Channel-forming proteins in the cell wall of amino acid-producing Corynebacteria

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades

der Fakultät für Biologie

an der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Peter Hünten

Würzburg

Würzburg 2005

Eingereicht am: .....

Prüfungskommision:

Vorsitzender: .....

1. Gutachter: Prof. Dr. R. Benz

2. Gutachter: Prof. Dr. J. Reidl

Tag der mündlichen Prüfung: .....

Promotionsurkunde ausgehändigt am: .....

Ich versichere, dass ich diese Dissertation selbständig angefertigt und keine anderen, als die von mir angegeben Quellen und Hilfsmittel benutzt habe.

PUBLICATIONS

- **Hünten, P., Schiffler, B., Lottspeich, F. & Benz, R. (2005).** PorH, a new channel-forming protein present in the cell wall of *Corynebacterium efficiens* and *Corynebacterium callunae*. (accepted)

- **Hünten, P., Costa-Riu, N., Palm, D., Lottspeich, F. & Benz, R. (2005).** Identification and characterization of PorH, a new cell wall channel of *Corynebacterium glutamicum.* (submitted)

CONTENTS

CHAPTER 1

Introduction

1.1 The *Prokariotae*

The *Prokariotae* consist of two big domains, the *Archaea* and the *Eubacteria*. The last can be divided in three big categories, Gram-negative, Gram-positive and the cell-wall-lacking mycoplasmas (Holt *et al*., 1994). The first two have in common, that above the cytoplasmic membrane exists a more or less complex cell wall that allows them to be classified by the Gram-staining technique, developed by Hans Christian Gram 1884.

1.2 The bacterial cell wall

1.2.1 The cytoplasmic membrane

The cytoplasmic membrane is a selectively permeable membrane that determines the uptake and release of substances (Brock *et al*., 2002). Nutrients must pass and waste has to leave the cell across this membrane. Water, dissolved gases such as carbon dioxide and oxygen and lipid-soluble molecules simply diffuse across the phospholipid bilayer. Water-soluble ions generally pass through small pores with a diameter less than 0.8 nm in the membrane. All other molecules require either carrier or porins - specific or general - to transport them across the membrane. Mechanisms by which materials move across the cytoplasmic membrane include simple diffusion, catalysed diffusion, osmosis, and transport of substances across the membrane by transport proteins.

The periplasmic space exists in between the cytoplasmic membrane and the peptidoglycan layer. It is thin, sometimes not existent in Gram-positive bacteria and quite large in Gram-

negatives. It contains hydrolytic enzymes required for metabolism of large substrates and binding proteins for nutrient processing and uptake. Proteins that destroy potentially dangerous foreign substances are also present in this space. In a matter of fact, it is an important reaction site for many substances that enter and leave cell.

1.2.2 Peptidoglycan (Murein)

Eubacteria, unlike the *Archaea* and the *Eukarya*, contain peptidoglycan. Peptidoglycan is a single bag-shaped, highly cross-linked macromolecule that surrounds the bacterial cell membrane, provides rigidity and protects bacteria from osmotic pressure (Schleifer & Kandler, 1972; Benz, 1994).

Peptidoglycan has a glycan (polysaccharide) backbone consisting of N-acetylmuramic acid and N-acetylglucosamine with peptide side chains containing D- and L-amino acids and in some instances diaminopimelic acid (Brock *et al.*, 2002; Alberts *et al*., 2002) (see Figure 1). The side chains are cross-linked by peptide bridges that are variable in structure among the bacterial species. While the cross-linking in Gram-positive is mediated by pentaglycine bridges, in Gram-negative the cross-linking is less frequent and between the meso-diaminopimelic and the D-alanine of the side chain peptide itself.
In Gram-positive cells the peptidoglycan is very thick, the percentage of the cellweight is 50 % and 90 % of the total cell wall. In comparison to the Gram-negative bacteria, it contains big variations in structure and composition (Schlegel, 1993). It is shown in electron micrographs that the peptidoglycan is about 20-80 nm thick. It is free permeable to most antibiotics.
The remainder of the cell wall consists of acidic polysaccharides. These polysaccharides, the teichoic acids, are repeating units of glycerol or ribitol joined by phosphodiester linkages. They are located throughout the wall. The mechanically strong cell wall of Gram-positive bacteria is hydrophilic as well as negatively charged, due to the presence of the teichoic acids (Brock *et al.,* 2002). It is believed that it does not form a permeability barrier for hydrophilic solutes up to a molecular mass of 100 kDa on the surface of Gram-positive bacteria, because it is rather porous (Nikaido, 1994).
The peptidoglycan in Gram-negative bacteria is only intermittently cross-linked and much thinner, the percentage of the cell wall is only about 15-20 %. It can be 10-20 nm thick and contains a middle layer of lipoprotein. It is selectively permeable to antibiotics. The backbone

of the peptidoglycan can be cleaved by lysozyme that is present in animal serum, tissue, secretions, and in the phagocytic lysosome (Imoto *et al.,* 1972; Prager *et al.,* 1974). Lysozyme cleaves specifically the β-1,4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine.

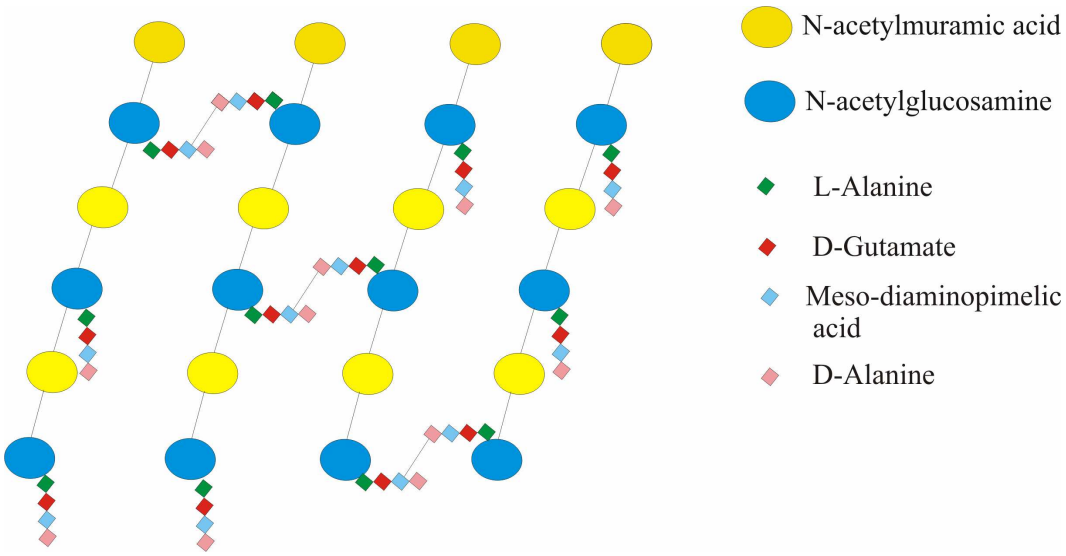

Figure 1. Peptidoglycan structure of a Gram-negative bacterium.

1.2.3 The outer membrane

The major difference between Gram-positive and Gram-negative bacteria, regarding the cell wall, is the restricted diffusion of hydrophilic solutes across the membrane. This is the result of a second permeability barrier besides the cytoplasmic membrane, the outer membrane of Gram-negative bacteria (Glauert & Thornley, 1969; Nikaido & Vaara, 1985) (see Figure 2B). It contains phospholipids, mostly phosphatidylethanolamine in its inner leaflet, whereas the outer leaflet is composed of lipopolysaccharides (LPS) (Kamio & Nikaido, 1976; Benz, 1994). LPS is a lipid-like amphiphilic molecule composed of lipid A, a diglucosamine phosphate dimer, linked to 5 or 6 fatty acids (also known as endotoxin (Galanos *et al*., 1977)). Oligosaccharides and a variable number of repeated tri- to pentasaccharide units called O-antigen are attached to the endotoxin. In the case of the enteric opportunist pathogen *Serratia marcescens* it is a virulence factor that contributes to the adherence of the bacterium (Palomar *et al.,* 1995). Lipopolysaccharides carry net negative charges resulting in a strong negatively charged cell surface (Galanos *et al*., 1977).

LPS is important for the function of most outer membrane proteins and it plays a special role in assembly and maintenance of the outer membrane as a permeability barrier for hydrophobic antibiotics, bile salts, detergents, proteases, lipases, and lysozyme (Benz & Bauer, 1988; Vaara *et al*., 1990; Plesiat & Nikaido, 1992). They interact with the LPS via hydrophobic interactions and by noncovalent cross-bridging of adjacent LPS molecules with divalent cations in such a way, that a tight network is produced. In some bacteria it has been seen, that LPS is the receptor for molecules such as phages (Montilla *et al.*, 1991).

LPS contains tightly packed saturated fatty acids, decreasing the mobility of the chains and the fluidity of the lipid interior (Cullis & Hope, 1985). For the permeation of small hydrophilic molecules, the outer membrane contains channel forming proteins, called porins, that form non-specific diffusion channels within the membrane (Nikaido & Vaara, 1985; Hancock, 1987; Benz & Bauer, 1988). But it may also contain solute specific channels that are specific for one class of solutes such as the carbohydrate-specific channels LamB and ScrY of enteric bacteria (Luckey & Nikaido, 1980; Benz *et al.*, 1986; Schmid *et al.*, 1991; Schülein *et al.*, 1991). Because of the special surface structure and the tight packing of the LPS, hydrophobic molecules are poorly permeable through the outer membrane. Their permeability is about 1 to 2 % of that of typical glycerophospholipid bilayers (Vaara *et al*., 1990; Plesiat & Nikaido, 1992). In addition, the LPS is considered as a virulence factor in adhesion processes, in activation of the inflammatory response and in appearance of resistance against a bactericidal serum (Tomas *et al.*, 1986).
In Figure 2 the schematic representation of the structures of the cell wall envelope from Gram-negative and Gram-positive bacteria are described.

A B

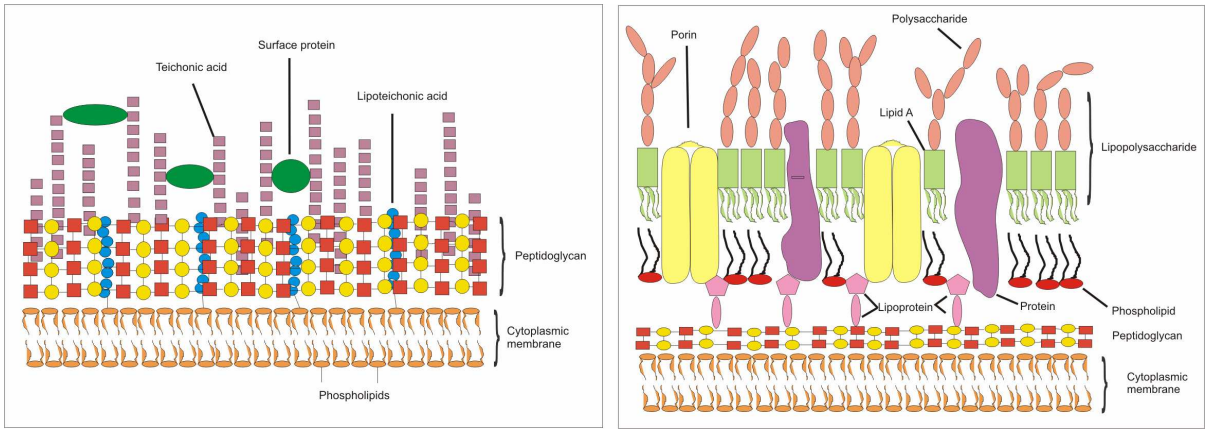

Figure 2. Schematic representation from cell wall structures of **(A)** Gram-positive and **(B)** Gram-negative bacteria. Notice the difference in thickness of the peptidoglycan layer and the additional outer membrane layer of Gram-negative bacteria.

1.3 The *Actinomycetales*

The Gram-positive bacteria can be divided in two major branches, the high G+C content (> 50 %) actinomycetes and the low G+C content (< 50 %) *Clostridium*, *Bacillus* and *Streptococcus* strains (Fox *et al*., 1980; Woese, 1987; Stackebrand *et al*., 1997;). The actinomycetes comprise a number of major separate lineages (Woese *et al*., 2000). Despite they stain as Gram-positive, they share the property of an outer barrier with Gram-negative, formed by their envelope, that is distinct from the plasma membrane. There is a large interest in biotechnology for the actinomycetes. The genus *Streptomyces* is a potent producer of the antibiotic streptomycin that inhibits translation initiation causing misreading of mRNA in protein synthesis (Zierhut *et al*., 1979). It belongs to the suborder *Streptomycineae* and it produces also other secondary metabolites, for instance, the sesquiterpene soil odour geosmin, which confers the typical musty or earthy smell to the soil, but it also causes a health problem in drinking water (Wood *et al*., 1983). In the suborder *Corynebacterineae* you can find pathogens which are responsible for deseases like tuberculosis, leprosy or diphtheria. Contrariwise, other organisms are the main producers of amino acids e.g. glutamate or lysine.

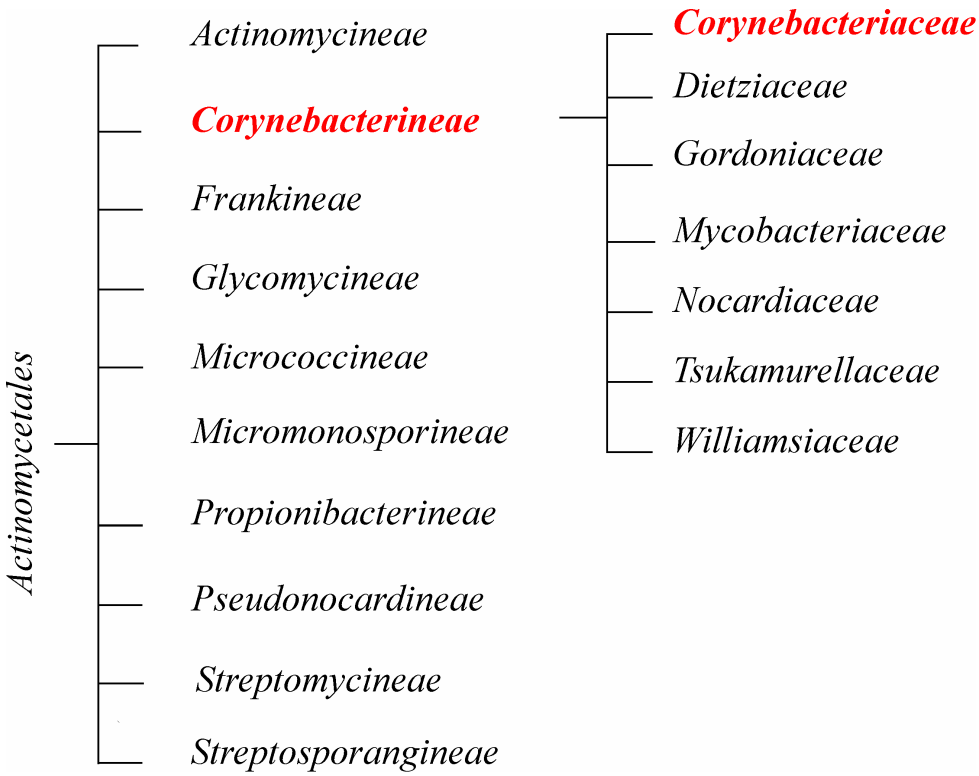

Figure 3. Members of the *Actinomycetales*. The interest of this work was focused on the red labelled suborder and family, respectively.

One part of the actinomycetes of particular interest is called mycolata. This is a broad and diverse group of mycolic-acid containing actinomycetes and encloses the genera *Rhodococcus*, *Gordona*, *Tsukamurella*, *Dietzia*, *Mycobacterium*, *Nocardia* and a part of *Corynebacterium.*

The relationship between these genera is further characterized by the chemical analysis of the cell wall and lipid structures (Minnikin *et al.*, 1978; Daffé & Drapper, 1998; Minnikin & Goodfellow, 1980; Minnikin *et al.*, 1984; Sutcliffe, 1997; Puech *et al.*, 2001). All the mycolata have in common that they have similar peptidoglycan structure, designated Alaγ (D-Ala in position γ of the tetrapeptide) by the nomenclature of Schleifer and Kandler (1972). This is formed by a thick *meso*-diaminopimelic acid-containing peptidoglycan covalently linked to arabinogalactan, which in turn is esterified by long-chain α-alkyl, β-hydroxy fatty acids. In all currently proposed models (Minnikin, 1982; Rastogi, 1991; Liu *et al.*, 1995) the outer membrane permeability barrier of mycobacteria consists of a monolayer of mycolil residues covalently linked to the arabinogalactan and peptidoglycan complex (MAPc) (Crick *et al.*, 2001) and a polysaccharide-rich capsule-like material.

The arabinogalactan strands are coiled and perpendicular to the plane of the plasma membrane (Liu *et al.*, 1999, Nikaido *et al*., 1993). In recent publications it has been indicated that not only the arabinogalactan, but also the peptidoglycan are perpendicular orientated to the plasma membrane (Dmitriev *et al.* 1999; 2000). This orientation is contradictory with the classical ones, where the arabinogalactan and the peptidoglycan layer are parallel to the plasma membrane (Ghuysen, 1968; McNeil & Brennan, 1991).
A summary of the structures and other taxonomically relevant features of some coryneform actinomycetes are listed in Table 1.

Genus	**Peptidoglycan**		**DNA**	**Mycolic Acids**	
	Type	Muramic acid group	% G+C	carbon atoms	double bonds
Corynebacterium	Alaγ	acetyl	51-69	22-38	0-2
Mycobacterium	Alaγ	glycolyl	62-70	60-90	1-2
Nocardia	Alaγ	glycolyl	64-69	46-60	0-3

Table 1. Summary of chemical analysis of some coryneform *Actinomycetes*. Taken from Schleifer and Kandler (1972); Uchida and Aida (1979); Goodfellow and Cross (1983) and Collins *et al.*, (1981).

The peptidoglycan is arranged parallel and the AG perpendicular to the cytoplasmic membrane, described by Liu *et al.* (1999). In the case of *Corynebacterium* the bacterial typical groups of N-acetylglucosamine and N-acetylmuramic are conserved but in *Mycobacterium*, the N-acetyl functions of the muramic acid of bacterial peptidoglycan are further oxidized to N-glycolyl functions (see Table 1). Another unique feature from mycolata is, that the muramic acid residues are modified by the tetrapeptide L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine side chains (Schleifer & Kandler, 1972; Eggeling & Sahm, 2001) and the cross-linking can occur in between two meso-diaminopimelic acids (DAP) as well as between DAP and D-alanine residues (Wietzerbin *et al*., 1974).

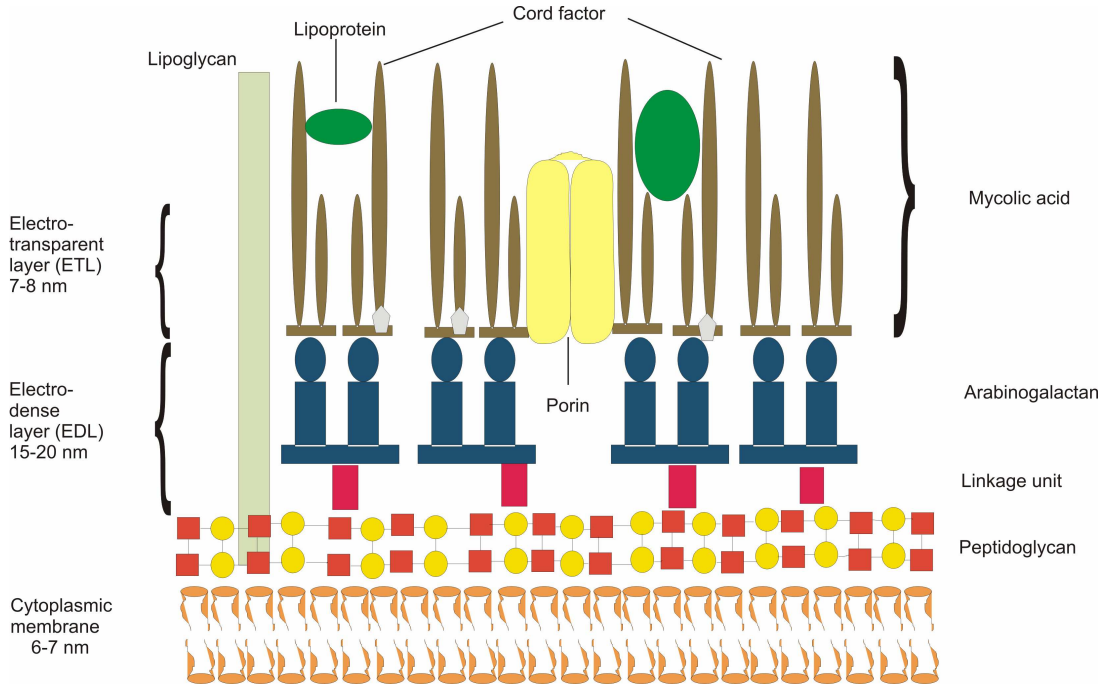

Figure 4. Schematic representation of the cell wall components from *Corynebacterineae*.

1.4 The genus *Corynebacterium*

In the end of the 19th century the genus *Corynebacterium*, consisting of Gram-positive, asporogeneous, pleomorphic bacteria, was first introduced to accommodate the type strain, *Corynebacterium diphteriae* (Lehmann & Neumann, 1896). The morphology of these microorganisms can fluctuate between rod and cocci forms depending on growth conditions, but generally it is club shaped and therefore its name derived from the Greek “coryne”. In the process of cell division, *Corynebacterium* adopt a “V” shaped structure which undergoes a characteristic snapping movement (probably due to their attachment by part of the cell wall layers) during fission of the two daughter cells.

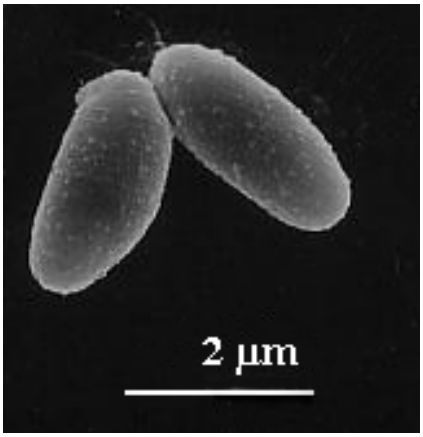

Figure 5. "V" shaped structure of *Corynebacterium efficiens*, taken and modified with courtesy of Ajinomoto CO., INC.

The genus was first described by Collins and Cummins (1986) as facultatively aerobic coryneform bacteria which contain meso-diaminopimelate, arabinose and galactose in the cell wall, a DNA base composition in the range of 51-63 % G+C content, and C_{22}-C_{36} corynomycolic acids. *Corynebacterium* can be differentiated from other coryneform taxa based on the following criteria:

- major peptidoglycan diamino acid
- peptidoglycan type
- N-glycolyl glycan moiety of the cell wall
- mol % of G+C
- presence of mycolic acids, fatty acid type
- major menaquinone isoprenologue(s)
- phosphatidylinositol and phosphatidylinositol mannoside(s).

Most of the members of *Corynebacterium* genus require one or more vitamins, amino acids, purines and pyrimidines. The range of temperature for the growth of most corynebacteria is between 30-37 °C with the exception of *C. efficiens* (Fudou *et al.*, 2002) which can grow at 45 °C. This feature is beneficial from an economic point of view, since fermentation using this bacterium reduce the need for a cooling system in industrial fermenters, decreasing the cost of production for L-glutamic acid. *C. glutamicum* is together with *C. callunae* and *C. efficiens* a potent producer of glutamate, lysine and other amino acids on industrial scale (see below).
In consequence of the application of modern taxonomic methods, it has been seen that species previously classified in other genera, like *Arthrobacter*, *Brevibacterium*, and *Microbacterium* are true *Corynebacterineae*. These include the glutamic acid producing bacteria *B.*

ammoniagenes, B. divaricatum, B. flavum and *B. lactofermentatum* all of them have been suggested to be synonyms for *C. glutamicum* (Goodfellow & Cross, 1983).

The cell wall organisation and structures have been described in the general properties of the Actinomycetes (see 1.3). Macromolecular components of the cell wall in *Corynebacterineae* appear to be arabinogalactan, peptidoglycan, lipomannan, and lipoarabinomannan. Their structure is only partially known because after extraction with organic solvents, the cell wall skeleton of *C. glutamicum* remains consisting of covalently linked arabinogalactan and peptidoglycan (Keddie *et al.*, 1977).

The two major mycolic acid types present in *C. glutamicum* are 32:0 3OH and 34:1 3OH (Collins *et al.*, 1981). This nomenclature gives the total number of carbon atoms with the degree of saturation and the fixed localization of the hydroxyl group in these special fatty acids. Mycolic acids are branched fatty acids with the general structure R1-CH(OH)-CH(R2)-COOH, with both, R1 and R2, representing alkyl chains. It is not known which of the structures of the 34:1 3OH mycolic acid is present or where the double bond is localized. There are traces of further species, but mycolic acids in *C. glutamicum* have a comparatively simple structure. Their total carbon number is in the range of 22-38 (see Table 1).

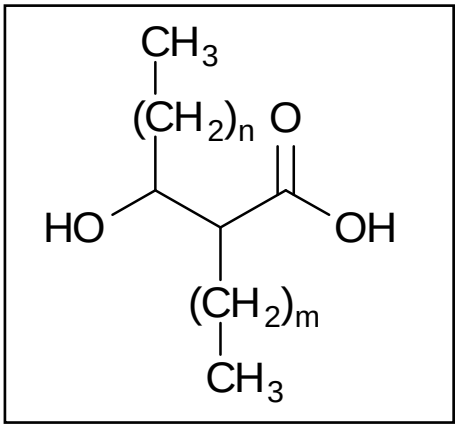

Figure 6. The general mycolic-acid structure.

In an electron transmission micrograph from *C. glutamicum*, the cell wall has a thickness of about 32 nm (Eggeling & Sahm, 2001; Puech *et al.*, 2001; Marienfeld *et al.*, 1997). The peptidoglycan layer gives values in between 15-18 nm depending on the way of preparing the sample, freeze-substitution or conventional fixation (Marienfeld *et al.*, 1997). Plasma membrane and peptidoglycan are followed by an electron-translucent region of 8.5 nm corresponding to a nonstainable outer layer, which could correspond to the mycolic acids esterified with the arabinogalactan together with non-covalently bound mycolic acid derivates.

Two findings indicate that the soluble mycolic acids also belong to this layer. First, the mycolic acids of *C. glutamicum* are only about 2 nm in length, second, in *C. glutamicum* only 1/10 of the covalently bound mycolic acids of *M. tuberculosis* are present (Puech *et al*., 2001). Together with the electron microscopy picture (see Figure 7), this leads to the assumption that large quantities of soluble lipids, such as trehalose dimycolate and trehalose monomycolate, are also localized in the outer lipid layer. Figure 7 reveals an ultrastructural cell wall envelope appearance similar to that found in mycobacteria (Daffé & Draper, 1998; Draper, 1982; Paul & Beveridge, 1992; Rastogi *et al.,* 1986). However, in contrast to mycobacteria, where a space is observed between the plasma membrane and the electron-dense layer corresponding to a hypothetical periplasmic space (Daffé & Draper, 1998), the plasma membrane is tightly associated to the electron-dense layer in thin sections of corynebacteria (Puech *et al.,* 2001), corresponding to the previous report from Marienfeld *et al.* (1997).

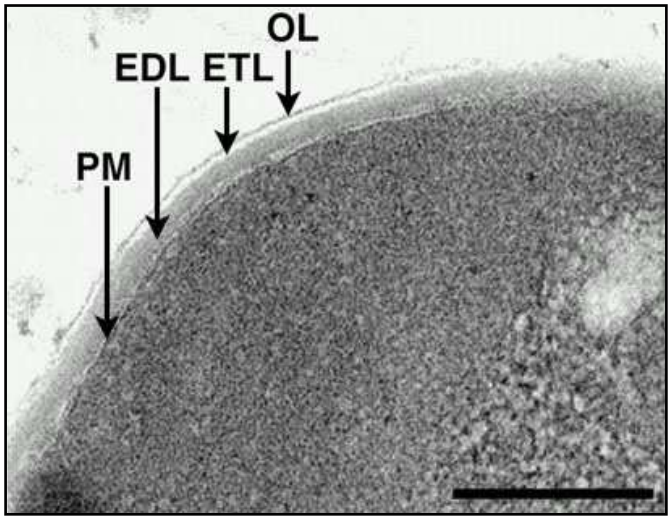

Figure 7. Electron microscopy picture from an ultrathin section of *C. glutamicum* prepared by conventional embedding. OL, outer layer; ETL, electron-transparent layer; EDL, electron-dense layer; PM, plasma membrane. Bar, 200nm. Taken and modified from Puech *et al*., 2001.

When grown on solid media, this region is followed by an outer layer, called surface layer (S-layer) containing PS2, an S-layer protein (Chami *et al.,* 1997). The S-layer of many bacteria consists of a single (glyco)protein species that is assembled into a two-dimensional crystalline array enveloping the cell (Sleytr *et al.,* 1996). The gene encoding the PS2 protein (*cspB*) has been characterized in *C. glutamicum* (Peyret *et al.,* 1993). Furthermore, the formation of the surface layer proteins is dependent on culture conditions (Chami *et al.,* 1995) and carbon sources (Soual-Hoebeke *et al*., 1999). Chami *et al*. (1995) showed that if *C. glutamicum* was grown on solid medium (34 mg PS2 protein/ (g bacterial dry weight)), the surface of cells was totally covered with a highly ordered, hexagonal surface layer, whereas if it was grown in

liquid medium (16 mg protein/ (g bacterial dry weight)) the cell and fracture surfaces were only partially covered by ordered arrays. This partial covering was correlated with less PS2 associated with the cell wall. This suggests that PS2 production depends on the physiological and metabolic state of the cell.

1.5 Porins in *Corynebacterineae*

Corynebacterineae feature a particular low permeable cell envelope. For *Mycobacterium chelonae* it has been demonstrated that the permeability of hydrophilic solutes is slightly lower than that of *Pseudomonas aeruginosa* and *E. coli* (Jarlier & Nikaido, 1990; Trias & Benz, 1994). As a result mycobacterial species are only susceptible to a few antibiotics.

The transport of ions, small solutes and macromolecules is an essential function of the bacterial cell wall. In the plasma membrane of *C. glutamicum*, which is the most important glutamate producer, there are several amino acid transporters (Eggeling & Sahm, 2001). Beyond the plasma membrane, the amino acids have to cross the hydrophobic barrier and they may cross the cell envelope through the hydrophilic pathway, formed by porins. In several bacteria belonging to the *Corynebacterineae*, porins were identified (Trias *et al*., 1992; Lichtinger *et al*., 1998; Niederweis *et al*., 1999, Riess *et. al*., 1998). Recently found porins from *C. glutamicum* are $PorA_{C.glut}$ (Lichtinger *et al*., 1998) and $PorB_{C.glut}$ (Costa-Riu *et al.*, 2003b). $PorA_{C.glut}$ is formed by a an oligomer of a small 45 amino acid polypeptide, the channel is cation-selective with a conductance of about 5.5 nS in 1 M KCl. $PorB_{C.glut}$ is a 99 amino acid long protein and forms anion-selective channels with a single-channel conductance of about 700 pS in 1 M KCl. The channel can be blocked by citrate, probably because of positively charged groups in or near the channel mouth that cause the anion selectivity.

