could enhance the excretion of amino acids. Furthermore, this strain facilitated to examine whether the permeability of the cell envelope and the excretion of amino acids could be correlated.
The *C. glutamicum* lysine producer strain ATCC 21527 was obtained by random mutation and selection. Requirement for leucine and homoserine and resistance to the threonine analogue α-amino-β-hydroxyvaleric acid indicate that lysine synthesis in ATCC 21527 was increased due to inactivated by-product synthesis and an aspartate-kinase resistant to feed-back inhibition. During fermentation this strain produced 38.2 g/L of L-lysine (Nakayama *et al.,* 1973).
Comparison of lysine producer *C. glutamicum* ATCC 21527 to the wild type ATCC 13032 should reveal how the mutations in the lysine producer influenced the general metabolism and especially trehalose metabolism to see whether the impact of trehalose metabolism on the mycolate layer in the wild type could be adapted to the lysine producer. Therefore, growth and cytoplasmic and external concentrations of lysine and trehalose were analysed with HPLC and GC, respectively. Since the behaviour of the wild type was well characterised under different osmotic conditions (Wolf, 2002), the two strains were compared in the absence of osmotic stress (0.2 osM) and under high osmolality (2.4 osM).

Introduction of amino acid auxotrophy in the lysine producer increased the generation time and the yield of biomass two times compared to the wild type in the absence of osmotic stress (Fig. 9). Besides the difference in growth velocity, increase of the osmolality had a similar effect on both strains since the growth rate of both strains divided in half under high osmolality.

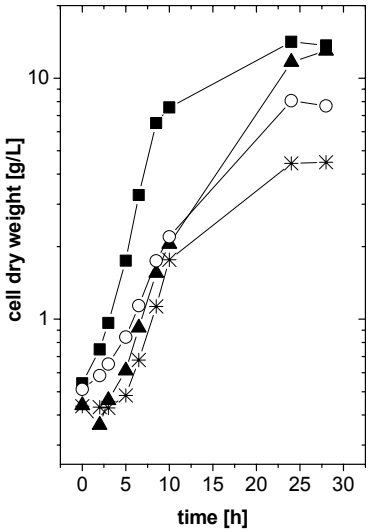


Fig. 9: Comparison of growth of wild type ATCC 13032 and L-lysine producer ATCC 21527 at constant osmolality of 0.2 osM or 2.4 osM. ■ ATCC 13032 (0.2 osM), ▲ ATCC 13032 (2.4 osM), O ATCC 21527 (0.2 osM); ∗ ATCC 21527 (2.4 osM).

Also trehalose metabolism was similar in wild type and lysine producer under the same cultivation conditions (Fig. 10). In the absence of osmotic stress, both strains accumulated about 50 µmoles/g cdw cytoplasmic trehalose and excreted about twice as much into the culture medium. Both, the cytoplasmic and the external trehalose concentration rose to the fivefold amount under hyperosmotic conditions in the wild type as well as in the lysine producer. Data for the wild type were consistent with results of the analysis of trehalose metabolism under different osmotic conditions by A. Wolf (2002).

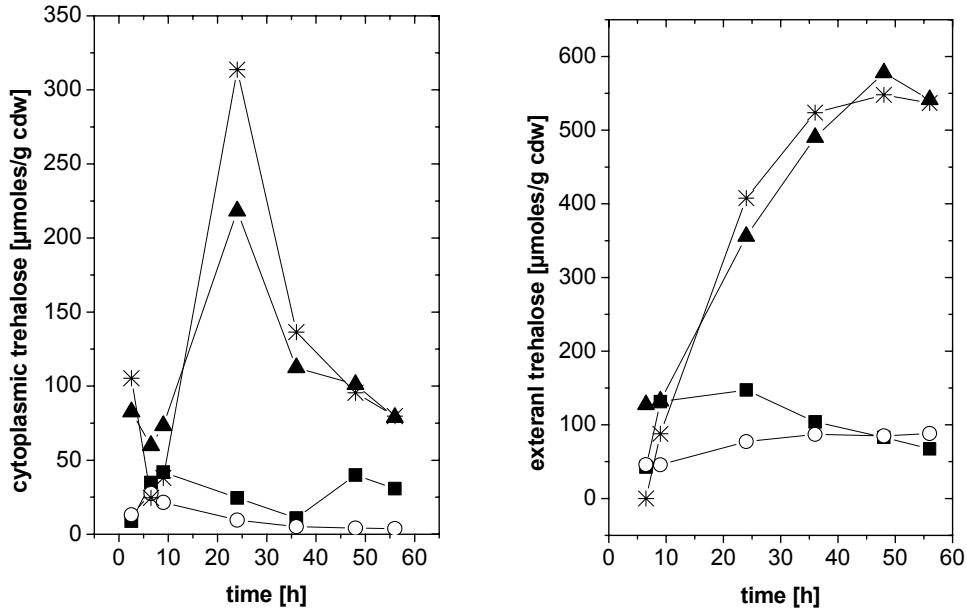


Fig. 10: Comparison of cytoplasmic and external trehalose accumulation by wild type ATCC 13032 and L-lysine producer ATCC 21527 at constant osmolality of 0.2 osM or 2.4 osM. ■ ATCC 13032 (0.2 osM), ▲ ATCC 13032 (2.4 osM), O ATCC 21527 (0.2 osM); ∗ ATCC 21527 (2.4 osM).

Lysine synthesis was different in both strains (Fig. 11). Whereas the wild type accumulated about 20 µmoles/g cdw cytoplasmic lysine which was not excreted, the lysine producer accumulated the 7 fold amount and excreted 50 mM (7.3 g/L) lysine after 50 h cultivation.

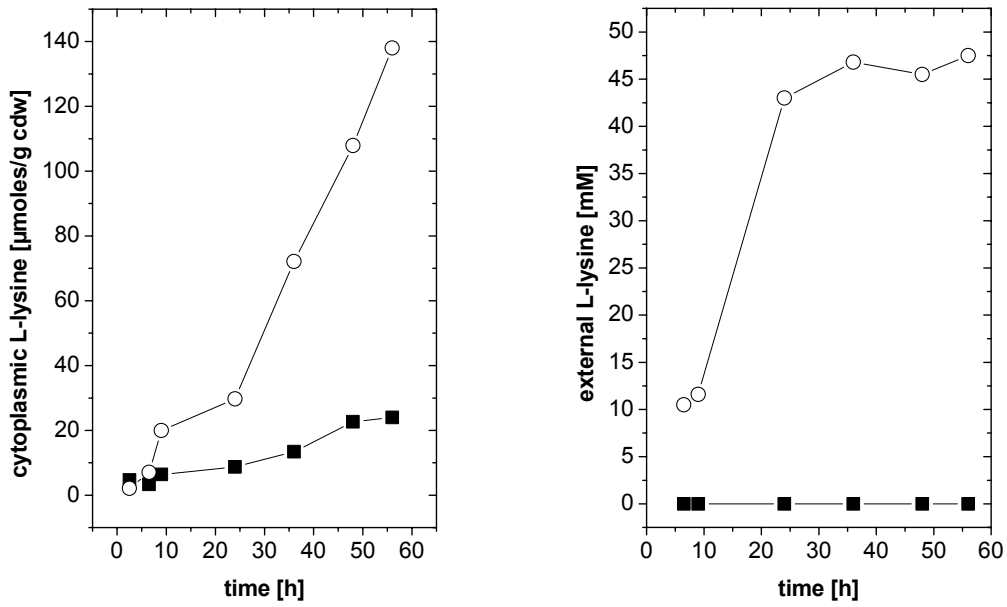


Fig. 11: Comparison of cytoplasmic and external L-lysine accumulation by wild type ATCC 13032 (■) and L-lysine producer ATCC 21527 (O) at an osmolality of 0.2 osM.

As expected, the lysine production strain excreted lysine in contrast to the wild type. Since cells were cultivated in shake flasks the lysine excretion was lower than excretion achieved by fermentation (Nakayama *et al.,* 1973).

Auxotrophy for homoserine and leucine limited growth rate and biomass production of the lysine producer compared to the wild type, but the overall growth behaviour was the same under the tested conditions. Trehalose metabolism was unaffected by the mutations in the lysine producer. As the influence of deletion of enzymes of trehalose metabolism might be similar in both strains, too, the lysine producer ATCC 21527 is a suitable strain to investigate the influence of trehalose deficiency on properties of the mycolate layer and on lysine excretion.

3.3.2 Construction of a *C. glutamicum* lysine production strain defective in trehalose biosynthesis

A *C. glutamicum* L-lysine production strain defective in all three trehalose synthesis pathways was the prerequisite to investigate the influence of trehalose metabolism on the mycolate layer and on the excretion of amino acids. Either the *otsA, treS* or *treY* gene encoding the first enzyme of one of the three trehalose synthesis pathways, respectively, was deleted in the chromosome of *C. glutamicum* lysine production strain ATCC 21527. Wild type alleles were exchanged against alleles comprising internal deletions by two events of homologous recombination (Schäfer *et al.,* 1994). Plasmids containing the *otsA, treS* or *treY* gene with internal deletions were described recently (Wolf *et al.,* 2003). Strains carrying multiple gene deletions were generated by further exchanges of alleles in single or double deletion strains. LP∆*treS*∆*otsA*∆*treY* defective in all three trehalose synthesis pathways was constructed. This strain was indeed unable to synthesise trehalose as demonstrated by GC analysis (data not shown).

3.3.3 Effect of trehalose deficiency on the growth behaviour of *C. glutamicum* lysine producer ATCC 21527

Since the trehalose deficient wild type mutant was mycolate deficient when cultured on sucrose as carbon source, but not on maltose, growth of LP∆*treS*∆*otsA*∆*treY* was analysed in medium supplemented with different carbon sources, i. e. with 4 % sucrose, 4 % fructose and 4 % glucose. Sucrose and fructose are of major importance for the industrial production of L-lysine which is carried out mostly on molasses, whereas in research, almost exclusively glucose is used as carbon source. The cultures were supplemented with 2 % trehalose to assay whether external trehalose could be used for mycolate synthesis and whether the phenotype of the parent strain ATCC 21527 could be restored, although *C. glutamicum* could not take up trehalose into the cytoplasm (cf. 3.2).

LP∆*treS*∆*otsA*∆*treY* grew on all three carbon sources, but growth rate and biomass accumulation were significantly decreased compared to the reference strain ATCC 21527 (ATCC 21527 $\mu = 0.3\ h^{-1}$; LP∆*treS*∆*otsA*∆*treY* $\mu = 0.2\ h^{-1}$; Fig. 12). LP∆*treS*∆*otsA*∆*treY* regained native growth behaviour supplemented with fructose or glucose in the presence of 2 % trehalose whereas cultivation on sucrose and trehalose only partially restored growth. Thus, trehalose added to the culture medium could replace at least partially trehalose biosynthesis. The level of restoration of growth behaviour depended not only on the availability of trehalose, but also on the nature of the carbon source.

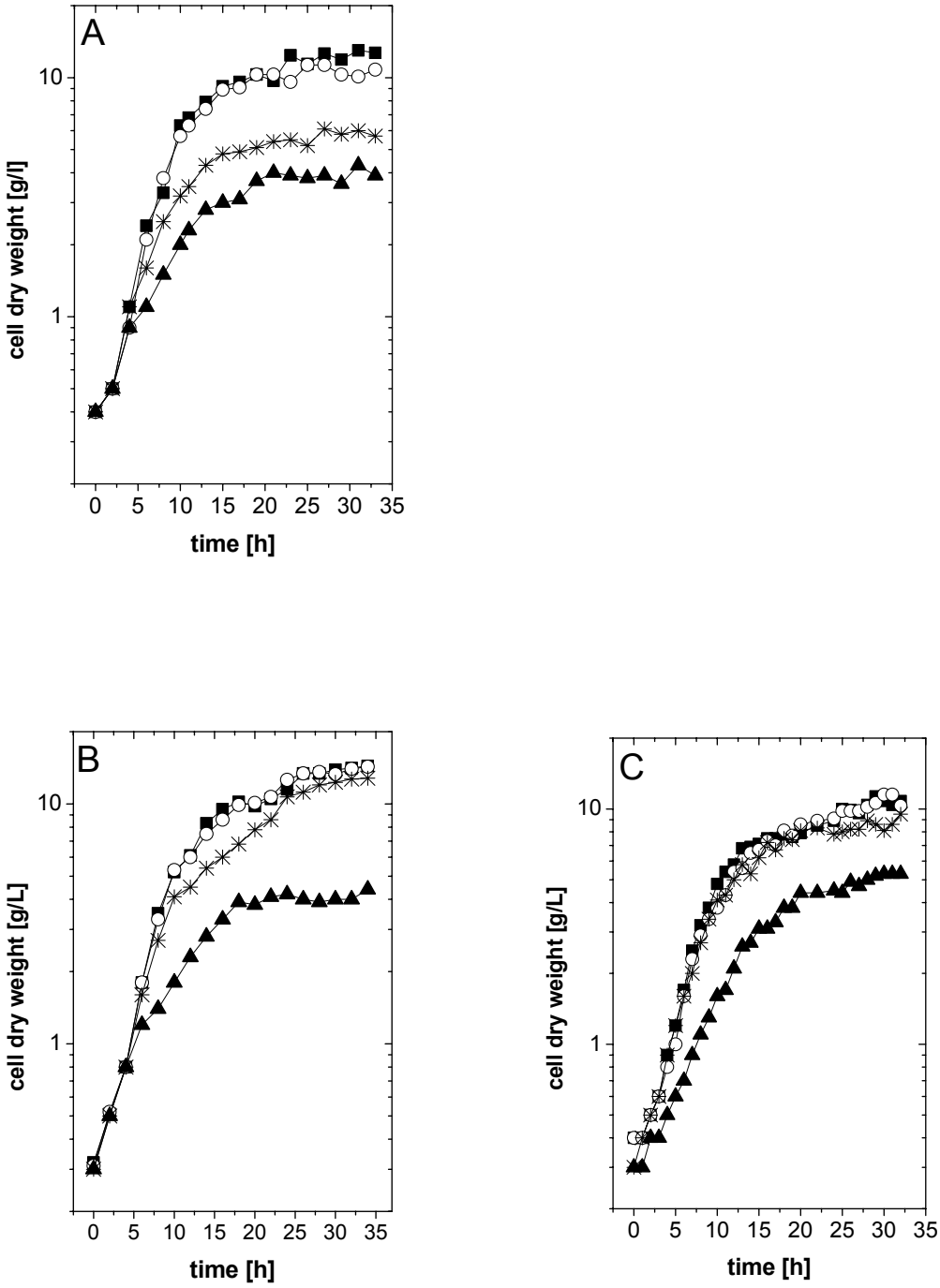


Fig. 12: Effect of external trehalose on growth of L-lysine producer ATCC 21527 and trehalose deficient LPΔ*treS*Δ*otsA*Δ*treY* in dependence of the carbon source of the medium. A, sucrose; B, glucose; C, fructose. ■ ATCC 21527 medium without trehalose; O ATCC 21527 medium with 2 % trehalose; ▲ LPΔ*treS*Δ*otsA*Δ*treY* medium without trehalose; ∗ LPΔ*treS*Δ*otsA*Δ*treY* medium with 2 % trehalose.

During cultivation, cells of LP∆*treS*∆*otsA*∆*treY* exhibited a phenotype distinct from the parent strain. They aggregated in liquid culture (BHI) and after centrifugation the cell-pellet was difficult to resuspend in medium. On agar plates (CgXII + sucrose) the colony surface was rough compared to the smooth bacterial lawn of ATCC 21527 and aggregation of cells was observed in the microscope. The tendency of the cells to adhesion indicated that trehalose deficiency altered the cell surface properties. Probably, the cell surface became more hydrophobic. External trehalose decreased the tendency to aggregation. A similar phenotype was described for the wild type based trehalose deficient strain Cgl*∆otsA∆treS∆treY* (Wolf, 2002).

3.3.4 Effect of trehalose availability and carbon source on the composition of the mycolate layer

The mycolate layer consists of mycolic acids covalently linked to arabinogalactan, the arabinogalactan mycolate (AGM), and mycolate extractible by organic solvents namely trehalose mono- and dimycolate (TMM and TDM). The composition of the mycolate layer of LP*∆treS∆otsA∆treY* and of ATCC 21527 cultivated with sucrose, glucose or fructose in combination with 0, 0.5 or 2 % of trehalose was analysed by means of thin-layer chromatography (TLC) to find out how the availability of trehalose and the type of carbon source influenced the mycolate composition in LP∆*treS*∆*otsA*∆*treY*. The trehalose deficient strain was supplemented with 0.5 % and 2 % trehalose to see whether externally supplied trehalose could be used for mycolate synthesis, although *C. glutamicum* could not take up trehalose into the cytoplasm (cf. 3.2).

ATCC 21527 synthesised arabinogalactan mycolate in equal amounts independent of trehalose supplementation and carbon source (Fig. 13.A-C, left). This strain synthesised both TMM and TDM under all tested conditions. The amount of trehalose mycolates increased with increasing trehalose supplementation (Fig. 13.A-C, right). Thus, the composition of the mycolate layer of ATCC 21527 was independent of the carbon source and depended only quantitatively on the supplementation with trehalose (cf. summary in Tab. 6).

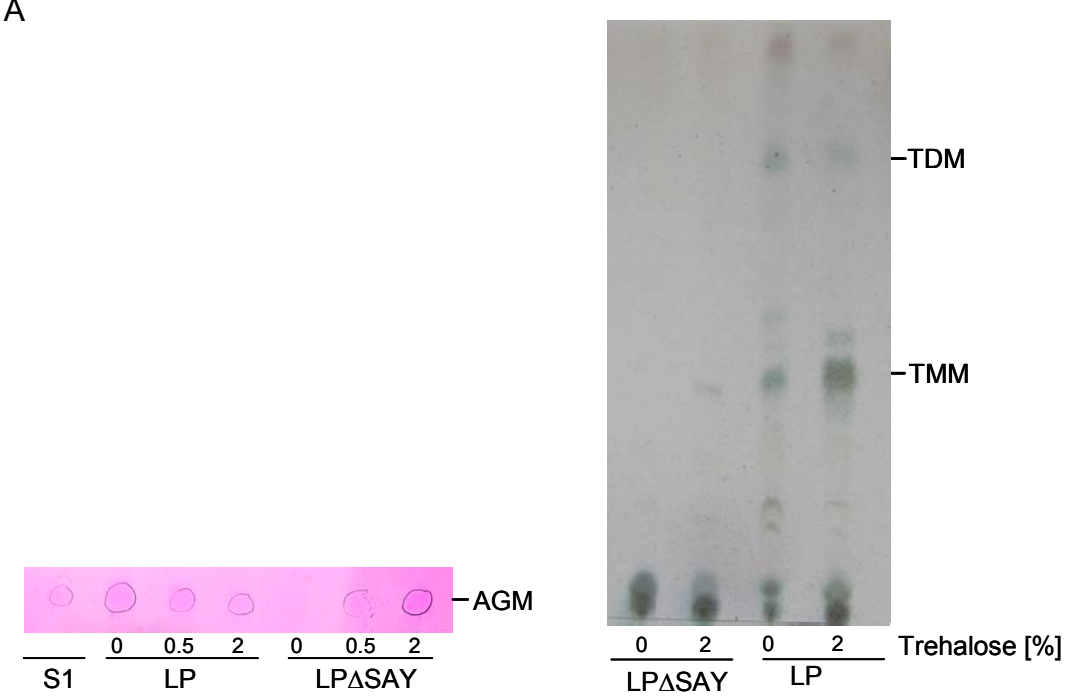
In contrast, the composition of the mycolate layer of LP*∆treS∆otsA∆treY* varied under different cultivation conditions. LP*∆treS∆otsA∆treY* grown on sucrose or fructose in the absence of trehalose did not synthesise arabinogalactan mycolate (Fig. 13.A+C, left), whereas on glucose in the absence of trehalose small amounts of arabinogalactan mycolate were detected (Fig. 13.B, left). Probably, glucose could substitute trehalose as translocator of mycolyl-residues to arabinogalactan whereas sucrose and fructose could not. This assumption was supported by results of the analysis of extractable lipids (Fig. 13.A-C, right).