MspA, found in *Mycobacterium smegmatis* (Niederweis *et al.*, 1999), is the best characterised and analysed outer membrane protein of *Corynebacterineae*. It is thought that porins influence the drug efficiency, because the three important drugs isoniazide, ethambutol, and pyrazinamide can easily permeate through the MspA channel (Lambert, 2002). A general cylindrical shape of MspA was established by electron microscopy (Engelhardt *et al.*, 2002). The atomic structure of this protein shows a β-structure (Faller *et al.*, 2004) that differs completely from its counterparts in Gram-negative bacteria (Weiss *et al*., 1990; Schulz,

2002). The porin represents a tightly interconnected octamer with eightfold rotation symmetry. It is described as a goblet consisting of a thick rim at the top, a stem consisting of the wide β-barrel, and a base at the bottom.

There is no significant sequence similarity between MspA and other known porins from the suborder *Corynebacterineae*.

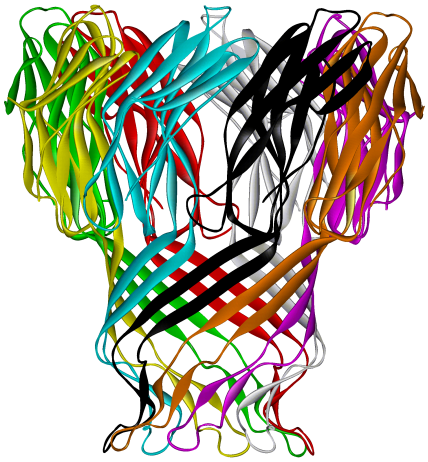

Figure 8. Structure of the octameric porin MspA from *M. smegmatis*. Each colour corresponds to one subunit. (created by WebLab viewer)

1.6 Amino acid production

C. glutamicum and *C. callunae* are used for industrial production of L-glutamate, L-lysine and other amino acids through fermentation processes (Udaka, 1960; Gutmann *et al.*, 1992, Keilhauer *et al.*, 1993, Sahm *et al.*, 1996, Eggeling & Sahm, 1999). In the year 2002 about 1×10^6 tons of L-glutamate and 5.5×10^5 tons of L-lysine were produced with *C. glutamicum* and used as a flavoring agent in food and animal feed supplements (Leuchtenberger, 1996; Hermann, 2003).

In addition, amino acids are important as seasonings and raw material for the synthesis of cosmetics, toothpaste, shampoo and detergents (Martin *et al.*, 1987). The development of the amino acid industry began 1908 in Japan. Kikuanae Ikeda isolated L-glutamic acid from konbu, a kelp-like seaweed used for seasoning. Based on this finding, Ajinomoto Co. initiated the industrial production of monosodium L-glutamate by the hydrolysis of wheat gluten or soybean protein (Hirose *et al.*, 1979). In the 1950's, two groups independently isolated

microbial strains which accumulated considerable quantities of L-glutamic acid when cultured in synthetic media containing glucose and ammonia (Kinoshita *et al.,* 1957). First *Micrococcus glutamicus* (later renamed *C. glutamicum*) was successfully used for the direct fermentative production of L-glutamic acid on industrial scale by Kyowa Hakko Kogyo Co. Ldt.

Two independent factors are required for the over-production and excretion of L-glutamate into the medium. The first is that *C. glutamicum* features a very little α-ketoglutarate dehydrogenase activity, although glutamate dehydrogenase activity is abundant (Kinoshita & Tanaka, 1972). Thus the tricarboxylic intermediate, α-ketoglutarate, is predominantly channelled to glutamic acid. Secondly, growth of a biotin requiring *C. glutamicum* strain in growth-limiting biotin results in a permeability change that allows glutamate excretion into the medium. Subsequent experiments demonstrated that the glutamate excretion could be induced by a number of treatments that all affect the phospholipid content of the membrane (Takinami *et al.,* 1965; Demain & Birnbaum, 1968; Nunheimer *et al.,* 1970). Although models suggesting a general increase in membrane permeability have been proposed, the increased efflux is glutamate specific and evidence for the specific efflux carrier of glutamate has been reported (Hoischen & Kramer 1990). Under optimal conditions this organism converts glucose into high yields of L-glutamic acid within a few days. Currently about 1×10^6 tons of this amino acid are produced annually as flavoring agent (Leuchtenberger, 1996). In the past 40 years various mutants of *C. glutamicum* have been isolated which are also able to produce significant high amounts of other L-amino acids. For example, nowadays L-lysine, mainly used as a feed additive, is produced with mutants deregulated in the biosynthetic pathway on a scale of 5.5×10^5 tons/year (Sahm *et al.,* 2000). While the breeding of overproducing strains by classical mutagenesis has been quite successful (Rowlands, 1984), the introduction of recombinant DNA technology to *C. glutamicum* has opened new ways to the genetic manipulation of these bacteria (Katsumata *et al.,* 1984; Santamaria *et al.,* 1984; Yoshihama *et al.,* 1985; Sahm *et al.,* 1995).

The improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the application of recombinant DNA technology is called metabolic engineering (Bailey, 1991). Introduction of genes into microorganisms via recombinant DNA techniques is a most powerful method for the construction of strains with the desired genotypes.

In Figure 9, the metabolism of amino acid production is schematized in order to have a global view of the pathways that are used and the point where glucose metabolism from *C. callunae*, *C. efficiens* and *C. glutamicum* is channelled to L-glutamate and the other amino acids.

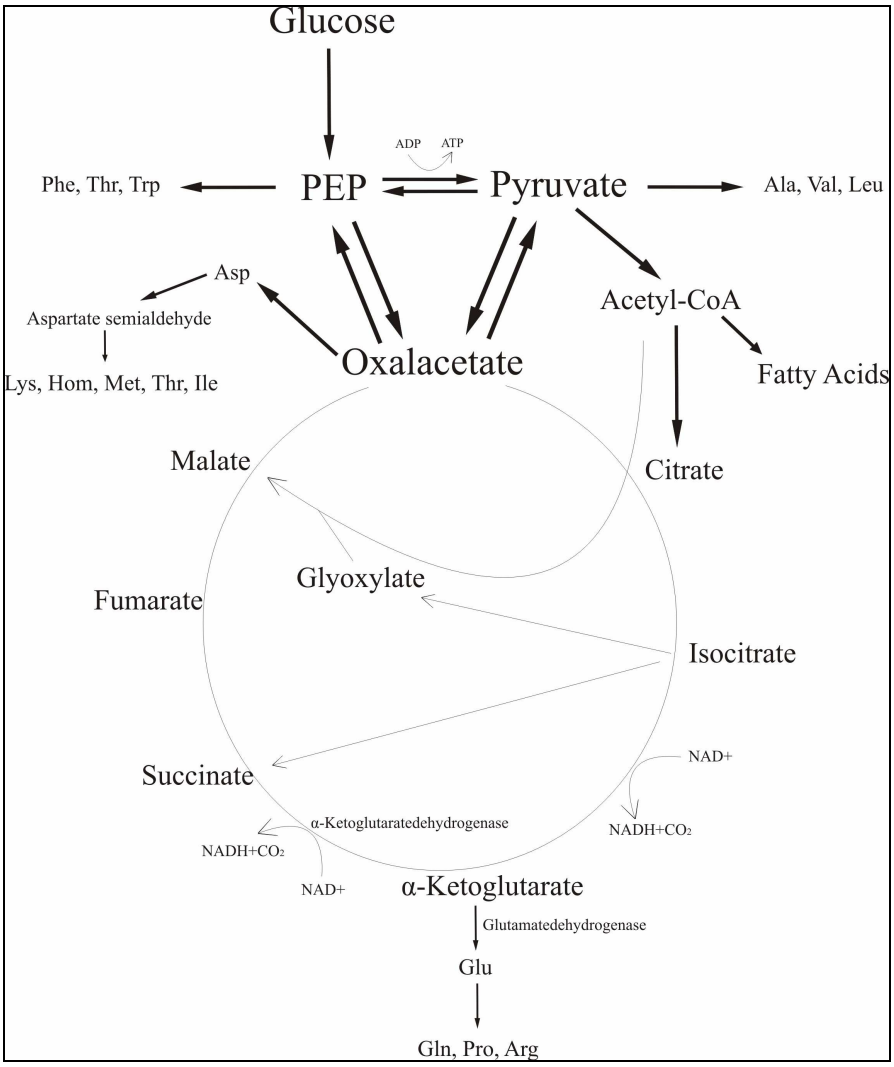

Figure 9. Simplified view of the Tricarboxylic Acid Cycle and the production of amino acids. Lysine and several other amino acids are produced from aspartate, whereas glutamate, proline and arginine are derived from α-ketoglutarate.

Beside producing amino acids, members of *Corynebacterium* are furthermore utilized in other biotechnological processes (Wohlleben *et al.*, 1993), such as:

- production of nucleotides (Ogata *et al*., 1976)
- production of antibiotics (Suzuki *et al*., 1972)
- production of surfactants (Zajic *et al.*, 1977)
- production of Vitamin C precursors (Anderson *et al*., 1985)

- cheese ripening (Lee *et al.*, 1985)
- bioconversion of steroids (Constantinides, 1980)
- terpenoid oxidation (Yamada *et al.*, 1985)
- degradation of carbohydrates (Cardini & Jurtshuk, 1970).

CHAPTER 2

PorH, a new channel-forming protein present in the cell wall of *Corynebacterium efficiens* and *Corynebacterium callunae*

2.1 Summary

Corynebacterium callunae and *Corynebacterium efficiens* are close relatives of the glutamate-producing mycolata *Corynebacterium glutamicum*. Organic solvent extracts of whole bacterial cells were used to study the permeability properties of the cell wall of the two bacteria. The cell extracts contained channel-forming proteins that formed ion-permeable channels with a single-channel conductance of about 2 to 3 nS in 1 M KCl in the lipid bilayer assay. The corresponding proteins from both corynebacteria were purified to homogeneity and were named $PorH_{C.call}$ and $PorH_{C.eff}$. Electrophysiological studies of the channels suggested that they are wide and water-filled. Channels formed by $PorH_{C.call}$ are cation-selective, whereas $PorH_{C.eff}$ forms slightly anion selective channels. Both proteins were partially sequenced. Multiple sequence alignment search within the known chromosome of *C. efficiens* demonstrated, that it contained a gene that fitted to the partial amino acid sequence of $PorH_{C.eff}$. The chromosome of *C. callunae* is not sequenced, but $PorH_{C.call}$ shows a high homology to $PorH_{C.eff}$. $PorH_{C.eff}$ is coded in the bacterial chromosome by a gene that is localized within the vicinity of the $porA_{C.eff}$ gene of *C. efficiens*. $PorH_{C.eff}$ has no signal sequence at the N-terminus, which means that it is not exported by the Sec-secretion pathway. The structure of PorH in the cell wall of the Corynebacteria is discussed.

2.2 Introduction

In 2002, three glutamic-acid-producing coryneform strains, belonging to the genus *Corynebacterium*, were isolated from soil and vegetable samples. Phylogenetic studies, based on 16S rRNA analysis, demonstrated that the nearest relatives of these strains were *Corynebacterium glutamicum* and *Corynebacterium callunae*, which are known as glutamic-acid-producing species. The most significant characteristics of the newly found strains termed *Corynebacterium efficiens* sp. nov. was the production of acid from dextrin (Fudou *et al.*, 2002). *C. glutamicum* is used for industrial production of L-glutamate, L-lysine and other amino acids through fermentation processes (Udaka, 1960; Gutmann *et al*., 1992; Keilhauer *et al*., 1993; Sahm *et al.*, 1996; Eggeling & Sahm, 1999). The range of temperature for the growth of most corynebacteria is between 30-37 °C with the exception of *C. efficiens* (Fudou *et al.*, 2002) that can grow up to 45 °C. This feature is beneficial from an economic point of view; the need for a cooling system in industrial fermenters is reduced using this bacterium, which means that the costs for the production of L-glutamic acid could be reduced.

C. efficiens, *C. callunae* and *C. glutamicum* share similar cell wall composition. The cell wall contains in addition to the thick peptidoglycan layer a second layer consisting of covalently bound mycolic acids and extractable lipids (Barksdale, 1981; Goodfellow *et al.*, 1976; Minnikin, 1987; Ochi, 1995). The mycolic acids are 2-branched, 3-hydroxylated fatty acids, variable in chain length. The cell wall of Corynebacteria contains mycolic acids with a chain length of about 22-38 carbon atoms, whereas other members of the mycolata contain much longer mycolic acids (Minnikin, 1987; Yano & Saito, 1972; Minnikin *et al.*, 1974; Daffé *et al.*, 1990; Holt *et al.*, 1994; Brennan & Nikaido, 1995). Similar to the outer membrane of Gram-negative bacteria, the mycolic acid layer represents a permeability barrier (Liu *et al*., 1995; 1996; Nikaido *et al*., 1993). To overcome this barrier, channel forming proteins, so-called porins, are necessary to allow the passage of hydrophilic solutes. With respect to transport of amino acids over this barrier it is of particular importance to characterize the hydrophilic pathways in the cell wall of Corynebacteria.

The first porin identified in the cell wall of a member of the mycolata was a 59 kDa cell wall protein with an average single-channel conductance of 2.7 nS in 1 M potassium chloride from *Mycobacterium chelonae* (Trias *et al.*, 1992; Trias & Benz, 1993). For this bacterium it has

been demonstrated that the permeability of the cell wall for hydrophilic solutes is slightly lower than that of *Pseudomonas aeruginosa* and much lower than that of *Escherichia coli* (Jarlier & Nikaido, 1990; Trias & Benz, 1993). This could explain why members of the mycolata have a low susceptibility towards certain antibiotics. Since the discovery of the first cell wall channel, several porins have been identified and characterized in members of the mycolata (Riess *et al.,* 1998; Lichtinger *et al.,* 1998; 1999; 2000; 2001; Costa-Riu *et al*., 2003b). Common to most of them is the formation of wide and water-filled pores that are cation-selective caused by negative point charges (Trias & Benz, 1993; 1994; Riess *et al.,* 1998; Lichtinger *et al.,* 1998; 1999; 2001).

PorA from *C. glutamicum* was the first pore-forming protein of Corynebacteria that was characterized. The channel is cation-selective with a single-channel conductance of about 5.5 nS in 1 M KCl and it is formed by an oligomer of a small 45 amino acid long polypeptide that is coded without leader sequence (Lichtinger *et al.,* 1998; 2001). Another porin, named PorB, comprising 99 amino acids was found after deleting the *porA* gene from the *C. glutamicum* chromosome (Costa-Riu *et al.,* 2003a; 2003b). PorB forms in lipid-bilayer experiments anion-selective channels with a single-channel conductance of about 700 pS in 1 M KCl. To extend the knowledge on channel-forming proteins of Corynebacteria we screened in this study the cell walls of two closely related coryneform strains *C. callunae* and *C. efficiens* for channel-forming proteins. Two homologous cell wall channel proteins, $PorH_{C.call}$ and $PorH_{C.eff}$ were identified in both species. The former is highly cation-selective and $PorH_{C.eff}$ is slightly anion-selective. Both are voltage-dependent and their single-channel conductance is similar in 1 M potassium chloride. The channel-forming proteins were purified to homogeneity and their biophysical properties were studied in detail. The proteins were partially sequenced. Sequence alignments search within the known chromosome of *C. efficiens* demonstrated that it contained a gene that fitted to the partial amino acid sequence of $PorH_{C.eff}$.

2.3 Materials and methods

2.3.1 Bacterial strains and growth conditions

C. efficiens AJ 12310 (obtained from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen) was routinely grown in BHI-medium (Brain Heart Infusion, Difco Laboratories), *C. callunae* ATCC 15991 (obtained from DSMZ) in double yeast tryptone (2xYT, BIO 101 Inc.) medium, both at 30 °C.

2.3.2 Isolation and purification of the channel-forming proteins

For the isolation of the channel-forming proteins from *C. efficiens* AJ 12310 and *C. callunae* ATCC 15991 a method was used that has been previously described for the isolation and purification of $PorA_{C.glut}$ of *C. glutamicum* (Lichtinger *et al.*, 1999). It is based on the extraction of whole cells with organic solvents and avoids the substantial loss of material caused by sucrose density centrifugation of the cell envelope to separate the cytoplasmic membrane from the cell wall fraction. For the extraction procedure 200 ml cells were grown to an OD of 10 and harvested by centrifugation (10 000 rpm for 10 min in Beckman J2-21M/E centrifuge). The cells were washed twice in 10 mM Tris-HCl (pH 8). The washed and centrifuged cells were extracted two times with organic solvent, a 1:2 mixture of chloroform/methanol in a proportion of 1 part cells and 5 to 8 parts chloroform/methanol. The duration of the extraction was about 3 hours at room temperature under stirring in a closed tube to avoid the loss of chloroform. Cells and chloroform/methanol solution were centrifuged for 15 minutes (10 000 rpm in Beckman J2-21M/E centrifuge). The pellet (cells) was discarded. The supernatant contained the channel-forming activity. It was mixed in a ratio of 1 part supernatant to 9 parts ether and kept over night at -20 °C. The precipitated protein was dissolved in a solution containing 0.4 % LDAO (*NN*-dimethyldodecylamine *N*-oxide) and 10 mM Tris-HCl (pH 8) and inspected for channel-forming activity. The protein was subjected to fast protein liquid chromatography (FPLC) across a HiTrap-Q column (Amersham Pharmacia Biotech, Freiburg, Germany). The column was washed first with a

buffer containing 0.4 % LDAO and 10 mM Tris-HCl (pH 8), then the protein was eluted with 0.4 % LDAO in 10 mM Tris-HCl (pH 8) using a linear gradient between 0 and 1 M NaCl.

2.3.3 SDS-PAGE

SDS-PAGE was performed with tricine containing gels (Schägger & von Jagow, 1987). The gels were stained with colloidal Coomassie brilliant blue (Neuhoff *et al*., 1988) or silver (Blum *et al*., 1987). Before separation, the samples were all incubated for 5 minutes at 100 °C with loading buffer (exempt preparative SDS-PAGE). Preparative SDS-PAGE was used for identification and purification of the channel-forming activity from the organic solvent extracts of whole *C. efficiens* cells.

2.3.4 Peptide sequencing

The precipitated protein pellet resulting from the active FPLC fractions or preparative SDS-PAGE was dissolved in 100 µl 70 % (by vol.) formic acid containing 10 % (mass/vol.) CNBr (Merck), and incubated in the dark at room temperature for 14 hours (Gross, 1967). After lyophilisation the CNBr peptides were dissolved in 20 % (by vol.) formic acid and separated by Reversed-phase-HPLC (SYCAM, Fürstenfeldbruck, Germany) applying a Luna C-18 column, 150 mm x 1 mm with a flow rate of 40 µl min^{-1} using a 120 min gradient from 100 % A (0.1 % trifluoroacetic acid/water (v/v)) to 80 % B (0.1 % trifluoroacetic acid in Acetonitril). Collected fractions were subjected to amino acid sequence analysis on a 492 protein sequencer (Applied Biosystems, Darmstadt, Germany) using the conditions recommended by the manufacturer. The major sequences for the precipitated protein pellet of *C. callunae* were DLSLLADNLDDYSTFGKNIGTAL and IPDLLKGIIAFFENFGDLAETT, the main sequence for the *C. efficiens* protein was DLSLLKDSLSDFATLGKN.

2.3.5 Lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously (Benz *et al*., 1978; Benz, 2003). The experimental set up consisted of a Teflon cell with two water-

filled compartments connected by a small circular hole. The hole had an area of about 0.4 mm^2. Membranes were formed across the hole using a 1 % solution of diphytanoyl phosphatidylcholine (PC; Avanti Polar Lipids, Alabaster, Ala.) dissolved in *n*-decane. The temperature was maintained at 20 °C during all experiments. All salts were obtained from Merck (Darmstadt, Germany, analytical grade). They were used unbuffered. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a home-made current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder.

The zero-current membrane potentials were measured as previously described (Benz *et al*., 1979). The membranes were formed in a 100 mM KCl solution containing a predetermined protein concentration so that the membrane conductance increased about 100- to 1,000-fold within 10 to 20 minutes after membrane formation. At this time the instrumentation was switched to the measurements of the zero-current potentials and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value between 2 to 5 minutes.

2.3.6 Effect of negatively charged groups attached to the channel mouth

Negative charges at the pore mouth result in substantial ionic strength-dependent surface potentials at the pore mouth that attract cations and repel anions. Accordingly, they influence both, single-channel conductance and zero-current membrane potential. A quantitative description of the effect of point charges on the single-channel conductance may be given by the following considerations. The first one is based on the Debeye-Hückel-theory describing the effect of point charges in an aqueous environment. The second treatment was proposed by Nelson & McQuarrie (1975) and describes the effect of point charges on the surface of a membrane and does not consider charges attached to a channel. However, this does not represent a serious restriction of its use and we assume here that the point charges are localized at the PorH channel. In case of a negative point charge, q, in an aqueous environment a potential Φ is created that is dependent on the distance, r, from the point charge:

$$\Phi = \frac{q \cdot e^{-\frac{r}{l_D}}}{4\pi \cdot \varepsilon_0 \cdot \varepsilon \cdot r} \qquad (1)$$

ε_0 (= 8.85 x 10^{-12} F m^{-1}) and ε (= 80) are the absolute dielectric constant of vacuum and the relative constant of water, respectively, and l_D is the so called Debeye length that controls the decay of the positive potential (and that of the accumulated positively charged ions) in the aqueous phase:

$$l_D^2 = \frac{\varepsilon \cdot \varepsilon_0 \cdot R \cdot T}{2F^2 \cdot c} \qquad (2)$$

c is the bulk aqueous salt concentration, and *R*, *T* and *F* ($RT\,F^{-1}$ = 25.2 mV at 20 °C) have the usual meaning. The potential Φ created by a negative point charge on the surface of a membrane is twice that of eqn. (1) caused by the generation of an image force on the opposite side of the membrane (Nelson & McQuarrie, 1975; Benz *et al.*, 1994). The concentration of the monovalent cations near the point charge increases because of the negative potential. Their concentration is in both cases (Debeye-Hückel or Nelson-McQuarie) dependent on the potential Φ and given by:

$$c_0^+ = c \cdot e^{\frac{-\phi \cdot F}{R \cdot T}} \qquad (3)$$

Similarly, the anion concentration c_0^-, near the point charge decreases according to:

$$c_0^- = c \cdot e^{\frac{\phi \cdot F}{R \cdot T}} \qquad (4)$$

In the following we assume that the negative point charge is attached to the channel. In such a case its conductance is limited by the accumulated positively charged ions and not by their bulk aqueous concentration. The cation concentration c_0^+ at the mouth of the pore can now be used for the calculation of the effective conductance-concentration curve:

$$G(c) = G_0 \cdot c_0^+ \qquad (5)$$

G_0 is the concentration independent conductance of the channel.

2.4 Results

2.4.1 Purification of PorH$_{C.call}$ and PorH$_{C.eff}$

Proteins within the organic solvent extract of whole *C. callunae* and *C. efficiens* cells were precipitated with ether in the cold. The precipitate was dissolved in the detergent LDAO and inspected for channel-forming activity using the lipid bilayer assay. The detergent solution had a high channel-forming activity and channels with a conductance between 2 and 6 nS in 1 M KCl were formed under these conditions. Purification of the channel-forming proteins from *C. callunae* and *C. efficiens* was performed by FPLC across a HiTrap Q column. Figure 1, lane 2 shows the protein composition of the organic solvent extract of *C. callunae* that was applied to the column.

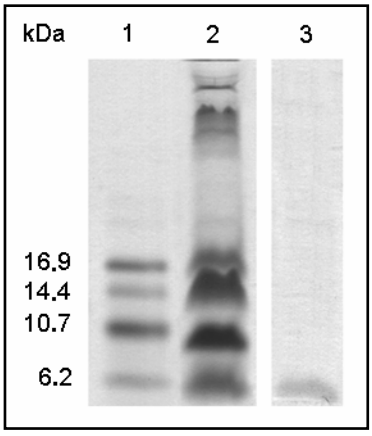

Figure 1. 10 % Tricine-SDS-PAGE according to (Schägger & von Jagow, 1987) of the purification procedure of PorH of *C. callunae*. The gel was stained with colloidal Coomassie.
Lane 1: Low Molecular mass marker 16.9 kDa, 14.4 kDa, 10.2 kDa and 6.2 kDa.
Lane 2: 15 µl of ether precipitated extract dissolved in 0.4 % LDAO, treated for 10 min at 100 °C with 5 µl sample buffer.
Lane 3: 15 µl of the fraction 17 of the Hitrap-Q FPLC column, treated for 10 min at 100 °C with 5 µl sample buffer.

The column was first washed with buffer and then eluted with buffer supplemented with increasing concentration of NaCl. Pure 6 kDa protein was eluted at a NaCl-concentration of 0.23 M. The 6 kDa protein of *C. efficiens* was not completely pure after FPLC across a

HiTrap Q column. Final purification was achieved by preparative SDS-PAGE of the precipitated organic solvent extract that contained the channel-forming activity (see Figure 2).

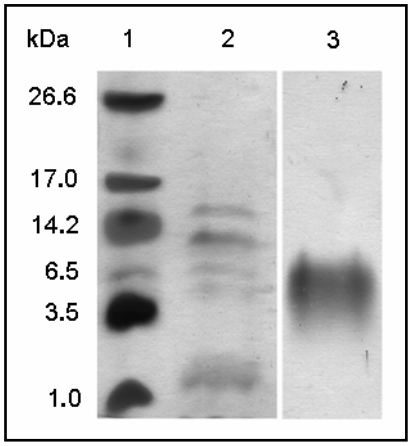

Figure 2. 12 % Tricine-SDS-PAGE according to (Schägger & von Jagow, 1987) of PorH of *C. efficiens* obtained by elution of the 6 kDa band from preparative SDS-PAGE. The gel was stained with silver (Blum *et al*., 1987).
Lane 1: Low Molecular mass marker: 26.6 kDa, 17.0 kDa, 14.2 kDa, 6.5 kDa, 3.5 kDa and 1.0 kDa.
Lane 2: 15 µl of ether precipitated extract dissolved in 0.4 % LDAO, treated for 10 min at 100 °C with 5 µl sample buffer.
Lane 3: 3 µg of the pure 6 kDa protein was solubilized for 10 min at 100 °C with 5 µl sample buffer.

2.4.2 Single-channel analysis of PorH$_{C.call}$ and PorH$_{C.eff}$

Figure 3A shows a single-channel recording of a diphytanoyl phosphatidylcholine membrane in the presence of the pure 6 kDa protein of *C. callunae*, which was added to a black membrane in a concentration of about 10 ng ml^{-1}. The single-channel recording demonstrates that PorH$_{C.call}$ formed defined channels. The single-channel conductance of most channels was about 3 nS in 1 M KCl.

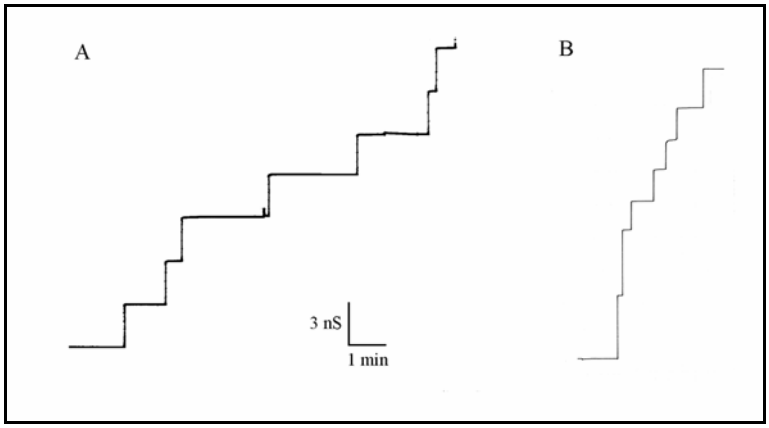

Figure 3. Single-channel recording of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of the pure 6 kDa proteins from (A) *C. callunae*, and (B) *C. efficiens*. The aqueous phase contained 1 M KCl (pH 6) and 10 ng ml^{-1} cell wall protein. The applied membrane potential was 20 mV; T = 20 °C.

Only a minor fraction of channels with other conductance was observed (see Figure 4A). It is noteworthy that the channels formed by $PorH_{C.call}$ of *C. callunae* had a long lifetime similar to those that have been detected previously for porins of gram-negative (Benz, 1994) and gram-positive bacteria (Lichtinger *et al.*, 1999). All these porins form channels in lipid bilayer membranes with long lifetimes at small transmembrane potential (mean lifetime at least 5 minutes). However, voltage-dependence closure was observed for $PorH_{C.call}$ of *C. callunae* for voltages higher than about 30 to 40 mV (see below). Channels fromed by $PorH_{C.eff}$ had a very similar single-channel conductance and lifetime compared to those formed by $PorH_{C.call}$ as the single-channel recording of Figure 3B clearly indicates. The only exception was the occurrence of two maxima in the histogram of the single-channel distribution (see Figure 4B). These two maxima (2.3 and 4.7 nS in 1 M KCl) reflect most probably the reconstitution of two channels at once, because the conductance of the right side maximum was always twice of that of the left side one.

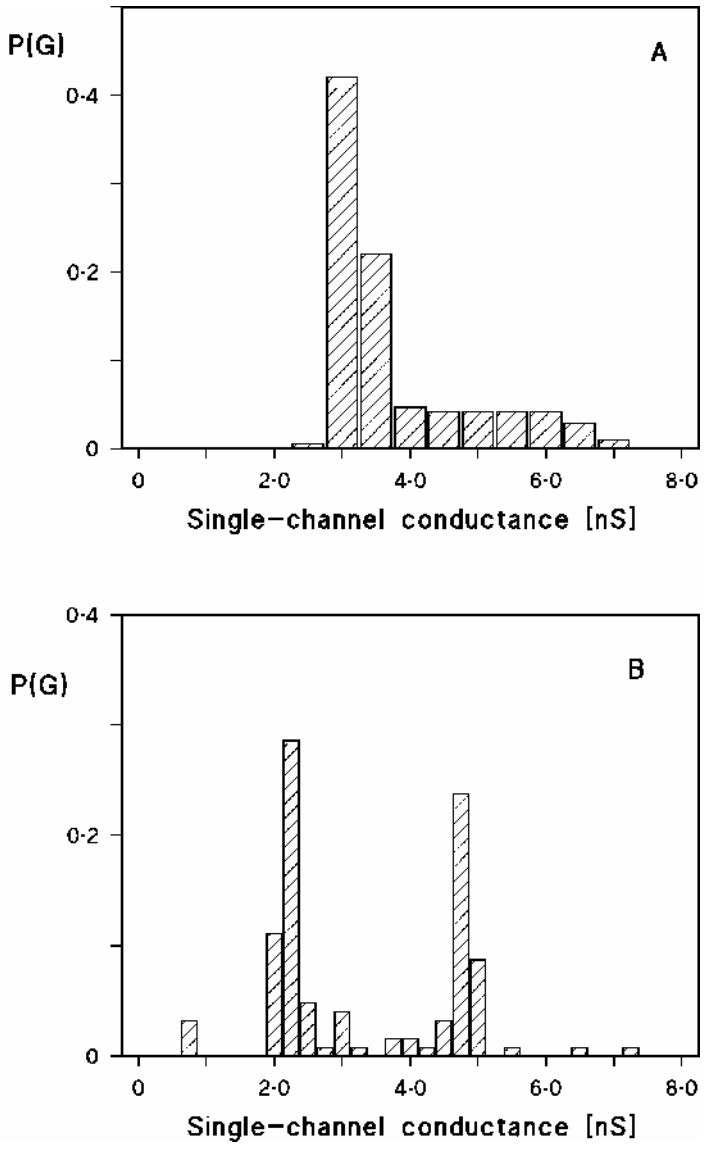

Figure 4. Histogram of the probability P(G) for the occurrence of a given conductivity unit observed with membranes formed of diphytanoyl phosphatidylcholine/n-decane in the presence of the pure cell wall proteins of (A) *C. callunae*, and (B) *C. efficiens*. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl and 10 ng ml^{-1} cell wall proteins. The applied membrane potential was 20 mV; T = 20 °C. The average single-channel conductances was 3.0 nS for 139 single-channel events of $PorH_{C.call}$ and 2.3 nS for 126 single-channel events (left-hand side maximum in Figure 4a) of $PorH_{C.eff}$ (steps similar as those of Figure 3).

Single-channel experiments were also performed with salts other than KCl to obtain some information on the size of the channels formed by $PorH_{C.call}$ and $PorH_{C.eff}$ and their ion selectivity. The results are summarized in Table 1. The conductance sequence of the different salts within the channel formed by $PorH_{C.call}$ was RbCl ≈ KCl > K acetate > NaCl > LiCl > $N(CH_3)_4Cl$ > $N(C_2H_5)_4Cl$, which means that the single-channel conductance followed approximately the aqueous mobility of the different cations in the aqueous phase. This means presumably that the influence of cations on the conductance of the channel in different salt solutions was more substantial than that of anions (see Table 1) suggesting a cation selectivity of the channel. Table 1 shows also the average single-channel conductance, G of $PorH_{C.call}$ as a function of the KCl concentration in the aqueous phase. Similarly, as in the case of many porin channels of gram-positive bacteria (Trias & Benz, 1993; 1994; Riess *et al*., 1998; Lichtinger *et al*., 1999) the conductance was not a linear function of the KCl-concentration, which is characteristic for the presence of point net charges in or near the channel (Trias & Benz, 1994; Lichtinger *et al*., 1999).

The single-channel conductance of the channels formed by $PorH_{C.eff}$ was approximately the same as that measured for $PorH_{C.call}$. However, it seems that the ion selectivity of $PorH_{C.eff}$ was somewhat different because the conductance of the channels in LiCl was higher than that in K-acetate (see Table 1). Furthermore, the conductance of salts containing tetraalkylammonium ions was more or less independent from the size of the cation. These results suggested that $PorH_{C.eff}$ could form anion selective channels.

Salt	**Concentration** [M]	**PorH$_{C.call}$ Single-channel conductance** G [nS]	**PorH$_{C.eff}$ Single-channel conductance** G [nS]
LiCl	1.0	1.25	1.50
NaCl	1.0	1.75	n.m.
KCl	0.01	n.m.	0.025
	0.03	0.35	0.075
	0.1	0.55	0.45
	0.3	1.10	0.70
	1.0	3.0	2.3
	3.0	7.0	6.5
RbCl	1.0	3.0	n.m.
$N(CH_3)_4Cl$	1.0	1.0	1.8
$N(C_2H_5)_4Cl$	1.0	0.70	1.7
KCH_3COO (pH 7)	1.0	2.0	1.0

Table 1. Average single-channel conductance, G, of PorH$_{C.call}$ and PorH$_{C.eff}$ in different salt solutions[a].

[a]The membranes were formed of diphytanoyl phosphatidylcholine dissolved in n-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The concentration of PorH$_{C.call}$ and PorH$_{C.eff}$ was about 10 ng ml^{-1}. Not that the average single-channel conductance of PorH$_{C.eff}$ corresponded always to the left side maximum of the histograms. The applied voltage was 20 mV, and the temperature was 20 °C. The average single-channel conductance, G, was calculated from at least 80 single events. n.m. means not measured.

Zero-current membrane potential measurements were performed in presence of KCl gradients to check such a possibility. Fivefold KCl gradients (100 mM versus 500 mM) were established across lipid bilayer membranes, which contained about 100 to 1000 PorH$_{C.eff}$ or PorH$_{C.call}$ channels. The measurements with PorH$_{C.eff}$ resulted in an asymmetry potential of about -6 mV at the more dilute side (average of 4 measurements). This result indicated some preferential movement of chloride over potassium ions through the PorH$_{C.eff}$ channel at neutral pH. Similar experiments with PorH$_{C.call}$ resulted in an asymmetry potential of about 28 mV at the more dilute side. The zero-current membrane potentials were analyzed using the Goldman-Hodgkin-Katz equation (Benz *et al*., 1979; 1985). The ratio of the potassium

permeability, P_K, divided by the chloride permeability, P_{Cl}, was about 0.7 and 7 for $PorH_{C.eff}$ and $PorH_{C.call}$, respectively, which indicated indeed a small anion selectivity for $PorH_{C.eff}$ and cation selectivity for $PorH_{C.call}$ (see also Discussion).

2.4.3 Voltage dependence

In single-channel recordings channels formed by $PorH_{C.call}$ and $PorH_{C.eff}$ exhibited some flickering at higher voltages, i.e. they showed rapid transitions between open and closed configurations. The voltage-dependent closure of the channels was studied in detail in multi-channel experiments (see Figure 5 for a multi-channel experiment with $PorH_{C.eff}$).

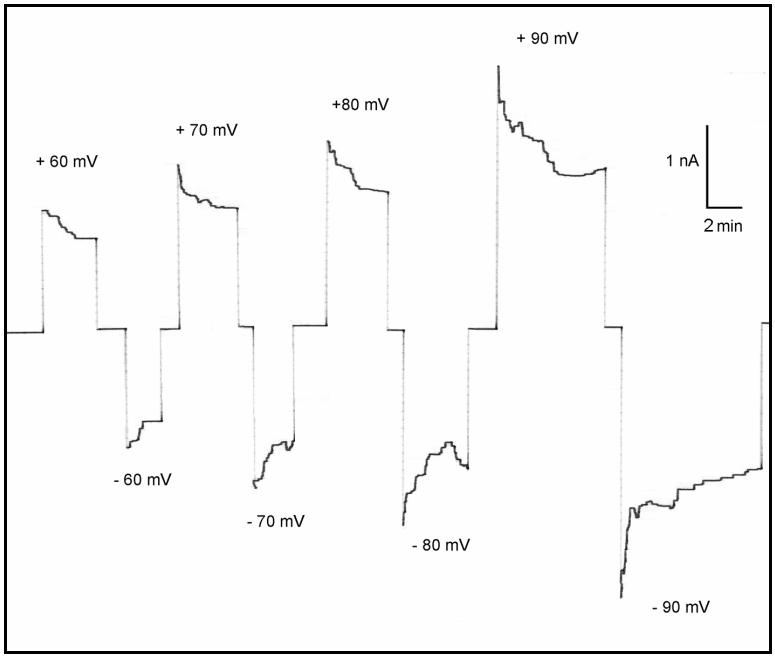

Figure 5. Study of the voltage dependence of $PorH_{C.eff}$ 500 ng ml^{-1} of the cell wall channel protein was added to the *cis*-side of a diphytanoyl phosphatidylcholine/n-decane membrane and the reconstitution of the channels was followed for about 30 min. Then increasing positive (60 to 90 mV; upper traces) and negative voltages (-60 to -90 mV; lower traces) were applied to the *cis*-side of the membrane, and the membrane current was measured as a function of time. The aqueous phase contained 1 M KCl; T = 20 °C.

The channel-forming protein was added in a concentration of 500 ng ml^{-1} to one side of a black diphytanoyl phosphatidylcholine/n-decane membrane (to the cis-side). After 30 min about 50 channels were reconstituted into the membrane. At that time different potentials were applied to the cis-side of the membrane: first 60 mV (upper trace of Figure 5) and then -

60 mV (lower trace of Figure 5). These experiments were repeated with 70, 80 and 90 mV. For both positive and negative potentials applied to the cis-side of the membrane the current decreased in an exponential fashion. This result indicated symmetric response of $PorH_{C.eff}$ to the voltage applied to the membranes. Similar experiments were performed with $PorH_{C.call}$, there a symmetrical response to the applied voltage was also observed (data not shown).

The experiment of Figure 5 and similar ones were analyzed in the following way: the membrane conductance (G) as a function of voltage, V_m, was measured when the closing of channels reached an equilibrium, i.e. after the exponential decay of the membrane current following the voltage step V_m. G was divided by the initial value of the conductance (G_o, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The data of Figure 6 (closed circles and squares, for $PorH_{C.eff}$ and $PorH_{C.call}$, respectively) correspond to the symmetric voltage-dependence of the two cell wall channels (mean of four membranes) when the proteins were exclusively added to the *cis*-side.

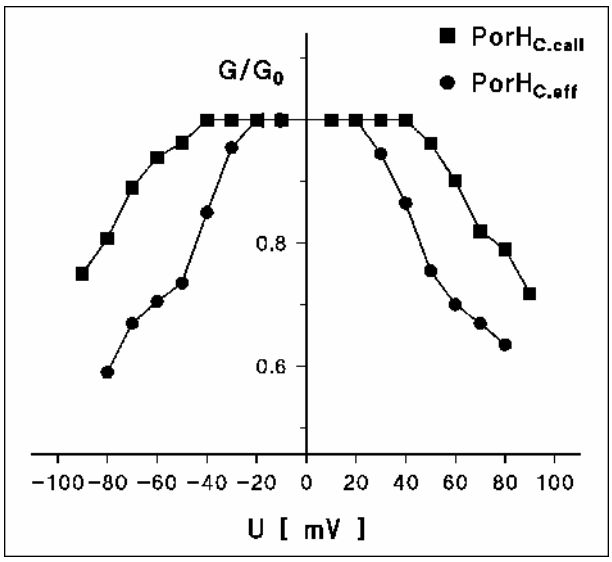

Figure 6. Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance Go at 10 mV as a function of the membrane potential V_m. The closed squares indicate the measurements in which $PorH_{C.call}$ was added to the cis-side of a membrane. The closed circles show results of measurements in which $PorH_{C.eff}$ was added to the cis-side of membranes. The membrane potential refers always to the cis-side of the membrane. The aqueous phase contained 1M KCl, and 500 ng porin per ml. The membranes were formed from diphytanoyl phosphatidylcholine dissolved in n-decane. T = 20 °C. Means of values obtained with four different membranes are shown.

The results suggest that $PorH_{C.eff}$ exhibited a somewhat higher voltage-dependence than $PorH_{C.call}$. The voltage-dependence of the data of Figure 6 was analyzed assuming a Boltzmann distribution between the number of open and closed channels, N_O and N_C, respectively (Ludwig *et al.*, 1986). This analysis allowed the calculation of the number of gating charges n (number of charges involved in the gating process) and the midpoint

potential V_O (potential at which the number of open and closed channels is identical) from a semilogarithmic plot of the ratio N_O/N_C, which is given by:

$$N_o/N_c = (G - G_{min})/(G_0 - G) \quad (6)$$

where G in this equation is the conductance at a given membrane potential V_m, G_0 and G_{min} are the conductance at zero voltage and very high potentials, respectively. The open to closed ratio of the channels, N_o/N_c, is given by:

$$N_o/N_c = \exp[-nF(V_m - V_0)/RT] \quad (7)$$

where F (Faraday's constant), R (gas constant) and T (absolute temperature) are standard symbols, n is the number of gating charges moving through the entire transmembrane potential gradient for channel gating (i.e. a measure for the strength of the interaction between the electric field and the open channel), and V_0 is the potential at which 50 % of the total number of channels are in the closed configuration (i.e. $N_o/N_c = 1$). Semilogarithmic plots of the data given in Figure 6 (see Figure 7) show that they could be fitted to straight lines with slopes of about 20 mV (for an e-fold change of V_m, because $RT/F = 25\ mV$). This result suggests that the number of charges involved in the gating process is approximately 1.5 for $PorH_{C.eff}$ and $PorH_{C.call}$ (see Figure 6). Whereas the voltage-dependence (the slope of the lines of Figure 6) was approximately the same for both channels, the midpoint potential V_0 (i.e. $N_o/N_c = 1$) differed somewhat for $PorH_{C.eff}$ ($V_0 \approx \pm 50$ mV) and $PorH_{C.call}$ ($V_0 \approx \pm 90$ mV) (see Figure 7).

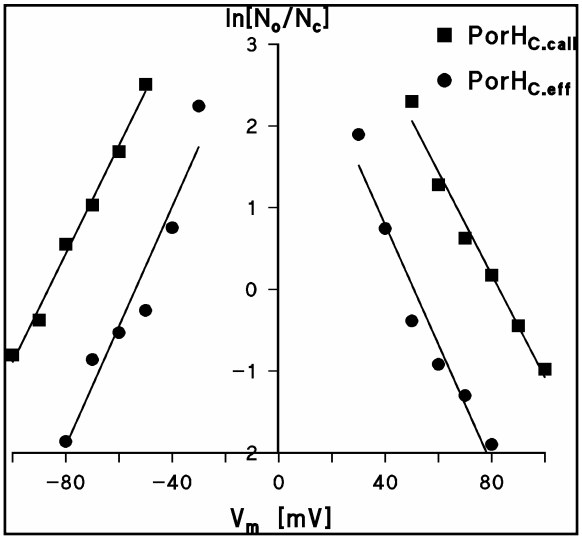

Figure 7. Semilogarithmic plot of the ratio, N_o/N_c as a function of the transmembrane potential V_m. The data were taken from Figure 6. The slope of the straight lines obtained by least squares fits is such that an e-fold change of N_o/N_c is produced by a change in V_m of about 15 to 16 mV (PorH$_{C.call}$) and about 14 mV (PorH$_{C.eff}$). The midpoint potential of the N_o/N_c distribution (i.e. $N_o = N_c$) is about ± 80 mV for PorH$_{C.call}$, and about ± 50 mV for PorH$_{C.eff}$. For further explanation see text.

2.4.4 Partial sequencing of the 6 kDa channel-forming proteins of *C. callunae* and *C. efficiens* and identification of *porH*$_{C.eff}$ within the chromosome of *C. efficiens*

The 6 kDa channel-forming proteins of *C. callunae* and *C. efficiens* were subjected to partial sequencing from the N-terminal end of the mature proteins after CNBr treatment using Edman-degradation. Three stretches of 22 and 23 amino acids (*C. callunae*) and 18 amino acids (*C. efficiens*) were resolved. Multiple sequence alignments were performed with the translated known nucleotide sequence of the complete *C. efficiens* genome (NCBI Reference Sequence accession number NC_004369). The NCBI BLAST-translation tool (Basic Local Alignment Search Tool, Zhang & Madden, 1997; Altschul *et al.*, 1990) showed that the 18 amino acids long stretch of *C. efficiens* is part of a 57 amino acid long hypothetical protein of *C. efficiens* (DDBJ/EMBL/GenBank accession number, AJ871586; see Figure 8), which we named PorH$_{C.eff}$. Interestingly, it exhibits only the inducer methionine at the N-terminal end but no N-terminal extension, which suggests that translation and assembly of the protein could be very similar to that of PorA$_{C.glut}$ of *C. glutamicum* (Lichtinger *et al*., 2001). Its gene *porH*$_{C.eff}$ comprises 174 bp and encodes for a 57 amino acid long acidic polypeptide (6 aspartic and glutamic acids as compared to 2 lysines). This means that PorH$_{C.eff}$ is not transported out of the cytoplasmic membrane using the Sec-apparatus as many other proteins from gram-positive bacteria but not the channel former PorA$_{C.glut}$ (Freudl, 1992; Lichtinger *et al.*, 2001).

```
          -   |  +-   -     +                          -  -   -
C.eff:   MDLSLLKDSLSDFATLGKNLGPALQGIPTLLNSIIAFFQNFGDLAETTGDAAGNLSS
          -   |   -  --     +            -  +      -   -  -
C.call:   DLSLLADNLDDYSTFGKNIGTALXXIPDLLKGIIAFFENFGDLAETT---
          -   |  +-     -                 -      -   -  -   -  -
C.glut:  MDLSLLKETLGNYETFGGNIGTALQSIPTLLDSILNFFDNFGDLADTTGENLDNFSS
```

Figure 8. Amino acid sequence of PorH$_{C.eff}$ and its comparison with the partial amino acid sequences of the N-terminus after CNBr cleavage derived peptide of PorH$_{C.call}$ and PorH$_{C.glut}$ (see Discussion). The result of the amino acid sequencing after CNBr cleavage of PorH$_{C.eff}$ using Edman degradation of the N-Terminus is underlined. The charged residues of the proteins (+/-) are specified on the top line. Conserved residues in both homolog proteins are shown in bold. The sequences within the vertical bars are supposed to fold in α-helices as shown in Figure 11. The sequence of PorH$_{C.eff}$ has been submitted to the DDBJ/EMBL/GenBank databases under the accession number: AJ871586.

Search within the chromosome of *C. efficiens* demonstrates that *porH*$_{C.eff}$ and the gene coding for PorA$_{C.eff}$ of *C. efficiens* are localized very close to one another (see Figure 9). The genes coding for both proteins were only separated by 77 bp and there was no indication for a transcription terminator between them. Thus it seems very likely that both proteins share a common way of export to the cell wall of *C. efficiens* and presumably also of *C. glutamicum* because the chromosome of the latter contains also a gene *porH*$_{C.glut}$ that has a high degree of homology to *porH*$_{C.eff}$ of *C. efficiens*.

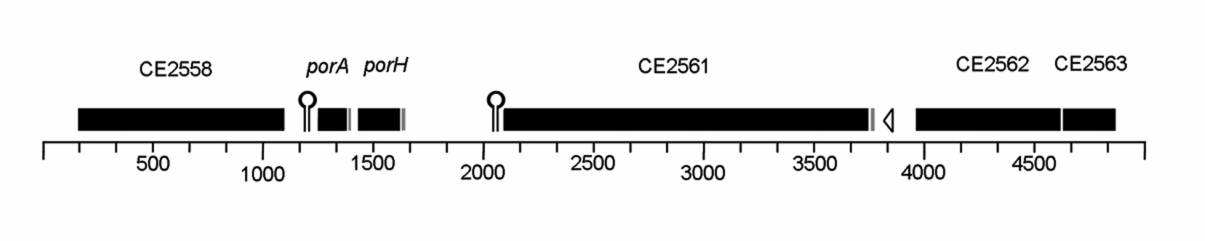

Figure 9. Overview of the *porH*$_{C.eff}$ gene locus and its flanking regions within the *C. efficiens* genome. Putative transcriptional terminators are shown by stem-loop structures, potential ribosome binding sites with the sequence AGGAG are shaded and a putative promoter is presented by a triangle. Gene names are specified, gene CE2561 encodes for the putative chaperonin GroEL2, CE2562 and CE 2563 are encoding for hypothetical proteins.

Comparison of the two amino acid stretches (22 and 23 amino acids) derived from sequencing of PorH$_{C.call}$ with the sequence of PorH$_{C.eff}$ and PorH$_{C.glut}$ suggests that the proteins are highly

homologous (see Figure 8). $PorH_{C.call}$ is also an acidic protein (8 aspartic and glutamic acids as compared to 2 lysines of the partial sequence). The interesting feature of the channels formed by the two homolog proteins is the observation, that one protein forms slightly anion-selective channels ($PorH_{C.eff}$) and the other one highly cation-selective channels ($PorH_{C.call}$). This means presumably that their arrangement in the channel-forming complexes may be responsible for their selectivity (see Discussion).

2.5 Discussion

2.5.1 The cell walls of *C. efficiens* and *C. callunae* contain ion-permeable channels formed by the 6 kDa PorH proteins

In previous studies we identified different cell wall channels in *C. glutamicum* (Lichtinger *et al.*, 1998; 2001; Costa-Riu *et al*., 2003a; 2003b). $PorA_{C.glut}$ forms a highly conductive cation-selective channel. Its deletion resulted in a much higher resistance of this bacterium versus neutral or positively charged antibiotics, which indicates a lower permeability of the cell wall of the deletion mutant (Costa-Riu *et al.*, 2003a). Nevertheless, growth of the mutant strain was only little impaired, in particular in rich media. Search for another cell wall channel revealed the existence of the anion-selective $PorB_{C.glut}$ channel (Costa-Riu *et al*., 2003a; 2003b). This result indicates that the cell wall of *C. glutamicum* contains several types of channels, as is the case in the outer membrane of gram-negative bacteria (Benz, 2001) and in the gram-positive *Rhodococcus equi*, which is also a member of the genus mycolata (Riess *et al.*, 2003). In this study, we inspected the cell walls of *C. efficiens* and *C. callunae*, which are closely related to *C. glutamicum* for the presence of cell wall channels using the lipid bilayer technique. In organic solvent extracts of whole cells, channels were observed for both organisms that had a molecular mass of about 6 kDa but were not identical to the well-studied $PorA_{C.glut}$ channels. The channel-forming proteins of *C. efficiens* and *C. callunae* were purified to homogeneity and were named $PorH_{C.call}$ and $PorH_{C.eff}$. Partial sequencing of the proteins from the N-terminus after CNBr cleavage resulted in three amino acid stretches that allowed the identification of the *porH* gene within the chromosome of *C. efficiens*. It codes

for a 57 amino acid long polypeptide without leader extension but starts with the inducer methionine, which is obviously cleaved during maturation. PorH$_{C.call}$ is highly homologous to PorH$_{C.eff}$ and the chromosome of *C. glutamicum* also contains the gene of a similar protein PorH$_{C.glut}$ (see Figure 8 and Chapter 3). The lack of an N-terminal leader extension suggested that PorH$_{C.eff}$ is not transported via the Sec-apparatus out of the cell to reach the cell wall. This is the same situation as for PorA$_{C.glut}$ and considering the genes within the flanking regions of *porA* and *porH* it seems likely that their gene products share the same way of translation, export and assembly, which represents a yet unknown secretion mechanism (Lichtinger *et al.*, 2001).

2.5.2 Effects of negative point net charges on the channel properties of PorH$_{C.call}$

The channels formed by PorH$_{C.call}$ and by PorH$_{C.eff}$ differ somewhat in the ionic selectivity. PorH$_{C.eff}$ forms slightly anion-selective channels despite the fact that the protein is in total acidic (6 negative charges as compared to 2 positive ones). Thus it seems that the lysine in position 6 of the mature protein plays a crucial role in the selectivity of the channel because it is absent in the primary sequence of the highly homologous PorH$_{C.call}$. The data of Table 1 demonstrate that the single-channel conductance of the channels formed by PorH$_{C.call}$ is not a linear function of the bulk aqueous concentration. Instead, we observed a dependence of the single-channel conductance on the square root of the salt concentration in the aqueous phase. This means (i) that the cation specificity of PorH$_{C.call}$ is not related to the presence of a binding site because saturation would be expected and (ii) that negative point charges are involved in ion selectivity as we and others have demonstrated previously for a variety of membrane channels (Menestrina & Antolini, 1981; Benz *et al.*, 1989; Benz, 1994), which includes also mycobacterial porins (Trias & Benz, 1993; 1994; Lichtinger *et al.*, 1998). When we apply eqs. (1) to (5) to the conductance of PorH$_{C.call}$ we receive a reasonable fit of the data of Table 1 if the channel has a diameter of about 2.2 nm and that 1.6 negative point charges (q = 2.4 10^{-19} As) are attached to the channel mouth. The results of this fit are shown in Figure 10.

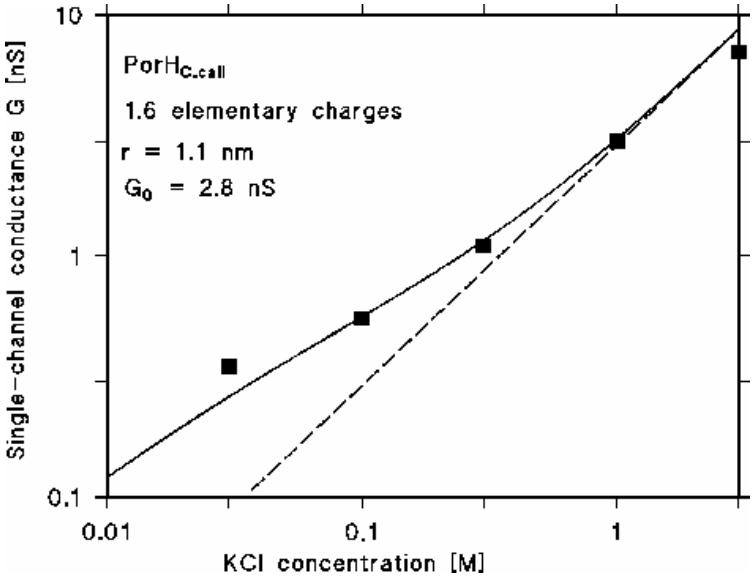

Figure 10. Single-channel conductance of PorH$_{C.call}$ as a function the KCl concentration in the aqueous phase (filled squares). The solid line represents the fit of the single-channel conductance data with the Nelson and McQuarrie (1975) formalism (eqs. (1) to (5)) assuming the presence of negative point charges (1.6 negative charges; q = -2.85 10^{-19} As) at the channel mouth and assuming a channel diameter of 2.2 nm. c, concentration of the KCl solution in M (molar); G, average single-channel conductance in nS. The broken (straight) line shows the single-channel conductance of the cell wall channel that would be expected without point charges. It corresponds to a linear function between channel conductance and bulk aqueous concentration.

The solid line represents the fit of the single-channel conductance versus concentration by using the Nelson and McQuarrie (1975) treatment and the parameters mentioned above together with a single-channel conductance, G_0 = 2.8 nS at 1 M KCl. The broken line corresponds to the single-channel conductance of PorH$_{C.call}$ without point net charges, i.e. it shows a linear relationship between the aqueous salt concentration and single-channel conductance. It is noteworthy, that the properties of PorA$_{C.glut}$ from *C. glutamicum* are also controlled by point charges (2 negative point charges; q = -3.2 10^{-19} As) using the same treatment. Interestingly, the diameter of channels formed by PorA$_{C.glut}$ is very similar to those of PorH$_{C.call}$.

2.5.3 Arrangement of PorH$_{C.call}$ and PorH$_{C.eff}$ in the cell wall

PorH$_{C.call}$ and PorH$_{C.eff}$ have a rather small molecular mass of about 6 kDa similar to that of PorA$_{C.glut}$ or PorB$_{C.glut}$. In general, the molecular masses of cell wall porins are rather small as

compared to those of gram-negative bacterial porins, which range between 30 and 60 kDa (Benz, 1994; Riess *et al*., 1998). This suggests that the cell wall channels are formed by oligomers. This has been demonstrated for the subunit of the cation-selective channel of *M. smegmatis*, which has a molecular mass of about 20 kDa (Niederweis *et al.*, 1999; Stahl *et al.*, 2001) and forms an octamer in the cell wall (Faller *et al.*, 2004). The monomers within the 3D-structure of the octamer are arranged in ß-sheet structure similar to that of gram-negative bacterial porins. However, such an arrangement is for the PorH$_{C.call}$ and PorH$_{C.eff}$ oligomers rather unlikely. Secondary structure predictions suggest, that a stretch of about 28 amino acids (see Figure 8) of both proteins form amphipathic α-helices with about 8 windings and a length of 4.2 nm in the mycolic acid layer (see Figure 11). The arrangement is such, that all hydrophilic amino acids are localized on one side of the helix and all hydrophobic ones on the other side. Comparison of the helical wheels from the two organisms indicate, that positively and negatively charged amino acids are balanced for PorH$_{C.eff}$, whereas the monomer of PorH$_{C.call}$ contains an excess of two negatively charged amino acids. It is noteworthy that this agrees nicely with the selectivity of both channels (see above).

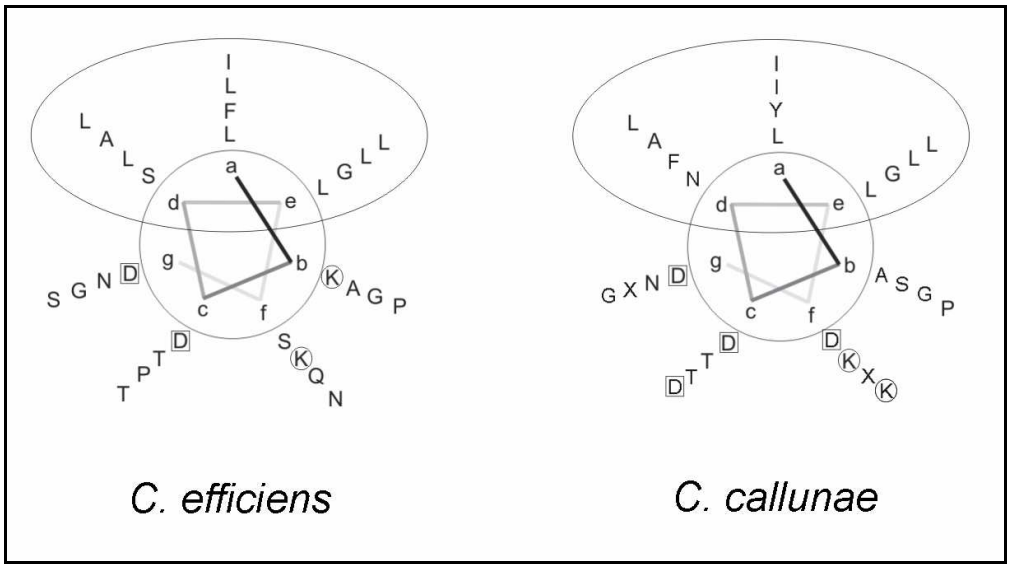

Figure 11. Schematic prediction of the PorH$_{C.eff}$ and PorH$_{C.call}$ secondary structures. Both molecules can form α-helices with 8 windings corresponding to an overall length of 4.2 nm based on secondary structure predictions of the primary sequence shown in Figure 8 between the two vertical bars. Residues of the heptameric repeats were labeled in the sequence as a-g. The hydrophobic residues are located at position a, e and d, indicating that they may be oriented towards the mycolic acids (indicated by oval rings). The hydrophilic residues are localized at the positions b, f, c and g and may face the channel lumen. Created with help of Helical Wheel Java Applet (http://www.site.uottawa.ca/~turcotte/resources/HelixWheel/).

This means that the arrangement of these cell wall channels is different to MspA of *M. smegmatis* (Faller *et al.*, 2004). It is possible that the arrangement of $PorH_{C.call}$ and $PorH_{C.eff}$ has to do with the thickness of the cell wall and the length of the mycolic acids of different mycolata. Thus, especially long mycolic acids have been found in Mycobacteria and *Tsukamurellae* (60-90 carbon atoms); they are medium-sized in *Gordonae*, *Nocardiae*, and *Rhodococci* (about 36-66 carbon atoms), and small in Corynebacteria (22-38 carbon atoms) (Yano & Saito 1972; Minnikin *et al.*, 1974; 1982; Minnikin 1987; 1991; Daffé *et al.*, 1990; Holt *et al.*, 1994; Ochi, 1995; Brennan & Nikaido 1995; Liu *et al.*, 1995; 1996; Yassin *et al.*, 1997). This means presumably that the cell walls of Corynebacteria are much thinner than those of other mycolata, which is in agreement with structural studies (Marienfeld *et al.*, 1997; Puech *et al.*, 2001). Smaller polypeptides arranged as α-helices are presumably sufficient to span the mycolic acid layer of Corynebacteria. However, this may be tentative and further investigation of the cell wall proteins of actinomycetes may be necessary to understand the structure and function of the cell wall channels of the mycolata.