LP*ΔtreSΔotsAΔtreY* cultured on sucrose or fructose in the absence of trehalose was devoid of mycolate. LP*ΔtreSΔotsAΔtreY* cultured on glucose in the absence of trehalose synthesised neither TMM nor TDM, but it synthesised a glycolipid migrating slightly slower than TDM. Recent analyses of the trehalose deficient wild type derivative Cgl*ΔotsAΔtreSΔtreY* showed that this strain synthesised neither TMM nor TDM grown on sucrose- or glucose-medium, but also a glycolipid migrating slightly slower than TDM which was identified as glucose monomycolate (GMM) by GC-MS analysis (Tropis *et al.*, 2005). Presumably, also the unidentified glycolipid in LP*ΔtreSΔotsAΔtreY* is GMM. In this case, also in this strain, glucose could be esterified by mycolic acids and consequently, glucose could replace trehalose as acceptor of mycolic acids and translocator of mycolyl-residues to arabinogalactan. Hence, trehalose was not essential for mycolate synthesis, when glucose was the carbon source.

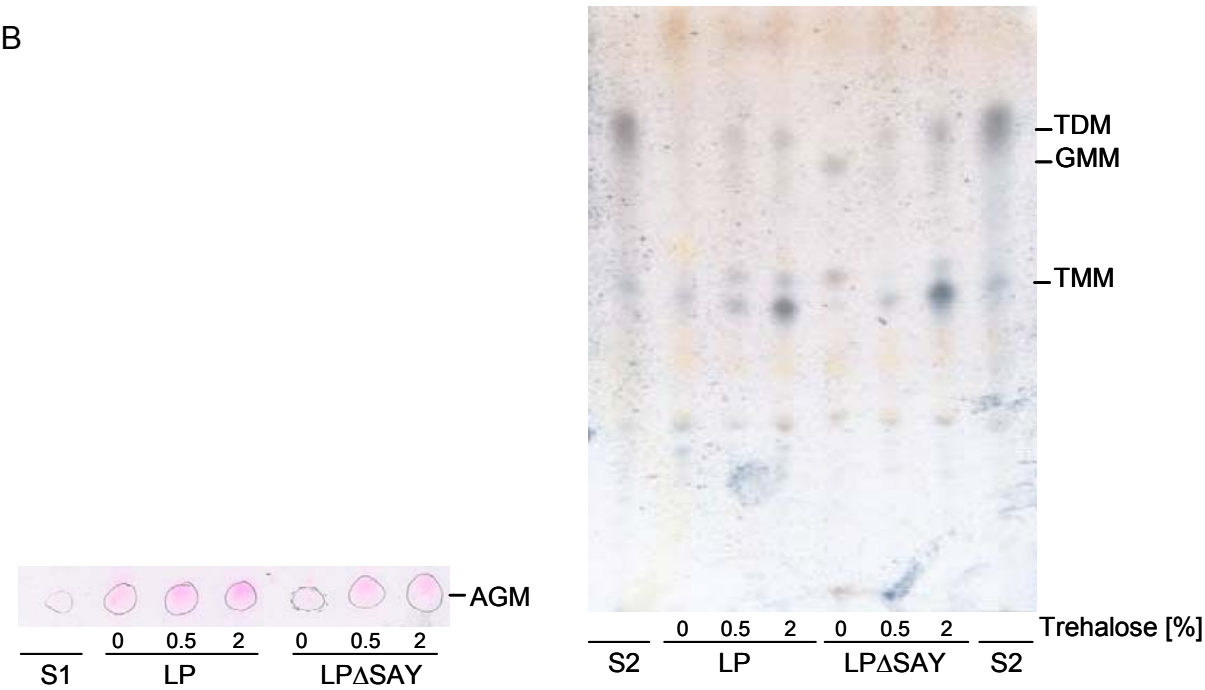
LP*ΔtreSΔotsAΔtreY* synthesised native arabinogalactan mycolate amounts supplemented with 2 % trehalose whereas cultivation with 0.5 % trehalose restored arabinogalactan mycolate synthesis partially, signalling a dose dependent effect of trehalose supplementation. The influence of trehalose supplementation on arabinogalactan mycolate synthesis was similar on all three carbon sources. In contrast, the composition of the extractable mycolate differed in dependence of the carbon source also in the presence of trehalose in the culture medium. The combination of trehalose and sucrose enabled LP*ΔtreSΔotsAΔtreY* to synthesise TMM, but not TDM. LP*ΔtreSΔotsAΔtreY* supplemented with fructose and trehalose synthesised TMM and TDM. Cultivated on glucose and trehalose this strain synthesised not only TMM and TDM, but also GMM. Although trehalose was not taken up into the cytoplasm by *C. glutamicum*, LP*ΔtreSΔotsAΔtreY* used trehalose supplied in the culture medium for mycolate synthesis, proving that mycolate synthesis is localized in the cell envelope.

TLC is not a suitable method for the exact quantification of mycolate, but the size of the lipid spots provided indications about the fractions of the different types of mycolates. While the composition of the mycolate layer depended on the carbon source, the quantity of mycolates could be correlated to the concentration of external trehalose. The amount of TMM and TDM increased with rising trehalose concentrations in combination with all three carbon sources. In glucose-medium, less GMM was synthesised when more trehalose was supplied indicating that trehalose was the preferred substrate for mycolate synthesis.

A


TDM
TMM
AGM
0 0.5 2 0 0.5 2
S1
LP
LPΔSAY
0 2 0 2
Trehalose [%]
LPΔSAY
LP

B


TDM
GMM
TMM
AGM
0 0.5 2 0 0.5 2
S1
LP
LPΔSAY
0 0.5 2 0 0.5 2
S2
LP
LPΔSAY
S2
Trehalose [%]

C

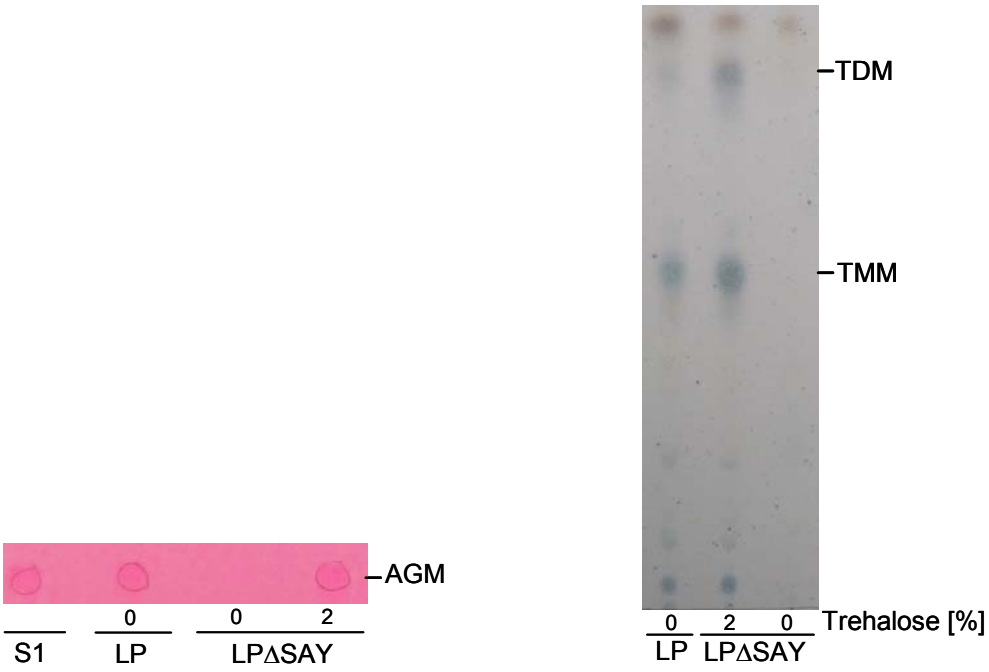


Fig. 13: TLC of arabinogalactan-bound lipids (left) and extractable lipids (right) of ATCC 21527 (*LP*) and of LP*ΔtreSΔotsAΔtreY* (*LPΔSAY*) culivated in CgXII with 4% sucrose (A), 4% glucose (B) or 4% fructose (C) and different concentrations of trehalose. *S1*, sample of arabinogalactan mycolate (*AGM*). *S2*, sample of trehalose dimycolate (*TDM*) and trehalose monomycolate (*TMM*). *GMM,* glucose monomycolate.

Results of the qualitative analysis of the composition of the mycolate layer are summarized in the following table (Tab. 6).

Tab. 6: Effect of trehalose and carbon source on the composition of the mycolate layer of L-lysine producer ATCC 21527 and trehalose deficient LP*ΔtreSΔotsAΔtreY*

Strain	C-source	Mycolate synthesis without external trehalose				Mycolate synthesis with external trehalose			
		AGM	TMM	TDM	GMM	AGM	TMM	TDM	GMM
LPΔSAY	sucrose	-	-	-	-	+	+	-	-
	glucose	+	-	-	+	+	+	+	+
	fructose	-	-	-	-	+	+	+	-
ATCC 21527	sucrose	+	+	+	-	+	+	+	-
	glucose	+	+	+	-	+	+	+	-
	fructose	+	+	+	-	+	+	+	-

External addition of trehalose was essential for mycolate synthesis in LP*ΔtreSΔotsAΔtreY* when sucrose or fructose were used as carbon source. In contrast, glucose substituted trehalose as acceptor and translocator of mycolic acids enabling the synthesis of arabinogalactan mycolate and of the novel glycolipid GMM in the absence of trehalose. These data were consistent with recently published results for the trehalose deficient wild type derivative Cgl*ΔotsAΔtreSΔtreY* (Tropis *et al.*, 2005). Supplementation with trehalose enabled LP*ΔtreSΔotsAΔtreY* to synthesise arabinogalactan mycolate, TMM and TDM similar to the parental strain supplemented with glucose and fructose, whereas with sucrose only arabinogalactan mycolate and TMM were synthesised. Thus, the mycolate layer of LP*ΔtreSΔotsAΔtreY* can be manipulated gradually from complete lack of mycolate to wild type like mycolate composition by the choice of the carbon source and the supplementation with trehalose.

3.3.5 Quantitative effect of trehalose supplementation on the amount of mycolate

The analysis of the mycolate composition of LP*ΔtreSΔotsAΔtreY* by means of thin-layer chromatography provided only qualitative information. However, the size of the lipid spots indicated that LP*ΔtreSΔotsAΔtreY* synthesised more mycolate if the medium was supplemented with 2 % trehalose instead of 0.5 % trehalose. The comparison of the amount of the different kinds of mycolate in LP*ΔtreSΔotsAΔtreY* with the corresponding amounts in the wild type could reveal whether the mycolate composition could not only be restored qualitatively, but also quantitatively. Moreover, the existence of a quantitative correlation of the mycolate concentration with the trehalose concentration could facilitate the manipulation of the grade of reconstitution of the mycolate layer by supplementing the medium with different concentrations of trehalose. For the quantitative analysis, mycolic acids were isolated by saponification of TMM, TDM, GMM and arabinogalactan bound mycolate and quantified by means of gas chromatography (GC).

In a typical chromatogram (Fig. 14) palmitic ($C_{16:0}$; peak 4) and octadecenoic acid ($C_{18:1}$; peak 7) had the retention time of about 9 min and 10.6 min, respectively, followed by the mycolic acids $C_{32:0}$ (20.4 min, peak 17), $C_{34:1}$ (21.6 min, peak 21) and $C_{34:0}$ (22.0 min, peak 22) (retention times according to standards of the group of M. Daffé, ipbs Toulouse). As palmitic and octadecenoic acid are part of the plasma membrane which is supposed to be unaffected by the different conditions tested, these fatty acids were taken as internal standard.

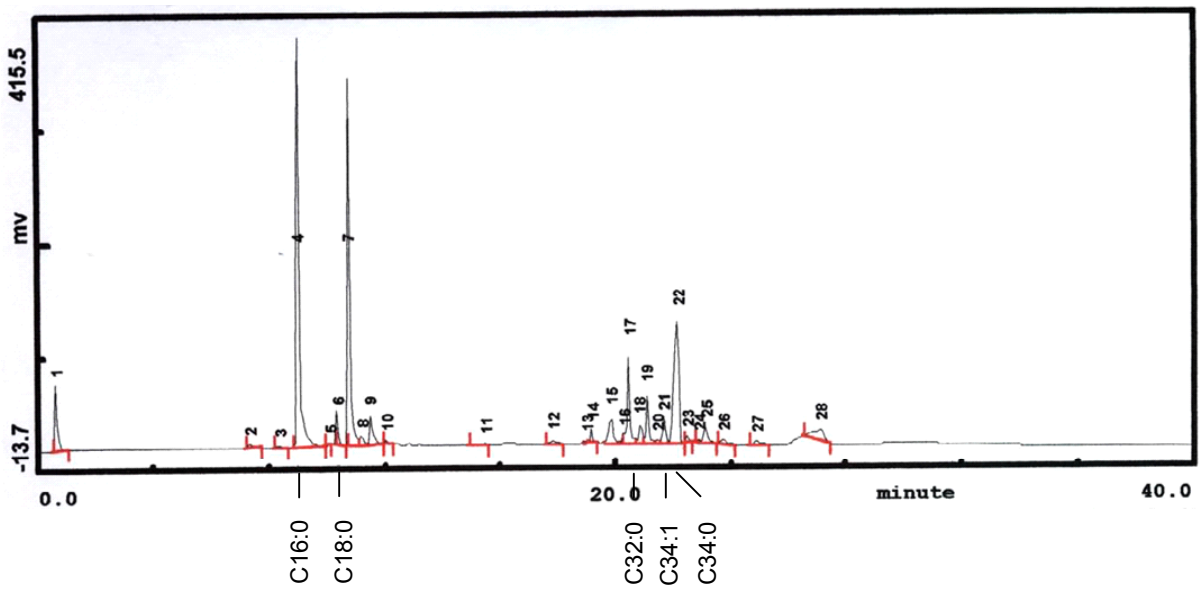


Fig. 14: GC chromatogram of lipid extracts from ATCC 21527 cultivated in CgXII with 4 % glucose and 2 % trehalose

The ratio between the sum of the peak areas of the mycolic acids and the sum of the peak areas of the fatty acids was calculated to compare the mycolate content under the different conditions. The resulting factors are summarized in the following Tab. 7.

Tab. 7: Quantitative effect of trehalose supplementation on the amount of mycolate. GC analysis of extractible and arabinogalactan bound lipids of ATCC 21527 and of LP*ΔtreSΔotsAΔtreY* cultured on 0, 0.5 or 2 % trehalose and 4 % glucose or 4 % sucrose as carbon source. The relative amount of mycolate was calculated by dividing the peak areas of mycolic acids by the peak areas of fatty acids.

C-source	Trehalose	ATCC 21527		LP*ΔtreSΔotsAΔtreY*	
		relative extractable mycolate	relative arabinogalactan-bound mycolate	relative extractable mycolate	relative arabinogalactan-bound mycolate
Glucose	**0 %**	0.450	0.329	0.1	0.175
	0.5 %	0.554	0.417	0.311	0.775
	2 %	0.405	0.456	0.894	0.913
Sucrose	**0 %**	0.350	0.474	0	0
	0.5 %	0.550	0.530	0.02	0.045
	2 %	0.504	0.503	0.075	0.195

For ATCC 21527, the ratio between mycolate and internal standard was similar on glucose and sucrose. The ratio increased slightly in the presence of trehalose. Probably, synthesis of mycolate had been limited by the amount of cytoplasmic trehalose or external trehalose was more accessible.

No mycolic acids were detected in LP*ΔtreSΔotsAΔtreY* grown with sucrose, proving results from TLC analysis. When LP*ΔtreSΔotsAΔtreY* was cultured on sucrose and 0.5 % trehalose, mycolic acids were synthesised. The ratio between trehalose mycolate and internal standard as well as the ratio between arabinogalactan mycolate and internal standard was fourfold higher when this strain was cultured on the fourfold concentration of trehalose (2 %). Thus, the mycolate content of the cell envelope of LP*ΔtreSΔotsAΔtreY* depended quantitatively on the concentration of trehalose, when sucrose was the carbon source confirming that under these conditions external trehalose was the only acceptor and translocator of mycolic acids.

Mycolic acids were detected among extractible lipids and in extracts of the cell wall bound mycolic acids of LP*ΔtreSΔotsAΔtreY* cultured on glucose in the absence of trehalose. Presumably, these mycolic acids derived from GMM. Supplementation of LP*ΔtreSΔotsAΔtreY* with trehalose increased the ratio between mycolate and internal standard, but mycolate and trehalose concentrations were not correlated quantitatively. With glucose as carbon source, mycolate synthesis was not determined by external trehalose alone proving that glucose could replace trehalose as acceptor and translocator of mycolic acids.

As the mycolate content could be correlated to the trehalose concentration in combination with sucrose, but not in combination with glucose, different trehalose concentrations can be used to manipulate the amount of mycolate only in LP*ΔtreSΔotsAΔtreY* cultured on sucrose. The relative concentration of extractible mycolate and of arabinogalactan mycolate of LP*ΔtreSΔotsAΔtreY* cultivated in sucrose-medium was lower than in ATCC 21527, even in the presence of 2 % trehalose. In contrast, LP*ΔtreSΔotsAΔtreY* cultivated in glucose-medium synthesised lower amounts of mycolate in the absence of trehalose, but similar or even higher mycolate concentrations than the parental strain when the medium was supplemented with 0.5 % or 2 % trehalose. Thus, on glucose, the amount of mycolic acids seemed to be sufficient to build a native mycolate layer. However, this method does not differentiate between mycolic acids derived from trehalose mycolate and from glucose mycolate.