CHAPTER 3

Identification and characterization of PorH, a new cell wall channel of *Corynebacterium glutamicum*

3.1 Summary

The cell wall of the mycolata *Corynebacterium glutamicum* contains the cation-selective channel (porin) $PorA_{C.glut}$ and the anion-selective channel $PorB_{C.glut}$ for the passage of hydrophilic solutes. Lipid bilayer experiments with organic solvent extracts of whole *C. glutamicum* cells cultivated in minimal medium suggested, that another cation-selective channel-forming protein, named $PorH_{C.glut}$ is present in *C. glutamicum*. The protein was purified to homogeneity by fast-protein liquid chromatography across a HiTrap-Q column. The pure protein had an apparent molecular mass of about 12 kDa on SDS-PAGE. Western blot analysis suggested, that the cell wall channel is presumably formed by protein oligomers. The purified protein forms cation-selective channels with a diameter of about 2.2 nm and an average single-channel conductance of about 2.5 nS in 1 M KCl in the lipid bilayer assay. The $PorH_{C.glut}$ protein was partially sequenced and based of the resulting amino acid sequence, the corresponding gene, which was designated as $porH_{C.glut}$, was identified in the published genome sequence of *C. glutamicum* ATCC13032. It contains only the inducer methionine but no N-terminal extension, which suggests, that secretion of the protein could be very similar to that of $PorA_{C.glut}$ of *C. glutamicum*, which also does not contain a signal sequence. $PorH_{C.glut}$ is coded in the bacterial chromosome by a gene that is localized in the vincinity of the $porA_{C.glut}$ gene, within a putative operon of 13 genes. RT-PCR and immunological detection experiments revealed that both porins are cotranscribed. They coexist in the cell wall of *C. glutamicum* together with $PorB_{C.glut}$ and $PorC_{C.glut}$.

3.2 Introduction

In 1957 a bacterium was isolated, which was shown to excrete large quantities of L-glutamic acid into the culture medium (Kinoshita *et al*., 1957). This bacterium, *Corynebacterium glutamicum*, was described as an aerobic, nonsporulating, gram-positive rod, capable of growing on a variety of sugars or organic acids. *C. glutamicum* is widely used for industrial production of L-glutamate, L-lysine and other amino acids through fermentation processes (Udaka, 1960; Gutmann *et al*., 1992, Keilhauer *et al*., 1993, Sahm *et al*., 1996, Eggeling & Sahm, 1999). During the year 2002 about 1×10^6 tons of L-glutamate and 5.5×10^5 tons of L-lysine were produced with *C. glutamicum* and used as a flavoring agent in food and as animal feed supplements (Leuchtenberger, 1996; Hermann, 2003). Little is known about the amino acid efflux properties of corynebacteria (Nikaido, 1995). Recently is has been shown that two genes are implicated in the efflux process of glutamate in *C. glutamicum* (Kimura *et al*., 1999; Hirasawa *et al*., 2000), but their function is still unknown. *C. glutamicum* is a member of the mycolic-acid containing actinomycetes, belonging to the mycolata. It was shown by 16S rRNA and in *rpoB* gene analysis that it is closed related to *C. efficiens* and *C. callunae* (Fudou *et al.,* 2002; Khamis *et al*., 2004).

The mycolata have in addition to a thick peptidoglycan layer a second surface layer, consisting of covalently bound mycolic acids and extractable lipids (Barksdale, 1981; Goodfellow *et al.,* 1976; Ochi, 1995). Ester bonds link the mycolic acids to the arabinogalactan, which is attached to the murein of the cell wall (Minnikin, 1987). The chain length of the mycolic acids varies considerably in different taxa of the mycolata. In Corynebacteria it consists of 22-38 carbon atoms (Minnikin, 1987; Yano & Saito, 1972; Minnikin *et al.,* 1974; Daffé *et al.,* 1990; Holt *et al.,* 1994; Brennan & Nikaido, 1995; Yague *et al.,* 2000; Ioneda, 1993). The outer layer of the mycolata functions as a permeability barrier (Nikaido *et al.,* 1993; Liu *et al.,* 1996), it affords for example resistance to cell lysis by egg white lysozyme (Hirasawa *et al*., 2001). This suggests that the cell wall of the mycolata has probably the same function as the outer membrane of Gram-negative bacteria. This membrane contains channel-forming proteins, the porins, which are required for the passage of hydrophilic solutes. Channels are also present in the mycolic acid layer of the mycobacterial

cell wall. With respect to transport of amino acids over this barrier it is of particular importance to understand the pathways present in the cell wall of Corynebacteria.

So far several types of cell wall channels of the mycolata have been identified and characterized (Rieß *et al.*, 1998; Lichtinger *et al.*, 1999; Niederweis, *et al.*, 1995; Lichtinger, *et al.*, 1998; Lichtinger, *et al.*, 2001; Costa-Riu, 2003a; 2003b). Similar to the porins of Gram-negative bacteria, these channels allow the permeation of hydrophilic solutes (Trias *et al.*, 1992; Trias & Benz, 1993). Common to them is, that the channels are wide and water-filled and that they contain point charges, which results in ion selectivity. The research performed with *C. glutamicum* can be of a great value, because some of the features from these cell wall channel-forming proteins can be expected for other mycolata. The *Corynebacterineae* family comprises microorganisms that cause very important infections worldwide such as *M. tuberculosis*, *M. leprae* and *C. diphtheriae*.

PorA$_{C.glut}$ from *C. glutamicum* was the first investigated pore-forming protein of corynebacteria. PorA$_{C.glut}$ is formed by an oligomer of a small 45 amino acid polypeptide and the channel is cation-selective with a single-channel conductance of about 5.5 nS in 1 M KCl (Lichtinger *et al.*, 1998, 2001). By deleting the *porA*$_{C.glut}$ gene from the *C. glutamicum* chromosome, an anion-selective pore-forming protein was discovered, named PorB$_{C.glut}$ (Costa-Riu *et al.*, 2003a; 2003b). It is a 99 amino acid long protein and forms anion-selective channels with a conductance of about 700 pS in 1 M KCl. Search for homologous genes showed, that the chromosome of *C. glutamicum* contained also another gene for a PorB$_{C.glut}$-like protein 138 bp downstream from *porB*$_{C.glut}$, which codes for PorC$_{C.glut}$. The arrangement of *porB*$_{C.glut}$ and *porC*$_{C.glut}$ suggested that both genes belong to the same cluster. RT-PCR from overlapping regions between both genes from wild-type *C. glutamicum* demonstrated that they are cotranscribed (Costa-Riu *et al.*, 2003b).

In this study we report the search for an additional cell wall channel of *C. glutamicum.* The organic solvent extract of whole cells cultivated in minimal medium contained another channel, which was found to be cation-selective. The channel-forming protein was purified to homogeneity and biophysical properties were studied in detail. The protein was partially sequenced and multiple sequence alignments search within the known chromosome demonstrated, that it contained a gene that fitted to the partial amino acid sequence of PorH$_{C.glut}$. Interestingly *porH*$_{C.glut}$ can be found in the *C. glutamicum* chromosome next to the

porA$_{C.glut}$. RT-PCR experiments from overlapping regions between *porA*$_{C.glut}$ and *porH*$_{C.glut}$ demonstrated that they are cotranscribed.

3.3 Materials and methods

3.3.1 Bacterial strain and growth conditions

C. glutamicum ATCC 13032 (DSMZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was routinely grown at 30 °C in minimal medium composed of 40 g glucose, 20 g $(NH_4)_2SO_4$, 5 g urea, 1 g KH_2PO_4 and 1.6 g K_2HPO_4, 42 g MOPS, 15 mg $CaCl_2$ 2 H_2O, 0.25 g $MgSO_4$ 7 H_2O, 10 mg $FeSO_4$, 10 mg $MnSO_4$ 2 H_2O, 1 mg $ZnSO_4$ 7 H_2O, 0.2 mg $CuSO_4$ 5 H_2O, 0.02 mg NiCl 6 H_2O and 0.2 mg biotin per 1 l distilled water, adjusted with NaOH to pH 7.0. For RT-PCR and immunological experiments *C. glutamicum* ATCC 13032 cells were routinely grown in BHI-medium (Brain Heart Infusion, Difco Laboratories).

3.3.2 Isolation and purification of the channel-forming proteins

For the isolation of the channel-forming proteins a method was used that has been previously devised for isolation and purification of PorA$_{C.glut}$ of *C. glutamicum* (Lichtinger *et al.*, 1999). This method uses the extraction of whole cells with organic solvents or detergents and avoids the substantial loss of material caused by sucrose density centrifugation of the cell envelope to separate the cytoplasmic membrane from the cell wall fraction. 200 ml cells were grown to an OD of 10 and harvested by centrifugation (10,000 rpm for 10 min in Beckman J2-21M/E centrifuge). The cells were washed twice in 10 mM Tris-HCl (pH 8). The final pellet (5 ml) was extracted with a 1:2 mixture of chloroform:methanol in a proportion of 1 part cells and 5 to 8 parts chloroform/methanol. The duration of the extraction was about 3 hours at room temperature under stirring in a closed tube to avoid loss of chloroform. Cells and chloroform/methanol solution were centrifuged for 15 minutes (10,000 rpm in Beckman J2-

21M/E centrifuge). The pellet (cells) was discarded. The supernatant contained the channel-forming activity. It was mixed in a ratio of 1 part supernatant to 9 parts ether and was kept over night at -20 °C. The precipitated protein was dissolved in a solution containing 0.4 % LDAO (*NN*-dimethyldodecylamine *N*-oxide) and 10 mM Tris-HCl (pH 8) and inspected for channel-forming activity. The protein was subjected to fast protein liquid chromatography (FPLC) across a HiTrap-Q column (Amersham Pharmacia Biotech, Freiburg, Germany). The column was washed first with a buffer containing 0.4 % LDAO and 10 mM Tris-HCl (pH 8) and the protein was eluted with 0.4 % LDAO in 10 mM Tris-HCl (pH 8) using a linear gradient between 0 and 1 M NaCl.

3.3.3 SDS-PAGE

SDS-PAGE was performed with tricine containing gels (Schägger & von Jagow, 1987). The gels were stained with colloidal Coomassie (Neuhoff *et al*., 1988) or silver stain (Blum *et al*., 1987). Before separation, the samples were all incubated for 5 min at 100 °C with loading buffer (exempt preparative SDS-PAGE). Preparative SDS-PAGE was used for identification and purification of the channel-forming activity from the organic solvent extracts of whole *C. glutamicum* cells.

3.3.4 Peptide sequencing

The precipitated protein pellet resulting from the extraction was dissolved in 100 µl 70 % (by vol.) formic acid containing 10 % (mass/vol.) CNBr (Merck), and incubated in the dark at room temperature for 14 h (Gross, 1967). After lyophilisation the CNBr peptides were dissolved in 20 % (by vol.) formic acid and separated by reversed - phase - HPLC (SYCAM, Fürstenfeldbruck, Germany) applying a Luna C-18 column, 150 mm x 1 mm with a flow rate of 40 µl/min using a 120 min gradient from 100 % A (0.1 % TFA in water) to 80 % B (0.1 % TFA in acetonitril). Collected fractions were subjected to amino acid sequence analysis on a 492 protein sequencer (Applied Biosystems, Darmstadt, Germany) using the conditions recommended by the manufacturer. The major sequence was DLSLLKETLGNYE beside small subsequences.

3.3.5 RT-PCR

Total mRNA was isolated from disrupted cells grown until mid-exponential growth phase using the RNeasy Kit according to the instructions of the manufacturer for the isolation of total RNA from bacteria (Qiagen, Hilden, Germany). Purified RNA was eluted with 50 µl of RNase-free water and treated with 2U of DNase I (Ambion, Huntingdon, Cambridgeshire, UK) in 0.1 volumes of 10x DNase buffer for 30 min at 37 °C to remove the DNA. After this treatment, DNase was inactivated with the inactivation reagent and 5 µl of the treated RNA were loaded in a 0.8 % agarose gel to test the integrity. Two sharp bands (both rRNA, 23S and 16S) were visible in each one of the samples. These samples reported A260/A280 ratios in the range of 1.7, as measured in unbuffered water. The reverse transcription (RT) was performed in a two-step reaction with the Enhanced Avian HS RT-PCR kit (Sigma, Deisenhofen, Germany). Random nonamers were used with 4 µg from total DNase-treated RNA to transcribe it into cDNA for 50 min at 45°C. The cDNA product was used for PCR reaction (Kohler *et al*., 1995) with all primers used for amplification of overlapping regions between genes of the $porA_{C.glut}$ and $porH_{C.glut}$ containing cluster (see Table 1). This putative cluster comprises the region between 2886331 and 2907251 of the chromosomal DNA of *C. glutamicum*. (NCBI Reference Sequence accession number NC_003450). The minus strand encodes for all genes inside this 20920 bp long fragment, which suggest that they could belong to the same transcription unit. The annealing temperature was 64 °C and elongation time with *Taq* polymerase was 2 min at 72 ºC. For negative control, 5µl of the DNase treated RNA was used for direct PCR with both specific primers for $porA_{C.glut}$ and $porH_{C.glut}$ and the same program used for PCR after RT reaction.

Oligonucleotide	**Position in the *C. glutamicum* genome**	**Sequence 5'- -3'**
Operon0-5'	2886331-2886351	AGCATGCTCGACGTCTTGCTC
Operon0-3'	2887096-2887116	GCGCTAAGGAAGAGCAGTTCC
Operon1-5'	2887601-2887621	CTTCAGCAGCTCGATCTGGAG
Operon1-3'	2887999-2888020	CGGCTACGTCTTCGACTTCCTC
Operon2-5'	2888040-2888060	GGACATCAAGGTTTCCAAGGA
Operon2-3'	2888289-2888311	CCTCGGCAACTACGAGACCTTC
Operon3-5'	2888260-2888280	CTCTGAAGAGCGGTACCGATG
Operon3-3'	2888950-2888970	TGCTGACAAGCCACAGCCTGC
Operon4-5'	2890501-2890521	CCACGACGTGCTTCCTCATCA
Operon4-3'	2890801-2890821	CTGGATCGGCACTGGCATTGC
Operon5-5'	2890890-2890910	AACCGCATCAAGCCTCACGCC
Operon5-3'	2892041-2892061	GATCCAGAAGCGACCTCATCA
Operon6-5'	2892117-2892137	GGGTAAACATCAGGAGCGGTC
Operon6-3'	2893079-2893099	GAACGTGATTCGACGGGATTG
Operon7-5'	2892971-2892991	CGGTTGGTTTCTGTGGAAGGA
Operon7-3'	2894000-2894020	CAGCACCTAAGGTGGAGCCTG
Operon8-5'	2894909-2894929	GGGTCACCGTTTCAATATGAG
Operon8-3'	2896201-2896221	CAGCACCGCGGCCGGGAGTAC
Operon9-5'	2897400-2897379	GTAGTCGCCGCCAGGTTTGAG
Operon9-3'	2898381-2898401	CAGCTCCGCATTCAAGTGGGC
Operon10-5'	2900301-2900321	GGTGTCCTGCGAATAGGCGGC
Operon10-3'	2901390-2901410	CTTTACGGCGATGAAGTCCGC
Operon11-5'	2903881-2903901	GCACCTGAACCACCACAGCCG
Operon11-3'	2904850-2904870	GTGCTCGGACTGGATAGCAG
Operon12-5'	2906491-2906511	TGACGCTCCGGCCTCAACTGC
Operon12-3'	2907088-2907108	GAATGGCTCGTTCGGCGGTTC

Table 1. Oligonucleotides used in this study. Sequences of the 13 couples of primers used for the amplification of regions that are overlapping two neighboring ORF from the putative $porA_{C.glut}$, $porH_{C.glut}$ cluster. The

sequences of the primers were derived from the region between 2886331 and 2907251 of the chromosomal DNA of *C. glutamicum* (NCBI Sequence accession number NC_003450).

3.3.6 Lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously (Benz *et al*., 1978; Benz, 2003). The experimental set up consisted of a Teflon cell with two water-filled compartments connected by a small circular hole. The hole had an area of about 0.4 mm^2. Membranes were formed across the hole using a 1 % solution of diphytanoyl phosphatidylcholine (PC; Avanti Polar Lipids, Alabaster, Ala.) dissolved in *n*-decane. The temperature was maintained at 20 °C during all experiments. All salts were obtained from Merck (Darmstadt, Germany, analytical grade). They were used unbuffered. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a homemade current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder. Zero current membrane potentials were measured with a Keithley 617 electrometer 5-10 minutes after five-fold salt gradients were established across the membranes (Benz et al., 1979).

3.3.7 Effect of negatively charged groups attached to the channel mouth

Negative charges at the pore mouth result in substantial ionic strength-dependent surface potentials at the pore mouth that attract cations and repel anions. Accordingly, they influence both single-channel conductance and zero-current membrane potential. A detailed quantitative description of the effect of point charges on the single-channel conductance was given in previous publications (Trias & Benz, 1993; Costa-Riu *et al.,* 2003b).

3.3.8 Immunological techniques

Synthetic polypeptides of part of the $PorB_{C.glut}$ sequence (KGEGFWTTQFPQIGD-WNEDQ), part of the $PorC_{C.glut}$ sequence (AHENSTRSELAANLRNSA) and part of the $PorH_{C.glut}$ sequence (DLSLLKETLGNYETFGGNIGTALQSIPTLL + SILNFFDNFGDLADTIGENL-

DNFSS) were synthesized and polyclonal antibodies against this peptides were raised in rabbits using the GEBRU 100 (GERBU Biochemicals, Gaiberg) Adjuvant System. The pre-immune serum was tested giving no reaction against cell extracts of *C. glutamicum*. The antiserum was used for Western blots of the extracted protein samples using standard procedures (Towbin *et al*., 1979). The blotting time was 5 minutes at 350 mA. The ECL Western Detection kit (Amersham Pharmacia Biotech) was used to detect binding of the antibody according to the instructions of the manufacturer. The exposure time was 30 seconds. The antibodies were highly specific for $PorB_{C.glut}$, $PorC_{C.glut}$ and $PorH_{C.glut}$ of *C. glutamicum*. The used $PorA_{C.glut}$ antibody was already described earlier (Lichtinger *et al.*, 2001).

For electron microscopy analysis whole *C. glutamicum* ATCC 13032 cells were fixed overnight at 4 °C in freshly made 4 % formaldehyde in PBS, pH 7. After dehydration in a graded series of ethanol, the cells were embedded in LR White, filled in gelatin capsules and cured at 40 °C for 3 days. Ultrathin sections were obtained and incubated with antibodies against $PorA_{C.glut}$, $PorB_{C.glut}$, $PorC_{C.glut}$, or $PorH_{C.glut}$. After that 12 nm Colloidal Gold–AffiniPure Goat Anti–Rabbit IgG antibodies were used in order to localize the porins.

3.4 Results

3.4.1 Identification of a new channel-forming protein in the cell wall extract of *C. glutamicum* ATCC 13032

Membrane experiments with organic solvent extracts of *C. glutamicum* ATCC 13032, grown in minimal medium, suggested, that the extracts contained besides $PorA_{C.glut}$ that forms channels with a single-channel conductance of 5.5 nS in 1 M KCl also another channel-forming protein that formed channels with a somewhat smaller conductance. To identify the protein, which is responsible for the additional channel-forming activity, the cell extracts were precipitated with ether in the cold. The pellet was suspended in 0.4 % LDAO, 10 mM Tris-HCl (pH 7), and subjected to lipid bilayer studies. The precipitate that showed several bands

on SDS-PAGE (see lane 1, Figure 1) contained the channel-forming activity. The purification of the additional channel-forming protein from *C. glutamicum* ATCC 13032 was achieved by FPLC. The pellet from the ether precipitation of the organic solvent extract was dissolved in 2 ml 0.4 % LDAO, 10 mM Tris-HCl (pH 8) and applied to a 1 ml HiTrap-Q FPLC column. The column was washed with 5 ml of the same buffer and then eluted with a linear NaCl-gradient between 0 and 1 M NaCl. The fraction eluted at 0.3 M NaCl showed a high channel-forming activity in black lipid bilayer membranes. SDS-PAGE of the corresponding fraction suggested that a 12 kDa protein could be responsible for the channel forming activity that was different to $PorA_{C.glut}$ (see lane 3, Figure 1).

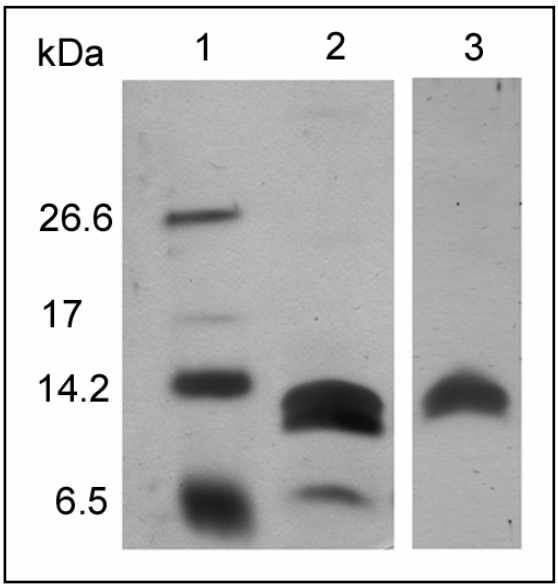

Figure 1. 12 % Tricine SDS-PAGE according to Schägger and von Jagow (1987) of the purification procedure of $PorH_{C.glut}$ of *C. glutamicum*. The gel was stained with silver.
Lane 1: Molecular mass marker.
Lane 2: 15 µl of ether precipitated organic solvent extract dissolved in 0.4 % LDAO, 10 mM Tris-HCl; pH 7 treated for 10 min at 100 °C with 5 µl sample buffer.
Lane 3: 15 µl of the fraction 23 of the Hitrap-Q FPLC column, treated for 10 min at 100 °C with 5 µl sample buffer.

To test whether the fraction containing the 12 kDa protein was really pure and did not contain another membrane active component, the pellet from the ether precipitation was subjected to preparative SDS-PAGE. The 12 kDa band was excised and eluted overnight at 4 °C with 1 % Genapol. Channel formation was found only in the band that corresponded to a molecular mass of 12 kDa indicating that no channel-forming impurities were present in the fraction of the HiTrap-Q column, eluted at 0.3 M NaCl. The channel forming protein was named $PorH_{C.glut}$.

3.4.2 $PorH_{C.glut}$ increases conductance of lipid bilayer membranes

$PorH_{C.glut}$ mediated channel formation was studied in detail. Small amounts of the protein were able to induce a substantial increase of the specific membrane conductance. About two

minutes after addition of the protein, the membrane conductance started to rise and increased by several orders of magnitude in approximately 20 minutes (data not shown). Only a small further increase, as compared to the initial one, occurred after that time. The time course of the conductance increase was similar irrespective of whether the protein was added to one or both sides of the membrane. Single-channel experiments revealed that the membrane activity of $PorH_{C.glut}$ was caused by the formation of ion-permeable channels. Figure 2 shows a single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane where $PorH_{C.glut}$ was added to a black membrane in a concentration of about 10 ng/ml.

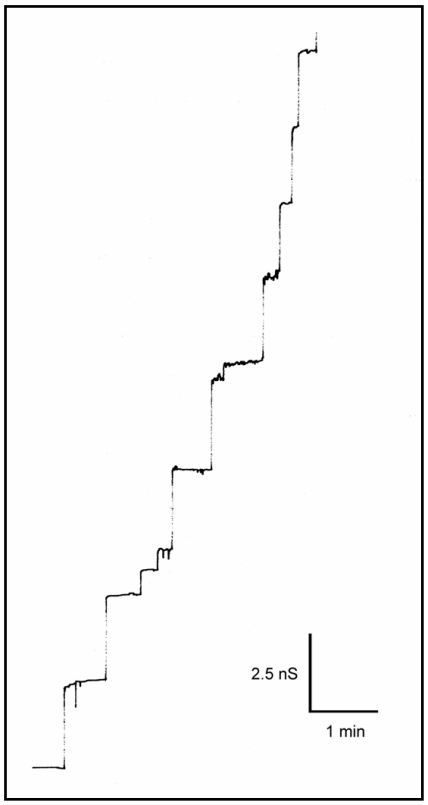

Figure 2. Single-channel recording of a PC/n-decane membrane in the presence of pure $PorH_{C.glut}$ of *C. glutamicum*. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml $PorH_{C.glut}$. The applied membrane potential was 20 mV; T = 20 °C.

The recording demonstrates that the protein formed defined channels. Their conductance was on average about 2.5 nS in 1 M KCl. Only a minor fraction of channels with other conductance was observed (see the histogram, Figure 3). It is noteworthy that the channels formed by $PorH_{C.glut}$ had a long lifetime similar to those that have been detected previously for cell wall proteins (porins) of *C. glutamicum* (Lichtinger et al., 1998; Costa Riu et al., 2003b) *Mycobacterium chelonae* (Trias & Benz, 1993) and *M. smegmatis* (Trias & Benz, 1994). All these proteins formed channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 minutes).

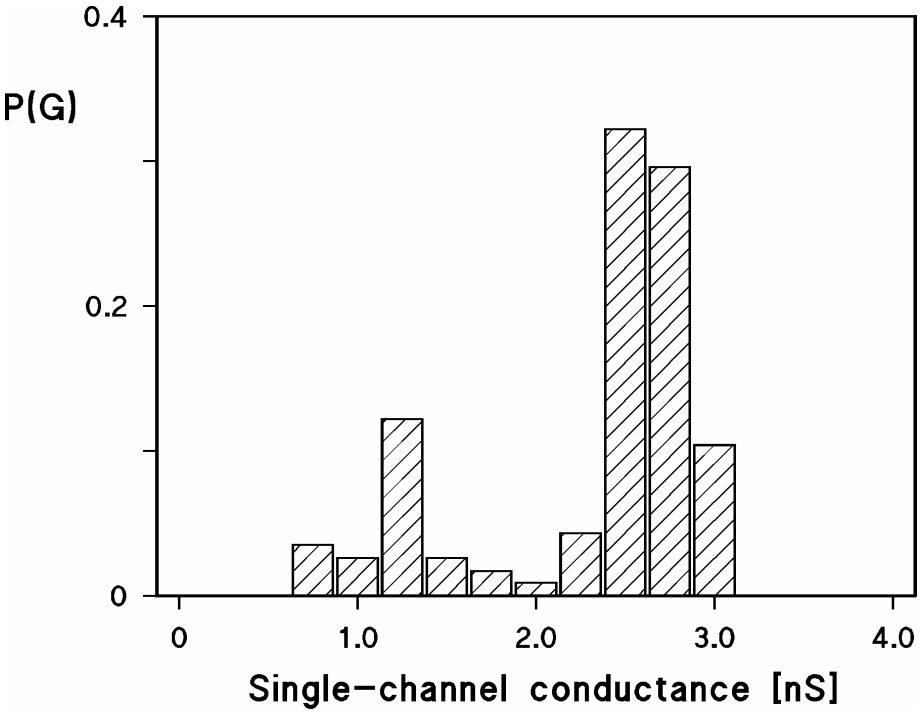

Figure 3. Histogram of the probability P(G) for the occurrence of a given conductivity unit observed with membranes formed of PC/n-decane in the presence of $PorH_{C.glut}$. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl, pH 6. The applied membrane potential was 20 mV; T = 20 °C. The average single-channel conductance was 2.5 nS for 115 single-channel events.

Single-channel experiments also performed with salts other than KCl to obtain some information on the properties of the channels formed by $PorH_{C.glut}$. The results summarized in Table 2 suggested that the channel is cation-selective. This can be derived from single-channel experiments, which demonstrated that the influence of the mobility of cations on the conductance was more substantial. Table 2 shows also the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase. Similarly, as in the case of other cell wall channels (Trias & Benz, 1993; 1994; Lichtinger et al., 1998), the relationship between conductance and KCl-concentration was not linear. Instead, the slope of the conductance versus concentration curves on a double logarithmic scale was approximately 0.5, which indicated the influence of point charges localized in or near the channel (see also Discussion and Figure 11).

Salt	Concentration c [M]	Single-channel conductance G [nS]
LiCl	1.0	1.0
KCl	0.01	0.15
	0.03	0.35
	0.1	0.4
	0.3	0.9
	1.0	2.5
	3.0	7.0
KCH_3COO(pH 7)	1.0	1.5

Table 2. Average single-channel conductance, G, of $PorH_{C.glut}$ in different salt solutions. The membranes were formed of diphytanoyl phosphatidylcholine dissolved in n-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20 °C. The average single-channel conductance, G, was calculated from at least 80 single events.

3.4.3 Selectivity of $PorH_{C.glut}$

Zero-current membrane potential measurements allow the calculation of the permeability ratio P_{cation} divided by P_{anion} in multichannel experiments. Membranes were formed in 100 mM KCl solution and concentrated $PorH_{C.glut}$ was added to the aqueous phase when the membranes were in the black state. After incorporation of 100 to 1000 channels into a membrane, five-fold KCl gradients were established by addition of small amounts of concentrated KCl solution to one side of the membrane. For all experiments with $PorH_{C.glut}$, the more diluted side of the membrane became positive, which indicated preferential movement of potassium ions through the channel. The zero-current membrane potential for KCl was on average about 25 mV (mean of four measurements). Analysis of the zero-current membrane potentials using the Goldman-Hodgkin-Katz equation (Benz *et al.*, 1979) revealed that $PorH_{C.glut}$ was cation-selective. On one hand it is possible that chloride has also certain permeability through the $PorH_{C.glut}$ channels because the ratio of the permeability coefficient P_{cation} divided by P_{anion} was 5.1. On the other hand, the negative point charges could influence the permeability ratio,

which means that cations could have a much higher permeability than anions through the PorH$_{C.glut}$ channels (see Discussion).

3.4.4 Partial sequencing of PorH$_{C.glut}$ and identification of *porH*$_{C.glut}$ within the chromosome of *C. glutamicum*

PorH$_{C.glut}$ of *C. glutamicum* was subjected to partial sequencing from the N-terminal end of the mature protein using Edman-degradation. One stretch of 13 amino acids was resolved. Multiple sequence alignment was performed with the translated known nucleotide sequence of the complete *C. glutamicum* genome (NCBI Reference Sequence accession number NC_003450). The NCBI BLAST-translation tool (Basic Local Alignment Search Tool, Zhang & Madden, 1997; Altschul et al., 1990) showed that the 13 amino acids long stretch of *C. glutamicum* is part of a 57 amino acid long hypothetical protein of *C. glutamicum* (see Figure 4), which is encoded by the gene *porH*$_{C.glut}$ comprising 174 bp. It has only the inducer methionine at the N-terminal end but no N-terminal leader extension, which suggests that translation and assembly of the protein could be very similar to that of PorA$_{C.glut}$ of *C. glutamicum* (Lichtinger et al., 2001). The total mass of the polypeptide is 6.1 kDa, which led to the assumption that the apparent molecular mass of ~12 kDa that was determined by SDS-PAGE (see Figure 4) may represent protein dimers. The mature protein contains 9 negatively charged residues (6 aspartic and 3 glutamic acids) and only 1 positively charged amino acid (lysine) and has a calculated pI of 3.5. Its overall charge agrees well with the cation selectivity of the channels formed by PorH$_{C.glut}$.

```
                 -    +-     -                    -       -   -   -   -   -
PorH C.glut      MDLSLLKETLGNYETFGGNIGTALQSIPTLLDSILNFFDNFGDLADTTGENLDNFSS
                 -    +-   -     +                          -   -   -
PorH C.eff       MDLSLLKDSLSDFATLGKNLGPALQGIPTLLNSIIAFFQNFGDLAETTGDAAGNLSS
```

Figure 4. Amino acid sequence of PorH$_{C.glut}$ and its comparison with the amino acid sequence of PorH$_{C.eff}$. The result of the amino acid sequencing of PorH$_{C.glut}$ using Edman degradation of the N-Terminus is underlined. The charged residues of the proteins (+/-) are specified on the top line. Conserved residues in both homologue proteins are shown in bold. The sequence of PorH$_{C.glut}$ has been submitted to the DDBJ/EMBL/GenBank databases under the accession number: AJ871585.