3.3.6 Influence of trehalose on the permeability of the cell envelope

In comparison to other Gram-positive as well as to Gram-negative cell envelopes, e. g. of *E. coli* or *Pseudomonas aeruginosa*, the permeability of the cell envelope of mycobacteria is extremely low for some hydrophilic as well as hydrophobic solutes e. g. antibiotics. The mycolate layer – a lipid bilayer of unusual thickness and low fluidity - constitutes the main permeation barrier (Brennan & Nikaido, 1995). Whereas hydrophobic solutes have to diffuse through the mycolate layer directly, small and hydrophilic solutes are believed to pass through porins – proteins forming hydrophilic channels. Lower permeability to small hydrophilic solutes was explained by a smaller number of porins and longer porin channels in *Mycobacterium smegmatis* compared to *E. coli* (Engelhardt *et al.,* 2002). However, the relative importance of porin mediated crossing or direct permeation of the mycolate layer is unknown for most substances.

Also in *C. glutamicum* the mycolate layer is believed to determine the permeability of the cell envelope, since *C. glutamicum* strains with reduced mycolate content due to inactivation of a mycolyltransferase took up faster glycerol and acetate (Puech *et al.,* 2000). Measurement of the permeability of the cell envelope of LP*ΔtreSΔotsAΔtreY* should reveal whether also an incomplete mycolate layer caused by trehalose deficiency rendered the cell envelope of *C. glutamicum* more permeable and whether the native phenotype was restored when the medium was supplemented with trehalose.

Therefore, three different methods were applied. The Zimmermann-Rosselet assay, which determines the diffusion of β-lactam antibiotics by measuring β-lactamase activity, is one of the most suitable methods to determine the permeability of the mycolate layer because it considers only diffusion through the outer layer, the mycolate layer and the arabinogalactan layer, but not through the plasma membrane, which exhibits a further permeation barrier. A further assay was based on an assay developed for mycobacteria. In mycobacteria the mycolate layer, is made responsible for the resistance against various antibiotics, thus in this assay cell wall permeability was correlated to the susceptibility to antibiotics (Liu and Nikaido, 1999). The third method was based on the observation that recombinant *C. glutamicum* strains with reduced mycolate content were characterized by an increased uptake rate for glycerol (Puech *et al.,* 2000).

3.3.6.1 Inspection of the Zimmermann - Rosselet assay for *C. glutamicum*

The Zimmermann - Rosselet assay determines rates of diffusion of β-lactam antibiotics through bacterial cell walls (Zimmermann and Rosselet, 1977). The permeability of the cell wall to a β-lactam antibiotic is measured using intact cells and periplasmic β-lactamases as a sink. Thus, diffusion is determined from the cell surface through the outer layer, the mycolate

layer and the arabinogalactan layer to the peptidoglycan layer, where β-lactamases are located. As the mycolate layer is the only permeation barrier limiting diffusion on this pathway, the Zimmermann - Rosselet assay provides a very accurate tool to determine the permeability of the mycolate layer. The assay is well established for *Mycobacterium smegmatis* (Stahl *et al.,* 2001), but for *C. glutamicum* no β-lactamase activity is reported. Although a putative β-lactamase gene was annotated in the genome of *C. glutamicum*, the existence of a functional β-lactamase has not been proven. Since the β-lactam antibiotic cephaloridine used by Stahl *et al.* (2001) could no longer be purchased, the related β-lactam antibiotic cephalothin was chosen for the permeability assay. Hydrolysis of the β-lactam antibiotic by the β-lactamase was monitored by determining UV absorption. The absorption maximum of cephalothin was 260 nm. Whereas the crude extract of *M. smegmatis* cells was able to hydrolyse cephalothin completely within 30 min, proving the experimental setup, an extract of *C. glutamicum* cells did not decrease the absorption of cephalothin. Cell extracts of *C. glutamicum* and of *M. smegmatis* were mixed to rule out a possible inhibition of β-lactamase activity by an unknown substance in the *C. glutamicum* cell extract. Cephalothin was hydrolysed by the mixture. This means that the cell extract of *C. glutamicum* is not inhibiting β-lactamase activity and furthermore that a functional β-lactamase is not present in *C. glutamicum* under the chosen cultivation conditions.

As no β-lactamase activity was detected in *C. glutamicum*, it was tested whether the heterologous expression of β-lactamase was possible in this bacterium. In a *C. glutamicum* strain comprising the coding sequence for β-lactamase integrated into the chromosome (kindly provided by O. Ley), no β-lactamase activity was detected. Thus, the Zimmermann - Rosselet assay is unfortunately not a suitable method to determine the permeability of the cell envelope in *C. glutamicum.*

3.3.6.2 Resistance to antibiotics as an indicator for the permeability of the cell envelope

As the Zimmermann-Rosselet assay could not be performed with *C. glutamicum,* a further assay developed for mycobacteria which correlated the resistance to antibiotics to the permeability of the cell envelope was applied. One disadvantage of this test was that the target of the antibiotic may be located in the cytoplasm of the cell so that diffusion of the antibiotic was determined by the permeability of both, the mycolate layer and the plasma membrane. Therefore, we chose two antibiotics which act on cell wall components. The β-lactam antibiotic penicillin G inhibits the synthesis of the peptidoglycan layer and the anti-tuberculosis drug ethambutol interferes with arabinogalactan synthesis. Both antibiotics are relatively small hydrophilic molecules. As charge and size of the molecule determine the

passage through the mycolate layer (Lambert, 2002), the large hydrophobic macrolide antibiotic erythromycin, which works on protein biosynthesis, was selected as an alternative. The minimal inhibitory concentration of these antibiotics for the lysine producer strain ATCC 21527 and its trehalose deficient derivative LP*ΔtreSΔotsAΔtreY* was determined by the use of a plastic strip bearing an immobilized antibiotic gradient on its back and the respective concentration scale on the front side which was laid on an agar plate inoculated with one of the *C. glutamicum* strains (Fig. 15). Antibiotic resistance of both strains was tested when cultivated on agar supplemented with 4 % glucose, 4 % fructose or 4 % sucrose and with 2 % trehalose if indicated.

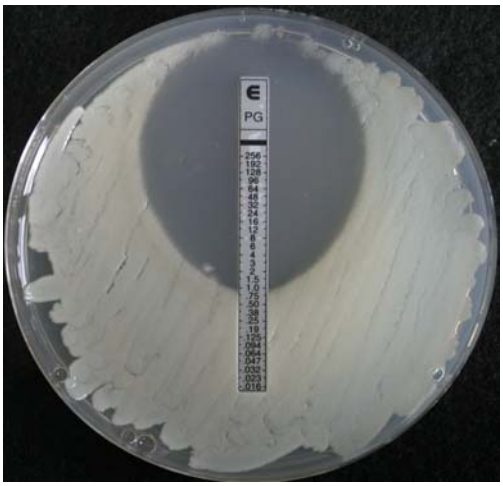


Fig. 15: Influence of trehalose on the inhibition of bacterial growth of ATCC 21527 by penicillin. The agar plates contained a gradient of penicillin concentrations due to antibiotic immobilized on the backside of the plastic strip.

The trehalose deficient LP*ΔtreSΔotsAΔtreY* was inhibited by a fourth of the concentration of penicillin (0.07-0.16 µg/ml) and ethambutol (0.5-1 µg/ml) and by a third of the concentration of erythromycin (0.38-0.46 µg/ml) that inhibited the parental strain (Fig. 16) indicating that the permeability of the cell envelope of LP*ΔtreSΔotsAΔtreY* was higher. In general, this strain was least susceptible to the three antibiotics when glucose was the carbon source. As under these cultivation conditions AGM and GMM were synthesised, while with sucrose or fructose no mycolate could be detected, uptake of antibiotics was probably hindered by the incomplete mycolate layer. When supplementation of the medium with trehalose facilitated mycolate synthesis in combination with all three carbon sources, LP*ΔtreSΔotsAΔtreY* became more resistant to all three antibiotics indicating that restoration of the mycolate layer decreased the permeability of the cell envelope. In spite of supplementation with trehalose, LP*ΔtreSΔotsAΔtreY* was still more susceptible to the antibiotics than ATCC 21527. Hence, external trehalose enabled mycolate synthesis by LP*ΔtreSΔotsAΔtreY*, but the extent of permeability of the cell envelope indicated that the structure of the mycolate layer still differed from the native mycolate layer.

An imperfect or missing mycolate layer affected the resistance to erythromycin less than that to penicillin or ethambutol. This could be due to the fact that erythromycin has to cross an additional permeability barrier, the plasma membrane, to access its target.

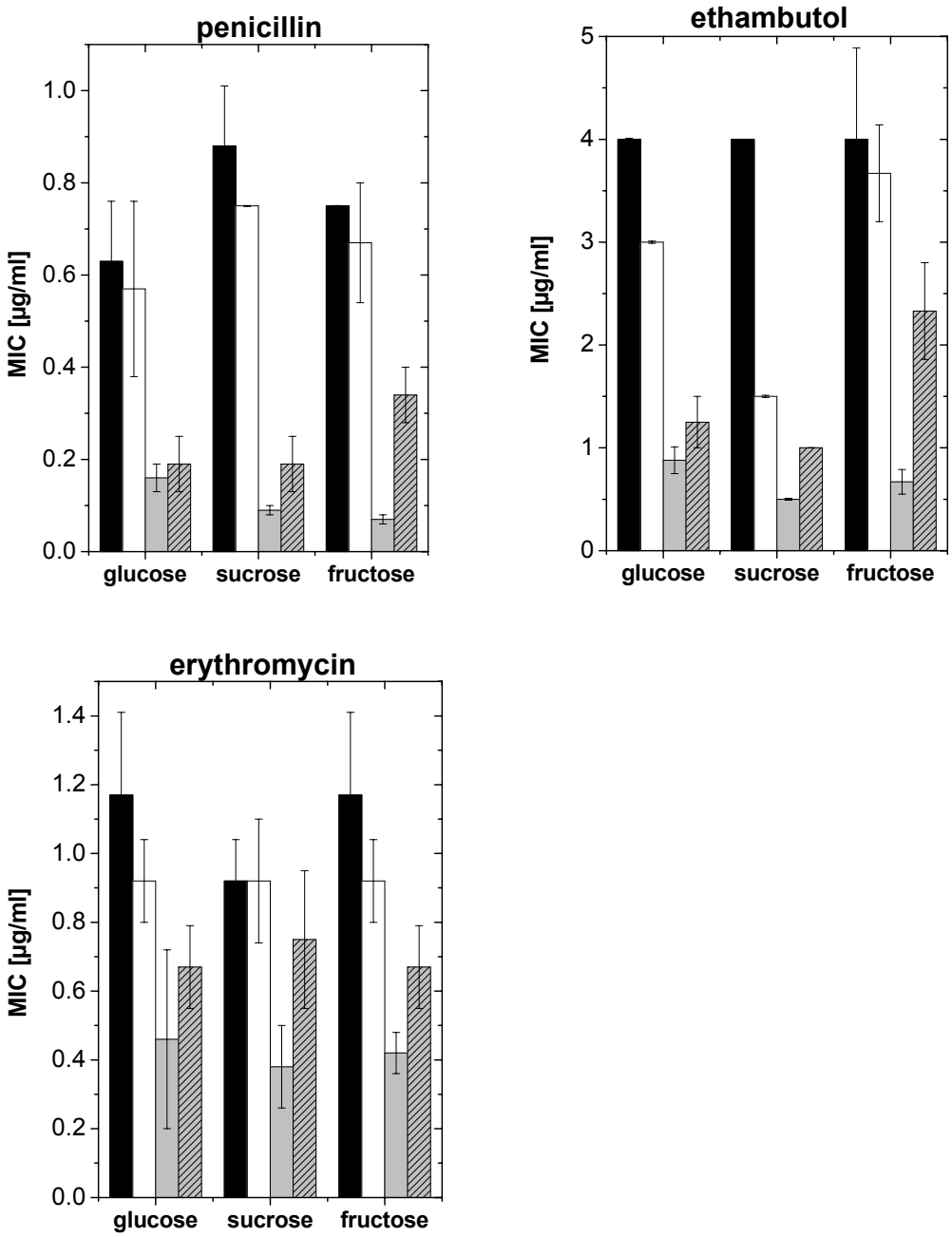


Fig. 16: Effect of external trehalose on the minimal inhibitory concentration (MIC) of antibiotics on L-lysine producer ATCC 21527 and on trehalose deficient LP*ΔtreSΔotsAΔtreY* in dependence of the carbon source of the medium. The values are means of three independent determinations. Black bars, ATCC 21527 medium without trehalose; white bars, ATCC 21527 medium with 2 % trehalose; grey bars, LP*ΔtreSΔotsAΔtreY* medium without trehalose; grey shaded bars, LP*ΔtreSΔotsAΔtreY* medium with 2 % trehalose.

3.3.6.3 Diffusion of glycerol as an indicator for the permeability of the cell envelope

A further method taking diffusion through the cell envelope as an indicator for the permeability of the cell envelope was to measure uptake rates of [^{14}C] labelled compounds. A feature of diffusion - in contrast to uptake by active transport - is that uptake rates rise proportionally to substrate concentrations. Recently, Puech *et al.* (2000) correlated glycerol uptake to the mycolate content.

Uptake rates of LP*ΔtreSΔotsAΔtreY* and ATCC 21527 were measured for different concentrations of [^{14}C]glycerol to determine the influence of an incomplete mycolate layer due to trehalose deficiency on the permeability of the cell envelope. Cells were grown on sucrose because under these conditions the mycolate layer was missing in LP*ΔtreSΔotsAΔtreY.* Supplementation of the medium with trehalose should demonstrate whether the restoration of the mycolate layer caused native permeability. Cells were harvested during stationary growth phase.

The uptake rates for glycerol of the two strains increased proportionally to the glycerol concentration, signalling that diffusion limited glycerol uptake (Fig. 17). Uptake of glycerol of the mycolate deficient LP*ΔtreSΔotsAΔtreY* was twice as fast as of the reference strain indicating that higher permeability of the envelope of LP*ΔtreSΔotsAΔtreY* accelerated diffusion. When LP*ΔtreSΔotsAΔtreY* was supplemented with trehalose, glycerol uptake rates were slightly lower than without trehalose, but not as low as for the parent strain. Thus, the partial restoration of the mycolate layer of LP*ΔtreSΔotsAΔtreY* due to the presence of external trehalose slightly decreased the permeability of the cell envelope, but the extent of permeability of the parent strain was not reached. These data were consistent with results of the measuring of resistance to antibiotics. Taken together, results obtained by these two methods confirmed that lack of mycolate made the cell envelope of the trehalose deficient strain more permeable compared to the cell envelope of ATCC 21527 and that the mycolate layer synthesised from trehalose supplemented in the medium did not have the same properties as the native mycolate layer.

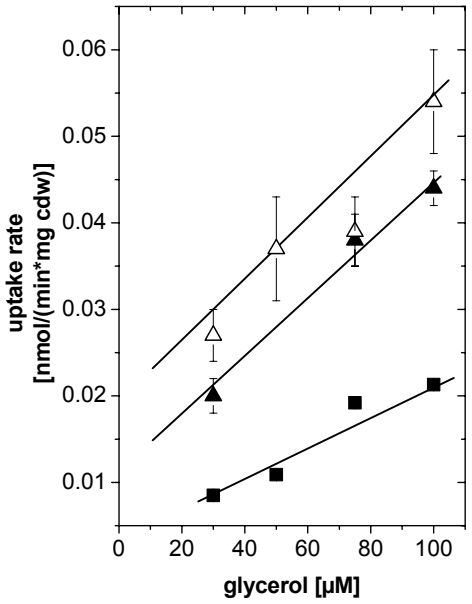


Fig. 17: Effect of external trehalose on uptake of [^{14}C]glycerol into stationary cells of L-lysine producer ATCC 21527 and of trehalose deficient LP*ΔtreSΔotsAΔtreY* cultivated with 4 % sucrose. The values are means of three independent determinations. ■ ATCC 21527 medium without trehalose, △ LPΔ*treS*Δ*otsA*Δ*treY* medium without trehalose, ▲ LPΔ*treS*Δ*otsA*Δ*treY* medium with 2 % trehalose.

Although the proportionality of glycerol concentration and uptake rates indicated that glycerol entered the cell by diffusion, an enzymatic assay for glycerol kinase - the key enzyme in glycerol metabolism - was established to verify that diffusion and not metabolism was the limiting factor for transport in stationary cells. Furthermore, this assay was utilized to compare the glycerol metabolism of LP*ΔtreSΔotsAΔtreY* and of ATCC 21527 to exclude that different uptake rates for glycerol were due to different metabolic activities.
Glycerol kinase activity was determined in a coupled assay based on the following reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L-glycerol-3-P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$

GK = Glycerol kinase
PK = Pyruvate kinase
L-LDH = Lactate dehydrogenase
PEP = Phosphoenolpyruvate

The amount of NADH oxidized in the above reaction is stochiometric to the amount of conversed glycerol. Two control experiments were performed. The enzymatic assay was

performed without extract or without the enzymes PK and L-LDH, to exclude that additional enzymes conversed NADH or that NADH was decaying for other reasons, respectively. As the concentration of NADH did not decrease under the control conditions (data not shown), this glycerol kinase assay was suitable to determine glycerol kinase activity.
The specific activity of the glycerol kinase was similar in the two strains (0.25 ± 0.01 µmoles * min^{-1} * mg^{-1} protein) indicating that trehalose deficiency did not alter glycerol metabolism. Hence, uptake rates determined for stationary cells of LP*ΔtreSΔotsAΔtreY* and of ATCC 21527 did not differ because of the metabolism, but because of cell envelope properties. A 100 fold higher glycerol kinase activity than glycerol uptake rates supported furthermore that glycerol uptake was not limited by glycerol kinase activity, but by diffusion.

3.3.7 Impact of the availability of trehalose and of the carbon source on the excretion of amino acids

An incomplete mycolate layer rendered the cell envelope of the *C. glutamicum* lysine producer ATCC 21527 more permeable as shown by measuring uptake of substances. The lysine producer strain had been chosen for this project since its ability to excrete lysine facilitated also the investigation of the impact of an incomplete mycolate layer on the efflux of solutes. Furthermore, comparison of the trehalose deficient lysine producer strain LP*ΔtreSΔotsAΔtreY* to its parental strain ATCC 21527 should reveal whether lack of mycolate rendered the production strain more powerful. The two strains were cultivated on 4 % sucrose, 4 % glucose or 4 % fructose, in the absence and presence of trehalose. Excretion of amino acids was determined by measuring the supernatant by means of HPLC.

The absolute concentration of lysine which accumulates during cultivation in the medium is one characteristic feature of a lysine production strain. LP*ΔtreSΔotsAΔtreY* and ATCC 21527 excreted the highest lysine concentration grown on sucrose whereas on glucose or fructose both strains excreted less than one third of this concentration (Fig. 18).

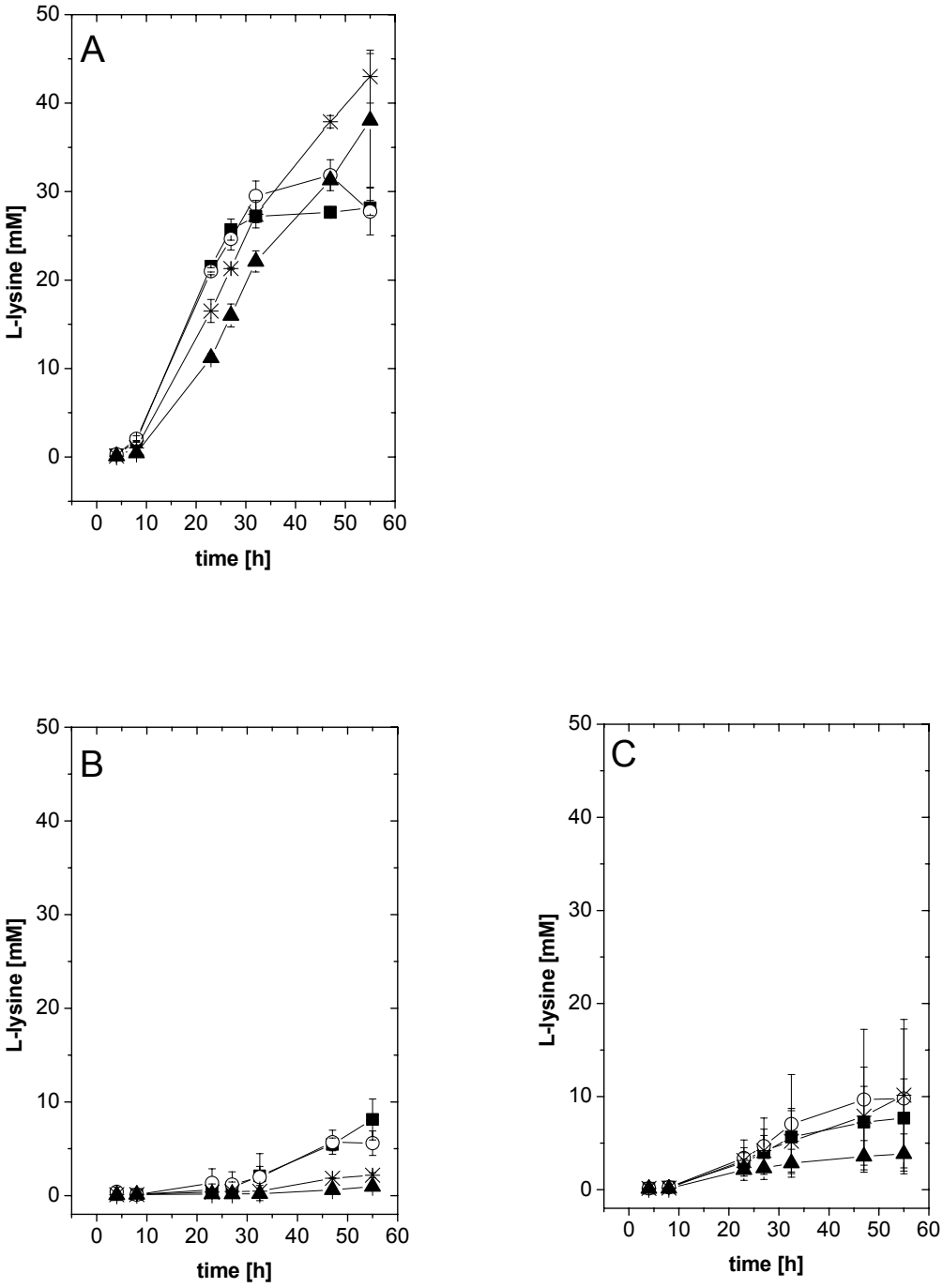


Fig. 18: Effect of external trehalose on L-lysine excretion of L-lysine producer ATCC 21527 and trehalose deficient LPΔ*treS*Δ*otsA*Δ*treY* in dependence of the carbon source of the medium. A, sucrose; B, glucose; C, fructose. ■ ATCC 21527 medium without trehalose; O ATCC 21527 medium with 2 % trehalose; ▲ LPΔ*treS*Δ*otsA*Δ*treY* medium without trehalose; ∗ LPΔ*treS*Δ*otsA*Δ*treY* medium with 2 % trehalose.

Biomass related lysine excretion (lysine accumulation in mM divided by cell dry weight) takes into account that LP∆*treS*∆*otsA*∆*treY* synthesised significantly less biomass than the parent strain (Fig. 12), therefore specific lysine excretion was used as a measure for the efflux of solutes. In sucrose-medium, biomass related lysine excretion of LP∆*treS*∆*otsA*∆*treY* was twice as high as that of the reference strain indicating that lack of mycolate facilitated lysine excretion by LP∆*treS*∆*otsA*∆*treY* (Fig. 19.A). When external trehalose enabled a partial restoration of the mycolate layer in LP∆*treS*∆*otsA*∆*treY*, the specific lysine excretion was only slightly higher than that of the reference strain. These data suggested that lack of mycolate was responsible for enhanced lysine excretion in the lysine producer strain, but results of the analysis of lysine excretion by the two strains in glucose- or fructose-medium were not conclusive (Fig. 19.B+C). Although LP∆*treS*∆*otsA*∆*treY* cultivated with fructose lacked the mycolate layer completely, biomass related lysine excretion was lower than that of ATCC 21527. In glucose-medium without trehalose LP∆*treS*∆*otsA*∆*treY* excreted slightly more lysine per biomass than ATCC 21527 in the first 30 h of cultivation. But afterwards, LP∆*treS*∆*otsA*∆*treY* and ATCC 21527 in trehalose supplemented medium, both synthesising the native mycolate composition, excreted more lysine than LP∆*treS*∆*otsA*∆*treY* in glucose-medium without trehalose which synthesised only AGM and GMM.

Probably, the composition of the mycolate layer is not the only factor which determines lysine excretion under these conditions. Kiefer *et al.* (2002) showed that the lysine productivity of the lysine producer ATCC 21253 was different on glucose, sucrose and fructose. In this strain the three carbon sources entered central metabolism at different levels guiding carbon fluxes to NADPH generating pathways of different efficiency thus providing different levels of NADPH for lysine synthesis (Georgi *et al.,* 2005). The lysine producer ATCC 21527 excreted three times more lysine in sucrose-medium than in fructose- or glucose-medium, although the composition of the mycolate layer was similar under all cultivation conditions. Hence, the carbon source influenced not only the composition of the mycolate layer and thus indirectly lysine excretion, but also lysine synthesis directly. Like its parental strain, LP∆*treS*∆*otsA*∆*treY* excreted more lysine on sucrose-medium than on glucose- or fructose-medium indicating that the metabolism of the cell depends on the carbon source also in this strain.

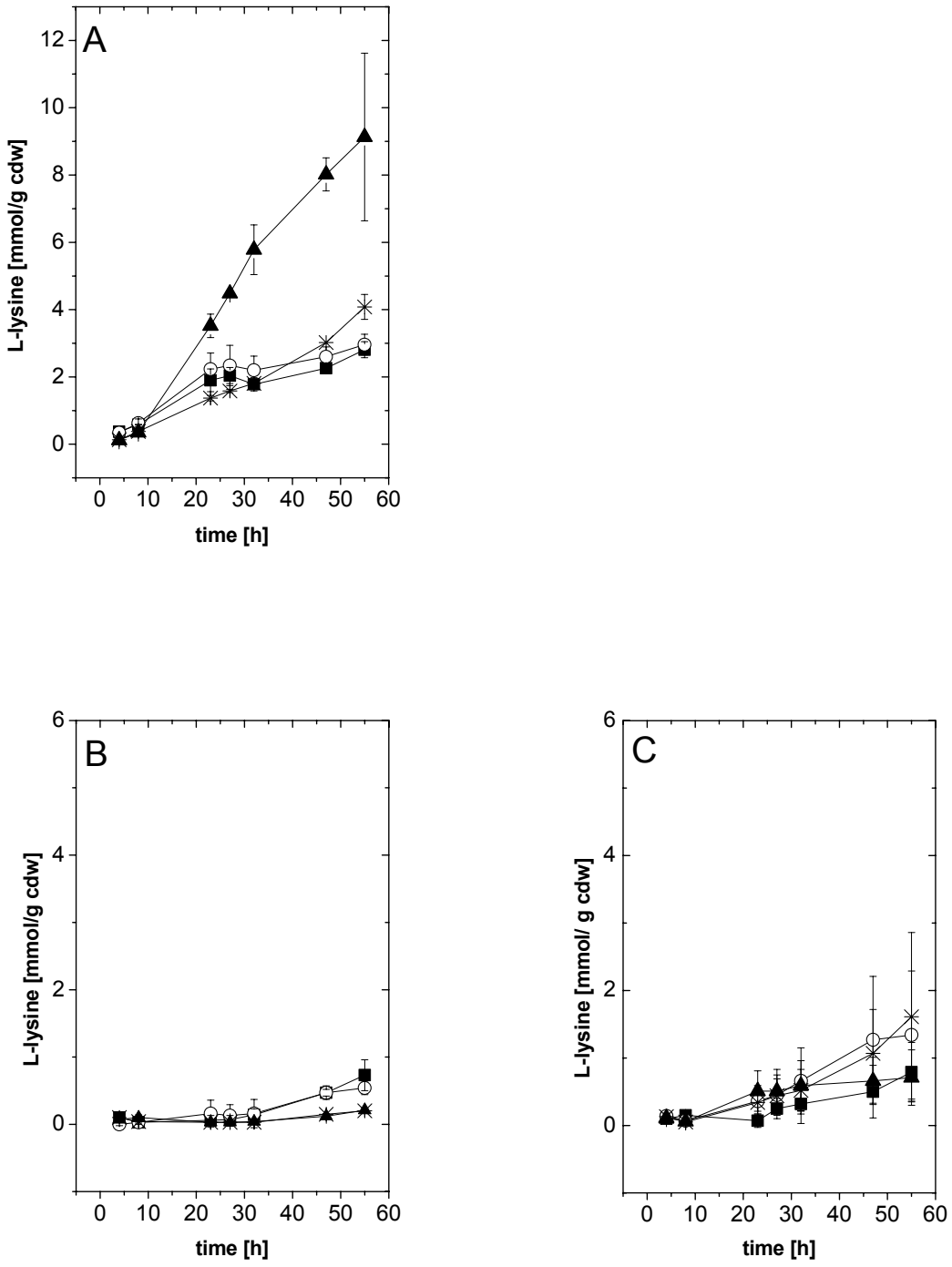


Fig. 19: Effect of external trehalose on specific L-lysine excretion of lysine producer ATCC 21527 and trehalose deficient LPΔ*treS*Δ*otsA*Δ*treY* in dependence of the carbon source of the medium. A, sucrose; B, glucose; C, fructose. ■ ATCC 21527 medium without trehalose; O ATCC 21527 medium with 2 % trehalose; ▲ LPΔ*treS*Δ*otsA*Δ*treY* medium without trehalose; ∗ LPΔ*treS*Δ*otsA*Δ*treY* medium with 2 % trehalose.

C. glutamicum is not only used for the industrial production of lysine, but also for the production of the amino acid glutamate. In the wild type strain the excretion of glutamate is caused by special treatments e.g. by biotin limitation or addition of detergents, which affect the lipid composition of the plasma membrane or the mycolate layer, but the exact mechanism triggering glutamate excretion is not known.

Surprisingly, glutamate was detected in the culture medium of LP∆*treS*∆*otsA*∆*treY* under normal cultivation conditions i. e. MM with sucrose, glucose or fructose as carbon source, whereas the parental strain did not excrete glutamate under the same conditions (Fig. 20). In contrast to the wild type, glutamate excretion by LP∆*treS*∆*otsA*∆*treY* required no special treatment. Obviously, the lacking or decomposed mycolate layer triggered glutamate excretion. This assumption was proven by the fact that cultivation of LP∆*treS*∆*otsA*∆*treY* with 2 % trehalose, which enabled the synthesis of a nearly native mycolate layer, inhibited glutamate excretion. Slightly higher glutamate excretion of LP∆*treS*∆*otsA*∆*treY* cultivated on glucose-medium compared to sucrose- or fructose-medium, could be due to higher glutamate synthesis on glucose.

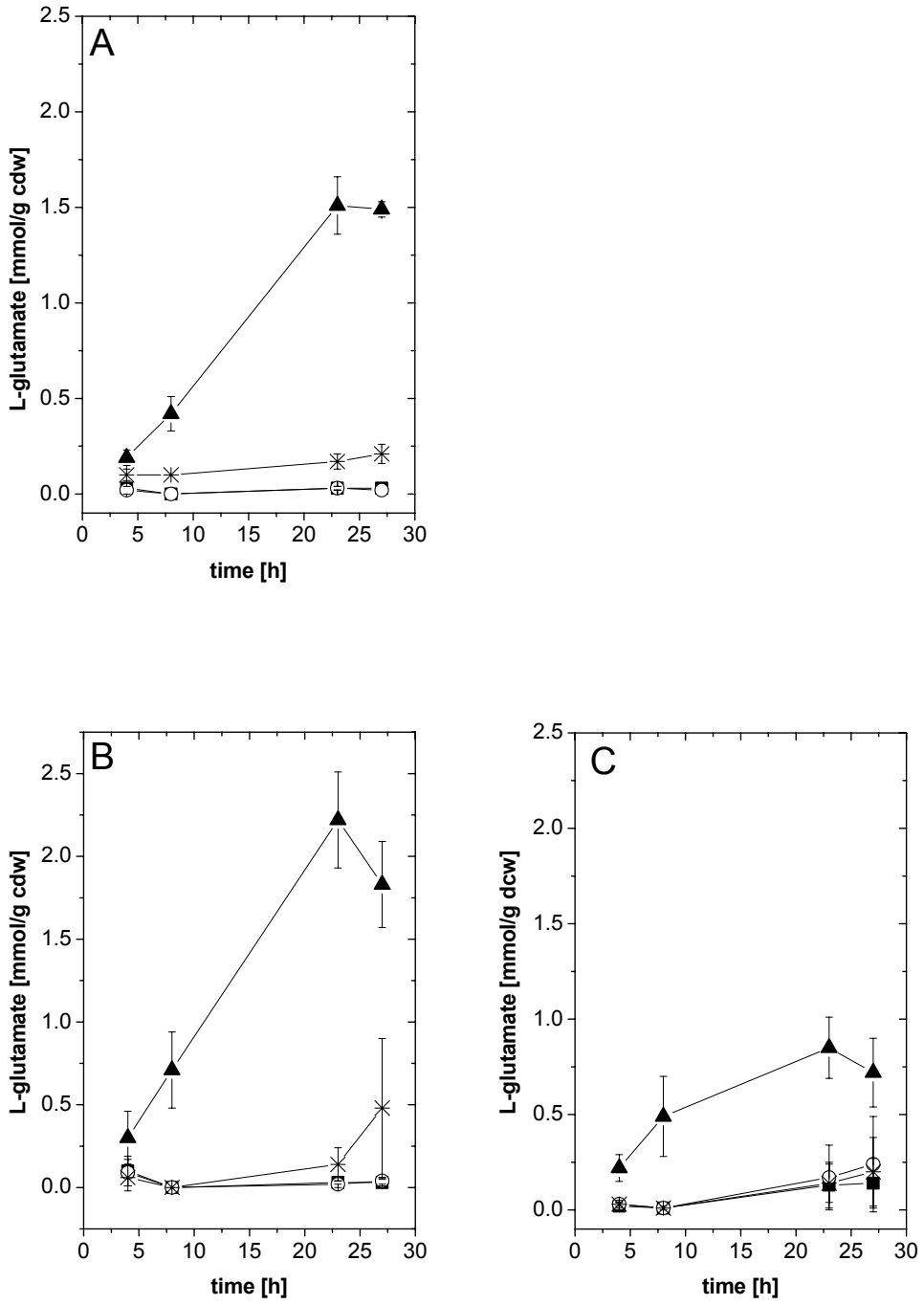


Fig. 20: Effect of external trehalose on spontaneous glutamate excretion of L-lysine producer ATCC 21527 and trehalose deficient LPΔ*treS*Δ*otsA*Δ*treY* in dependence of the carbon source. A, sucrose; B, glucose; C, fructose. ■ ATCC 21527 medium without trehalose; O ATCC 21527 medium with 2 % trehalose; ▲ LPΔ*treS*Δ*otsA*Δ*treY* medium without trehalose; ∗ LPΔ*treS*Δ*otsA*Δ*treY* medium with 2 % trehalose.

3.3.8 Effect of the carbon source on the metabolism of LPΔ*treS*Δ*otsA*Δ*treY* and of ATCC 21527

Analysis of the efflux of the amino acids lysine and glutamate revealed that their excretion was not only influenced by the state of the mycolate layer, but that it might be determined furthermore by the energy metabolism of the cell. In lysine producer ATCC 21526 sucrose and glucose directed the carbon fluxes differently, so that depending on the carbon source different levels of NADPH were available for the synthesis of lysine (Wittmann *et al*., 2004; Kiefer *et al.,* 2003).

Biomass yield $Y_{X/S}$ (g dcw / g carbon source) and product yields $Y_{Glu/S}$ for glutamate (g glutamate / g carbon source) and $Y_{Lys/S}$ for lysine (g lysine / g carbon source) were calculated to get an insight into carbon and amino acid metabolism of LPΔ*treS*Δ*otsA*Δ*treY* compared to ATCC 21527. Substrate concentrations in the medium were measured at different time points to determine the consumption of the carbon source.

LPΔ*treS*Δ*otsA*Δ*treY* as well as ATCC 21527 spent 14 % less glucose than sucrose on biomass and amino acid production indicating that by-product formation or overflow metabolism restricted energy for amino acid production on glucose-medium (Fig. 21). The unassigned substrate consumption of LPΔ*treS*Δ*otsA*Δ*treY* was higher than that of ATCC 21527 with both carbon sources, probably due to a higher maintenance coefficient. Whereas ATCC 21527 excreted lysine, but not glutamate, LPΔ*treS*Δ*otsA*Δ*treY* excreted both, glutamate and lysine. The amounts of these amino acids depended on the carbon source. Whereas LPΔ*treS*Δ*otsA*Δ*treY* utilized more glucose for glutamate than for lysine synthesis, the reverse was found on sucrose-medium. Since lysine synthesis requires more NADPH than glutamate synthesis, on glucose less NADPH might be available for lysine synthesis due to the high unassigned substrate consumption. In contrast on sucrose-medium, LPΔ*treS*Δ*otsA*Δ*treY* has sufficient NADPH to synthesise lysine at its disposal.