Search within the chromosome of *C. glutamicum* demonstrated that $porH_{C.glut}$ and $porA_{C.glut}$ are localized very close to one another (see Figure 5). Both genes are only separated by 83 bp; there is no indication for a transcription terminator between them. Thus it seems very likely that both proteins share a common way of export to the cell wall of *C. glutamicum* and presumably also of *C. efficiens* because the chromosome of the latter contains also a gene that has a high degree of homology to $porH_{C.glut}$ of *C. glutamicum*. Comparison of the amino acid sequence of $PorH_{C.eff}$ with the sequence of $PorH_{C.glut}$ suggests that the former protein is highly homologous to the latter (see Figure 4).

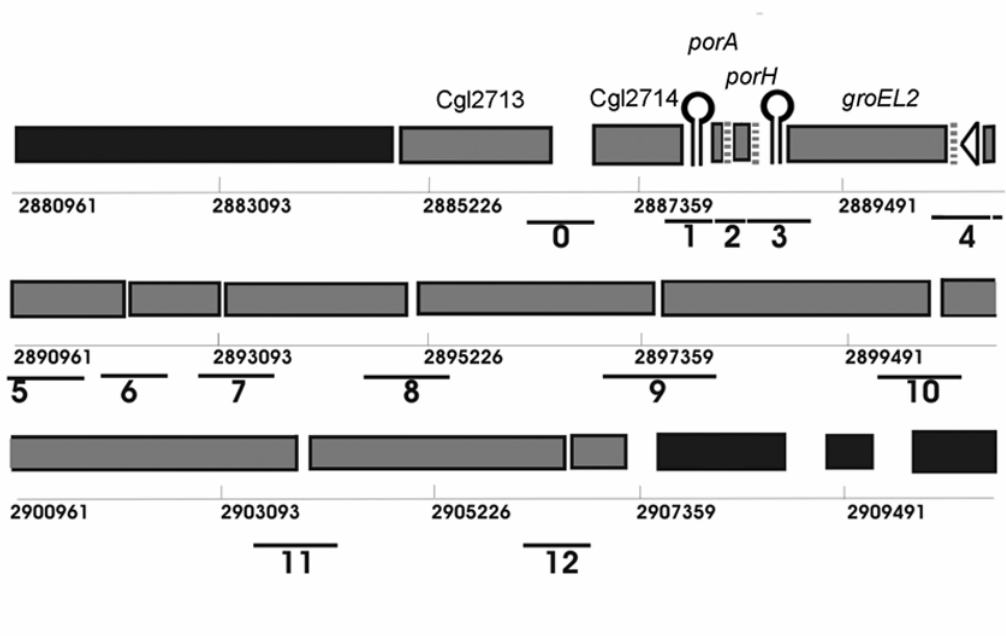

Figure 5. Overview of the $porH_{C.glut}$ gene locus and its flanking regions within the *C. glutamicum* genome. Putative transcriptional terminators are shown by stem-loop structures, potential ribosome binding sites with the sequence AGGAG are shaded and a known promoter is presented by a triangle (Barreiro *et al*., 2004). Gene names are specified, gene *Cgl2714* encodes for a putative RNA polymerase sigma factor, *Cgl2713* encodes for a putative aldehyde dehydrogenase. Light-gray colored genes belong to the same putative operon. The lines are representing the regions amplified by the pair of primers 0-12 designed in between overlapping regions (see Table 1).

3.4.5 RT-RCR

Analysis of the gene region coding for $PorH_{C.glut}$ and $PorA_{C.glut}$ suggested that $porH_{C.glut}$ and $porA_{C.glut}$ of are part of a putative gene cluster that is responsible for the transcription and

translation of the channel-forming proteins in the cell wall. This putative cluster is localized within the region from bp 2886331 to bp 2907251 of the chromosomal DNA of *C. glutamicum*. (GenBank Accesion number NC_003450). Interestingly, all genes of this 20920 bp long stretch are encoded by the minus strand. To see if the different genes are transcribed together, total RNA was isolated from *C. glutamicum* ATCC 13032 and treated with DNase to completely digest genomic DNA (data not shown). Via reverse transcription, the mRNA of *C. glutamicum* wildtype was converted into cDNA with random nonamers and afterwards amplified with the primers specific for the different genes within the putative cluster (see Table 1). As shown in Figure 6, the mRNA of *C. glutamicum* contained signals that indicated amplification of the overlapping regions between the genes of the putative *porH* cluster with the different primers. This result suggested that $porH_{C.glut}$ and $porA_{C.glut}$ could be part of a transcriptional unit that contains up to 13 genes.

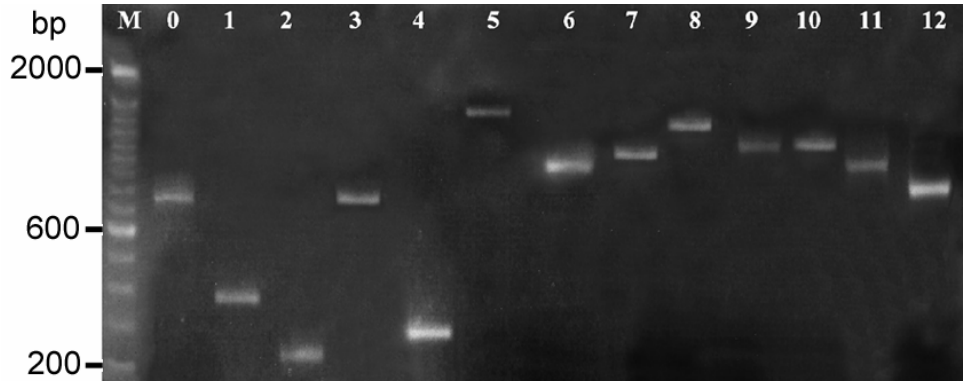

Figure 6. 0.8 % agarose gel from RT- PCR experiments. For each pair of primers from Table 1, a product is shown. The length of the products is: 0: 790 bp; 1: 410 bp; 2: 250 bp; 3: 710 bp; 4: 280 bp; 5: 1430 bp; 6: 1010 bp;7: 1030 bp; 8: 1310 bp; 9: 1020 bp; 10: 1100 bp; 11: 1000 bp; 12: 630 bp. M: 100 bp ladder. Taken from Costa-Riu, N. (2003), unpublished data.

3.4.6 Western blots of $PorH_{C.glut}$ indicate its oligomeric form

$PorH_{C.glut}$ is probably too small to account alone for a transmembrane channel. To check whether $PorH_{C.glut}$ forms oligomers, we performed Western blots with different crude cell extracts of *C. glutamicum*. One of them was performed with 2 % LDAO; the other two consisted of extraction of the cells for three hours either with 8 M urea or with a 1:2 mixture of chloroform/methanol (see Material and methods). After extraction according to the

different methods the cells were centrifuged for 10 minutes at 14 000 rpm, and the supernatant was subjected to SDS-PAGE followed by Western blot with anti $PorH_{C.glut}$ antibodies. Figure 7 shows the results of the experiments. Lane 1 demonstrates that $PorH_{C.glut}$ could form oligomers, possibly hexamers and others if it is extracted with the detergent LDAO and solubilized at room temperature. Interestingly, the oligomers seem to resist boiling for 5 minutes in sample buffer (lane 2, Figure 7). Extraction of the cells with 8 M urea or organic solvent tends to destroy the oligomers and only monomers or dimers of $PorH_{C.glut}$ were detected on the Western blot (see lanes 3 and 4, respectively).

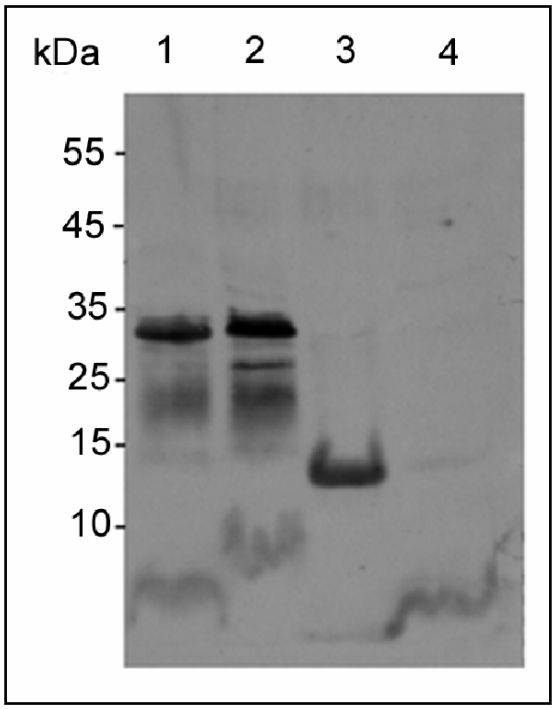

Figure 7. Western Blot analysis of $PorH_{C.glut}$. The samples were separated on a Tricine containing 10 % SDS-PAGE and blotted onto a nitrocellulose membrane. Proteins were visualized using anti-$PorH_{C.glut}$ antibodies and a chemoluminescence reaction.
Lane 1: 15 µl supernatant of 2 % LDAO extraction of whole cells were solubilized with 5 µl sample buffer without mercaptoethanol at room temperature;
Lane 2: 15 µl supernatant of 2 % LDAO extraction of whole cells were solubilized with 5 µl sample buffer at 100 °C;
Lane 3: 15 µl supernatant of 8 M urea extraction treated for 5 min at 100 °C with 5 µl sample buffer;
Lane 4: 15 µl of ether precipitated organic solvent extract dissolved in 0.4 % LDAO, 10 mM Tris-HCl; pH 7 treated for 10 min at 100 °C with 5 µl sample buffer.

3.4.7 Immunological detection of $PorA_{C.glut}$, $PorH_{C.glut}$, $PorB_{C.glut}$ and $PorC_{C.glut}$

Immunological detection of the different cell wall channels was performed to check if they were all expressed in the cell wall of *C. glutamicum*. Whole cells, grown in BHI-medium, were fixed with formaldehyde and incubated with antibodies against $PorA_{C.glut}$, $PorH_{C.glut}$, $PorB_{C.glut}$ and $PorC_{C.glut}$. Then the cells were treated with gold-labeled (12 nm diameter) goat anti rabbit antibodies. Figure 8 shows electron micrographs of cells treated with anti-$PorA_{C.glut}$ (A), anti- $PorH_{C.glut}$ (B), anti- $PorB_{C.glut}$ (C) and anti- $PorC_{C.glut}$ (D) antibodies. The immuno gold particles were in all cases only visible in the region of the envelope of *C. glutamicum*

cells. The results indicated that the channels coexist in the *C. glutamicum* cell wall. However, the labeling with immuno gold particles was different for the different pore-forming proteins. Whereas $PorA_{C.glut}$ (A) and $PorH_{C.glut}$ (B) were well labeled, only a few immuno gold particles were visible in the case of $PorB_{C.glut}$ (C) and $PorC_{C.glut}$ (B). Possibly, the antigenic determinants, chosen for the generation of the corresponding antibodies, were not well accessible from the surface of the cells.

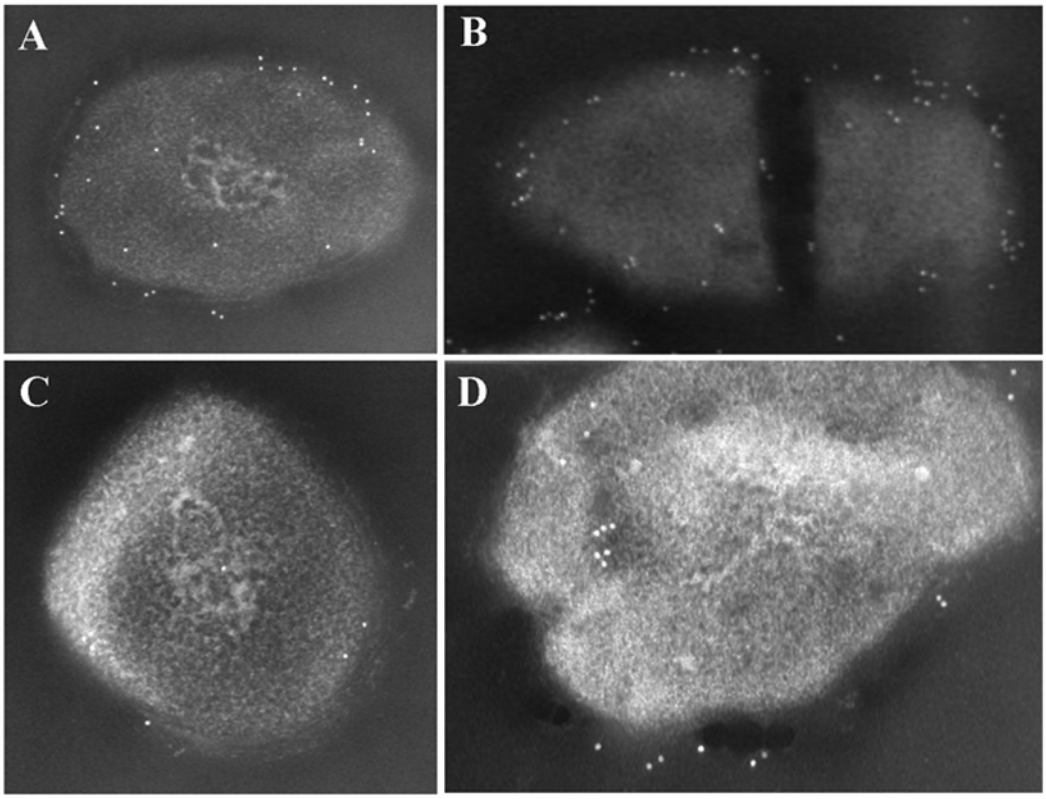

Figure 8. Electron micrograph of *C. glutamicum* cells, grown in BHI-medium, fixed with formaldehyde, treated with anti-$PorA_{C.glut}$ (A), anti-$PorH_{C.glut}$ (B), anti-$PorB_{C.glut}$ (C) and anti-$PorC_{C.glut}$ (D) antibodies and then with 12 nm colloidal Gold–AffiniPure goat Anti–Rabbit IgG antibodies. Original magnification 63 000x.

SDS-PAGE of total cell extracts using organic solvent followed by immunodecoration with antibodies against all four channel-forming proteins demonstrated that they all were present in the cells (see Figure 9). This provides evidence that $PorC_{C.glut}$ is also expressed in *C. glutamicum* besides the other porins because we could only show in a previous publication that the corresponding gene is transcribed (Costa-Riu *et al.*, 2003b).

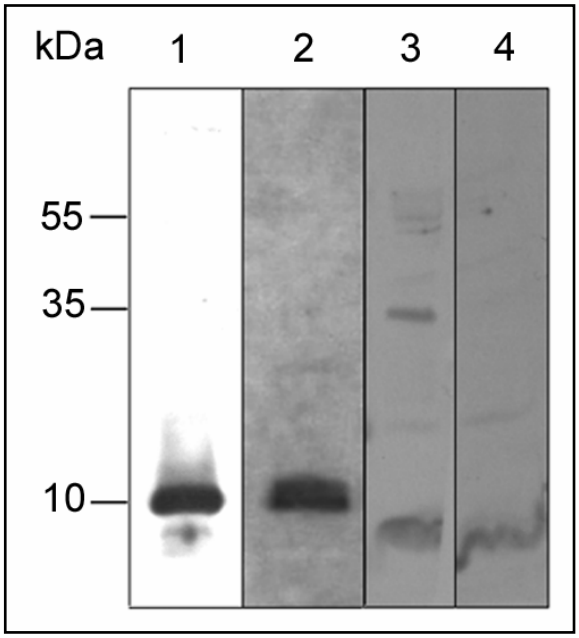

Figure 9. Western blot analysis of proteins obtained by organic solvent extract of whole *C. glutamicum* cells. The ether precipitates dissolved in 0.4 % LDAO were separated on a 10 % Tricin SDS-PAGE and blotted on a nitrocellulose membrane. Proteins were visualized using anti-PorB$_{C.glut}$ (lane 1) anti-PorC$_{C.glut}$ (lane 2), anti-PorA$_{C.glut}$ (lane 3) and anti-PorH$_{C.glut}$ (lane 4) antibodies and a chemoluminescence reaction.

3.5 Discussion

3.5.1 The cell wall of *C. glutamicum* contains another cation-selective channel beside PorA$_{C.glut}$

PorA$_{C.glut}$ was the first pore-forming protein from the cell wall of *C. glutamicum* that was investigated in detail (Lichtinger *et al.*, 1998; 2001). It is a small 45 amino acids long polypeptide forming an oligomeric, cation-selective channel of very high ion permeability. The deletion of PorA$_{C.glut}$ allowed the discovery of another cell wall channel, PorB$_{C.glut}$, of much lower permeability that is anion-selective (Costa-Riu *et al.*, 2003a; 2003b). This result indicated that the cell wall of *C. glutamicum* contains several types of channels similar to the situation in the outer membrane of gram-negative bacteria (Benz, 2001) and also in the gram-positive *Rhodococcus equi*, which is likewise a member of the genus mycolata (Riess *et al.*, 2003). Here we were able to detect in organic solvent extracts of whole cells an additional cation-selective channel in the cell wall of *C. glutamicum*, PorH$_{C.glut}$. PorH$_{C.glut}$ seems to be particularly frequent when the cells are cultivated in minimal medium; it has an apparent molecular mass of 12 kDa on SDS-PAGE and forms in the lipid bilayer assay highly cation-selective channels with a single-channel conductance of about 2.5 nS in 1 M KCl. The

channel-forming protein was purified to homogeneity. Partial sequencing of the 12 kDa protein resulted in a stretch of 13 amino acids, which allowed the identification of the $porH_{C.glut}$ gene within the chromosome of *C. glutamicum* that codes for a 57 amino acid long polypeptide (molecular mass 6.1 kDa) without leader sequence at the N-terminus for protein sorting. This suggests that $PorH_{C.glut}$ is not transported via the *Sec*-apparatus out of the cell to reach the cell wall similar as in the case of $PorA_{C.glut}$ (Freudl, 1992; Lichtinger *et al.*, 2001).

The existence of two cation-selective channels of high permeability in the cell wall of *C. glutamicum* seems to be contradictory. So far the role of $PorH_{C.glut}$ and $PorH_{C.glut}$ is not clear. However, *E. coli* also exhibits two cation-selective porins and the expression of these major porins of *E. coli* is regulated through the OmpR-EnvZ system (Pratt *et al*., 1996; Walthers *et al.* 2004). Under high osmotic strength and high temperature conditions, OmpC is expressed and the OmpF expression is repressed whereas under low solute concentrations, OmpF is expressed. OmpF produces a slightly larger channel than OmpC, thus bigger substrates diffuse far better through the channel. In consequence of the increased production of OmpF under low-osmolarity conditions *E. coli* will benefit by facilitating the influx of scarce nutrients (Nikaido, 2003).
The situation in *C. glutamicum* is somewhat different because $PorH_{C.glut}$ and $PorA_{C.glut}$ seem to be cotranscribed and coexpressed, which means that they are coexisting in the cell wall. This has only qualitative significance. The quantitative ratio of the porins in the cell wall is not known and may change dependent on unknown regulatory mechanisms in *C. glutamicum* and growth conditions. It is conceivable, that the translation of the two porins and their channel-forming activity is linked to the modification of the proteins. For this we have to keep in mind that synthetically produced $PorH_{C.glut}$ possesses only very low pore-forming activity (see Chapter 4).

3.5.2 The $porH_{C.glut}$ and $porA_{C.glut}$ genes belong to the same putative operon

Interestingly, $porH_{C.glut}$ can be found next to $porA_{C.glut}$ in the *C. glutamicum* chromosome, both genes are separated by only 83 bp (see Figure 5). The results of the RT-PCR experiments suggests that they are cotranscribed and the immunological detection experiments present evidence, that the proteins are also expressed together. Another interesting result of the RT-RCR experiments was, that $porA_{C.glut}$ and $porH_{C.glut}$ could be part of a gene cluster comprising

13 genes although putative transcriptional terminators may be localized within the cluster (see Figure 5 and Figure 6). It is possible that there exist different transcripts, one may correspond to the entire operon, and others related to the cluster containing only the *groEL2*, $porA_{C.glut}$ and $porH_{C.glut}$ genes, terminated at the stem loop structures. Barreiro *et al.* (2004) demonstrated in Northern blot analysis that there exists in *C. glutamicum* a *groEL2* transcript of 1.8 kb that corresponds to the *groEL2* open reading frame of 1647 nucleotides. The *groEL2* promoter is heat shock inducible because it can be induced by a temperature shift from 30 °C to 40 °C (Barreiro *et al*., 2004). This result provides some indication for another promoter in front of the porin genes. This stretch contains also a potential ribosome-binding site with the sequence AGGAG. Further investigation is needed to test which genes of this cluster are transcribed together into a single mRNA. Northern blot analysis with specific probes against $porA_{C.glut}$, $porH_{C.glut}$, *groEL2* and the other gene transcripts of this putative cluster could provide more information about such a possibility.

When the export of $PorH_{C.glut}$ and $PorA_{C.glut}$ to the cell wall is considered, it is possible that a similar export mechanism may exist in *C. glutamicum* as has been reported for the ESAT-6/CFP10 gene families of *Mycobacterium tuberculosis* H37Rv (Pym *et al*., 2003). ESAT-6 and CFP10 are low molecular weight antigens that also lack signal peptides, but are transported to the cell surface. The genes encoding the ESAT-6 and CFP10 proteins lie in a cluster of 12 other genes. Among them are genes coding for different proteins that could be involved in export, such as putative ABC transporters, ATP-binding proteins, subtilisin-like membrane-anchored cell-wall-associated serine proteases and other amino-terminal membrane-associated proteins. Pym *et al.* (2003) provide evidence that the flanking genes are required for the secretion of the antigens. The genome of *M. tuberculosis* H37Rv exhibits five copies of this cluster and this cluster is also conserved in other mycobacteria like in the genome of *Corynebacterium diphteriae*. It is possible that these membrane associated and energy-providing proteins may function to secrete members of the ESAT-6/CFP10 protein families, and the proteases may be involved in the procession the secreted peptides (Gey van Pittius *et al*., 2001). Lichtinger *et al.* (2001) described a modification for the serine at position 15 of $PorA_{C.glut}$, which could fit in a similar export mechanism. Synthetic $PorA_{C.glut}$ has almost no channel-forming activity (Costa-Riu *et al.*, 2003a). Interestingly, we found also some evidence for a modification of $PorH_{C.glut}$ too. $PorH_{C.glut}$ synthesized by solid phase synthesis shows only low pore-forming activity in black lipid bilayer experiments (results not shown), which suggests that the mature protein could be modified. The possible functional similarity

of the ESAT-6 gene cluster with the $PorH_{C.glut}$, $PorA_{C.glut}$ gene cluster is up to now only hypothetical, but it cannot be excluded that a similar way of protein export may exist. The putative gene cluster in *C. glutamicum* consists of totally 13 genes. $PorA_{C.glut}$, $PorH_{C.glut}$, GroEL2, a putative RNA polymerase sigma factor and a putative aldehyde dehydrogenase are known from the proteins coded by these genes, but the others remain still unknown.

3.5.3 Arrangement of $PorH_{C.glut}$ in the cell wall

$PorH_{C.glut}$ has a rather small molecular mass of about 6 kDa similar to that of $PorA_{C.glut}$ or $PorB_{C.glut}$. In general, the molecular masses of corynebacterial cell wall porins are rather small as compared to those of Gram-negative bacterial porins, which range between 30 and 60 kDa (Benz, 1994; Lichtinger *et al*., 1998; Costa-Riu *et al.*, 2003b). This suggests that the cell wall channels are formed by oligomers. A possibly hexameric form of $PorH_{C.glut}$ was found in Western blot analysis with anti-$PorH_{C.glut}$ antibodies (see Figure 7). Secondary structure predictions for $PorH_{C.glut}$ suggest that a stretch of about 42 amino acids forms amphipathic α-helices with about 12 windings and a total length of 6.3 nm (see Figure 10). This should be sufficient to cross the mycolic acid layer. The arrangement of $PorH_{C.glut}$ in the putative oligomer is such that all hydrophilic amino acids are localized on one side of the helix and all hydrophobic ones on the other side. It is noteworthy that this agrees nicely with the selectivity of the channel, because the charged amino acids are located to the channel lumen (see below).

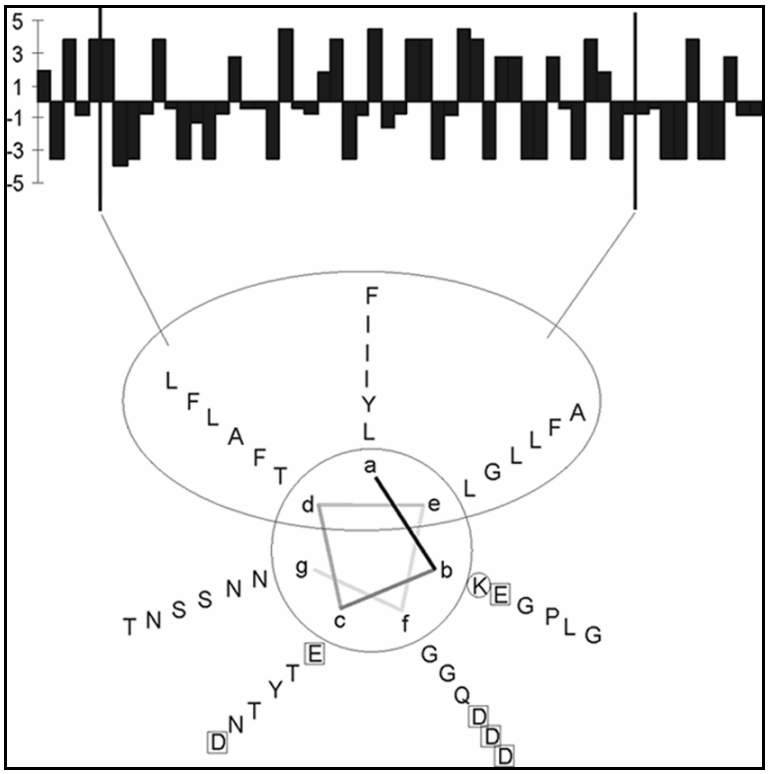

Figure 10. Scheme of the PorH$_{C.glut}$ secondary structure. The upper panel shows hydrophobicity indices of the individual amino acids according to Kyte and Doolittle (1982). The molecule can form, according to secondary structure predictions, α-helices with 12 windings corresponding to an overall length of 6.3 nm. Amino acid residues of the heptameric repeats were labeled in the sequence as a-g. The hydrophobic residues are located at position a, e and d, (presumably oriented towards the mycolic acids; indicated by the oval ring). The hydrophilic residues are localized at the positions b, f, c and g and may face the channel lumen, positive residues are encircled, negative residues are framed.

It is noteworthy that the possible α-helical arrangement of PorH$_{C.glut}$ does not agree with the 3D-structure of the cation-selective cell wall channel of *M. smegmatis*, which is formed by an MspA octamer (Faller *et al.*, 2004). MspA monomers (molecular mass of about 20 kDa; Niederweis *et al.*, 1999) contain in the mycolic acid layer spanning part ß-sheet structure similar to the structure of Gram-negative bacterial porins (Benz, 2001). On the other hand, the latter porins form trimers with three individual channels, whereas MspA forms an octamer with only one central channel. It is possible that the arrangement of PorH$_{C.glut}$ has to do with the thickness of the cell wall and the length of the mycolic acids of different mycolata. Mycobacteria contain rather long mycolic acids (60-90 carbon atoms; Minnikin 1987; 1991; Daffé *et al.*, 1990; Ochi, 1995; Brennan & Nikaido 1995), whereas they are short in Corynebacteria (22-38 carbon atoms; Yague *et al.;* 2000, Ioneda, 1993). On the other hand, it is also possible that the arrangement of PorH$_{C.glut}$ has to do with its biosynthetic pathway and

its export to the cell wall. Export and assembly of the cell wall channels of Corynebacteria are not well understood and further investigation of the proteins are necessary to understand their structure and function.

3.5.4 Effects of negative point net charges on the channel properties of PorH$_{C.glut}$

Possible arrangement of PorH$_{C.glut}$ in the cell wall and the data of Table 2 suggest, that point charges influence the conductance of the PorH$_{C.glut}$ channel because the single-channel conductance of PorH$_{C.glut}$ is not a linear function of the bulk aqueous concentration. This means, that the cation specificity of PorH$_{C.glut}$ is caused by negative point charges and not by a binding site, which has been demonstrated in a number of studies (Menestrina & Antolini, 1981; Benz *et al.*, 1989; Benz, 1994) including mycobacterial porins and PorA$_{C.glut}$ of *C. glutamicum* (Trias & Benz, 1993; 1994; Lichtinger *et al.*, 1998). A quantitative description of the effect of point charges on the single-channel conductance may be given with the considerations of Nelson and McQuarrie (Nelson & McQuarrie, 1975), as previously described (Trias & Benz, 1993; Costa-Riu *et al.*, 2003b). A best fit of the data of Table 2 was obtained by assuming that 2 negative point charges (q = -3.2×10^{-19} A·s) are attached to the channel mouth and if the channel has a diameter of about 2.2 nm. The results of this fit are shown in Figure 11.