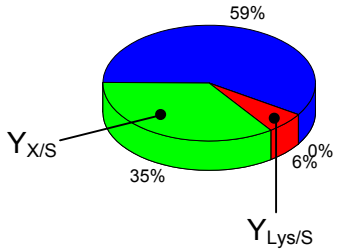
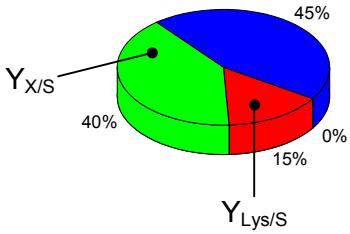
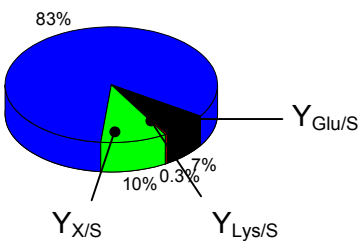
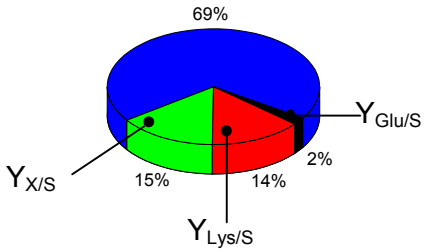
	glucose	sucrose
ATCC 21527		
LP*ΔtreSΔotsAΔtreY*		

Fig. 21: Influence of the carbon source on the biomass yield and the yields for lysine and glutamate of ATCC 21527 and LP*ΔtreSΔotsAΔtreY* after 24 cultivation with 4 % glucose or 4 % sucrose. Light grey sector, biomass yield $Y_{X/S}$; dark grey sector, lysine yield $Y_{P/S\ lys}$; black sector, glutamate yield $Y_{P/S\ glu}$; anthracite sector, unassigned substrate consumption.

In summary, there are at least two possibilities how the carbon source directs amino acid excretion in LP*ΔtreSΔotsAΔtreY*. (i) The carbon source influences the condition of the mycolate layer, facilitating efflux of amino acids. (ii) The carbon source directs carbon fluxes in the metabolism of the cell affecting amino acid metabolism.

4 Discussion

4.1 Function of the trehalose synthesis pathway OtsAB

In its natural habitat as well as during industrial production *C. glutamicum* has to cope with changes of osmolality to which it adapts by accumulation or synthesis of compatible solutes e. g. trehalose. Wolf *et al.* (2003) showed that trehalose synthesis increased under hyperosmotic conditions in *C. glutamicum*. The level of trehalose accumulation depended on the nutrient supply, namely carbon and nitrogen availability. Whereas in the presence of excess nitrogen proline was the predominant compatible solute, under nitrogen limitation the amino acid synthesis was lower in favour of trehalose synthesis.

C. glutamicum harbours two different trehalose synthesis pathways: OtsAB and TreYZ. Whereas the TreYZ-pathway is responsible for the accumulation of trehalose as a compatible solute after hyperosmotic shock, the function of the OtsAB-pathway in *C. glutamicum* was unknown. The suggestion that the OtsAB-pathway is involved in mycolate synthesis was ruled out by the fact that a *C. glutamicum* strain deleted in the *otsA* gene synthesised trehalose mycolate (Wolf *et al.,* 2003). Upregulation of the *otsA* transcript by a factor of five 15 min after a hyperosmotic shock indicated a function of the OtsAB-pathway in the osmostress response of *C. glutamicum* (Wolf *et al*., 2003). As an immotile soil bacterium *C. glutamicum* is not only exposed to changes of external osmolality, but it has to cope furthermore with limitation of nutrients. Since glycogen, the substrate for the TreYZ-pathway, becomes limiting under conditions of carbon limitation, the OtsAB-pathway could be necessary to synthesise trehalose when it is simultaneously exposed to carbon limitation and an osmotic upshift.

Before the relevance of trehalose synthesis by the OtsAB-pathway after hyperosmotic shock could be studied, the experimental setup to achieve glycogen limitation had to be developed. The first approach was based on the observation that the quality of the carbon source influenced the intracellular concentration of glycogen (B. Eikmanns, personal communication). Testing of different carbon sources revealed that cultivation with 2 % lactate caused glycogen deficient cells. The deletion mutants Cgl*ΔtreYΔtreS* and Cgl*ΔotsAΔtreS* harbouring only the OtsAB-pathway or the TreYZ-pathway, respectively, were grown on 2 % lactate to quantify the contribution of each pathway to trehalose synthesis under glycogen limitation. Under these conditions the OtsAB-pathway was the predominant trehalose synthesis pathway, whereas under carbon surplus (4 % glucose) TreYZ is more important (Wolf *et al*., 2003). Thus, the quality of the carbon source determined the pool of precursors which in turn decided which pathway was used for the synthesis of trehalose.

Although cells were glycogen depleted after cultivation in medium supplemented with 2 % lactate for 24 h, these conditions were not suitable to determine trehalose synthesis under glycogen limitation after hyperosmotic shock, since Cgl*ΔtreYΔtreS,* Cgl*ΔotsAΔtreS* and the wild type started to accumulate glycogen 15 min after the osmotic upshift. The TreYZ-pathway became the predominant pathway for trehalose synthesis, as soon as glycogen was available, so that under these conditions the OtsAB-pathway was of minor importance. Accumulation of glycogen was unexpected since it is not the typical response to osmotic stress and carbon limitation was supposed rather to suppress the synthesis of the storage carbohydrate. The residual amount of 0.5 % lactate was a possible resource for glycogen accumulation in the cell which had been glycogen depleted before. A connection between glycogen synthesis and osmotic shock was also observed in other organisms. *Bradyrhizobium japonicum* cells produced glycogen under high osmolality (Pfeffer *et al.,* 1994) and in yeast glycogen metabolism was induced by an osmotic shock (Hohmann *et al.,* 2002). Probably, in *C. glutamicum* the accumulation of glycogen was triggered by the cumulating effects of the two different stresses, carbon limitation and osmotic upshift.

Since cultivation on lactate was not a suitable tool to provide glycogen starvation conditions after a hyperosmotic shock, glycogen biosynthesis in Cgl*ΔtreYΔtreS,* Cgl*ΔotsAΔtreS* and the wild type was inactivated to elucidate the function of the OtsAB-pathway. Therefore the *glgC* gene encoding ADP-glucose-pyrophosphorylase, the first enzyme of the glycogen synthesis pathway, was disrupted (Eikmanns, personal communication). The insertion strains Cgl*ΔglgC*, Cgl*ΔotsAΔtreSΔglgC* and Cgl*ΔtreYΔtreSΔglgC* were glycogen deficient. Lower growth rate and decreased biomass accumulation of the *ΔglgC* insertion strains compared to the wild type signalled that inactivation of glycogen biosynthesis weakened the general metabolism of the cell.

After a hyperosmotic shock, growth of the glycogen deficient mutant Cgl*ΔtreYΔtreSΔglgC,* harbouring the OtsAB-pathway as single trehalose synthesis pathway, recovered fastest suggesting that trehalose synthesised by the enzymes of the OtsAB-pathway was the most important compatible solute. Although Cgl*ΔtreYΔtreSΔglgC* synthesised significantly more trehalose than Cgl*ΔotsAΔtreSΔglgC,* the amount of trehalose was still 50 fold lower than the amount synthesised by the wild type when trehalose replaced proline as the main compatible solute under N-limitation (Wolf *et al*., 2003). Considering the low amount of trehalose, it was unlikely that it was responsible for the fast growth recovery of Cgl*ΔtreYΔtreSΔglgC*. Since proline is the most important compatible solute in the wild type (Rönsch *et al*., 2002; Ley, 2005), it was verified whether it could also be accounted for protection against osmotic stress in the *ΔglgC* insertion mutants. Similar proline accumulation in Cgl*ΔotsAΔtreSΔglgC* and Cgl*ΔtreYΔtreSΔglgC* and in the wild type confirmed that proline was the most important compatible solute also in the glycogen deficient strains. The predominant role of the amino

acid proline as protectant against osmotic stress in contrast to the sugar trehalose seems to be reasonable since under the tested conditions nitrogen was available in surplus whereas carbon limitation might change the whole sugar metabolism. An altered energy metabolism may explain furthermore, why the three *ΔglgC* insertion mutants synthesised much lower amounts of trehalose and why they were growing slower than the wild type.

The important role of proline as a compatible solute in the glycogen deficient strains was proven by Cgl*ΔglgC* which behaved differently from the other *ΔglgC* insertion strains. For unknown reasons, this strain synthesised significantly less proline than the wild type. Consistently, it did not resume growth after hyperosmotic shock. Since low proline synthesis in Cgl*ΔglgC* was surprising, a *ΔglgC* clone derived by a different round of mutagenesis was tested to exclude that the first clone contained secondary mutations. But both clones exhibited the same phenotype.

Taken together these results indicate that the OtsAB-pathway was necessary for trehalose synthesis under carbon limitation when the TreYZ-pathway was not working. Why the *otsA* gene was upregulated after hyperosmotic shock remains unclear because under glycogen limitation trehalose was not the most important compatible solute, but proline was responsible for protection against osmotic stress. However, the conditions of the natural habitat of *C. glutamicum* are difficult to imitate in the laboratory. In the soil the bacterium may be constantly exposed to a variety of limitations. Since trehalose is the predominant compatible solute under nitrogen-limitation, the OtsAB-pathway might be necessary for the synthesis of trehalose as compatible solute after an osmotic upshift when the cells suffer from simultaneous carbon- and nitrogen-limitation.

4.2 Localisation of the synthesis of trehalose monomycolate

The detailed mechanism of mycolate biosynthesis is unknown in *Corynebacterineae*. Already the first reaction step, the condensation of a molecule of trehalose with a mycolyl-residue to TMM, raises several questions. It is neither clear where the synthesis of TMM takes place, in the cytoplasm or in the cell envelope, nor which mycolyltransferase catalyses this reaction. Whereas Takayama *et al.* (2005) recently suggested for *Mycobacterium tuberculosis* that TMM is synthesised in the cytoplasm, we assumed that synthesis of TMM is located in the cell envelope of *C. glutamicum*. This hypothesis was based on the correlation of the following two observations. The *C. glutamicum* wild type strain ATCC 13032 could not grow on trehalose as carbon source indicating that the disaccharide cannot be taken up by *C. glutamicum* (Wolf, 2002), and a trehalose deficient mutant of *C. glutamicum* could synthesise TMM when it was cultivated in medium supplemented with external trehalose (Tzvetkov *et al.,* 2003).

Uptake of [^{14}C]trehalose into cells of *C. glutamicum* ATCC 13032 after short time incubation (10 min) was determined to verify the assumption that external trehalose was integrated into the mycolate layer by the activity of external enzymes and not via uptake into the cytoplasm followed by export of TMM. The use of Cgl*ΔtreYΔtreSΔotsA* and wild type allowed the differentiation between these two possibilities, because after cultivation in minimal medium supplemented with sucrose no mycolate was present in the trehalose deficient Cgl*ΔtreYΔtreSΔotsA.* Furthermore, accumulation of [^{14}C]trehalose in isolated cell envelopes, obtained by permeabilisation of wild type cells followed by washing, was compared with accumulation of [^{14}C]trehalose in intact wild type cells. Accumulation of [^{14}C]glucose and of [^{14}C]betaine was quantified as control for the energy state of the cell and the isolation conditions of the cell envelopes.

Whereas a very low, but significant uptake rate for [^{14}C]trehalose was measured in the wild type, no uptake of [^{14}C]trehalose into the mycolate deficient Cgl*ΔtreYΔtreSΔotsA* cells was detected. Since similar uptake rates for [^{14}C]glucose excluded that the energy state of Cgl*ΔtreYΔtreSΔotsA* was unfavourable for sugar uptake, the different uptake activities for [^{14}C]trehalose of these two strains indicated that [^{14}C]trehalose was incorporated into the mycolate layer in the wild type rather than taken up into the cytoplasm. This assumption was proven by the integration of equal amounts of [^{14}C]trehalose into both, isolated cell envelopes and intact cells. In other words, [^{14}C]label determined in the whole cell derived exclusively from [^{14}C]label in the cell envelope. Since [^{14}C]betaine was washed away completely whereas the partial incorporation of [^{14}C]glucose into the cell envelope was detected, the method was suitable to distinguish between incorporation and adsorption of [^{14}C]trehalose.

Thus, determination of uptake of [^{14}C]trehalose after short time incubation confirmed the assumption that *C. glutamicum* cells cannot take up trehalose into the cytoplasm. Consequently, trehalose supplemented in the medium remains outside the plasma membrane in the cell envelope. Since a trehalose deficient *C. glutamicum* strain cultivated with external trehalose was able to synthesise TMM, TMM synthesis has to be localized in the cell envelope. Accordingly, in the wild type, which synthesises trehalose in the cytoplasm, a transporter which exports trehalose across the plasma membrane into the cell envelope has to be postulated. Since the second building block of TMM, the mycolic acids, are also synthesised in the cytoplasm a further exporter might be required. Recently, Tropis *et al*. (2005) suggested that a mycolyl-residue might be linked to a phospholipid of the plasma membrane which transfers the mycolyl-residue into the cell envelope.

Furthermore, Tropis *et al*. (2005) reported that apart from trehalose also glucose, maltose and maltotriose were esterified by mycolic acids and enabled the synthesis of AGM and of the respective glycosyl monomycolate in the trehalose deficient Cgl*ΔtreYΔtreSΔotsA,* whereas other carbon sources such as sucrose, fructose or pyruvate did not facilitate mycolate synthesis. The three carbon sources glucose, maltose and maltotriose all have a structure in common with trehalose: a terminal α-glucosyl sugar residue. This α-glucosyl sugar unit was identified as the residue esterfied by mycolic acids (Tropis *et al*., 2005). Consequently, the still unknown mycolyltransferase catalysing the condensation of a sugar with a mycolyl-residue to glycosyl monomycolate can only use sugars comprising α-glucosyl sugar residues as substrate. Since in *C. glutamicum* ATCC 13032 six mycolyltransferases were identified which are partially redundant, the analysis of deletion mutants with a single functional mycolyltransferase could help to identify the mycolyltransferase synthesising TMM.

4.3 Importance of trehalose for the cell envelope of a *C. glutamicum* L-lysine production strain

Exceptionally for Gram-positive bacteria, the cell envelope of the *Corynebacterineae* contains a second lipid bilayer apart from the plasma membrane, named mycolate layer. In mycobacteria, which belong to the suborder of *Corynebacterineae,* the mycolate layer is supposed to make the cell envelope impermeable to most of the common antibiotics (Brennan & Nikaido, 1995; Puech *et al.,* 2001). Since *C. glutamicum* strains with reduced mycolate content exhibited higher uptake rates for glycerol and acetate, the mycolate layer could determine the permeability of the cell envelope also in corynebacteria (Puech *et al.* 2000). Mycolate deficiency of trehalose deficient *C. glutamicum* strains which were cultured on sucrose indicated that trehalose is essential for mycolate synthesis (Wolf *et al.,* 2003). However, maltose and glucose were identified as alternative acceptors of mycolyl-residues (Wolf, 2002; M. Daffé, personal communication; Puech *et al.,* 2000).

In this project the question was addressed how the carbon source and the absence of trehalose specifically influence the composition of the mycolate layer of a *C. glutamicum* strain inactivated in trehalose synthesis. Additionally, the impact of the supplementation of the medium with trehalose on the synthesis of the mycolate layer was examined. Strains harbouring differently composed mycolate layers were compared to investigate how the composition determines the properties of the mycolate layer, especially its permeability. Since *C. glutamicum* is one of the most important industrial producers of amino acids, a trehalose deficient strain based on the *C. glutamicum* L-lysine production strain ATCC 21527 was chosen for these experiments. This strain was used to test whether the alteration of the cell envelope could improve lysine production. Moreover, it was examined whether lysine excretion as a measure of the efflux of solutes could be correlated to the permeability of the cell envelope. Measuring the uptake of substances was a method to determine the permeability of the mycolate layer.

4.3.1 Impact of the availability of trehalose and of the carbon source on the composition of the mycolate layer

All three trehalose synthesis pathways were inactivated in a *C. glutamicum* lysine production strain to investigate the influence of trehalose and of the carbon source on the composition of the mycolate layer. The mycolate composition of this trehalose deficient strain was analysed quantitatively and qualitatively under six different cultivation conditions: supplemented with sucrose, fructose or glucose as carbon source, in the absence or presence of external

trehalose, respectively. Whereas the trehalose deficient strain LP∆*treS*∆*otsA*∆*treY* did not synthesise any mycolate grown on sucrose- or fructose-medium, the same strain synthesised AGM and GMM grown on glucose-medium signalling that glucose could replace trehalose as acceptor and translocator of mycolic acids. These data were consistent with results of the analysis of the trehalose deficient strain based on the wild type (Wolf *et al*., 2003; Tropis *et al*., 2005). Since glucose could replace trehalose for mycolate synthesis, it was surprising that no mycolate was synthesised with sucrose and fructose as carbon source because these sugars are metabolized into glucose phosphates inside the cytoplasm. Since all carbon sources were provided in excess, it could be excluded that glucose was not available for mycolate synthesis because of rapid consumption for the energy metabolism or other compounds. Hence, the externally available carbon source seemed to determine monomycolate synthesis indicating that synthesis of GMM was localized in the cell envelope and not inside the cytoplasm. These data confirmed again that synthesis of TMM was located in the cell envelope (cf. 4.2). The major proof of the localisation of the synthesis of glycosyl monomycolates in the cell envelope consisted of the result that supplementation of the culture medium with trehalose facilitated TMM synthesis in the trehalose deficient LP∆*treS*∆*otsA*∆*treY,* although trehalose was shown not to enter the cytoplasm of *C. glutamicum* (cf. 3.2). Since TLC analysis showed that GMM synthesis in LP∆*treS*∆*otsA*∆*treY* decreased in favour of TMM synthesis when the medium was supplemented with trehalose, GMM and TMM were likely to be synthesised by the same mycolyltransferase which then should have a higher affinity for trehalose than for glucose.

Supplementation of medium with fructose and trehalose facilitated not only TMM, but also TDM and AGM synthesis in LP∆*treS*∆*otsA*∆*treY* restoring qualitatively a native mycolate composition. Also LP∆*treS*∆*otsA*∆*treY* cultured on glucose and trehalose synthesised the native mycolate composition including TMM, TDM and AGM, but additionally, GMM. Quantification of mycolic acids showed that LP∆*treS*∆*otsA*∆*treY* grown on glucose and 2 % trehalose synthesised more arabinogalactan mycolate and extractible mycolate than the parental strain. Since the method applied for the quantification of extractable mycolate did not differentiate between mycolic acids originating from glucose monomycolate or trehalose mycolate, and since arabinogalactan mycolate can be synthesised from GMM as well as from TMM as mycolyl-donor, the additional amount of mycolic acids could derive from glucose monomycolate which is not synthesised in ATCC 21527. These data indicated that external glucose participated efficiently in mycolate synthesis.

Surprisingly, LP∆*treS*∆*otsA*∆*treY* grown on sucrose-medium supplemented with trehalose synthesised only AGM and TMM, but no TDM. Under these conditions the amount of arabinogalactan mycolate as well as the amount of extractible mycolate was significantly lower than in the parental strain. Possibly, TDM is absent because the disaccharide sucrose

inhibits TDM synthesis by blocking the catalytic site of the mycolyltransferase. With this explanation in mind it is surprising that ATCC 21527 could synthesise TDM when it was grown on sucrose. Since ATCC 21527 synthesises trehalose in the cytoplasm, TDM synthesis in this strain could indicate that cytoplasmic trehalose is more efficient for mycolate synthesis than external trehalose. This assumption was supported by the observation that on sucrose mycolate synthesis in LP∆*treS*∆*otsA*∆*treY* was very low compared to the wild type. Probably, the amount of external trehalose was not sufficient to compete with sucrose for the catalytic site of the mycolyltransferase, or access of external trehalose to the catalytic site was limited.

In summary, utilisation of a *C. glutamicum* L-Lysine production strain inactivated in trehalose synthesis facilitated a specific manipulation of the composition of the mycolate layer. Supplementation of this strain with different carbon sources and external trehalose caused various stages of restoration of the mycolate layer, from completely missing to parental strain composition. The mycolate layer was lacking completely when this strain was grown on sucrose or fructose, while an incomplete mycolate layer, consisting of AGM and GMM was synthesised during cultivation on glucose. Thus, trehalose was essential for mycolate synthesis in the combination with sucrose or fructose as carbon source, whereas glucose could partially substitute trehalose as acceptor and translocator of mycolic acids. In the presence of external trehalose this strain synthesised on sucrose-medium neither the native composition nor the native amount of mycolate, whereas on glucose- and fructose-medium all native components were synthesised. However, the composition of the mycolate layer of LP∆*treS*∆*otsA*∆*treY* grown on glucose and trehalose differed from the native composition in two aspects. Additionally to the native mycolate composition, LP∆*treS*∆*otsA*∆*treY* synthesised GMM, furthermore, the amount of mycolate in the trehalose deficient strain was higher. These data indicated that the proportions of AGM, TMM and TDM might not be equal in the native mycolate layer and the mycolate layer of LP∆*treS*∆*otsA*∆*treY*. Since AGM, GMM, TMM and TDM have different structures, diverging fractions of these components in LP∆*treS*∆*otsA*∆*treY* could change the packing of the mycolate layer compared to ATCC 21527.

Since either the amount of mycolate in LP∆*treS*∆*otsA*∆*treY* was lower than in the native strain or the fractions of the different types of mycolate varied between ATCC 21527 and the trehalose deficient strain, these results indicate that external trehalose could only partially substitute trehalose synthesised in the cytoplasm for the synthesis of the mycolate layer. How can the inefficiency of external trehalose be explained? Different substrate affinities of the mycolyltransferases can be excluded since the molecule is the same, but the access of external and cytoplasmic trehalose to the enzyme might differ. Since TMM synthesis is located in the cell envelope an exporter for trehalose is postulated which translocates

cytoplasmic trehalose across the plasma membrane. Probably, the mycolyltransferase catalysing the condensation to TMM is attached to this exporter, so that cytoplasmic trehalose can be metabolised efficiently. In contrast, the catalytic site may be difficult to access for trehalose passing through the cell envelope. The impediment of access for sugar molecules diffusing through the cell envelope might be necessary to ensure efficient mycolate synthesis under physiological conditions, since glucose was shown to compete with trehalose for mycolate synthesis and sucrose may even block the catalytic site of the mycolyltransferase.

4.3.2 Impact of the composition of the mycolate layer on growth of a *C. glutamicum* L-lysine production strain

Trehalose deficiency reduced significantly growth rate and biomass production in LP∆*treS*∆*otsA*∆*treY* compared to the parental strain ATCC 21527 indicating that inhibition of mycolate synthesis in LP∆*treS*∆*otsA*∆*treY* may slow down the synthesis of a new cell wall after division of the cell. LP∆*treS*∆*otsA*∆*treY* grown on sucrose or fructose being mycolate deficient exhibited an equally slow growth phenotype as LP∆*treS*∆*otsA*∆*treY* grown on glucose which could synthesise GMM and AGM. Obviously, AGM and GMM were not sufficient to restore a mycolate layer with properties similar to that of the parental strain. Probably, TMM and TDM were necessary for the appropriate arraying of the lipid bilayer. Furthermore, under these conditions the amount of mycolate synthesised in LP∆*treS*∆*otsA*∆*treY* was lower than in ATCC 21527 indicating that the amount of mycolate was not sufficient to form a lipid bilayer covering the whole bacterial surface of LP∆*treS*∆*otsA*∆*treY*. In coincidence with these results, LP∆*treS*∆*otsA*∆*treY* cultured on glucose and 2 % trehalose exhibited a growth phenotype similar to ATCC 21527 probably caused by the ability to synthesise AGM, TMM and TDM in higher quantities than the parental strain. Also LP∆*treS*∆*otsA*∆*treY* cultured on a mixture of fructose and trehalose synthesised the native mycolate composition (TMM, TDM and AGM) and grew like the parental strain. Consistently, LP∆*treS*∆*otsA*∆*treY* cultured on a mixture of sucrose and trehalose which synthesised lower quantities of mycolate than the parental strain and lacked TDM, had a slower growth rate than ATCC 21527. Taken together these results indicate that with respect to growth behaviour TMM and especially TDM may be important structural components of the mycolate layer. Furthermore, the quantity of the different types of mycolate could be decisive for the restoration of a mycolate layer facilitating native growth.

4.3.3 Impact of the composition of the mycolate layer on the permeability of the cell envelope

The permeability of the cell envelope of the *Corynebacterineae* is extremely low for some hydrophilic as well as hydrophobic solutes, e. g. antibiotics (Brennan & Nikaido, 1995). Exceptionally for Gram-positive bacteria, the cell envelope of this suborder comprises a second lipid bilayer apart from the plasma membrane, named mycolate layer, which constitutes the main permeation barrier. In mycobacteria the mycolate layer is of unusual thickness and low fluidity and is supposed to render these bacteria resistant to most of the common antibiotics (Brennan & Nikaido, 1995). Although the mycolate layer in *C. glutamicum* is thinner than in mycobacteria due to shorter mycolic acids constituting this lipid bilayer, the mycolate layer was correlated to the permeability of the cell envelope, since *C. glutamicum* strains with reduced mycolate content due to inactivation of a mycolyltransferase took up glycerol and acetate faster (Puech *et al.,* 2000).

This project investigated how different levels of restoration of the mycolate layer altered the permeability of the cell envelope. The analysis of the mycolate layer of a trehalose deficient strain showed that the composition of the mycolate layer could be manipulated by cultivating this strain on different carbon sources, in the absence or presence of external trehalose. The Zimmermann-Rosselet assay would have been the best method to measure the permeability of the mycolate layer. This assay, which was established in mycobacteria, determines the diffusion of β-lactam antibiotics by measuring β-lactamase activity. Thus, only diffusion through the outer layer, the mycolate layer and the arabinogalactan layer, but not through the plasma membrane, which exhibits a further permeation barrier, is determined. Unfortunately, no β-lactamase activity was detected in *C. glutamicum* under the tested conditions making this test unusable for this bacterium.

A further assay developed for mycobacteria correlated the resistance to antibiotics to the permeability of the cell envelope. With penicillin G and ethambutol we chose two hydrophilic antibiotics which act on cell wall components to ensure that their diffusion is only determined by the mycolate layer and not by the plasma membrane. The macrolid antibiotic erythromycin was selected as an example for a large hydrophobic molecule. A lacking or imperfect mycolate layer increased the permeability of the cell envelope significantly as shown by lower resistance of LPΔ*treS*Δ*otsA*Δ*treY* compared to ATCC 21527 to the antibiotics penicillin G, erythromycin and ethambutol. The cultivation of LPΔ*treS*Δ*otsA*Δ*treY* in medium supplemented with trehalose decreased the permeability of the cell envelope, but not to the level of permeability of the cell envelope of ATCC 21527.

Since LPΔ*treS*Δ*otsA*Δ*treY* grown on sucrose synthesised neither the native mycolate composition nor the native mycolate amount, it was not astonishing that external trehalose did not restore native permeability of the cell envelope. The mycolate layer was incomplete,

causing higher susceptibility to the antibiotics. However, it was surprising that the native permeability was not restored when LP∆*treS*∆*otsA*∆*treY* was cultured on fructose- or glucose-medium supplemented with trehalose, since under these conditions the native mycolate composition was synthesised, and at least in glucose-medium the amount of mycolate was even higher than in ATCC 21527. Furthermore, growth of LP∆*treS*∆*otsA*∆*treY* was similar to the parental strain under these conditions. Perhaps, not the mere amount of mycolic acids decides whether the mycolate layer obtains the appropriate structure, but the proper fractions of the different components have to be available. For example LP∆*treS*∆*otsA*∆*treY* grown on glucose and 2 % trehalose synthesised twice of the native amount of AGM. In the parental strain AGM constitutes the inner layer of the mycolate layer together with TDM and TMM. A higher amount of AGM in LP∆*treS*∆*otsA*∆*treY* may displace TDM and TMM from the inner layer, thus the fluidity of the lipid bilayer may be altered facilitating uptake of the tested substance. Furthermore, under these conditions LP∆*treS*∆*otsA*∆*treY* synthesised GMM additionally to the native mycolate composition. Probably, also the presence of GMM impeded the proper arrangement of the mycolate layer. Taken together these data indicated that the different types of mycolate have to be synthesised in specific fractions to ensure the appropriate packing of the mycolate layer. The importance of the packing of the mycolate layer for its permeability had already been demonstrated by measuring the fluidity of mycolate layers with different structures (Liu *et al.,* 1996).

However, the tested substances may either pass directly through the lipid domains of the mycolate layer, or cross it through porins. Whereas erythromycin is likely to cross the mycolate layer directly because it is too large and too hydrophobic to diffuse through a porin channel (Stephan *et al.*, 2004) the acidic β-lactam antibiotic penicillin G was supposed to pass the mycolate layer by porins. Costa-Riu *et al.* (2003) showed that deletion of *porA,* the gene encoding the major porin in *C. glutamicum,* decreased the susceptibility of the mutant to the positively charged penicillin ampicillin. As ethambutol is a small hydrophilic solute it should penetrate the mycolate layer by the help of porins (Lambert, 2002), but inactivation of MspA the major porin in *M. smegmatis* did not increase resistance to this drug indicating that either other porins were responsible for ethambutol uptake or a further mechanism e. g. direct permeation through the mycolate layer (Stephan *et al.*, 2004). Although the tested antibiotics may use different pathways to cross the mycolate layer in the native strain, mycolate deficiency in LP∆*treS*∆*otsA*∆*treY* moved away the permeation barrier for all three tested antibiotics. This result can be correlated with the observation that no porins, which are believed to be integrated into the mycolate layer, could be detected in the mycolate deficient Cgl*∆treY∆treS∆otsA* (R. Benz, personal communication). The partially restored, probably not well packed mycolate layer, due to supplementation of LP∆*treS*∆*otsA*∆*treY* with trehalose,

facilitated higher permeation rates for all substances than the cell envelope of the native strain. If porins are present under these conditions, they may not represent the only passage over the mycolate layer for substances using porins in the native strain.

Since the Zimmermann-Rosselet assay, which provides a very accurate tool to determine the permeability of the mycolate layer by measuring diffusion of β-lactam antibiotics, was unusable for *C. glutamicum,* alternatively, [^{14}C]glycerol uptake rates of cells of LP∆*treS*∆*otsA*∆*treY* and of ATCC 21527 were determined as a measure of the permeability of the cell envelope. Since no genes comprising sequence similarities to genes encoding a glycerol diffusion facilitator protein were identified in the genome of *C. glutamicum*, uptake of [^{14}C]glycerol is supposed to be determined by the diffusion through the cell envelope, i. e. by diffusion through the mycolate layer and through the plasma membrane. The velocity of diffusion in turn is determined by the permeability of the cell envelope. Since no difference between the two strains was observed for the uptake rates of [^{14}C]glucose (cf. 3.2), which is mediated by a phosphotransferase system, the plasma membrane is supposed to be similar in these strains.

However, it had to be excluded that the uptake rates of the two strains differed because of different glycerol metabolisms. Similar activities of glycerol-kinases in both strains proved that their glycerol metabolism was comparable. Since glycerol-kinase activity was 100 times higher than the [^{14}C]glycerol uptake rates, the glycerol metabolism was ruled out as a limiting factor for [^{14}C]glycerol uptake under the tested conditions. Furthermore, the uptake rates of [^{14}C]glycerol of the two strains increased proportionally to the glycerol concentration, indicating that diffusion limited [^{14}C]glycerol uptake. Higher uptake rates of [^{14}C]glycerol of LP∆*treS*∆*otsA*∆*treY* compared to ATCC 21527 indicated that the cell envelope of the trehalose deficient strain was more permeable than that of the parental strain. Since LP∆*treS*∆*otsA*∆*treY* and ATCC 21527 were cultivated with sucrose as carbon source the mycolate layer lacked completely in LP∆*treS*∆*otsA*∆*treY*. Also porins are believed to be absent when the mycolate layer is missing (B. Eikmanns, personal communication). Since lack of mycolate and of porins increased [^{14}C]glycerol uptake rates, the mycolate layer even including porins seems to constitute a permeation barrier for glycerol. Supplementation of the medium with trehalose reduced the uptake rates by LP∆*treS*∆*otsA*∆*treY* slightly, but not to the low level of the native strain indicating that external trehalose could not completely replace cytoplasmic trehalose for the synthesis of a mycolate layer exhibiting native permeability.

In summary, measuring the resistance to antibiotics as well as the uptake of [^{14}C]glycerol showed that under conditions of lacking or incomplete mycolate synthesis the permeability of the cell envelope in the trehalose deficient strain was higher than in the parental strain.

These results proved the assumption that similar to mycobacteria also in *C. glutamicum*, the mycolate layer determines the permeability of the cell envelope for antibiotics and glycerol.
Although the presence of external trehalose in combination with glucose or fructose facilitated the synthesis of all native compounds of the mycolate layer in the trehalose deficient strain, the permeability of its cell envelope remained significantly higher than that of the parent strain. The quantitative and qualitative analysis of the composition of the mycolate layer already indicated that, in spite of the presence of all native components due to supplementation with trehalose, the mycolate layer of the trehalose deficient strain might differ from the native mycolate layer, since the fractions of AGM, TMM and TDM might not be equal in both strains. AGM, GMM, TMM and TDM might have to be available in appropriate amount and proportion to restore the permeation barrier.

4.3.4 Impact of the availability of trehalose and of the carbon source on the excretion of amino acids

Whereas in mycobacteria the influence of the cell envelope on the uptake of antibiotics is important, in *C. glutamicum* the influence of the cell envelope on the efflux of substances is more relevant as it is the sole industrial producer of the amino acids L-lysine and L-glutamate. The discovery of the lysine exporter LysE in *C. glutamicum* revealed that lysine production requires efficient efflux of the amino acid (Broer *et al*., 1991a/b; Vrljic *et al*., 1995; Vrljic *et al*., 1996; Bellmann *et al*., 2001). LysE exports lysine across the plasma membrane into the cell envelope where lysine has to cross a second lipid bilayer - the mycolate layer.
Measurement of the permeability of the cell envelope of LP∆*treS*∆*otsA*∆*treY* and ATCC 21527 containing differently composed mycolate layers demonstrated that an imperfect mycolate layer increased the permeability of the cell envelope for the uptake of the tested antibiotics and of glycerol. The following part of the project investigated whether also the efflux of substances, especially of amino acids, was influenced by different compositions of the mycolate layer caused by the absence or presence of trehalose and by the type of carbon source.
Lysine excretion by the trehalose deficient LP∆*treS*∆*otsA*∆*treY* was similar to ATCC 21527 except for one condition. Only LP∆*treS*∆*otsA*∆*treY* grown on sucrose in the absence of trehalose excreted significantly more lysine than the parent strain. Since LP∆*treS*∆*otsA*∆*treY* was mycolate deficient under these conditions, the elimination of the permeation barrier could be responsible for enhanced lysine excretion. Supplementation of the medium with trehalose decreased lysine excretion nearly to the level of the parent strain. Since under these conditions the mycolate layer is partially restored, it could impede lysine excretion.

However, the measurement of uptake of antibiotics and of glycerol indicated that the native permeability is not restored under these conditions.

A further restriction for the relevance of the permeability of the mycolate layer for the excretion of lysine was the fact that LP∆*treS*∆*otsA*∆*treY* was also mycolate deficient cultured on fructose in the absence of trehalose, but excreted lysine concentrations similar to the parent strain. Lysine excretion of LP∆*treS*∆*otsA*∆*treY* as well as of ATCC 21527 was significantly lower on fructose than on sucrose. Hence, the carbon source influenced amino acid excretion also of ATCC 21527 which exhibited a similar composition of the mycolate layer on all three carbon sources. This in turn indicated that apart from the mycolate layer, a further factor, which was also influenced by the carbon source, determined lysine excretion.

Similarly, cultivation of the *C. glutamicum* lysine producer strain ATCC 21253 on sucrose, glucose and fructose caused different levels of lysine production (Kiefer *et al.,* 2002). Metabolic flux analysis showed that the carbon sources caused different fluxes through the pentose-phosphate-pathway (PPP) and also through the tricarboxylic acid cycle (Kiefer *et al.,* 2003; Wittmann *et al*., 2004). Since these two pathways are the main sources of NADPH in *C. glutamicum* and an excess of NADPH is considered to be essential for efficient lysine production, the different metabolic fluxes could explain the different levels of lysine excretion. This assumption was confirmed by metabolic flux analysis of ATCC 21526 which showed that the NADPH budget within the cell depended on the carbon source (Kiefer *et al.,* 2003; Wittmann *et al*., 2004). Higher lysine synthesis from sucrose than from glucose or fructose in ATCC 21527, the lysine producer used in this study, could indicate that during cultivation on sucrose excess of NADPH facilitated lysine synthesis whereas on glucose and fructose NADPH was limiting. However, ATCC 21527 excreted more lysine cultured on sucrose than cultured on glucose or fructose, while the other two lysine production strains excreted more lysine grown on glucose than grown on the two other carbon sources. Since all three strains were generated by random mutagenesis, this divergence could be due to unidentified secondary mutations in carbon metabolism.

A further support for the hypothesis that NADPH was limiting for lysine synthesis in ATCC 21527 grown on glucose was the fact that under these cultivation conditions this strain exhibited a higher amount of unassigned substrate consumption. This unassigned substrate consumption could be caused by higher by-product formation and/or overflow metabolism which in turn consumes NADPH. Also LP∆*treS*∆*otsA*∆*treY* cultured on glucose showed a higher unassigned substrate consumption than cultured on sucrose indicating by-product formation and/or overflow metabolism similar to the parent strain.

In summary, when ATCC 21527 was grown on sucrose an excess of NADPH was generated enabling a high level of lysine production. Enhanced lysine excretion by LP∆*treS*∆*otsA*∆*treY* on sucrose may be interpreted as indication that lysine production by ATCC 21527 had been

limited by the permeation barrier and that spare NADPH was available for additional lysine production. Taking the enhanced excretion of lysine as an indicator for higher permeability of the cell envelope these results support the permeability measurements (cf. 4.3.3). In contrast, the imperfect mycolate layer did not enhance lysine excretion when LPΔ*treS*Δ*otsA*Δ*treY* was grown on fructose or glucose indicating that lysine excretion was not limited by the permeation barrier under these conditions, but by an additional factor, presumable a low NADPH pool.
In spite of enhanced lysine excretion on sucrose LPΔ*treS*Δ*otsA*Δ*treY* is not suitable for industrial amino acid production. Fermentation of the trehalose deficient strain is not efficient because of low biomass production and low growth rates. Furthermore, slow growth makes LPΔ*treS*Δ*otsA*Δ*treY* very susceptible for contamination.