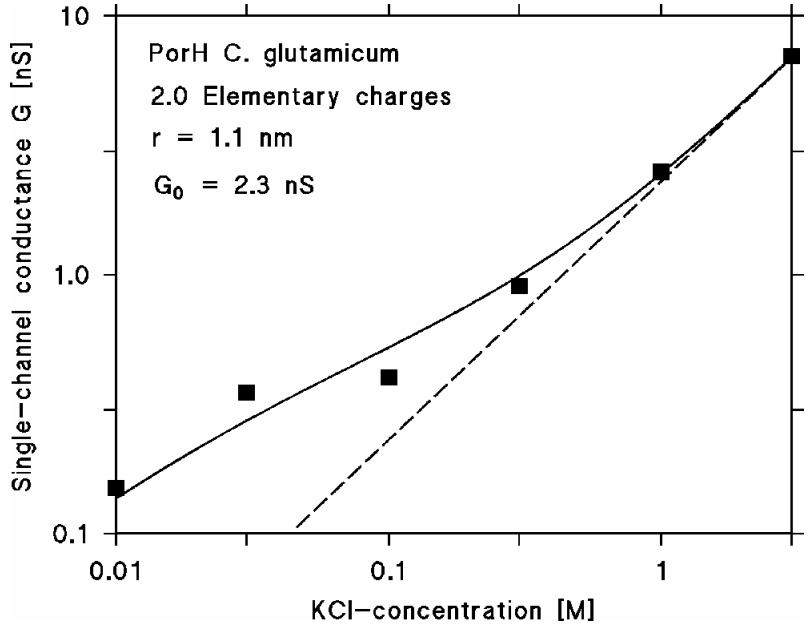

Figure 11. Single-channel conductance of $PorH_{C.glut}$ of *C. glutamicum* as a function the KCl-concentration in the aqueous phase (full squares). The solid line represents the fit of the single-channel conductance data (see Costa-Riu *et al.*, 2003b for details) assuming the presence of negative point charges (2.0 negative charges; q = -3.2 x 10^{-19} A s) at the channel mouth on both sides of the membrane and assuming a channel diameter of 2.2 nm (radius r = 1.1 nm). c, concentration of the KCl-solution in M (molar); G, average single-channel conductance in nS. The broken (straight) line shows the single-channel conductance of $PorH_{C.glut}$ without the effect of point charges and corresponds to a linear function between channel conductance and bulk aqueous concentration.

The solid line represents the fit of the single-channel conductance versus concentration by using the Nelson and McQuarrie (1975) treatment and the parameters mentioned above together with a single-channel conductance, G_0 = 2.3 nS at 1 M salt. The broken line corresponds to the single-channel conductance of the cell wall channel without point net charges, i.e. it shows a linear relationship between the cation concentration in the aqueous phase and single-channel conductance.

CHAPTER 4

Heterologous expression of corynebacterial channel-forming proteins

4.1 Summary

The cell wall of the Gram-positive bacterium *Corynebacterium glutamicum* contains the cation-selective porins $PorA_{C.glut}$ and $PorH_{C.glut}$ as well as the anion-selective channel $PorB_{C.glut}$ for the passage of hydrophilic solutes. In *C. efficiens* the $PorH_{C.glut}$ homologue porin $PorH_{C.eff}$ was identified and characterized in this work (see Chapter2).

Multiple sequence alignments search within the known chromosomes of *C. glutamicum* and *C. efficiens* demonstrated that both organisms contain the genes for PorA, PorH and PorB. Further search for homologous genes showed, that the chromosome of *C. glutamicum* contains also another gene for a PorB-like protein 138 bp downstream from *porB*, this protein is called $PorC_{C.glut}$. The arrangement of *porB* and *porC* as well as *porA* and *porH* suggested that both genes belong to the same cluster.

The aim of this work was to establish an heterologous expression system for corynebacterial channel forming proteins. We could demonstrate with recombinant expression experiments in *E. coli* that $porB_{C.eff}$ and $porC_{C.eff}$ encode for channel forming proteins. They are, like $PorB_{C.glut}$, anion selective with a similar single-channel conductance of 1 nS in 1 M KCl. A more precise characterization of the channel forming properties was not accomplishable because of the low channel forming activity of the heterologous expressed porins. Lipid bilayer experiments with $PorH_{C.glut}$ synthesized by solid phase synthesis were in accordance with this results, only low channel-forming activity was observed. It is conjecturable that the recombinant, and the synthetic porin respectively, are not right folded, perhaps because of a missing translational modification, that is not proceeded in *E. coli*.

4.2 Introduction

4.2.1 Cell wall channels of mycolic-acid containing actinomycetes

A part of the actinomycetes of particular interest is called the mycolata. That is a broad and diverse group of actinomycetes and encloses, the genera *Rhodococcus, Gordona, Tsukamurella, Dietzia, Mycobacterium, Nocardia* and a part from the genera *Corynebacterium*. The relationship between these genera is further established by the chemical analysis of the cell wall and lipid structures (Minnikin *et al.,* 1978; Daffé & Drapper, 1998; Minnikin & Goodfellow, 1980; Minnikin *et al*., 1984; Sutcliffe, 1997; Puech *et al.,* 2001).

The mycolic acid layer on the surface of mycolata represents a permeability barrier (Jarlier & Nikaido, 1990, Brennan & Nikaido, 1995) so that water-filled channels will be needed to allow the permeation of hydrophilic solutes. In agreement with this, channels have been identified in the cell wall of mycolata. Till present the channel-forming proteins of the organisms listed in Table 1 are identified and characterized. Among the displayed microorganisms are ones that cause dangerous infections like *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae* and such that are potent producers of amino acids on industrial scale like *C. glutamicum, C. callunae* and *C. efficiens*. *M. smegmatis* is used as model-system for the research on pathogenisis of tuberculosis. These channels are wide, water-filled and contain point charges, which results in selectivity of the cell wall channels. Our observations could mean that cell wall porins may be present in all members of the mycolata. Whereas some cell wall channels have been characterised on functional basis by reconstitution experiments in lipid bilayer membranes, relatively little is known about the proteins forming the channels.

Cell wall porin; MW monomer/oligomer	**G [nS]** in 1 M KCl	**Selectivity** P_c/P_a in KCl 0.05/3 M[b]	**Point charges** at the channel mouth	**Channel Diameter** [nm]	**Reference**
C. glutamicum $PorA_{C.glut}$; 5 kDa/n.d.	5.50	8.10	-2.0	2.2 [1,2]	Lichtinger *et al.*, 1998
C. glutamicum $PorB_{C.glut;}$ 10 kDa/n.d.	0,7	0,12	+1.5	1.4 [1]	Costa-Riu *et al.*, 2003b
C. glutamicum $PorH_{C.glut}$; 6 kDa/36 kDa	2.50	5.10	-2	2.2 [2]	Hünten *et al.*, 2005 this work
C. efficiens $PorH_{C.eff}$; 6 kDa/n.d.	2.25	0.7	n.d.	n.d.	Hünten *et al.*, 2005 this work
C. callunae $PorH_{C.call}$; 6 kDa/n.d.	3.0	7.1	-1.6	2.2 [2]	Hünten *et al.*, 2005 this work
C. diphteriae 6 kDa/67 kDa	2.2	1.2	n.d.	n.d.	unpublished data
M. chelonae; n.d./59 kD	2,7	6.3	-2.5	2.0 [1] 2.2 [a]	Trias and Benz, 1992; 1993
M. smegmatis MspA; 20 kDa/100 kDa	4.1	9.7	-4.0	1.8 [1] 3.0 [2]	Trias and Benz, 1994 Niederweis *et al.*, 1999

M. bovis BCG PorA*M.bo*; n.d./100 kDa PorB*M.bo*; 10 kDa/60 kDa	4.30 0.78	>1 <1	n.d. n.d.	n.d. n.d.	Lichtinger *et al.*, 1999
M. phlei MppA; 22 kDa/135 kDa	4.5	14.9	-2.2	1.8 [1] 2.0 [2]	Rieß *et al.*, 2001 Dörner *et al*., 2004
N. farcinica; 20 kDa/87 kDa	3.0	8.2	-1.3	1.4 [1] 1.6 [2]	Rieß *et al.*, 1998
N. asteroides n.d./84 kDa	3.0	20.0	-1.0	1.3 [1]	Rieß *et al.*, 1999
N. corynebacteroides; 23 kDa/134 kDa	5.50	3.80	-2.7	2.0 [1] 2.2 [2]	Rieß and Benz, 2000
R. erythropolis; 8 kDa/67 kDa	6.00	11.80	-2.7	2.0 [1,2]	Lichtinger *et al.*, 2001
R. equi PorA*R.eq;* 67 kDa PorB*R.eq;* 11 kDa	4.00 0.30	9.0 0.16	-1.5 +1.5	1.8 [1] 2.0 [2] 1.4 [1,2]	Rieß *et al.*, 2003
T. inchonensis 33 kDa/n.d.	4.5	4.0	-2.2	2.0 [1]	Dörner *et al*., 2004

Table 1. Comparison of the channel properties of the cell wall channels from the mycolata that are known to date**.** **a**The channel diameters were estimated from liposome swelling assay. The channel diameters were estimated from the single-channel conductance as a function of the hydrated ion radii[1] or the effect of negative point charges on single-channel conductance[2]; **b** concentration from the beginning and at the end of the experiment; n.d. not determined.

MspA, found in *M. smegmatis* (Niederweis *et al*., 1999), is the best characterized and analysed outer membrane protein of *Corynebacterineae*. The protein was cloned and the *mspA* gene sequenced. It is encoding a 184 amino acid protein with an N-terminal signal sequence. MALDI mass spectrometry of the purified porin revealed a mass of 19 406 Da, in agreement with the predicted mass of mature MspA. Three important drugs, isoniazide,

ethambutol, and pyrazinamide can easily permeate the channel MspA which leads to the assumption, that porins influence the drug efficiency (Lambert, 2002). A general cylindrical shape of MspA was established by electron microscopy (Engelhardt *et al.*, 2002). The atomic structure of this protein shows a β-structure (Faller *et al*., 2004) that differs completely from its counterparts in Gram-negative bacteria (Weiss *et al*., 1990; Schulz, 2002). The porin represents a tightly interconnected octamer with eightfold rotation symmetry. It is described as a goblet consisting of a thick rim at the top, a stem consisting of the wide β-barrel, and a base at the bottom. There is no significant sequence similarity between MspA and other known porins from different *Corynebacterineae*.

In the cell wall of the fast growing mycobacterium, *M. phlei* and in the slow growing *Tsukamurella inchonensis* channel forming proteins were isolated and characterised with similar properties to MspA (Rieß *et al*., 2001; Dörner *et al*., 2004). The genes of both cell wall channels were cloned and sequenced. The somewhat surprising result was that the two genes *tip*A and *mpp*A coding for the cell wall channels were found to be identical to *msp*A of *M. smegmatis*. This means that the major cell wall channel of *T. inchonensis* and *M. phlei* are identical to MspA, which has previously been identified in *M. smegmatis* (Trias & Benz, 1994; Niederweis *et al*., 1999). This means that the *msp*A gene appears to be specific for mycolata with long mycolic acids as has already been discussed by Niederweis *et al*. (1999) and Riess *et al*. (2001). This has already been suggested by the observation that chromosomal DNA of other mycolata, such as *T. inchonensis* or *Nocardia farcinica* contains genes that are very closely related to *msp*A of *M. smegmatis* (Riess *et al.*, 2001).

Channel-forming proteins have also been identified in *C. glutamicum* (Lichtinger *et al*., 1998; Costa-Riu *et al*., 2003b), *C. efficiens* and *C. callunae* (see Chapter 2). In this case the channel subunits have a molecular mass of about 5-10 kDa, and channel-forming oligomers have been observed for $PorH_{C.glut}$ of *C. glutamicum* (see Chapter 3). Since these values are unusually small for a transmembrane channel this argues for an oligomerisation. The proteins were cloned and their genes sequenced. The sequences of the cation selective porins $PorA_{C.glut}$, $PorH_{C.glut,}$ and $PorH_{C.eff}$ are not containing any signal peptide that allows the mature proteins to be exported by the Sec-system. This means that the export of the proteins over the cytoplasmic membrane and the insertion in the cell wall follows an other way as it takes place in *M. smegmatis*. Whereas the anion selective $PorB_{C.glut}$ protein exhibits an N-terminal extension of 27 amino acids, and is, as a result, exported by the Sec system. While sequencing the $PorA_{C.glut}$ protein problems in detecting position 15 assumed, only the phenylthiohydantoin derivate of dehydroalanine was determined (Lichtinger *et al*., 2001).

The knowledge of the primary sequence of PorA$_{C.glut}$, PorH$_{C.glut,}$, PorH$_{C.call}$ and PorH$_{C.eff}$ allowed also secondary structure predictions for the proteins (Lichtinger *et al*., 2001; Chapter 2 and 3). The search for α-helical structures and for amphipathic β-strands suggested that both are possible for PorA$_{C.glut}$. Amino acids 9 to 37 can form a transmembrane helix and amino acids 8 to 15 and 27 to 35 can form amphipathic β-strands. But they are probably not long enough to cross the mycolic acid layer of *C. glutamicum*, because its thickness is 6.2 ± 1.7 nm according to electron microscopy analysis (Marienfeld *et al*., 1997). So far it is not known how many PorA$_{C.glut}$ monomers are needed to form a cylinder with an inner diameter of 2.2 nm (Lichtinger *et al*., 1998), but in correlation to Gram-negative bacterial porins about 16 monomers would be sufficient to form such a channel. Secondary structure predictions for PorH$_{C.glut,}$, PorH$_{C.call}$ and PorH$_{C.eff}$ suggest that part of the proteins forms amphipathic α-helices with a length sufficient to cross the mycolic acid layer (see Chapter 2 and 3).

PorA$_{C.glut}$, PorH$_{C.glut}$ and PorB$_{C.glut}$ from *C. glutamicum* show no homology to the first cloned mycobacterial cell wall channel OmpATb from *M. tuberculosis* (Senaratne *et al*., 1998) or the subunit MspA of the cell wall channel from *M. smegmatis* (Niederweis *et al*., 1999). There the cell wall channel proteins have considerably higher molecular masses than PorA$_{C.glut}$, PorH$_{C.glut}$ and PorB$_{C.glut}$. Furthermore, also no homology exists to the partial amino acid sequence of the subunit of the cell wall channel of *R. erythropolis* (Lichtinger *et al*., 2000), although this protein has a similarly small molecular mass as PorA$_{C.glut}$ and the thickness of the mycolic acid layers of both bacteria are also very similar as judged from the length of their mycolic acids.

Multiple sequence alignments search within the known chromosomes of *C. glutamicum* and *C. efficiens* demonstrated that both organisms contain the genes for PorA, PorH and PorB. Further search for homologous genes showed that the chromosome of *C. glutamicum* contained also another gene for a PorB$_{C.glut}$-like protein 138 bp downstream from *porB*, this protein is called PorC$_{C.glut}$ (see Figure 2; Costa-Riu *et al*., 2003b). The arrangement of *porB* and *porC* as well as *porA* and *porH* suggested that both genes belong to the same cluster . An alignment of the genes is shown in Figure 1 and 2.

```
C.glut.       TTTCTTTG CTGGTCCTCT CAAGGAGATT TCTCATGGAT CTTTCCCTTC TCAAG
C.eff.        CTTATTTG CTGGTCCTCT CTAGGAGATT TCTCATGGAT CTTTCCCTTC TCAAG

C.glut.     GAAACCCTCG GCAACTACGA GACCTTCGGT GGCAACATCG GTACCGCTCT TCAGA
C.eff.      GACTCCCTGT CCGACTTCGC TACCCTCGGC AAGAACCTGG GACCTGCTCT CCAGG

C.glut.     GCATCCCAAC CCTGCTCGAT TCCATCCTTA ACTTCTTCGA CAACTTCGGA GATCT
C.eff.      GCATCCCGAC CCTGCTGAAC AGCATCATCG CCTTCTTCCA GAACTTCGGT GACCT
```

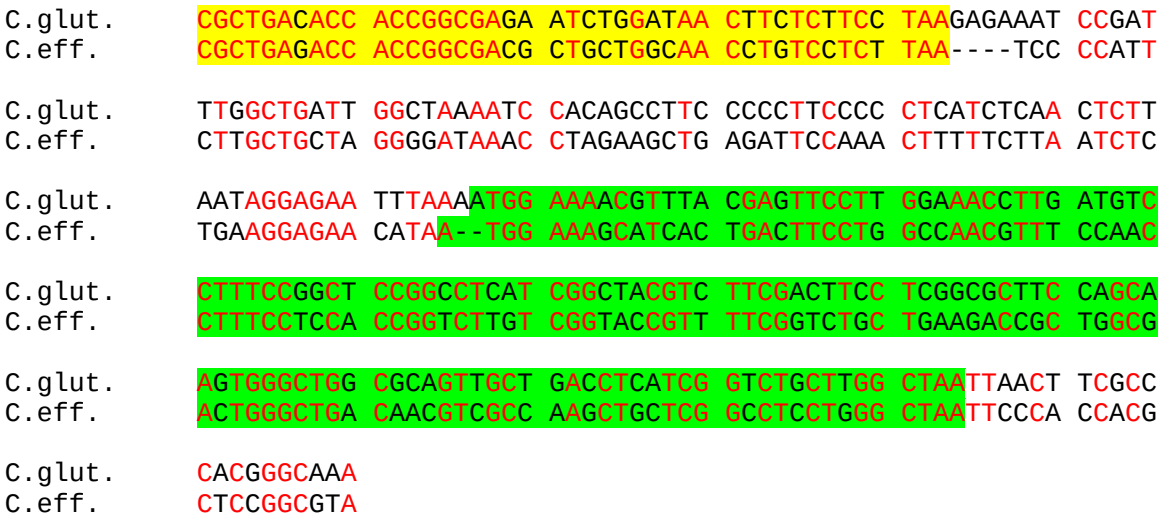

Figure 1. Alignment of the *porH* (shown in yellow) and *porA* (shown in green) gene cluster of *C. glutamicum* (NCBI Reference Sequence accession number NC_003450, region 2887853-2888368) and *C. efficiens* (NCBI Reference Sequence accession number NC_004369, region 2727449-2727840). The NCBI accession numbers of the protein genes are: $porA_{C.glut.}$: AJ238703; $porH_{C.glut.}$: AJ871585; $porA_{C.eff.}$ is up to now not listed; $porH_{C.eff.}$: AJ871586; Homologues are shown in red.

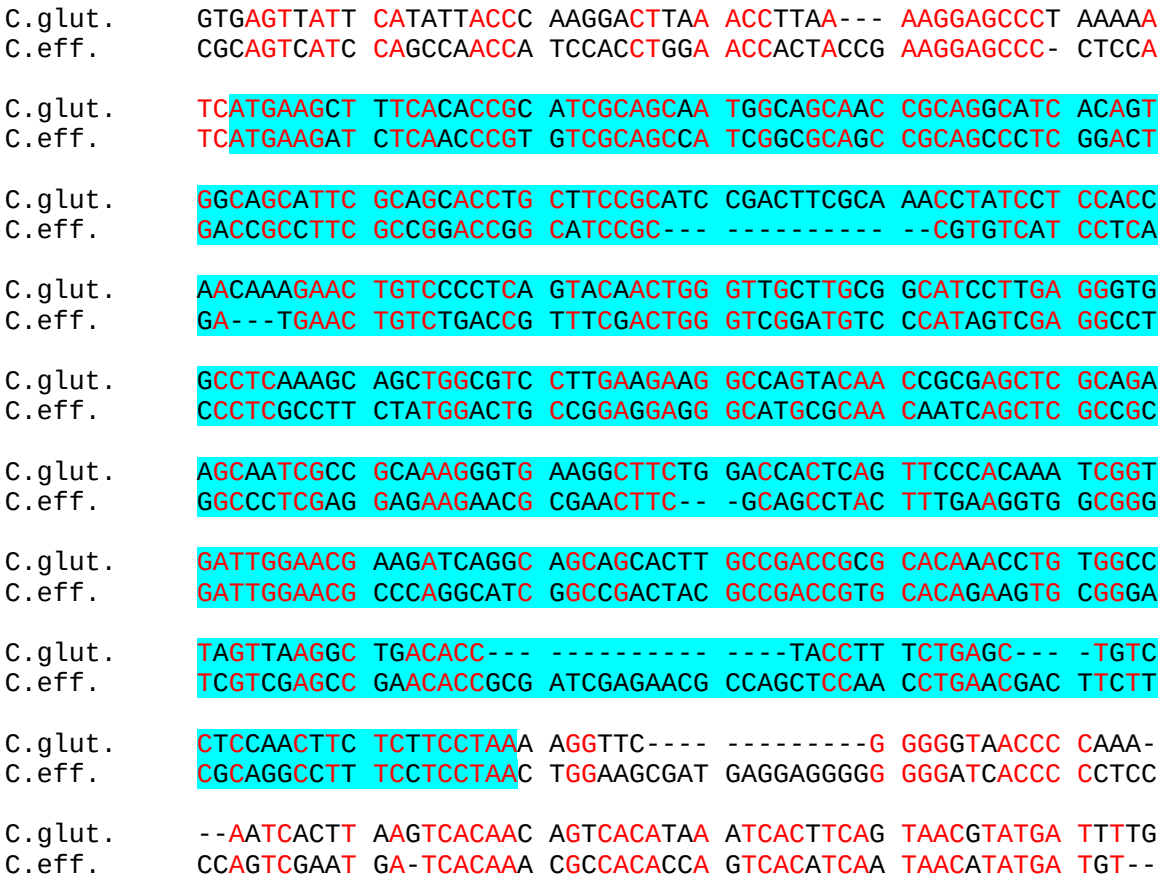

Figure 2. Alignment of the *porB* (shown in blue) and *porC* (shown in grey) gene cluster of *C. glutamicum* (NCBI Reference Sequence accession number NC_003450, region 1032726-1033619) and *C. efficiens* (NCBI Reference Sequence accession number NC_004369, region 1099197-1100062). The NCBI accession numbers of the protein genes are: $porB_{C.glut.}$: BK001251; $porC_{C.glut.}$: BK001268; $porB_{C.eff.}$: AJ555471; $porC_{C.eff.}$: AJ555472; Homologues are shown in red.

The aim of this work was to establish an heterologous expression system for corynebacterial channel forming proteins. It would provide the opportunity to investigate the channel forming properties of the up to now only hypothetical porins $PorA_{C.eff}$, $PorB_{C.eff}$, $PorC_{C.eff}$ from *C. efficiens* and $PorC_{C.glut}$ from *C. glutamicum*. On the basis of the homologies, identifiable in Figure 1 and 2, it may be expected that the homologue genes of the *C. glutamicum* porins vote also for pore-forming proteins in *C. efficiens*. Furthermore structural analyses would be possible by the construction and expression of point mutants, or if high amounts of the porins are producible, by X-ray structure analysis.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions

E. coli Top 10F' (Invitrogen, Karlsruhe, Germany) were used for all cloning experiments. For expression of the recombinant porins *E. coli* BL21(DE3)Omp8 and *E. coli* Top10F' (Invitrogen, Karlsruhe, Germany) cells were used. These strains were routinely grown in LB (Luria Bertani) medium at 37 °C. Ampicillin was used when required at a concentration of 25 µg/ml.

4.3.2 Construction of the expression vector

The porin gene sequences were obtained by polymerase chain reaction (PCR) with the corresponding primers (see Table 2) using a proof reading polymerase, Easy-A (Stratagene, Heidelberg, Germany). The assembling reaction was carried out in a volume of 50 µl using the following temperature profile: 3 minutes denaturation at 95 °C followed by 35 cycles 1 minute 95 °C, 1 minute annealing at T_a, 2 minutes elongation at 68 °C completed with 10 minutes extension at 68 °C. The annealing temperature T_a is different for each primer-couple.

Oligonucleotide	**Position in the *C. efficiens* and *C. glutamicum* genome respectively**	**Sequence 5'- -3'**
$PorA_{C.eff}$ - fwd	2727543-2727578	ATGGAAAGCATCACTGACTTCCTGGCCAACGTTTCC
$PorA_{C.eff}$ - rev	2727441-2727470	TTAGCCCAGGAGGCCGAGCAGCTTGGCGAC
$PorB_{C.eff}$ - fwd	1100006-1100038	GTGTCATCCTCAGATGAACTGTCTGACCGTTTC
$PorB_{C.eff}$ - rev	1099739-1099766	TTAGGAGGAAAGGCCTGCGAAGAAGTCG
$PorC_{C.eff}$ - fwd	1099472-1099501	CAGAATGCGGACATCGTCAGCGGCATCAAC
$PorC_{C.eff}$ - rev	1099202-1099232	TTAGGAGGACAGGTTGCTGGAGAGGTTCTGC
$PorH_{C.eff}$ - fwd	2727806-2727829	ATGGATCTTTCCCTTCTCAAGGAC

PorH$_{C.eff}$ - rev	2727656-2727679	TTAAGAGGACAGGTTGCCAGCAGC
PorB$_{C.glut}$ - fwd	1033536-1033565	TCCGACTTCGCAAACCTATCCTCCACCAAC
PorB$_{C.glut}$ - rev	1033266-1033303	TTAGGAAGAGAAGTTGGAGGACAGCTCAGAAAGGTAGG
PorC$_{C.glut}$ - fwd	1033030-1033050	CAGGATTTCAACCAAATCATC
PorC$_{C.glut}$ - rev	1032757-1032779	CTAAGCAGTGAAGAAGGAAGAAG
PorH$_{C.glut}$ - fwd	2888315-2888336	ATGGATCTTTCCCTTCTCAAGG
PorH$_{C.glut}$ - rev	2888163-2888184	TTAGGAAGAGAAGTTATCCAGA

Table 2. Sequences of the 7 couples of primers used for the amplification of the porin genes *porA, porB, porC* and *porH* from *C. glutamicum* and *C. efficiens*.

Two different plasmids were used for the following cloning of the genes. First the PCR products were cloned with the TOPO TA Cloning® Kit into the pCR2.1 vector (Invitrogen, Karlsruhe, Germany). Then these plasmids were used as a template for a second PCR with the following primers (see Table 3).

Oligonucleotide	**Sequence** **5'-** **-3'**
PorA$_{C.eff}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTATGGAAAGCATCACTGACTTCCTGGCCAACG
PorA$_{C.eff}$ - rev + *Eco*R1	GATATCTGCA***GAA TTC***GCCCTTTTAGCCCAGGAGG
PorB$_{C.eff}$ - fwd + *Bam*H1	GCTGGAAT**GGATCC**TTGTGTCATCCTCAGATGAACTGTCTGACCG
PorB$_{C.eff}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTTTAGGAGGAAAGGC
PorC$_{C.eff}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTCAGAATGCGGACATCGTCAGCGGCATC
PorC$_{C.eff}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTTTAGGAGGACAGGTTGC
PorH$_{C.eff}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTATGGATCTTTCCCTTCTCAAGGACTCCCTG

PorH$_{C.eff}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTTTAAGAGGACAGGTT G
PorB$_{C.glut}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTTCCGACTTCGCAAACCTATCCTCC ACCAAC
PorB$_{C.glut}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTTTAGGAAGAGAAGTTGG
PorC$_{C.glut}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTCAGGATTTCAACCAAATCATCGAC AAC
PorC$_{C.glut}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTCTAAGCAGTGAAG
PorH$_{C.glut}$ - fwd + *Bam*H1	GCTGGAAT***GGATCC***TTATGGATCTTTCCCTTCTCAAGGAA AC
PorH$_{C.glut}$ - rev + *Eco*R1	GATATCTGCA***GAATTC***GCCCTTTTAGGAAGAGAAGTTATC C

Table 3. Sequences of the 7 couples of primers used for the amplification of the porin genes *porA, porB, porC* and *porH* from *C. glutamicum* and *C. efficiens* with restriction sites (bold letters).

The primers provide *Bam*HI and *Eco*RI cloning sites (italic) for cloning the PCR products in frame with the glutathione *S*-transferase (GST) of the pGEX-3X (Amersham Pharmacia Biosciences, Freiburg, Germany) expression vector.

With the pGEX-3X vector, fusion proteins of the 26 kDa glutathione S-transferase and the respective porin are produced. The expression is under control of the *tac* promoter, which is induced by isopropyl β-D thiogalactoside (IPTG). In addition an internal *lac*I^q gene is engineered which encodes for a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG. The vector encodes a recognition sequence for a specific protease, Factor Xa (Amersham Pharmacia Biosciences, Freiburg, Germany), that allows the removal of the affinity tags from the synthesized proteins.

After digesting the PCR fragments and the pGEX-3X vector with the restriction enzymes *Bam*HI and *Eco*RI, the porin genes were ligated with T4DNA ligase (MBI Fermentas, St. Leon-Roth, Germany) at 16 °C overnight into the expression vector. The obtained product was transformed in *E. coli* Top 10F' cells and plated on LB agar plates containing ampicillin at a concentration of 25 µg/ml. Some of the grown colonies were picked, grown in LB

medium and the plasmid was isolated (Miniprep Kit, Qiagen, Hilden, Germany). The presence of correct inserts was confirmed by sequence analysis (Seqlab, Göttingen, Germany).

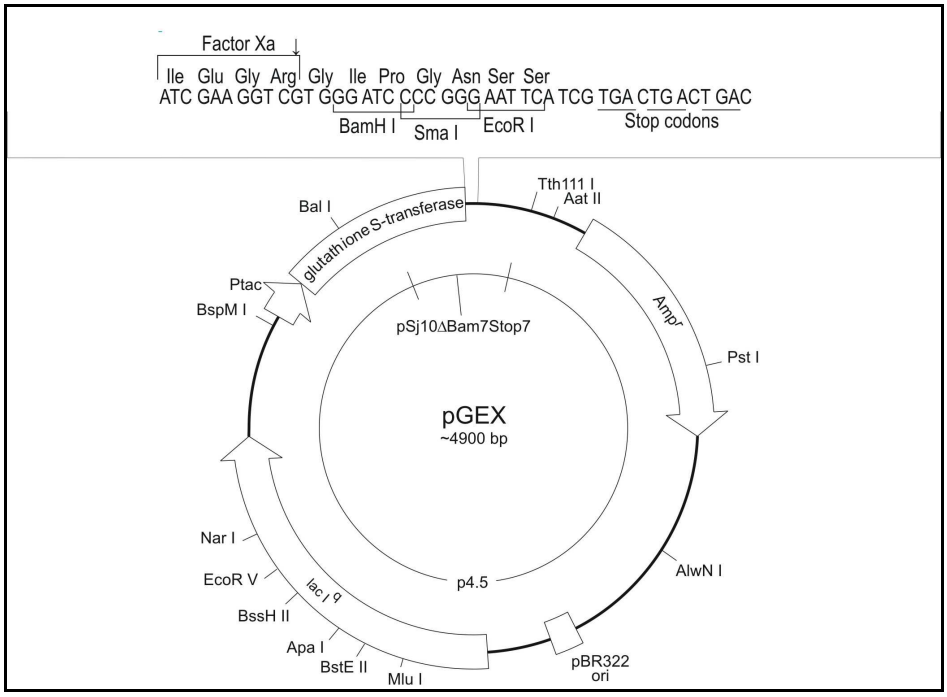

Figure 3. pGEX-3X expression vector (Amersham Pharmacia Biosciences, Freiburg, Germany). The fusion-proteins are transcribed from the *tac* promoter under control of the *lac* operator. The *lacI* gene encodes for the lactose repressor. The vector offers resistance to ampicillin.

4.3.3 Porin-expression in *E. coli*

E. coli BL21(DE3)Omp8 and *E. coli* Top10F' (Invitrogen, Karlsruhe, Germany) cells were transformed with the expression vector pGEX-3X + porin and grown in LB medium containing ampicillin at a concentration of 25 µg/ml. At OD_{600} of 0.8 the cells were induced with a final concentration of 0.1 mM isopropyl β-D-thiogalactoside (IPTG). At different times samples were withdrawn from the culture. After growing overnight at 30 °C the cells were harvested by centrifugation (6,000 rpm for 15 min in Beckman J2-21M/E centrifuge) and washed twice in 10 mM Tris-HCl (pH 8.0). The cell pellet was resuspended in 5 ml 10 mM Tris pH 8 and passed three times through a French pressure cell at 900 psi. Unbroken cells were removed by centrifugation at 12,000 rpm for 15 minutes. The supernatant contained the expressed GST-fusion protein.