Unlike lysine excretion, the excretion of L-glutamate requires special treatments of the cell such as biotin limitation, temperature upshift or addition of penicillin or of detergents (Kimura, 2003). Two different mechanisms that may trigger the excretion of glutamate are discussed. Eggeling and Sahm (2001) suggest that the structure of the cell envelope, especially the permeability of the mycolate layer is crucial for glutamate efflux. Kimura (2003) favours the "metabolic flux model" which is based on the 30 years old observation that the activity of the ODHC enzyme complex plays a central role in glutamate production (Shingu & Terui, 1971).
The cultivation conditions applied in this project did not include a special treatment to trigger glutamate excretion. Consequently, ATCC 21527 did not excrete glutamate. Surprisingly, the trehalose deficient LPΔ*treS*Δ*otsA*Δ*treY* spontaneously excreted glutamate cultured on all three carbon sources. Since the main difference between LPΔ*treS*Δ*otsA*Δ*treY* and ATCC 21527 was the composition of the mycolate layer, the changed lipid composition of this second lipid bilayer could be the trigger for glutamate excretion by LPΔ*treS*Δ*otsA*Δ*treY*.
This assumption was supported by recent experiments indicating a function of the cell wall skeleton for glutamate excretion. The cell wall skeleton consists of peptidoglycan linked covalently to arabinogalactan which in turn is esterified by mycolic acids. Radmacher *et al.* (2005b) showed that glutamate excretion was triggered by treatment with ethambutol, which inhibits arabinogalactan synthesis. Consequently, the ethambutol treated cells exhibited a reduced content of arabinogalactan mycolate which should impede the appropriate formation of the mycolate layer. These recent results are consistent with the old observation that penicillin treatment, which inhibits peptidoglycan synthesis, triggers glutamate excretion (Nunheimer *et al.,* 1970). Furthermore, glutamate excretion was induced by inactivation of the fatty acid synthases FAS-IA and FAS-IB, which synthesise building blocks for phospholipids constituting the plasma membrane as well as for mycolic acids. Manipulation of fatty acid synthesis could generally influence the constitution of both lipid bilayers since

the *fasA* and *fasB* inactivation mutants exhibited an altered phospholipid composition as well as an altered mycolate composition (Radmacher *et al.,* 2005a). In summary, inhibition of the synthesis of any of the components of the cell wall skeleton induced the excretion of glutamate supporting the hypothesis of Eggeling and Sahm (2001). As all the components of the cell wall skeleton are covalently linked, elimination of any of them causes finally lack of arabinogalactan mycolate. Since trehalose deficiency is supposed to affect only the mycolate layer and no other component of the cell wall skeleton, glutamate excretion by LP∆*treS*∆*otsA*∆*treY* proved that the alteration of the mycolate layer alone was sufficient to induce glutamate excretion.

In contrast to lysine excretion, glutamate excretion was similar on all three carbon sources, and only slightly higher when LP∆*treS*∆*otsA*∆*treY* was grown on glucose as carbon source. Recently, Georgi *et al*. (2005) reported that glutamate production by ATCC 13032 was not affected by the carbon source and explained this with the fact that only 1 mol NADPH is required for synthesis of 1 mol glutamate. In contrast, 4 mol NADPH are needed for 1 mol lysine. Consequently, glutamate synthesis depends not as much on the energy metabolism as lysine synthesis, so that the effect of the carbon source on the energy pool is not relevant.

Supplementation of the medium with trehalose almost completely impeded the excretion of glutamate by LP∆*treS*∆*otsA*∆*treY*. Similarly, LP∆*treS*∆*otsA*∆*treY* excreted lysine concentrations as low as ATCC 21527 when the medium was supplemented with trehalose. Under these conditions the partially restored mycolate layer could constitute a permeation barrier for glutamate and lysine. However, the measurement of uptake of antibiotics and of glycerol indicated that the native permeability of the mycolate layer is not restored under these conditions. Probably, trehalose deficiency influences amino acid excretion on the level of metabolism. Whether increase of amino acid excretion in the absence of trehalose is due to higher permeability of the mycolate layer or due to changes of metabolism cannot be definitely answered based on the results of this project.

5 Summary

The first part of this project focused on the role of trehalose as a protectant against osmotic stress. Since an involvement of the OtsAB trehalose synthesis pathway in mycolate synthesis, as suggested by Shimakata and Minatogawa (2000) could be ruled out (Wolf *et al.*, 2003), the function of the OtsAB-pathway remained unknown. RNA-hybridisation experiments indicated a role of this pathway in the response to osmotic stress in *Corynebacterium glutamicum* (Wolf *et al.,* 2003). Analysis of trehalose synthesis in strains defective in individual trehalose synthesis pathways disclosed that the OtsAB-pathway was the predominant trehalose synthesis pathway under carbon limiting conditions in the absence and presence of osmotic stress. However, trehalose was not the most important protectant against osmotic stress under these conditions. Nor is trehalose the predominant compatible solute during the usual cultivation in minimal medium providing abundant carbon and nitrogen, but trehalose is the most important protectant against osmotic stress under nitrogen limiting conditions (Wolf *et al.,* 2003). Therefore, the OtsAB-pathway might be necessary to synthesise trehalose as compatible solute, when *C. glutamicum* is exposed to the coincidental limitation of carbon and nitrogen, which occurs frequently in its natural soil habitat.

In contrast to other Gram-positive bacteria all members of the suborder of *Corynebacterineae*, including *C. glutamicum,* contain a cell envelope that comprises a second lipid bilayer apart from the plasma membrane, the mycolate layer, which is considered as a permeability barrier (Puech *et al.,* 2001). Trehalose is important for the biosynthesis of all main components of the mycolate layer since trehalose monomycolate (TMM) serves as a precursor for arabinogalactan mycolate (AGM) and trehalose dimycolate (TDM). A *C. glutamicum* strain inactivated in trehalose synthesis was utilized to investigate the importance of trehalose for the corynebacterial mycolate layer, with special focus on the permeability of the mycolate layer. The *C. glutamicum* L-lysine production strain ATCC 21527 deficient in trehalose biosynthesis was chosen for these experiments to test whether lysine excretion could be correlated with the permeability of the cell envelope. Moreover, this strain was tested for improved lysine production due to the alteration of the mycolate layer.

Analysis of this *C. glutamicum* L-lysine production strain inactivated in trehalose synthesis showed that the mycolate layer lacked completely when this strain was grown on sucrose or fructose, while grown on glucose it synthesised AGM and glucose monomycolate. Thus, trehalose was only essential for mycolate synthesis, when sucrose or fructose were the carbon source, whereas glucose could replace trehalose as acceptor and translocator of mycolic acids. Under these conditions of lacking or incomplete mycolate synthesis, the growth rate of the trehalose deficient strain was lower compared to ATCC 21527 and the

permeability of the cell envelope was higher compared to the parent strain proving that the mycolate layer determines the permeability of the cell envelope.

Trehalose could not be taken up into the cytoplasm of *C. glutamicum* as demonstrated by the determination of [^{14}C]trehalose uptake rates, and the trehalose deficient strain could synthesize TMM when trehalose was supplied in the medium. Hence, the condensation to TMM had to be located in the cell envelope. External trehalose replaced cytoplasmic trehalose for mycolate synthesis only partially, since the trehalose deficient strain did not synthesise TDM and contained lower quantities of mycolate than ATCC 21527 cultured on a mixture of sucrose and trehalose, while it synthesised the native mycolate composition cultured on a mixture of fructose or glucose and trehalose. Since growth rate and biomass production were restored to the level of the parent strain when the trehalose deficient strain was grown on a mixture of glucose or fructose and trehalose, but not on a mixture of sucrose and trehalose, the native composition and/or the native amount of the mycolate layer was important for the growth behaviour. In contrast, the mere presence of all the components of the native mycolate layer in the trehalose deficient strain was not sufficient to restore the extent of permeability of the mycolate layer of the parent strain. Presumably, the components of the mycolate layer have to be available in appropriate amount and proportion to restore the permeation barrier. An imperfect mycolate layer enhanced the excretion of lysine only when the trehalose deficient strain was cultured on sucrose. Dependency of lysine excretion on the carbon source confirmed the significance of carbon fluxes through the central metabolism for amino acid production. An incomplete mycolate layer was identified as trigger for glutamate excretion because, in contrast to the parent strain, the trehalose deficient strain spontaneously excreted glutamate.

6 References

Abe, S., Takayama, K. und Kinoshita, S. (1967) Taxonomical studies on glutamic acid producing bacteria. *J Gen Appl Microbiol.* **13**: 279-301.

Alderwick, LJ., Radmacher, E., Seidel, M., Gande, R., Hitchen, PG., Dell, A., Sahm, H., Eggeling, L., Besra, GS. (2005) Deletion of Cg-*emb* in *Corynebacterianeae* leads to a novel truncated cell wall arabinogalactan, whereas inactivation of Cg-*ubiA* results in an arabinan deficient mutant with a cell wall galactan core. *J Biol Chem.* [Epub ahead of print]

Ankri, S., Serebrijski, I., Reyes, O. und Leblon, G. (1996) Mutations in the *Corynebacterium glutamicum* proline biosynthetic pathway: a natural bypass of the ProA step. *J Bacteriol* **178**: 4412-4419.

Arakawa, T. & Timasheff, S. N. (1985) The stabilization of proteins by osmolytes. *Biophys J.* **47**: 411-414.

Argüelles, J. C. (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Arch Microbiol.* **174**: 217-224.

Asselineau, J. (1966) *The bacterial lipids*. Hermann-Editeurs des Sciences et des Arts, Paris.

Barry, C. E., Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A. und Yuan, Y. (1998) Mycolic acids: structure, biosynthesis and physiological functions. *Prog. Lipid Res*. **37**: 143-179.

Bayan, N., Houssin, C., Chami, M., Leblon, G. (2003) Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. *J Biotechnol*. **104:**55-67. Review.

Becker, A., Schloder, P., Steele, J. E. und Wegener, G. (1996) The regulation of trehalose metabolism in insects. *Experientia* **52**: 433-439.

Belisle, J. T., Varalakshmi, D. V., Sievert, T., Takayama, K., Brennan, P. und Besra, G. S. (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* **276**: 1420-1422.

Bellmann, A., Vrljic, M., Patek, M., Sahm, H., Krämer, R., Eggeling, L. (2001) Expression control and specificity of the basic amino acid exporter LysE of *Corynebacterium glutamicum. Microbiology*. **147:** 1765-74.

Benaroudj, N., Lee, D. H. und Goldberg, A. L. (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *J Biol Chem.* **276**: 24261-24267.

Besra, G. S. und Chatterjee, D. (1994) Lipids and carbohydrates of *Mycobacterium tuberculosis*. In Bloom, B. R. (ed.) *Tuberculosis: Pathogenesis, protection and control*: 285-306. American Society for Microbiology. Washington, D.C.

Blazquez, M. A., Santos, E., Flores, C. L., Martinez-Zapater, J. M., Salinas, J. und Gancedo, J. (1998) Isolation and molecular characterization of the *Arabidopsis* TPS1 gene, encoding trehalose-6-phosphate synthase. *Plant J.* **13**: 685-689.

Boos, W., Ehmann, U., Bremer, E., Middendorf, A., Postma, P. (1987) Trehalase of *Escherichia coli*. Mapping and cloning of its structural gene and identification of the enzyme as a periplasmic protein induced under high osmolarity growth conditions. *J Biol Chem*. **262:**13212-8.

Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmele, M. und Postma, P. (1990) Trehalose transport and metabolism in *Escherichia coli*. *J. Bacteriol*. **172**: 3450-3461.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72:** 248-254.

Brand, S., Niehaus, K., Pühler, A., Kalinowski, J. (2003) Identification and functional analysis of six mycolyltransferase genes of *Corynebacterium glutamicum* ATCC 13032: the genes *cop1, cmt1*, and *cmt2* can replace each other in the synthesis of trehalose dicorynomycolate, a component of the mycolic acid layer of the cell envelope. *Arch Microbiol. **180:*** 33-44.

Brennan, P. J. & Nikaido, H. (1995) The envelope of mycobacteria. *Annu. Rev. Microbiol*. **64:** 29-63.

Broer, S., Krämer, R. (1991a) Lysine excretion by *Corynebacterium glutamicum*. 1. Identification of a specific secretion carrier system. *Eur J Biochem*. **202:** 131-5.

Broer, S., Krämer, R. (1991b) Lysine excretion by *Corynebacterium glutamicum*. 2. Energetics and mechanism of the transport system. *Eur J Biochem*. **202:** 137-43.

Carpenter, JF. & Crowe, JH. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry*. **28:** 3916-22.

Chami, M., Bayan, N., Peyret, JL., Gulik-Krzywicki, T., Leblon, G., Shechter, E. (1997) The S-layer protein of *Corynebacterium glutamicum* is anchored to the cell wall by its C-terminal hydrophobic domain. *Mol Microbiol*. **23:** 483-92.

Costa-Riu, N., Burkovski, A., Krämer, R., Benz, R. (2003a) PorA represents the major cell wall channel of the Gram-positive bacterium *Corynebacterium glutamicum. J Bacteriol.* **185:** 4779-86.

Costa-Riu, N., Maier, E., Burkovski, A., Krämer, R., Lottspeich, F., Benz, R. (2003b) Identification of an anion-specific channel in the cell wall of the Gram-positive bacterium *Corynebacterium glutamicum. Mol Microbiol*. **50:** 1295-308.

Cowan, SW., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, RA., Jansonius, JN., Rosenbusch, JP. (1992) Crystal structures explain functional properties of two *E. coli* porins. *Nature.* **358:** 727-33.

Daffé, M. (2005) The cell envelope of corynebacteria. *Handbook of Corynebacterium glutamicum.* 121-147.

Daffé, M. und Draper, P. (1998) The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol*. **39:** 131-203.

De Smet, K. A. L., Weston, A., Brown, I. N., Young, D. B. und Robertson, B. D. (2000) Three pathways for trehalose biosynthesis in mycobacteria. *Microbiol.* **146**: 199-208.

De Sousa-D'Auria, C., Kacem, R., Puech, V., Tropis, M., Leblon, G., Houssin, C., Daffe, M. (2003) New insights into the biogenesis of the cell envelope of corynebacteria: identification and functional characterization of five new mycoloyltransferase genes in *Corynebacterium glutamicum*. *FEMS Microbiol Lett*. **224:** 35-44.

De Virgilio, C., Bürckert, N., Bell, W. Jenö, W., Boller, T. und Wiemken, A. (1993) Disruption of *TPS2*, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. *Eur J Biochem.* **212**: 315-323.

Dominguez, H. und Lindley, N. D. (1996) Complete sucrose metabolism requires fructose phosphotransferase activity to ensure phosphorylation of liberated fructose. *Appl. Env. Microbiol.* **62**: 3878-3880.

Eggeling, L. & Sahm, H. (2001) The cell wall barrier of *Corynebacterium glutamicum* and amino acid efflux. *J Bio Bioeng.* **92:** 201-213.

Eggeling, L., Oberle, S., Sahm, H. (1998) Improved L-lysine yield with *Corynebacterium glutamicum*: use of *dapA* resulting in increased flux combined with growth limitation. *Appl Microbiol Biotechnol*. **49:** 24-30.

Eikmanns, BJ., Metzger, M., Reinscheid, D., Kircher, M., Sahm, H. (1991) Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. *Appl Microbiol Biotechnol*. **34:** 617-22.

Elbein, AD., Pan, YT., Pastuszak, I., Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology.* **13(4):** 17R-27R. Epub 2003 Jan 22. Review.

Engelhardt, H., Heinz, C., Niederweis, M. (2002) A tetrameric porin limits the cell wall permeability of *Mycobacterium smegmatis. J Biol Chem.* **277:** 37567-72.

Fillinger, S., Chaveroche, M. K., van Dijck, P., de Vries, R., Ruijter, G., Thevelein, J. und d'Enfert, C. (2001) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiol.* **147**: 1851-1862.

Gande, R., Gibson, KJ., Brown, AK., Krumbach, K., Dover, LG., Sahm, H., Shioyama, S., Oikawa, T., Besra, GS., Eggeling, L. (2004) Acyl-CoA carboxylases (*accD2* and *accD3*), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in *Corynebacterianeae* such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. *J Biol Chem*. **279:** 44847-57.

Georgi, T., Rittmann, D., Wendisch, VF. (2005) Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: Roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab Eng*. Jun 24; [Epub ahead of print]

Goren, M. B., D'Arcy Hart, P., Young, M. R. und Armstrong, J. A. (1976) Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA* **73**: 2510-2514.

Gutmann, M., Hoischen, C., Krämer, R. (1992) Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. *Biochim Biophys Acta*. **1112:** 115-23.

Han, EK., Cotty, F., Sottas, C., Jiang, H., Michels, CA. (1995) Characterization of AGT1 encoding a general alpha-glucoside transporter from *Saccharomyces*. *Mol Microbiol.* **17:** 1093-107.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev*. **66:** 300-72. Review.

Hoischen, C., Krämer, R. (1990) Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. *J Bacteriol.* **172:** 3409-16.

Hounsa, C.-G., Brandt, E. V., Thevelein, J., Hohmann, S. und Prior, B. A. (1998) Role of trehalose in survival of Saccharomyces cerevisiae under osmotic stress. *Microbiol.* **144:** 671-680.

Hunten, P., Costa-Riu, N., Palm, D., Lottspeich, F., Benz, R. (2005) Identification and characterization of PorH, a new cell wall channel of *Corynebacterium glutamicum*. *Biochim Biophys Acta*. **1715:** 25-36.

Inoue, H., Nojima, H. und Okayama, H. (1990) High efficiency transformation of *Escherichia col*i with plasmids. Gene **96:** 23-28.

Jarlier, V. & Nikaido, H. (1994) Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol Lett*. **123:** 11-8. Review.

Joliff, G., Mathieu, L., Hahn, V., Bayan, N., Duchiron, F., Renaud, M., Schechter, E., Leblon, G. (1992) Cloning and nucleotide sequence of the *csp1* gene encoding PS1, one of the two major secreted proteins of *Corynebacterium glutamicum*: the deduced N-terminal region of PS1 is similar to the Mycobacterium antigen 85 complex. *Mol Microbiol*. **6:** 2349-62.

Jules, M., Guillou, V., Francois, J., Parrou, JL. (2004) Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae. Appl Environ Microbiol*. **70**: 2771-8.

Kacem, R., De Sousa-D'Auria, C., Tropis, M., Chami, M., Gounon, P., Leblon, G., Houssin, C., Daffe, M. (2004) Importance of mycoloyltransferases on the physiology of *Corynebacterium glutamicum. Microbiology.* **150:** 73-84.

Kartmann, B., Stenger, S., Niederweis, M. (1999) Porins in the cell wall of *Mycobacterium tuberculosis*. *J Bacteriol.* **181:** 6543-6. Erratum in: *J Bacteriol* **181(24):**7650. Stengler S [corrected to Stenger S].