4.3.4 Purification and cleavage of the GST-fusion protein

Two different purification methods were used to obtain the fusion protein. Either the GST-fusion protein containing supernatant was applied to fast protein liquid chromatography (FPLC) for loading onto a 1 ml GSTrap FF (Amersham Pharmacia Biosciences, Freiburg, Germany) column, or the fusion protein was cleaned by batch purification with Glutathione Sepharose 4B (Amersham Pharmacia Biosciences, Freiburg, Germany). The following buffers were used for both methods:

- Binding buffer: 1 × PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3)
- Elution buffer: 50 mM Tris HCL, 10 mM reduced gluthathione, pH 8

For column purification the following procedure was applied: The column was equilibrated with five column volumes of binding buffer, then 5 ml of the centrifuged supernatant (resulting from 200 ml cell culture) was loaded. Next the column was washed with binding buffer until no protein appeared in the flow-through, and finally the bound fusion protein was eluted with 5–10 volumes of elution buffer. The fractions containing the fusion protein were afterwards enzymatically cleaved with factor Xa protease, to remove the GST carrier protein from the fusion protein.

The following protocol was used for the batch purification: 1.5 ml of the Glutathione Sepharose medium was prepared by washing it 3 times with binding buffer. Then 10 ml supernatant (resulting from 200 ml cell culture) were added and incubated gently shaking 30 minutes at room temperature. The medium was washed again 5 times using binding buffer (1 % Genapol added), the supernatant was discarded. To proceed, the GST carrier protein must be removed from the fusion protein by enzymatic cleavage with factor Xa. The sepharose was washed before with factor Xa cleavage buffer (50 mM Tris HCL, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5), and then incubated for 2 hours at room temperature with 4 µl factor Xa protease added to 200 µl cleavage buffer. The protein of interest was found in the supernatant together with factor Xa. 20 µl of the supernatant were charged with loading buffer and incubated 5 minutes at 100 °C before gel electrophoresis.

Preparative SDS-PAGE was used for separation of the expressed porins, the GST-carrier protein and the factor Xa protease.

4.3.5 SDS-PAGE

SDS-PAGE was performed with tricine containing gels (Schägger & von Jagow, 1987). The gels were stained with Coomassie brilliant blue (Neuhoff *et al*., 1988) or silver (Blum *et al*., 1987). Before separation, the samples were all incubated for 5 minutes at 100 °C with loading buffer.

4.3.6 Immunological techniques

Western blots of the expressed protein samples using the Anti GST antibody (Amersham Pharmacia Biosciences, Freiburg, Germany) were performed following standard procedures (Towbin *et al.*, 1979). The blotting time was 5 minutes at 350 mA. The ECL Western Detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) was used to detect binding of the antibody according to the instructions of the manufacturer. The exposure time was 10 seconds.

4.3.7 Lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously (Benz *et al*., 1978; Benz, 2003). The experimental set up consisted of a Teflon cell with two water-filled compartments connected by a small circular hole. The hole had an area of about 0.4 mm^2. Membranes were formed across the hole using a 1 % solution of diphytanoyl phosphatidylcholine (PC; Avanti Polar Lipids, Alabaster, Ala.) dissolved in *n*-decane. The temperature was maintained at 20 $^{\circ}C$ during all experiments. All salts were obtained from Merck (Darmstadt, Germany, analytical grade). They were used unbuffered. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a home-made current-to-voltage converter made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder.

4.3.8 Cell wall protein extraction of *C. efficiens*

For the preparation of cell wall extracts from *C. efficiens* a method was used that has been previously devised for isolation and purification of $PorA_{C.glut}$ of *C. glutamicum* (Lichtinger *et al.*, 1999). This method uses the extraction of whole cells with organic solvents or detergents and avoids the substantial loss of material caused by sucrose density centrifugation of the cell envelope to separate the cytoplasmic membrane from the cell wall fraction. For this extraction 200 ml cell suspension was washed twice in 10 mM Tris-HCl (pH 8). The final pellet (5 ml) was extracted with a 1:2 mixture of chloroform:methanol in a proportion of 1 part cells and 5 to 8 parts chloroform/methanol. The duration of the extraction was about 3 h at room temperature under stirring in a closed tube to avoid loss of chloroform. Cells and chloroform/methanol solution were centrifuged for 15 minutes (10,000 rpm in Beckman LH20). The pellet (cells) was discarded. The supernatant contained the channel-forming activity. It was mixed in a ratio of 1 part supernatant to 9 parts ether and was kept over night at -20 °C. The precipitated protein was dissolved in a solution containing 0.4 % LDAO and 10 mM Tris-HCl (pH 8) and inspected for channel-forming activity.

4.3.9 Preparation of synthetic *C. glutamicum* PorH for the lipid bilayer assay

The PorH peptide of *C. glutamicum* synthesized by solid phase synthesis was produced by Prof. Dr. Palm (Rudolf-Virchow-Zentrum, Würzburg, Germany). For the lipid bilayer experiments, the peptide was treated in different conditions to allow the right folding of the protein and obtain channel forming. 1 mg was resuspended in:

- 1 M NaCl, 10 mM Tris and 0.4 % LDAO
- 1 M NaCl, 10 mM Tris, 1 % mycolic acids from *Mycobacterium tuberculosis* (Sigma Aldrich, Steinheim, Germany) and 0.4 % LDAO
- 1 M NaCl, 10 mM Tris and 0.4 % LDAO, incubated at 80 °C for 5 min

and stored at 4 °C over night before the measurements.

4.4 Results

4.4.1 Constructed expression vectors

Figure 4 displays the pGEX-3X expression vector with the inserted porin gene. The following porin genes have been cloned and transformed in *E. coli* BL21(DE3)Omp8 and *E. coli* Top10F' cells:

- ***C. glutamicum*:** pGEX-3X + *porH*
  pGEX-3X + *porB*
  pGEX-3X + *porC*
- ***C. efficiens*:** pGEX-3X + *porA*
  pGEX-3X + *porH*
  pGEX-3X + *porB*
  pGEX-3X + *porC*

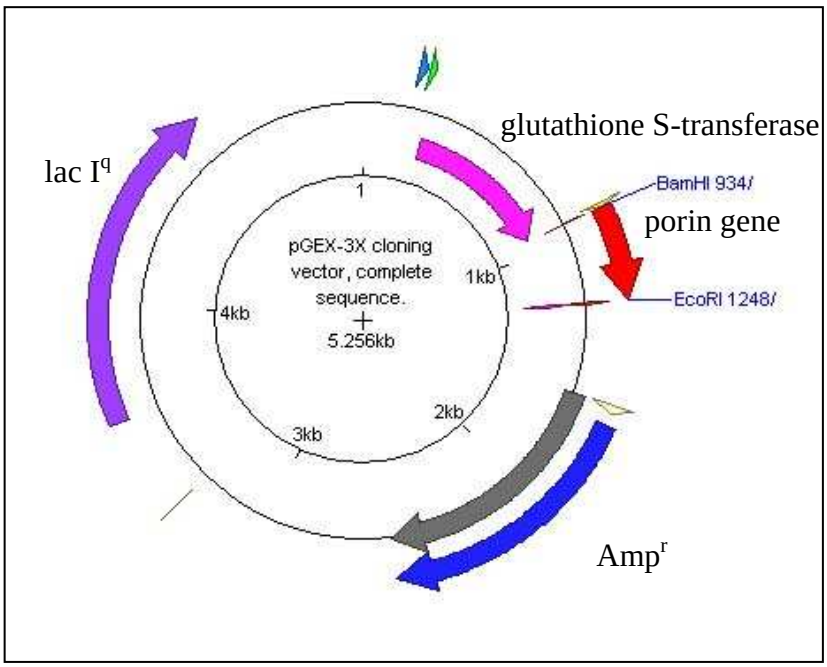

Figure 4. Map of glutathione S-transferase fusion vector pGEX-3X showing the main features and the inserted porin gene.

4.4.2 Expression analysis in *E. coli*

To express the porins from *C. glutamicum* and *C. efficiens* in *E. coli* the porin genes without signal sequence were cloned into the pGEX-3X expression vector. In order to see whether the porin expression took place, *E .coli* BL21(DE3)Omp8 and *E. coli* Top10F' (Invitrogen, Karlsruhe, Germany) cells containing the plasmid were induced with 0.1 mM IPTG at OD_{600} of 0.8 and harvested after growth over night at 30 °C. In Figure 5 the expression of $PorH_{C.eff}$ (A) and $PorC_{C.glut}$ (B) in *E. coli* Top10F' is demonstrated, the expression pattern of the other porins look similar (data not shown). To achieve similar amounts of protein, the cells were diluted with loading buffer, depending on the reached OD_{600}, and incubated at 100 °C for 5 minutes, to be analyzed by SDS page.

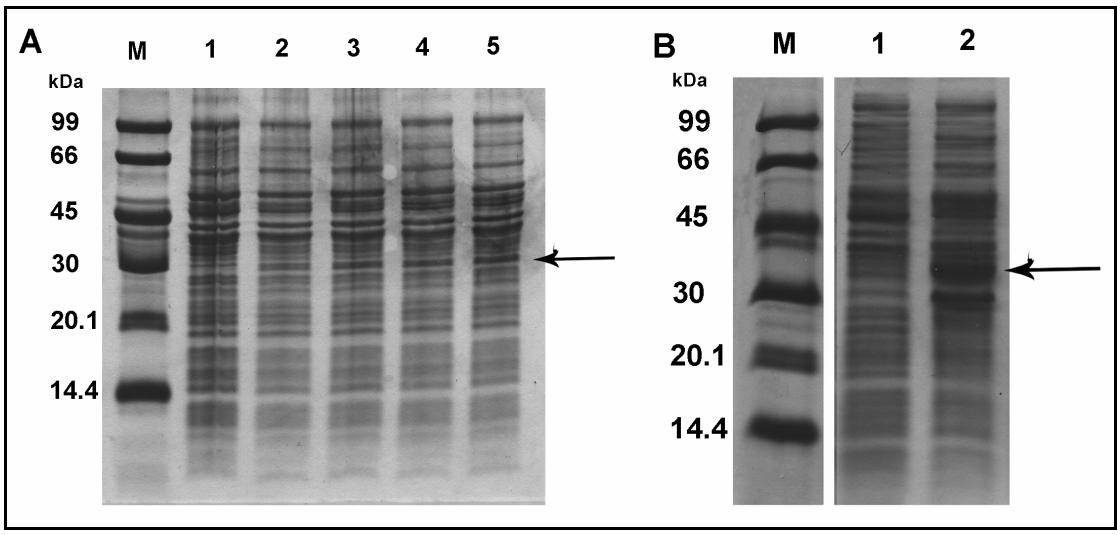

Figure 5. Tricine SDS PAGE demonstrating the expression of $PorH_{C.eff}$ and $PorC_{C.glut}$ in *E. coli* Top10F'. (A) 12 % Tricine SDS PAGE; Vector pGEX-3X + $porH_{C.eff}$, growth at 37 °C. Lane M: molecular mass marker; Lane 1: before induction; Lane 2: after 2 hours induction; Lane 3: after 3 hours induction; Lane 4: after 4 hours induction; Lane 5: after 5 hours induction; (B) 10 % Tricine SDS PAGE; Vector pGEX-3X + $porC_{C.glut}$. Lane M: molecular mass marker; Lane 1: before induction; Lane 2: induction over night at 30 °C. The gels are stained with Comassie blue G 250. Similar amounts of protein were loaded in all lanes.

The expected molecular weight for the fusion proteins are about 32 kDa for PorH–GST and about 36 kDa for the PorC-GST protein, this values become apparent in Figure 5.

4.4.3 Purification and cleavage of the GST-fusion protein

The method of fast protein liquid chromatography (FPLC) was applied first for the purification of the fusion proteins. The cells were disrupted by french press and the supernatant after centrifugation was loaded onto a 1 ml GSTrap FF (Amersham Biosciences, Freiburg, Germany) column. The fractions containing the fusion protein were likewise analysed by SDS PAGE, the results are demonstrated in Figure 6.

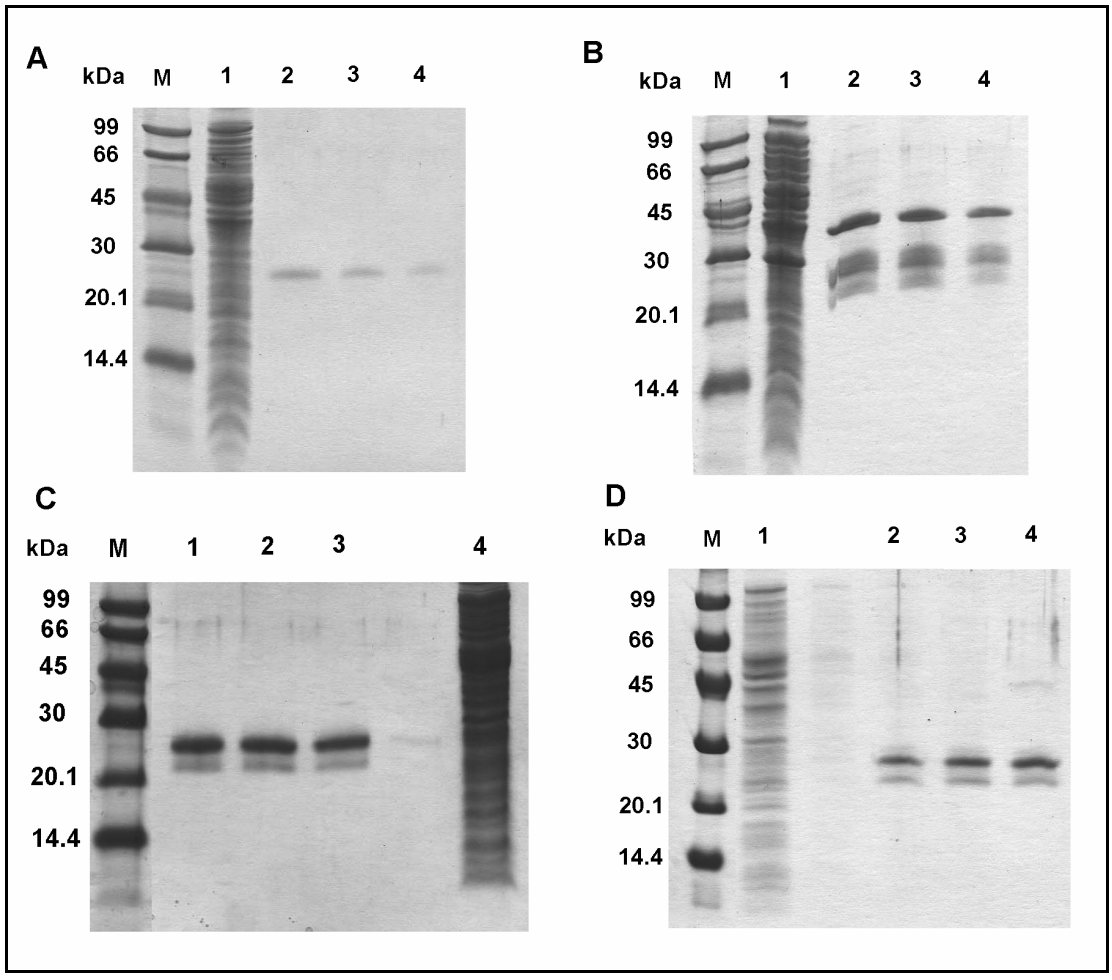

Figure 6. Tricine SDS PAGE illustrating the expression of $PorA_{C.eff}$, $PorB_{C.eff}$ $PorH_{C.eff}$ and $PorA_{C.glut}$ in *E. coli* Top10F' cells (exept C, in this experiment *E. coli* BL21(DE3)Omp8 cells were used) after FPLC purification with a 1 ml GSTrap FF column. To 15 µl of the fractions containing the fusion protein 5µl loading buffer were added, incubated 5 min at 100 °C. (A) 10 % Tricine SDS PAGE; Vector pGEX-3X + $porA_{C.eff}$, Lane M: molecular mass marker; Lane1: flow through; Lanes 2, 3, and 4: fractions containing PorA + GST; (B) 10 % Tricine SDS PAGE; Vector pGEX-3X + $porB_{C.eff}$. Lane M: molecular mass marker; Lane 1: flow through; Lanes 2, 3, and 4: fractions containing $PorB_{C.eff}$ + GST; (C) 12 % Tricine SDS PAGE; Vector pGEX-3X + $porH_{C.eff}$, Lane M: molecular mass marker; Lane 4: flow through; Lanes 1, 2, and 3: fractions containing $PorH_{C.eff}$ + GST;

(D) 10 % Tricine SDS PAGE; Vector pGEX-3X + *porH*$_{C.glut}$, Lane M: molecular mass marker; Lane 1: flow through; Lanes 2, 3, and 4: fractions containing PorH$_{C.glut}$ + GST; The gels are stained with Comassie blue G 250.

The presence of the expressed fusion proteins is readily identifiable. The molecular weight of the PorA- and PorH–GST fusion proteins is less than the PorB- and PorC–GST proteins, this is cognizable in Figure 6.

To remove the glutathione S-transferase the vector encodes a recognition sequence for a specific protease, Factor Xa, that allows the removal of the affinity tags from the synthesized proteins. Hence the FPLC fractions containing the fusion protein were cleaved with Factor Xa protease, shown in Figure 7 for PorH$_{C.eff}$ and PorH$_{C.glut}$. The expression was performed with *E. coli* Top10F' cells, and after disrupting the cells with the French pressure cell, the supernatant was subjected to FPLC using a 1 ml GSTrap FF column. 1µl Factor Xa was added to 100µl FPLC fraction and incubated at 37 °C for 5.3 hours and over night.

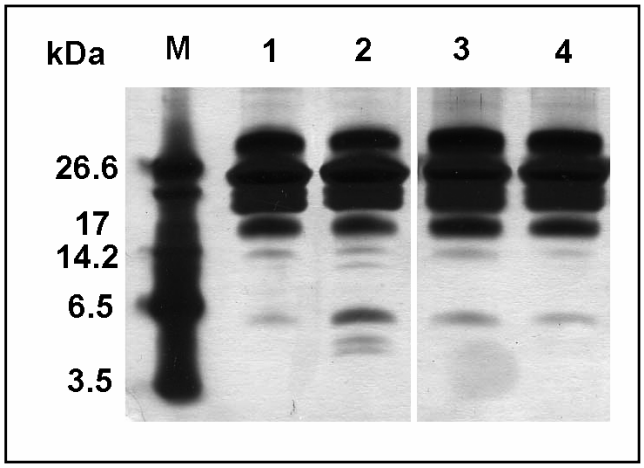

Figure 7. 10 % Tricine SDS PAGE displaying the cleavage with Factor Xa. Lane M: molecular mass marker; Lane 1: GST–PorH$_{C.eff}$ digested 5.3 hours at 37°C; Lane 2: GST–PorH$_{C.eff}$ digested over night at 37 °C; Lane 3: GST–PorH$_{C.glut}$ digested 5.3 hours at 37 °C; Lane 4: GST–PorH$_{C.glut}$ digested over night at 37 °C.

If large amounts of Factor Xa protease are used, two bands appear on the gel, at 17–20 kDa and 28-30 kDa, these can be observed in Figure 7. Beside these two bands the GST-fusion protein with about 32 kDa and the 26 kDa glutathione S-transferase can be found. The cleaved PorH porins are localized around 6 kDa, additional bands result from unspecific cleavage. Similar cleaving results were obtained with GST-PorB and GST-PorC fusion proteins.

In Figure 8 the purification and cleavage using the batch method is shown. *E. coli* BL21(DE3)Omp8 cells have been used for the expression. After disrupting the cells, the

supernatant was purified as described (see Material and methods). The SDS-PAGE in Figure 8 represents the cleavage of the *C. efficiens* porins $PorB_{C.eff}$ and $PorC_{C.eff}$.

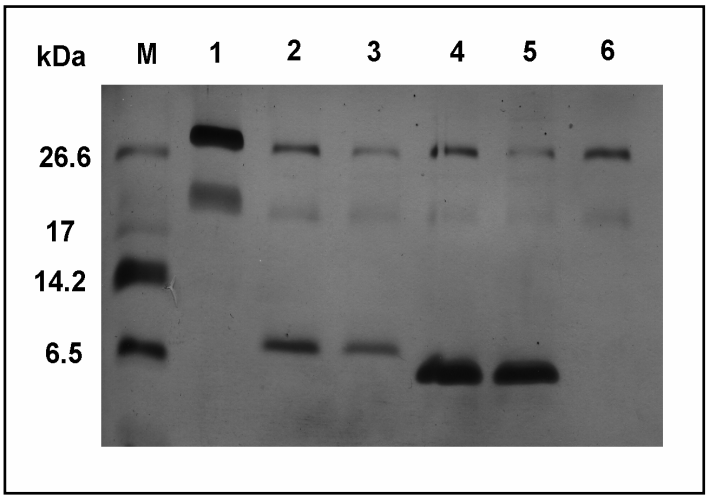

Figure 8. 12 % Tricine SDS PAGE representing the recombinant expressed *C. efficiens* porins $PorB_{C.eff}$ and $PorC_{C.eff}$. Lane M: molecular mass marker; Lane 1: Factor Xa protease 0.4 units; Lane 2: $PorB_{C.eff}$ cleaved over night at room temperature; Lane 3: first washing step of the sepharose with PBS; Lane 4: $PorC_{C.eff}$ cleaved over night at room temperature; Lane 5: first washing step of the sepharose with PBS; Lane 6: control, *E. coli* BL21(DE3)Omp8 cells without expression vector, treated like transformed cells.

The bands deriving from Factor Xa protease are clearly observable in lane 1 of Figure 8. These bands must be substrated in the following lanes, to recognise the clean purified recombinant *C. efficiens* porins $PorB_{C.eff}$ and $PorC_{C.eff}$.

4.4.4 Western blot experiments

The GST-fusion proteins were recognized in Western-blot experiments using Anti-GST HRP Conjugate (Amersham Biosciences, Freiburg, Germany) and visualized using a chemoluminescence reaction (ECLplus detection system, Amersham Biosciences, Freiburg, Germany). In the following Western-blot (see Figure 9) the detection of the glutathione S-transferase tagged porins is visible.

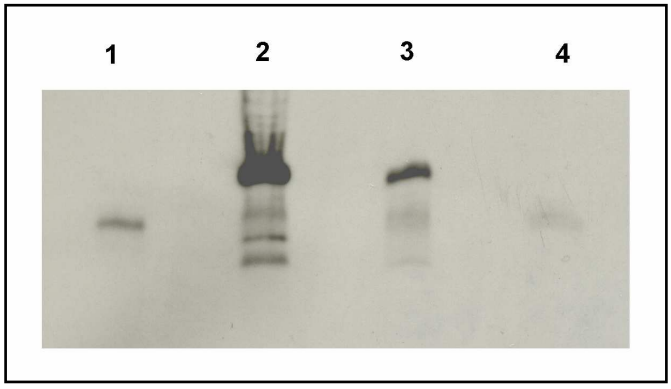

Figure 9. Western-blot of whole *E. coli* Top10F' cells after 5 hours induction transformed with:
Lane 1: pGEX-3X + *porA*$_{C.eff}$; Lane 2: pGEX-3X + *porB*$_{C.eff}$; Lane 3: pGEX-3X + *porC*$_{C.eff}$; Lane 4: pGEX-3X + *porH*$_{C.eff}$; 10 % Tricine SDS PAGE, blotted onto a nitrocellulose membrane, Anti-GST HRP Conjugate concentration 1:5000. In all lanes similar amounts of protein were loaded.

In previous studies we have demonstrated that polyclonal anti-PorB$_{C.glut}$ antibodies bound highly specific to PorB$_{C.glut}$ (see Chapter 3). In this study we checked if organic solvent cell wall extract of *C. efficiens* contains protein that cross-reacts with the anti-PorB$_{C.glut}$ antibodies, to do the same experiment with the recombinant expressed PorB$_{C.eff}$ from *C. efficiens*. The results are shown in Figure 10, both, the cell wall extracts of *C. efficiens* and the recombinant expressed PorB$_{C.eff}$ from *C. efficiens* shows cross-reactivity with the anti-PorB$_{C.glut}$ antibodies.

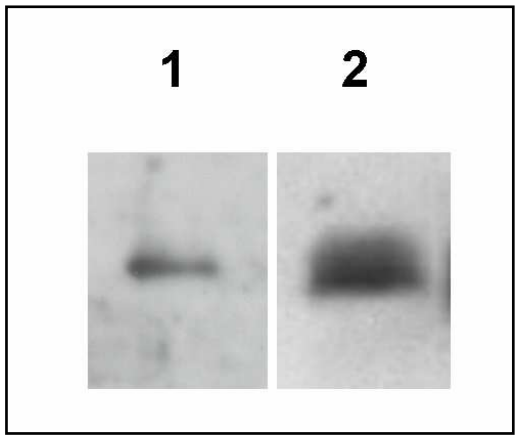

Figure 10. Western blot of organic solvent extract of *C. efficiens* and recombinant PorB$_{C.eff}$.
Lane 1: PorB$_{C.eff}$, isolated from *E. coli* BL21(DE3)Omp8 + pGEX-3X + *porB*$_{C.eff}$; Lane 2: organic solvent extract of *C. efficiens*; 10 % Tricine SDS PAGE blotted onto a nitrocellulose membrane, anti-PorB$_{C.glut}$ antibody concentration 1:200.

4.4.5 Single-channel experiments

Single-channel experiments have been performed with the recombinant expressed porins. After purification and cleavage of the GST-fusion proteins, preparative SDS-PAGE was used for separation the GST-carrier protein, the factor Xa protease and the expressed porins. 1 % genapol was added to allow the refolding of the proteins and to keep them soluble.

Expression experiments with *E. coli* Top10F' cells resulted in channel forming activity, but most of the observed channels resulted from *E. coli* membrane proteins. This was verified in control experiments done with in *E. coli* BL21(DE3)Omp8 and *E. coli* Top10F' cells without the expression plasmids. The cells were passed through a French pressure cell, 1 % genapol was added to the supernatant and subjected to black lipid bilayer experiments. The most frequent observed single-channel conductance was about 2.8 nS for *E. coli* Top10F' cells and 2 nS for *E. coli* BL21(DE3)Omp8 cells. These pore-forming activity of *E. coli* Top10F' cells overlaied the channel-forming of the expressed porins. *E. coli* BL21(DE3)Omp8 cells, which lack all major porins, exhibited less activity in lipid bilayer experiments and were therefore used for the following expression.

Channels deviating from the control experiments have been observed for $PorB_{C.eff}$ and $PorC_{C.eff}$ expressed in *E. coli* BL21(DE3)Omp8 cells. For $PorH_{C.glut,}$ $PorC_{C.glut\ ,}$ $PorA_{C.eff}$ and $PorH_{C.eff}$ no single-channel conductance could be determined.

Figure 11 shows a single-channel recording of $PorB_{C.eff}$, expressed in *E. coli* BL21(DE3)Omp8 cells, added to a black membrane in a concentration of about 10 ng/ml. The single-channel recording demonstrates that the protein formed defined channels.

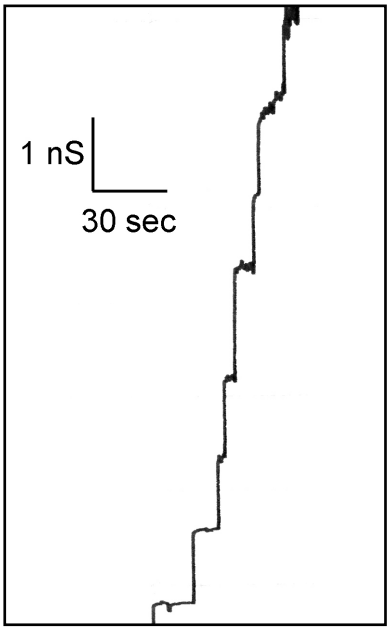

Figure 11. Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of 20 ng/ml expressed cell wall protein $PorB_{C.eff}$. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T: 20 °C.

The average single-channel conductance of most of the channels formed by the cell wall protein was 1 nS in 1 M KCl. Only a minor fraction of channels with other conductance was observed (see Figure 12).

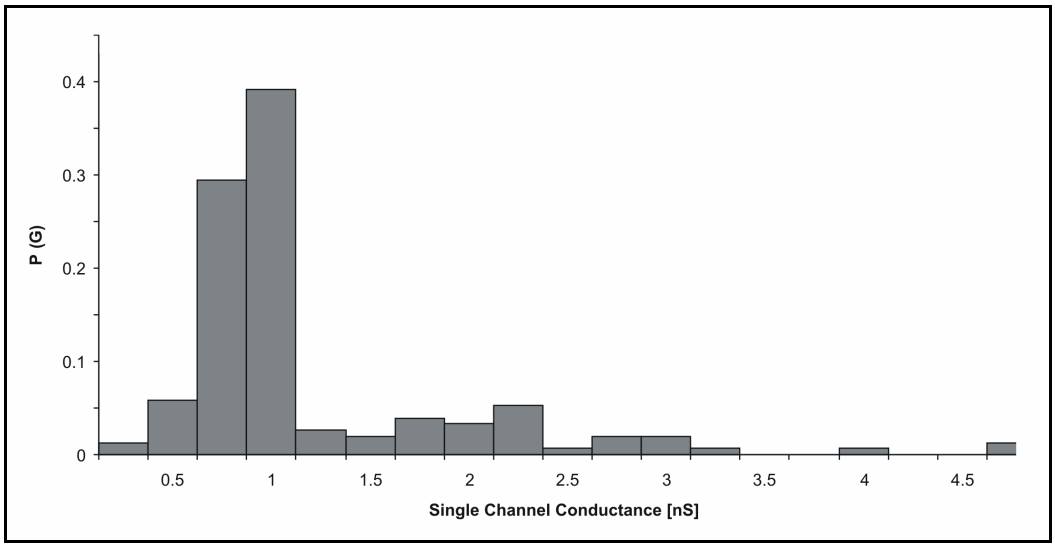

Figure 12. Histogram of the occurrence probability of certain conductivity units observed with membranes formed of PC dissolved in n-decane in the presence of 20 ng/ml of the recombinant cell wall protein $PorB_{C.eff}$. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T: 20 °C. The average single-channel conductance was 1 nS for 149 single-channel events.

It is important that the channels formed by the recombinant expressed cell wall protein $PorB_{C.eff}$ had a long lifetime similar to those that have been detected previously for cell wall proteins (porins) of *C. glutamicum* (Lichtinger *et al.*, 1998), *Mycobacterium chelonae* (Trias & Benz, 1993) and *M. smegmatis* (Trias & Benz, 1994). All these proteins formed channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 minutes).

Single-channel experiments were therefore performed with salts other than KCl to obtain some information on the selectivity of the channels formed by the recombinant expressed cell wall protein $PorB_{C.eff}$ of *C. efficiens*. Measurements with 1 M LiCl and 1 M KCH_3COO have been performed, the results are summarized in Table 4.

Table 4. Average single-channel conductance, G, of $PorB_{C.eff}$ in different salt solutions**.** The membranes were formed of PC dissolved in n-decane. The aqueous solutions of KCl and LiCl were unbuffered and had a pH of 6, the KCH_3COO solution was adjusted to pH 7. The applied voltage was 20 mV, and the temperature was 20 °C. The average single-channel conductance, G, was calculated from at least 80 single-channel events.