Kawahara, Y., Takahashi-Fuke, K., Shimizu, E., Nakamatsu, T., Nakamori, S. (1997) Relationship between the glutamate production and the activity of 2-oxoglutarate dehydrogenase in *Brevibacterium lactofermentum. Biosci Biotechnol Biochem.* **61:** 1109-12.

Keilhauer, C., Eggeling, L. und Sahm, H. (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol.* **175**: 5595-5603.

Kiefer, P., Heinzle, E., Wittmann, C. (2002) Influence of glucose, fructose and sucrose as carbon sources on kinetics and stoichiometry of lysine production by *Corynebacterium glutamicum. J Ind Microbiol Biotechnol.* **28:** 338-43.

Kim, Y. H., Kwon, T. K., Park, S., Seo, H. S., Cheong, J.-J., Kim, C. H., Kim, J.-K., Lee, J. S. und Choi, Y. D. (2000) Trehalose synthesis by sequential reactions of recombinant maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase from *Brevibacterium helvolum. Appl. Env Microbiol.* **66**: 4620-4624.

Kimura, E. (2003) Metabolic engineering of glutamate production. *Adv Biochem Eng Biotechnol.* **79:** 37-57. Review.

Kimura, E. (2005) L-glutamate production. *Handbook of Corynebacterium glutamicum.* 439-463.

Kimura, E., Abe, C., Kawahara, Y., Nakamatsu, T. (1996) Molecular cloning of a novel gene*, dtsR*, which rescues the detergent sensitivity of a mutant derived from *Brevibacterium lactofermentum. Biosci Biotechnol Biochem*. **60:** 1565-70.

Kimura, E., Abe, C., Kawahara, Y., Nakamatsu, T., Tokuda, H. (1997) A *dtsR* gene-disrupted mutant of *Brevibacterium lactofermentum* requires fatty acids for growth and efficiently produces L-glutamate in the presence of an excess of biotin. *Biochem Biophys Res Commun.* **234:** 157-61.

Krömer, JO., Sorgenfrei, O., Klopprogge, K., Heinzle, E., Wittmann, C. (2004) In-depth profiling of lysine-producing *Corynebacterium glutamicum* by combined analysis of the transcriptome, metabolome, and fluxome. *J Bacteriol*. **186:** 1769-84.

Kwakman, JH., Postma, PW. (1994) Glucose kinase has a regulatory role in carbon catabolite repression in *Streptomyces coelicolor. J Bacteriol*. **176:** 2694-8.

Lambert, PA. (2002) Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. *J Appl Microbiol*. **92** Suppl:46S-54S. Review.

Ley, O. (2005) Charakterisierung der Regulation des Prolinbiosyntheseweges in *Corynebacterium glutamicum.* PhD thesis, Universität zu Köln.

Lichtinger, T., Burkovski, A., Niederweis, M., Krämer, R., Benz, R. (1998) Biochemical and biophysical characterization of the cell wall porin of *Corynebacterium glutamicum*: the channel is formed by a low molecular mass polypeptide. *Biochemistry.* **37:** 15024-32.

Lichtinger, T., Rieß, F., Burkovski, A., Engelbrecht, F., Hesse, D., Kratzin, H. D., Krämer, R. und Benz, R. (2001) The low-molecular-mass subunit of the cell wall channel of the Gram-positive *Corynebacterium glutamicum. Eur. J. Biochem*. **268:** 462-469.

Liu, J. & Nikaido, H. (1999) A mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids accumulates meromycolates. *Proc Natl Acad Sci U S A*. **96:** 4011-6.

Lopez-Marin, LM., Quemard, A., Laneelle, G., Lacave, C. (1991) Ethylenic mycolic acid biosynthesis: extension of the biosynthetic model using cell-free extracts of *Mycobacterium aurum* and *Mycobacterium smegmatis*. *Biochim Biophys Acta.* **1086:** 22-8.

Mailaender, C., Reiling, N., Engelhardt, H., Bossmann, S., Ehlers, S., Niederweis, M. (2004) The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis. Microbiology*. **150**: 853-64.

Makihara, F., Tsuzuki, M., Sato, K., Masuda, S., Nagashima, KV., Abo, M., Okubo, A. (2005) Role of trehalose synthesis pathways in salt tolerance mechanism of *Rhodobacter sphaeroides* f. sp. denitrificans IL106. *Arch Microbiol*. Jul 29: 1-10 [Epub ahead of print]

Maruta, K., Hattori, K., Nakada, T., Kubota, M., Sugimoto, T. und Kurimoto, M. (1996a) Cloning and sequencing of trehalose biosynthesis genes from *Rhizobium sp.* M-11. *Biosci Biotechnol Biochem.* **60**: 717-720.

Maruta, K., Hattori, K., Nakada, T., Kubota, M., Sugimoto, T. und Kurimoto, M. (1996b) Cloning and sequencing of trehalose biosynthesis genes from *Arthrobacter sp.* Q36. *Biochim Biophys Acta.* **1289**: 10-13.

Maruta, K., Mitsuzumi, H., Nakada, T., Kubota, M., Chaen, H., Fukuda, S., Sugimoto, T. und Kurimoto, M. (1996c) Cloning and sequencing of a cluster of genes encoding novel enzymes of trehalose biosynthesis from thermophilic archaebacterium *Sulfolobus acidocaldarius*. *Biochim Biophys Acta.* **1291**: 177-181.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. und Erlich, H. (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp Quant Biol*. **51:** 263-273.

Nakayama, K., Sagamihara, K., Araki, K. (1973) Process for producing L-lysine. US Patent No 3708395.

Nampoothiri, KM., Hoischen, C., Bathe, B., Mockel, B., Pfefferle, W., Krumbach, K., Sahm, H., Eggeling, L. (2002) Expression of genes of lipid synthesis and altered lipid composition modulates L-glutamate efflux of *Corynebacterium glutamicum. Appl Microbiol Biotechnol*. **58:** 89-96.

Niederweis, M. (2003) Mycobacterial porins-new channel proteins in unique outer membranes. *Mol Microbiol*. **49:** 1167-77. Review.

Nunheimer, TD., Birnbaum, J., Ihnen, ED., Demain, AL. (1970) Product inhibition of the fermentative formation of glutamic acid. *Appl Microbiol*. **20:** 215-7.

Pan, YT., Koroth Edavana, V., Jourdian, WJ., Edmondson, R., Carroll, JD., Pastuszak, I., Elbein, AD. (2004) Trehalose synthase of *Mycobacterium smegmatis*: purification, cloning, expression, and properties of the enzyme. *Eur J Biochem*. **271(21):**4259-69.

Parrou JL, Francois J. (1997) A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Anal Biochem*. **248:** 186-8.

Peters-Wendisch, PG., Schiel, B., Wendisch, VF., Katsoulidis, E., Mockel, B., Sahm, H., Eikmanns, BJ. (2001) Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum. J Mol Microbiol Biotechnol.* **3:** 295-300.

Pfeffer, PE., Becard, G., Rolin, DB., Uknalis, J., Cooke, P., Tu, S. (1994) In vivo nuclear magnetic resonance study of the osmoregulation of phosphocholine-substituted beta-1,3;1,6 cyclic glucan and its associated carbon metabolism in *Bradyrhizobium japonicum* USDA 110. *Appl Environ Microbiol.* **60:** 2137-46.

Pfefferle, W., Mockel, B., Bathe, B., Marx, A. (2003) Biotechnological manufacture of lysine. *Adv Biochem Eng Biotechnol*. **79:** 59-112. Review.

Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffé, M., Guilhot, C. (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proc Natl Acad Sci U S A.* **101:** 314-9.

Portevin, D., de Sousa-D'Auria, C., Montrozier, H., Houssin, C., Stella, A., Laneelle, MA., Bardou, F., Guilhot, C., Daffé, M. (2005) The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. *J Biol Chem*. **280:** 8862-74.

Puech, V., Bayan, N., Salim, K., Leblon, G. und Daffé, M. (2000) Characterization of the *in vivo* acceptors of the mycolyl residues transferred by the corynebacterial PS1 and the related mycobacterial antigens 85. *Mol. Microbiol*. **35:** 1026-1041.

Puech, V., Chami, M., Lemassu, A., Laneelle, M. A., Schiffler, B., Gounon, P., Bayan, N., Benz, R. und Daffé, M. (2001) Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. *Microbiology* **147:** 1365-1382.

Qu, Q., Lee, SJ., Boos, W. (2004) TreT, a novel trehalose glycosyltransferring synthase of the hyperthermophilic archaeon *Thermococcus litoralis. J Biol Chem.* **279:** 47890-7. Epub 2004 Sep 13.

Qureshi, N., Sathyamoorthy, N., Takayama, K. (1984) Biosynthesis of C30 to C56 fatty acids by an extract of *Mycobacterium tuberculosis* H37Ra. *J Bacteriol*. **157:** 46-52.

Radmacher, E., Alderwick, LJ., Besra, GS., Brown, AK., Gibson, KJ., Sahm, H., Eggeling, L. (2005a) Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum. Microbiology*. **151:**2421-7.

Radmacher, E., Stansen, KC., Besra, GS., Alderwick, LJ., Maughan, WN., Hollweg, G., Sahm, H., Wendisch, VF., Eggeling, L. (2005b) Ethambutol, a cell wall inhibitor of Mycobacterium tuberculosis, elicits L-glutamate efflux of *Corynebacterium glutamicum. Microbiology.* **151:** 1359-68.

Reinders, A., Bürckert, N., Hohmann, S., Thevelein, J. M., Boller, T., Wiemken, A. und De Virgilio, C. (1997) Structural analysis of the subunits of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae* and their function during heat shock. *Mol. Microbiol*. **24:** 687-695.

Rest, van der, M. E., Lange, C. und Molenaar, D. (1999) A heat shock following electroporation induces highly efficient transformation of Corynebacterium glutamicum with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* **52:** 541-545.

Riess, FG., Lichtinger, T., Cseh, R., Yassin, AF., Schaal, KP., Benz, R. (1998) The cell wall porin of *Nocardia farcinica*: biochemical identification of the channel-forming protein and biophysical characterization of the channel properties. *Mol Microbiol*. **29:** 139-50.

Ronning, DR., Klabunde, T., Besra, GS., Vissa, VD., Belisle, JT., Sacchettini, JC. (2000) Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. *Nat Struct Biol*. **7:** 141-6.

Ronning, DR., Vissa, V., Besra, GS., Belisle, JT., Sacchettini, JC. (2004) *Mycobacterium tuberculosis* antigen 85A and 85C structures confirm binding orientation and conserved substrate specificity. *J Biol Chem.* **279:** 36771-7.

Rönsch, H., Krämer, R., Morbach, S. (2003) Impact of osmotic stress on volume regulation, cytoplasmic solute composition and lysine production in *Corynebacterium glutamicum* MH20-22B. *J Biotechnol*. **104:** 87-97.

Ryu, SI., Park, CS., Cha, J., Woo, EJ., Lee, SB. (2005) A novel trehalose-synthesizing glycosyltransferase from *Pyrococcus horikoshii*: molecular cloning and characterization. *Biochem Biophys Res Commun*. **329:** 429-36.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. und Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* **239:** 487-491.

Sambrook, J., Fritsch, E. F. und Maniatis, T (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. und Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum. Gene.* **145:** 69-73.

Schleifer, KH. & Kandler. O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev.* **36:** 407-77. Review.

Schuster, R. (1988) Determination of amino acids in biological, pharmaceutical, plant and food samples by automated pre-column derivatization and high performance liquid chromatography. *J. Chromatogr*. **431:** 271-284.

Sharbati-Tehrani, S., Stephan, J., Holland, G., Appel, B., Niederweis, M., Lewin, A. (2005) Porins limit the intracellular persistence of *Mycobacterium smegmatis. Microbiology.* **151:** 2403-10.

Shimakata, T. & Minatogawa, Y. (2000) Essential role of trehalose in the synthesis and subsequent metabolism of corynomycolic acid in *Corynebacterium matruchotii*. *Arch. Biochem. Biophys.* **380:** 331-338.

Shimakata, T., Iwaki, M. und Kusaka, T. (1984) In vitro synthesis of mycolic acids by the fluffy layer fraction of *Bacterionema matruchotii. Arch. Biochem. Biophys*. **229:** 329-339.

Shingu, H., & Terui, G. (1971) Studies on the process of glutamic acid fermentation at the enzyme level: I. On the changes of α-ketoglutaric acid dehydrogenase in the course of culture. *J Ferment Technol.* **49:** 400-405

Shirai, T., Nakato, A., Izutani, N., Nagahisa, K., Shioya, S., Kimura, E., Kawarabayasi, Y., Yamagishi, A., Gojobori, T., Shimizu, H. (2005) Comparative study of flux redistribution of metabolic pathway in glutamate production by two coryneform bacteria. *Metab Eng*. **7:** 59-69.

Spargo, B. J., Crowe, L. M., Ioneda, T., Beaman, B. L. und Crowe, J. H. (1991) Cord factor (alpha, alpha-trehalose 6,6'-dimycolate) inhibits fusion between phospholipid vesicles. *Proc. Natl. Acad. Sci. USA* **88:** 737-740.

Stahl, C., Kubetzko, S., Kaps, I., Seeber, S., Engelhardt, H., Niederweis, M. (2001) MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*. *Mol Microbiol.* **40:** 451-64.

Stambuk, BU., De Araujo, PS., Panek, AD., Serrano, R. (1996) Kinetics and energetics of trehalose transport in *Saccharomyces cerevisiae. Eur J Biochem*. **237:** 876-81.

Stephan, J., Mailaender, C., Etienne, G., Daffé, M., Niederweis, M. (2004) Multidrug resistance of a porin deletion mutant of *Mycobacterium smegmatis*. *Antimicrob Agents Chemother*. **48:** 4163-70.

Sweeley, C. C., Bentley, R., Makita, M. und Wells, W. W. (1963) Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J Am Chem Soc.* **85:** 2497-2507.

Takayama, K., Wang, C., Besra, GS. (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis. Clin Microbiol Rev.* **18:** 81-101.

Thierbach, G., Kalinowski, J., Bachmann, B., Pühler, A. (1990) Cloning of a DNA fragment from *Corynebacterium glutamicum* conferring aminoethyl cysteine resistance and feedback resistance to aspartokinase. *Appl Microbiol Biotechnol.* **32:** 443-8.

Trias, J. & Benz, R. (1994) Permeability of the cell wall of *Mycobacterium smegmatis. Mol Microbiol.* **14:** 283-90.

Trivedi, OA., Arora, P., Sridharan, V., Tickoo, R., Mohanty, D., Gokhale, RS. (2004) Enzymic activation and transfer of fatty acids as acyl-adenylates in mycobacteria. *Nature.* **428:** 441-5. Erratum in: *Nature*. (2004); **428:** 963. *Nature*. (2004) **430:** 810.

Tropis, M., Meniche, X., Wolf, A., Gebhardt, H., Strelkov, S., Chami, M., Schomburg, D., Krämer, R., Morbach, S., Daffé, M. (2005) The crucial role of trehalose and structurally related oligosaccharides in the biosynthesis and transfer of mycolic acids in *Corynebacterineae*. *J Biol Chem.* **280:** 26573-85.

Tzvetkov, M., Klopprogge, C., Zelder, O., Liebl, W. (2003) Genetic dissection of trehalose biosynthesis in *Corynebacterium glutamicum*: inactivation of trehalose production leads to impaired growth and an altered cell wall lipid composition. *Microbiology*. **149:** 1659-73.

Vieira, J. und Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19:** 259-268.

Vrljic, M., Kronemeyer, W., Sahm, H., Eggeling, L. (1995) Unbalance of L-lysine flux in *Corynebacterium glutamicum* and its use for the isolation of excretion-defective mutants. *J Bacteriol*. **177:** 4021-7.

Vrljic, M., Sahm, H., Eggeling, L. (1996) A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum. Mol Microbiol.* **22:** 815-26.

Walker, R. W., Prome, J. C. und Lacave, C. S. (1973) Biosynthesis of mycolic acids. Formation of a C32 beta-keto ester from palmitic acid in a cell-free system of *Corynebacterium diphtheriae. Biochim. Biophys. Acta* **326:** 52-62.

Wilson WA, Wang Z, Roach PJ. (2005) Regulation of yeast glycogen phosphorylase by the cyclin-dependent protein kinase Pho85p. *Biochem Biophys Res Commun*. **329(1):**161-7.

Wittmann, C., Kiefer, P., Zelder, O. (2004) Metabolic fluxes in *Corynebacterium glutamicum* during lysine production with sucrose as carbon source. *Appl Environ Microbiol*. **70:** 7277-87.

Wolf, A., (2002) Trehalose metabolism in *Corynebacterium glutamicum.* PhD thesis, Universität zu Köln.

Wolf, A., Krämer, R., Morbach, S. (2003) Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC13032 and their significance in response to osmotic stress. *Mol Microbiol*. **49:** 1119-34.

Yanisch-Perron, C., Vieira J. und Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103-119.

Zaborosch, C. & Schlegel, HG. (1992) Allgemeine Mikrobiologie. *Georg Thieme Verlag Stuttgart-New York*

Zevenhuizen, LP. (1992) Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. *Antonie Van Leeuwenhoek*. **61:** 61-8.

Zimmermann, W. & Rosselet, A. (1977) Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. *Antimicrob Agents Chemother*. **12:** 368-72.

Danksagung

Bei Herrn Prof. Dr. Reinhard Krämer möchte ich mich für die engagierte Betreuung, insbesondere die wertvollen Tipps zur Präsentationsgestaltung, das spannende Arbeitsthema und die Übernahme des Hauptreferats herzlich bedanken.

Herrn Prof. Dr. Thomas Langer danke ich für die Übernahme des Koreferates.

Meiner Betreuerin Dr. Susanne Morbach danke ich herzlich für die hervorragende Betreuung, der Arbeit, insbesondere für ihre stete Bereitschaft jede Frage zu beantworten, und ihre genauen und hilfreichen Korrekturen.

Mein besonderer Dank gilt Dr. Marielle Tropis und Prof. Dr. Mammadou Daffé, die mich am ipbs (*Institut de Pharmacologie et Biologie Structurale*) in Toulouse bei der Lipidextraktion betreut haben und auch später immer für Fragen offen waren.

Meinen direkten Laborkollegen Nina, OL, Ute und Eva danke ich für die entspannte und lustige Arbeitsatmosphäre und allen meinen Kollegen für die Hilfsbereitschaft und das gute Arbeitsklima. Mein besonderer Dank gilt Andreas Wolf für die Einweihung in die Geheimnisse der Trehaloseforschung und der GC.

Außerdem danke ich meinen IMPRS-Kollegen für den - nicht nur wissenschaftlichen - Blick über den Tellerrand und Dr. Ralf Petri danke für sein Engagement für die IMPRS auch an der Uni.

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Teilpublikationen:

Tropis, M., Meniche, X., Wolf, A., Gebhardt, H., Strelkov, S., Chami, M., Schomburg, D., Krämer, R., Morbach, S., Daffé, M. (2005) The crucial role of trehalose and structurally related oligosaccharides in the biosynthesis and transfer of mycolic acids in *Corynebacterineae*. *J Biol Chem.* **280:** 26573-85.

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