Salt	**Single-channel conductance G[nS]**
1 M KCl	1
1M LiCl	1
1M KCH_3COO	0.25/1.75

The replacement of potassium by the less mobile lithium ion had no influence on the conductance of the channels. The influence of the anion on the single-channel conductance in KCH_3COO was more substantial, which suggests that the cell wall channel was anion-selective. The conductance of 250 pS in KCH_3COO is equivalent to the value of $PorB_{C.glut}$ (Costa-Riu *et al.* 2003b), the 1.75 nS peak results assumedly from *E. coli* porin contamination.

Zero-current membrane potential measurements, which allow the calculation of the permeability ratio P_{cation} divided by P_{anion} in multichannel experiments, were not realisable. There was no incorporation of channels into the membrane, formed of PC dissolved in n-decane, in 100 mM KCl solution. The pore-forming activity of $PorB_{C.eff}$ was generally low thereby single-channel experiments were not easily accomplishable.

Expressed $PorC_{C.eff}$ in *E. coli* BL21(DE3)Omp8 cells subjected to lipid bilayer experiments also resulted in pore-forming activity, but the activity of $PorC_{C.eff}$ was even worse than the pore formation of $PorB_{C.eff}$**.** The obtained single-channel conductances are shown in Table 5.

Table 5. Average single-channel conductance, G, of $PorC_{C.eff}$ in different salt solutions**.** The membranes were formed of PC dissolved in n-decane. The aqueous solutions of KCl was unbuffered and had a pH of 6, the KCH_3COO solution was adjusted to pH 7. The applied voltage was 20 mV, and the temperature was 20 °C. The average single-channel conductance, G, was calculated from at least 90 single events.

Salt	**Single-channel conductance G[nS]**
1 M KCl	1
1M KCH_3COO	0.5/1.25

The measurable single-channel conductances for KCl and KCH_3COO (see Table 5) indicate that $PorC_{C.eff}$ behaves like $PorB_{C.eff}$. More experiments to proof this assumption were

conducted, but because of the low channel-forming activity of PorC$_{C.eff}$ no more expedient datas could be obtained.

4.4.6 Interaction of PorH$_{C.glut}$ of *C. glutamicum* synthesized by solid phase synthesis with lipid bilayer membranes

The measurements with the synthetic produced PorH$_{C.glut}$ are in accordance with the results of the single-channel experiments described before.

The channel-forming activity of PorH$_{C.glut}$ synthesized by solid phase synthesis with lipid bilayers was studied by the addition of small amounts of peptide to the aqueous phase bathing black PC membranes. In these experiments we observed a substantial increase of the specific membrane conductance in the presence of the synthetic PorH$_{C.glut}$ peptide. About one minute after addition of the protein, the membrane conductance started to rise and increased by several orders of magnitude (see Figure 13). In all approaches the increase of conductance could be observed, but defined channels were infrequent to notice, in Figure 14 the channel formation is demonstrated. The single-channel conductance from PorH$_{C.glut}$, isolated from *C. glutamicum*, is about 2.5 nS in 1 M KCl (see Chapter 3), this value is agreeing with this results. A histogram of the occurrence probability was impossible to create, because of the undefined channels distinguishable in Figure 13 B. It is important that the channels formed by the synthetic PorH$_{C.glut}$ peptide had a long lifetime if the concentration was low, similar to those that have been detected previously for cell wall proteins (porins) of *C. glutamicum* (Lichtinger *et al.*, 1998), *M. chelonae* (Trias & Benz, 1993) and *M. smegmatis* (Trias & Benz, 1994). All these proteins formed channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 minutes).

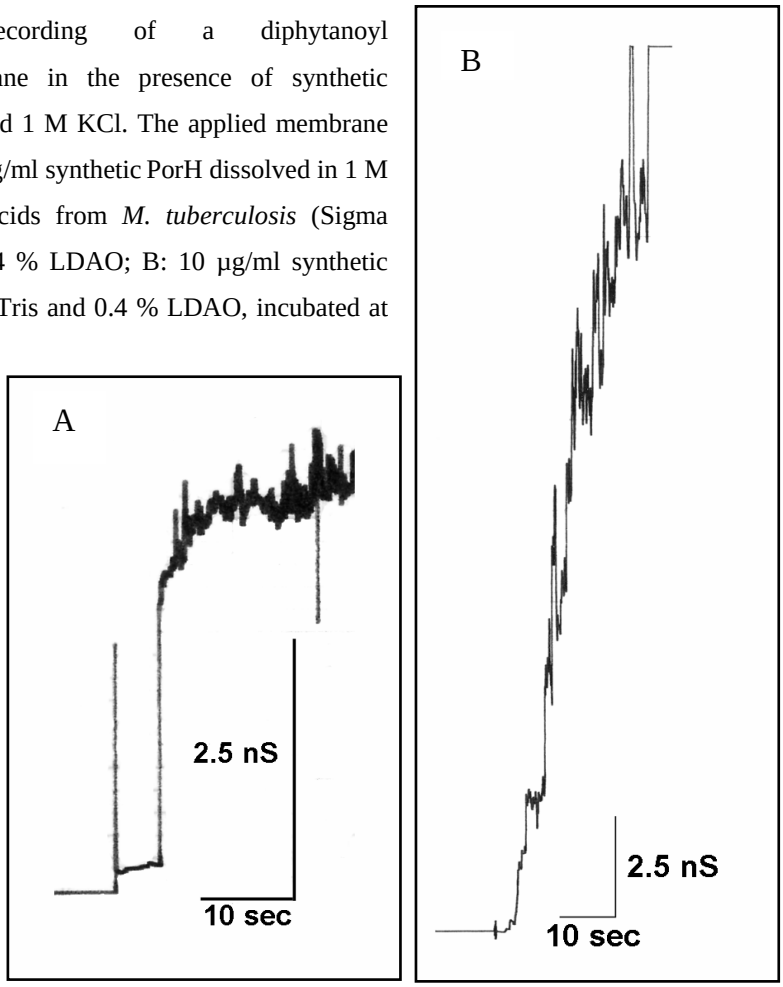

Figure 13. Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of synthetic $PorH_{C.glut}$. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T: 20 °C. A: 1 µg/ml synthetic PorH dissolved in 1 M NaCl, 10 mM Tris, 1 % mycolic acids from *M. tuberculosis* (Sigma Aldrich, Steinheim, Germany) and 0.4 % LDAO; B: 10 µg/ml synthetic PorH dissolved in 1 M NaCl, 10 mM Tris and 0.4 % LDAO, incubated at 80 °C for 5 min.

4.5 Discussion

With the heterologous expression of corynebacterial porins we wanted to tap new possibilities in porin research. Because of the biotechnological relevance of *C. glutamicum* and *C. efficiens* in amino acid production, it is important to understand, how the export across the outer membrane takes place. It is speculated, that the transport across the cell wall is the limiting step in industrial amino acid production (Eggeling & Sahm, 2001). Understanding the molecular basis of the pore-forming proteins in the corynebacterial cell wall is the clue for a more effective production of amino acids.

Since overexpression systems are not available for Corynebacteria, heterologous production of corynebacterial proteins seem to be the only alternative. To achieve this goal, the porin genes were cloned into the expression vector pGEX-3X. Expression in *E. coli* yielded fusion proteins with the Glutathione S-transferase (GST) moiety at the amino terminus, and the pore-forming proteins at the carboxyl terminus. GST is a naturally occurring 26 kDa water soluble protein and we expected that the fusion proteins still remain water soluble, in spite of the hydrophobe porin part. The fusion proteins accumulated in the cell's cytoplasm and could be purified from bacterial lysates by affinity chromatography using immobilized glutathione. It is noticeable, that in Western blot experiments with Anti-GST antibodies different amounts of expressed proteins are present. In all our experiments the amounts of expressed PorA and PorH fusion proteins were less than PorB and PorC. It is possible that the expression is hampered by the different codon usages of Corynebacteria and *E. coli* as reflected by their G+C contents of about 51 % for *E. coli* (Blattner *et al*. 1997), 63.4 % for *C. efficiens* and 53.8 % for *C. glutamicum* (Nishio *et al*., 2003). Differences in codon usage among organisms can lead to a variety of problems concerning heterologous gene expression. The percentage of codons of the porin genes with a usage of less than 20 % in *E .coli* is displayed in Table 6. It is cognizable that the *porB* and *porC* genes are more suitable for the heterologous expression in *E. coli*.

Table 6. The porin sequences have been splitted in codons and the fraction of codon usage in *E. coli* has been calculated. Represented is the percentage of codons with a usage of less than 20 % in *E. coli*.

	C. glutamicum	***C. efficiens***
PorA	30 %	22 %
PorH	28 %	31 %
PorB	22 %	19 %
PorC	30 %	17 %

Similar problems have been described by Heinz *et al*. 2003. They demonstrate the overexpression of *mspA* from *M. smegmatis* in *E. coli* after synthesis of the gene with codons preferentially used in highly expressed *E. coli* genes. Lakey *et al*. 2000 also achieved an enhanced production of recombinant *M. tuberculosis* antigens in *E. coli* by replacement of low-usage codons (Lakey *et al*. 2000). To attain higher amounts of expressed pore-forming proteins it is possible that this codon usage adaptation would also be a promising attempt.

In our approach we wanted to investigate the pore-forming activity of the heterologous expressed pore-forming proteins, but single-channel experiments were not easily accomplishable. In all our black lipid bilayer experiments contaminations of *E. coli* porins were present, even while using *E. coli* BL21(DE3)Omp8 cells, which lack all major porins. By dint of control experiments we could distinguish the *E. coli* porins from the expressed porins. In Table 7 the acquired single-channel datas are summarized and compared to the known datas.

Salt solution	**1 M KCl**	**1 M LiCl**	**1 M KCH_3COO** (pH 7)
	Single-channel conductance G [nS]		
$PorB_{C.glut}$ [1]	0.7	0.7	0.25
recombinant $PorB_{C.eff}$	1.0	1.0	0.25/1.75
recombinant $PorC_{C.eff}$	1.0	-	0.5/1.3

Table 7. Average single-channel conductance, G, of $PorB_{C.glut}$ (taken from [1] Costa Riu *et al*. 2003b), in *E. coli* BL21(DE3)Omp8 heterologous expressed $PorB_{C.eff}$ and $PorC_{C.eff}$ in different salt solutions**.**
The membranes were formed of PC dissolved in n-decane. The aqueous solutions were unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV, and the temperature was 20 °C. The average single-channel conductances, G, were calculated from at least 80 single events.

A contamination of *E. coli* porins is visible in the single-channel experiments of recombinant $PorB_{C.eff}$ and $PorC_{C.eff}$ in 1 M KCH_3COO. There two values are mentioned, the expected conductance for the *C. efficiens* porins should be 0.25 nS, respectively 0.5 nS. We suggest that the cell wall channels are anion-selective, so that the influence of the anion on the single-channel conductance is more substantial. The higher conductances argue for *E. coli* porin. In control measurements in 1 M KCl solution we detected channel forming activity with a average single-channel conductance of 2 nS in detergent extracts of *E. coli* BL21(DE3)Omp8 cells. The conductances of 1.75 nS and 1.3 nS in 1 M KCH_3COO solution argue for a cation selective channel, because the anion has no substantial effect on the single-channel conductances.

On the basis of the homology of 42.4 % between the amino acid sequence of $PorB_{C.glut}$ and $PorB_{C.eff}$ we expected similar single-channel conductances. This assumption could be

evidenced in our experiments, the comparison of the amino acid sequences is shown in Figure 15.

```
                      10         20         30         40         50         60
                      |          |          |          |          |          |
PorB_C.glut     MKLSHRIAAMAATAGITVAAFAAPASASDFANLSSTNKELSPQYNWVACGILEGGLKAAG
PorB_C.eff      MKISTRVAAIGAAAALGLTAFAGPASAV------SSSDELSDRFDWVGCPIVEASLAFYG
PorC_C.eff      MNL-RRTLAVAAASVMALTATIAPAQAQN-ADIVSGINNLIDTFD---CDLLRTGLTQTG

                     70         80         90        100        110        120
                      |          |          |          |          |          |
PorB_C.glut     VLEEGQYNRELAEAIAAK-GEGFWTTQFPQIGDWNEDQAAALADRAQTCGLVKADTYLSE
PorB_C.eff      LPEEGMRNNQLAAALEEK-NANF-AAYFEGGGDWNAQASADYADRAQKCGIVEPNTAIEN
PorC_C.eff      LVTPETTRSELAATLRTTANLGEIDVAFAFVG---SAYAGRIADRAQTCGIVQPDPE-QD
                       130
                        |
PorB_C.glut     LSSNFSS-------
PorB_C.eff      ASSNLNDFFAGLSS
PorC_C.eff      ILTQLQNLSSNLSS
```

Identity:

$PorB_{C.glut}$	:	$PorB_{C.eff}$:	42.4 %
$PorB_{C.eff}$	:	$PorC_{C.eff}$:	31.3 %
$PorB_{C.glut}$	:	$PorC_{C.eff}$:	32.8 %

Figure 15. Amino acid sequence of $PorB_{Cglut}$ and its comparison with $PorB_{C.eff}$ and $PorC_{c.eff}$. The signal sequence is underlined, the conserved residues in at least three of the four homologues are specified in red. The conserved residues in two of the four homologues are marked in green. The NCBI GenBank Accesion numbers are: $porB_{C.glut}$.: BK001251; $porB_{C.eff}$.: AJ555471; $porC_{C.eff}$.: AJ555472.

The homology between $PorB_{C.glut}$ and $PorB_{C.eff}$ is higher than between $PorB_{C.eff}$ and $PorC_{C.eff}$, but in our single-channel experiments we determined for both the same single-channel conductance. *porC*$_{C.glut}$ is localized only 138 bp downstream of *porB*$_{C.glut}$, they are cotranscribed, and *porC*$_{C.glut}$ is presumably the result of a gene duplication (Costa Riu *et al.*, 2003b). The same gene arrangement is present in *C. efficiens*, *porC*$_{C.eff}$ is localized 160 bp downstream of *porB*$_{C.eff}$ (see Figure 2) and they are presumably cotranscribed too. $PorC_{C.eff}$ forms anion-selective channels similar to $PorB_{C.eff}$, and it is probable that $PorC_{C.glut}$ also represents an anion selective channel. It is noteworthy that both proteins, PorB and PorC, are acidic and contain a smaller number of positively charged amino acids than negatively charged ones. It seems that the organization of PorB and PorC in secondary, tertiary and quaternary structure is responsible for its function.

One major problem was the low channel forming activity of the heterologous expressed corynebacterial porins. This leads to the assumption, that the recombinant porins are not right folded, perhaps because of a missing translational modification, that is not proceeded in *E. coli*. Lichtinger *et al.* described 2001 a similar attribute for $PorA_{C.glut}$ from *C. glutamicum*. While sequencing the $PorA_{C.glut}$ protein there have been problems in detecting position 15, there only the phenylthiohydantoin derivate of dehydroalanine was determined (Lichtinger *et al*., 2001). This supposition would also be in agreement with the obtained results from the channel forming experiments of synthetic $PorH_{C.glut}$. In all our approaches the increase of conductance could be observed, but defined channels were infrequent to notice.

The problems we had, caused by the different codon usage and the presumably missing translational modification, resulted in the decision to look for other possibilities of the expression of corynebacterial porins. To create porin deficient mutants of *C. glutamicum* and *C. efficiens* to express the pore-forming proteins in their host organism would probably be the best way to overcome the discussed problems. On the other hand succeeded Heinz *et al.* 2003 in the high-level expression of the mycobacterial porin MspA in *E .coli* by using a designed *E. coli* adapted gene of MspA. This, in *E. coli* expressed MspA protein, was used by Faller *et al.* 2004 for X-ray analysis and the first structure of a mycobacterial outer membrane protein was demonstrated. It seems that the porin is in the right conformation but the evidence of pore-forming activity is missing. It could be possible that the presumably translational modification is not necessary for the right folding, but for the channel forming activity and thus for the functionality of the porin.

CHAPTER 5

Summary

5.1 Summary

Corynebacterium glutamicum is together with *C. callunae* and *C. efficiens* a member of the diverse group of mycolic-acid containing actinomycetes, the mycolata. These bacteria are potent producer of glutamate, lysine and other amino acids on industrial scale. But beside these microorganisms of industrial importance the *Corynebacterineae* family comprises also microorganisms which cause the most dangerous infections worldwide like *Mycobacterium tuberculosis* (TBC, at present 3 millions deaths/a), *M. leprae* (lepra), *Nocardia farcinica* (nocardiosis) and *C. diphteriae* (diphtheria). The cell walls of most actinomycetes contain besides an arabinogalactan-peptidoglycan complex large amounts of mycolic acids. This three-layer envelope is called MAP (mycolyl-arabinogalactan-peptidoglycan) complex and it represents a second permeability barrier beside the cytoplasmic membrane similar to the outer membrane of Gram-negative bacteria. In analogy to the situation in the outer membrane of Gram-negative bacteria, channels are present in the mycolic acid layer of the mycobacterial cell wall for the passage of hydrophilic solutes.

Molecular studies have provided far-reaching findings on the amino acid flux and its balance in *C. glutamicum* in general, but the L-glutamate export still remains unknown. The properties of the outer layers, typical of mycolata, seem to be of major importance in this process, and diffusion seems to play a key role for this part of the cell wall. With respect to transport of amino acids over this barrier it is of particular importance to understand the pathways present in the cell wall of Corynebacteria. Some of the features from the cell wall channel-forming proteins investigated in this thesis can be expected for other mycolata and improve strategies for example in antimicrobial drug design. Due to their heterogeneity, that on one hand microorganisms which cause the most dangerous infections worldwide and on the other hand organisms of high industrial interest are members of the *Corynebacterineae*, they have evolved to an interesting and challenging research object.

The major aim of this thesis was to identify and study novel channel-forming proteins of the amino acid producers *C. glutamicum, C. callunae* and *C. efficiens*. Cell wall extracts of the organisms were investigated and a novel pore-forming protein, named PorH, that is homologue in all three organisms, was detected and characterized.

PorH$_{C.glut}$ was isolated from *C. glutamicum* cells cultivated in minimal medium. The protein was identified in lipid bilayer experiments and purified to homogeneity by fast-protein liquid chromatography across a HiTrap-Q column. The purified protein forms cation-selective channels with a diameter of about 2.2 nm and an average single-channel conductance of about 2.5 nS in 1 M KCl in the lipid bilayer assay.

Organic solvent extracts were used to study the permeability properties of the cell wall of *C. callunae* and *C.efficiens*. The cell extracts contained channel-forming activity, the corresponding proteins were purified to homogeneity by fast-protein liquid chromatography across a HiTrap-Q column and named PorH$_{C.call}$ and PorH$_{C.eff}$. Channels formed by PorH$_{C.call}$ are cation-selective with a diameter of about 2.2 nm and an average single-channel conductance of 3 nS, whereas PorH$_{C.eff}$ forms slightly anion selective channels with an average single-channel conductance of 2.3 nS in 1 M KCl in the lipid bilayer assay.

The PorH proteins were partially sequenced and the corresponding genes, which were designated as *porH*, were identified in the published genome sequence of *C. glutamicum* and *C. efficiens*. The chromosome of *C. callunae* is not sequenced, but PorH$_{C.call}$ shows a high homology to PorH$_{C.eff}$ and PorH$_{C.glut}$. The proteins have no N-terminal extension, only the inducer methionine, which suggests that secretion of the proteins could be very similar to that of PorA$_{C.glut}$ of *C. glutamicum*. PorH$_{C.glut}$ is coded in the bacterial chromosome by a gene that is localized in the vincinity of the *porA*$_{C.glut}$ gene, within a putative operon formed by 13 genes that are encoded by the minus strand. Both porins are cotranscribed and coexist in the cell wall, which was demonstrated in RT-PCR and immunological detection experiments. The arrangement of *porH*$_{C.glut}$ and *porA*$_{C.glut}$ on the chromosome is similar to that of *porB*$_{C.glut}$ and *porC*$_{C.glut}$ and it was found that PorA$_{C.glut}$, PorH$_{C.glut}$, PorB$_{C.glut}$ and PorC$_{C.glut}$ coexist in the cell wall of *C. glutamicum*.

The molecular mass of about 6 kDa of the PorH channel forming proteins is rather small and suggests that the cell wall channels are formed by oligomers. A possibly hexameric form was demonstrated for PorH$_{C.glut}$ in Western blot analysis with anti- PorH$_{C.glut}$ antibodies. Secondary structure predictions for PorH$_{C.glut}$, PorH$_{C.call}$ and PorH$_{C.eff}$ predict that a stretch of about 42 amino acids of PorH$_{C.glut}$ and 28 amino acids of PorH$_{C.call}$ and PorH$_{C.eff}$ forms

amphipathic α-helices with a total length of 6.3 nm and 4.2 nm respectively. This should be sufficient to cross the mycolic acid layer.

Another objective of this work was to establish an heterologous expression system for corynebacterial channel-forming proteins, to investigate the channel-forming properties of the up to now only hypothetical porins PorA, PorB, PorC from *C. efficiens* and PorC from *C. glutamicum*. We could demonstrate with recombinant expression experiments in *E. coli* that $porB_{C.eff}$ and $porC_{C.eff}$ encode for channel-forming proteins. They are, like $PorB_{C.glut}$, anion-selective with a similar single-channel conductance of 1 nS in 1 M KCl.

It is demonstrated in this thesis, that the pore-forming proteins $PorH_{C.glut}$, $PorH_{C.eff}$ and $PorH_{C.call}$ of the closely related Corynebacteria *C. glutamicum*, *C. efficiens* and *C. callunae* have similar properties and are highly conserved. Translation of PorH, and also of PorA, is up to now not known, but there is evidence that the flanking genes are required. The presumptive secondary structure of the PorH channel is α-helical, what is likewise a novelty for porins. The mycolic acid layer spanning part of MspA of *M. smegmatis* and gram-negative bacterial porins feature ß-sheet secondary structure. It could be furthermore confirmed in recombinant expression experiments, that $PorB_{C.eff}$ and $PorC_{C.eff}$ of *C. efficiens* represent like $PorB_{C.glut}$ of *C. glutamicum* anion-selective channel-forming proteins, with similar single-channel conductance.

5.2 Zusammenfassung

C. glutamicum gehört zusammen mit *C. efficiens* und *C. callunae* zu den bedeutensten Aminosäureproduzenten weltweit. Industriell werden hauptsächlich Glutamat und Lysin produziert und als Geschmacksverstärker bzw. Futtermittelzusatz verwendet. Sie sind Mitglieder der heterogenen Gruppe der mykolsäurehaltigen Aktinomyceten, den Mycolaten. Neben Mikroorganismen von industrieller Bedeutung beinhaltet die Familie der *Corynebacterineae* auch solche, die weltweit die gefährlichsten Infektionskrankheiten auslösen, wie *M. tuberculosis* (TBC, 3 Millionen Tote pro Jahr), *M. leprae* (Lepra), *N. farcinica* (Nocardiose) und *C. diphteriae* (Diphtherie). Die Zellwände der meisten Aktinomyceten weisen neben einem Arabinogalactan-Peptidoglycankomplex große Mengen an Mycolsäuren auf. Diese MAP (Mycolyl-Arabinogalactan-Peptidoglycan) genannte dreischichtige Hülle stellt neben der Cytoplasmamembran eine zweite Permeabilitätsbarriere dar, und übernimmt somit die gleiche Funktion wie die äußere Membran der Gram-negativen Bakterien was zur Folge hat, dass für den Transport von hydrophilen Stoffen kanalbildende Proteine benötigt werden. Analog zur Situation in Gram-negativen Bakterien sind in der Mykolsäureschicht von *C. efficiens, C. callunae* und *C. glutamicum* porenbildende Transmembranproteine vorhanden.
Es ist bisher vieles bekannt über den Aminosäurefluss über die Cytoplasmamembran und dessen Regulation in *C. glutamicum,* aber der Export von L-Glutamat ist noch ungeklärt. Die besondere Beschaffenheit der äußeren Membran von Mycolaten scheint in diesem Zusammenhang eine wichtige Rolle zu spielen, da man annimmt, dass die Diffusion im Bezug auf den Transport über die Mycolsäureschicht eine Schlüsselrolle einnimmt. Im Bezug auf den Transport von Aminosäuren über die Zellwand von Corynebakterien ist die Erforschung der Transportwege über diese Barriere von besonderem Interesse.

Das Ziel dieser Arbeit war es neue porenbildende Proteine der Aminosäureproduzenten *C. glutamicum, C. efficiens* und *C. callunae* zu identifizieren und zu charakterisieren. Bei Untersuchungen von Zellwandextrakten wurde ein neuer, in allen drei Organismen homologer, Zellwandkanal, PorH, gefunden und charakterisiert.
$PorH_{C.glut}$ wurde aus in Minimalmedium kultivierten *C. glutamicum* Zellen isoliert. Das Protein wurde in Lipid-Bilayer Experimenten gefunden und mit Hilfe der Ionenaustauscher-

chromotographie über eine HiTrap-Q Säule aufgereinigt. PorH$_{C.glut}$ bildet in Black-Lipid Bilayer Experimenten kationenselektive Kanäle mit einem Durchmesser von 2,2 nm und hat eine Einzelkanalleitfähigkeit von 2,5 nS in 1 M KCl. Aus organischen Zellwandextrakten von *C. callunae* und *C. efficiens* wurden PorH$_{C.call}$ und PorH$_{C.eff}$ isoliert. PorH$_{C.call}$ bildet einen kationenselektiven Kanal mit einem Durchmesser von 2,2 nm und einer Einzelkanalleitfähigkeit von 3 nS, PorH$_{C.eff}$ hingegen bildet einen leicht anionenselektiven Kanal mit einer Leitfähigkeit von 2,3 nS in 1 M KCl.

Über die Sequenzierung der PorH Proteine wurden die entsprechenden *porH* Gene im Genom von *C. glutamicum* und *C. efficiens* identifiziert. Das Genom von *C. callunae* ist nicht bekannt, jedoch weisen die PorH Proteinsequenzen eine hohe Homologie untereinander auf. Es ist keine Signalsequenz vorhanden, so dass man annimmt, dass der Export über die Plasmamembran ähnlich funktioniert wie bei PorA$_{C.glut}$ aus *C. glutamicum*. Das Gen *porH*$_{C.glut}$ aus *C. glutamicum* befindet sich in unmittelbarer Nähe zu *porA*$_{C.glut}$ in einem möglichen Cluster das aus 13 Genen besteht. In RT-RCR Experimenten und immunologischen Reaktionen mit polyklonalen Antikörpern konnte gezeigt werden, dass die Gene *porA*$_{C.glut}$ und *porH*$_{C.glut}$ zusammen transkribiert werden und die Porine in der Zellwand coexistieren. Die Anordnung von *porA*$_{C.glut}$ und *porH*$_{C.glut}$ im Genom von *C. glutamicum* ist ähnlich der von *porB*$_{C.glut}$ und *porC*$_{C.glut}$, und es konnte gezeigt werden, dass die vier Porine in der Zellwand gleichzeitig vorhanden sind.

Das Molekulargewicht von PorH ist mit 6 kDa sehr klein für Kanalproteine und man nimmt an, dass die Kanäle von Oligomeren gebildet werden. In Western Blots wurde eine mögliche hexamere Form von PorH$_{C.glut}$ nachgewiesen. Sekundärstrukturvorhersagen für PorH sagen aus, dass 42 Aminosäuren von PorH$_{C.glut}$ und 28 von PorH$_{C.eff}$ und PorH$_{C.call}$ amphiphatische α-Helices mit einer Länge von 6,3 nm, bzw. 4,2 nm ausbilden. Diese Länge würde ausreichen, um die Mykolsäureschicht zu durchspannen.

Ein weiteres Ziel dieser Arbeit war es, ein heterologes Expressionssystem für corynebakterielle Kanalproteine zu erstellen, um die kanalbildenden Eigenschaften von bisher nur hypothetischen Porinen wie PorA$_{C.eff}$, PorB$_{C.eff}$ und PorC$_{C.eff}$ von *C. efficiens*, oder PorC$_{C.glut}$ von *C. glutamicum* zu bestimmen. In rekombinanten Expressions-Experimenten in *E. coli* konnte gezeigt werden, dass die Gene *porB*$_{C.eff}$ und *porC*$_{C.eff}$ für Proteine mit kanalbildender Aktivität kodieren. Die Kanäle sind anionenselekitv, mit einer Einzelkanalleitfähigkeit von 1 nS in 1 M KCl, vergleichbar mit den Eigenschaften von PorB$_{C.glut}$ aus *C. glutamicum*.

In dieser Arbeit konnte gezeigt werden, dass die Kanalproteine $PorH_{C.glut}$,$PorH_{C.eff}$ und $PorH_{C.call}$ der eng verwandten Corynebakterien *C. glutamicum, C. efficiens* und *C. callunae* hoch konserviert sind und ähnliche Eigenschaften haben. Der Exportmechanismus von PorH sowie von PorA über die Plasmamembran ist noch unklar, jedoch bestehen Hinweise darauf, dass möglicherweise die Gene, die in der Umbebung von *porA* und *porH* lokalisiert sind, an diesem beteiligt sind. Ein weiteres Novum ist die für Porine ungewöhnliche α-helikale Sekundärstruktur von PorH. Der die Mykolsäureschicht durchspannende Teil von MspA aus *M. smegmatis*, sowie die Porine aus Gram-negativen Bakterien weisen β-Faltblatt Sekundärstrukturen auf. Es konnte weiterhin mit Hilfe von rekombinanten Expressionsexperimenten bewiesen werden, dass es sich bei $PorB_{C.eff}$ und $PorC_{C.eff}$ aus *C. efficiens* ebenfalls um porenbildende Proteine handelt.

Unter der Familie der *Corynebacterineae* befinden sich auch oben genannte pathogene Mikroorganismen, und man kann davon ausgehen, dass einige Eigenschaften der untersuchten Kanalproteine auch auf andere Proteine der mykolsäurehaltigen Bakterien übertragen werden können und so zu Fortschritten im Bereich der Entwicklung z.B. von neuen antimikrobiellen Substanzen führen. Diese Heterogenität der Familie der *Corynebacterineae*, dass sich einerseits Erreger der weltweit gefährlichsten Infektionskrankheiten und andererseits Bakterien von großem industriellen Interesse unter ihnen befinden macht sie zu einem sehr interessanten Forschungsgebiet.

CHAPTER 6

Appendix

6.1 References

Alberts, B., Roberts, K., Lewis, J., Raff, M., Johnson, A. & Walter, P. (2002). Molecular biology of the cell, 4th edition. New York (USA): Garland Publishing Inc.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403-410.

Anderson, S., Marks, C. B., Lazarus, R., Miller, J., Stafford, K., Seymour, S., Light, D., Rastetter, W. & Estell, D. (1985). Production of 2-keto-L-gluconate, an intermediate in L-ascorbate synthesis by a genetically modified *Erwinia herbicola*. *Science* 230, 144-149.

Bailey, J. E. (1991). Towards a science of metabolic engineering. *Science* 252, 1668-1691.

Barksdale, L. (1981). The genus *Corynebacterium*. In *The Prokaryotes*, pp. 1827-1837. Edited by M. P. Starr, H. Stoll, H. G. Trüper, A. Balows & H. G. Schlegel. Berlin: Springer-Verlag.

Barreiro, C., Gonzalez-Lavado, E., Patek, M. & Martin, J. F. (2004). Transcriptional analysis of the *groES-groEL1*, *groEL2*, and *dnaK* genes in *Corynebacterium glutamicum*: characterization of heat shock-induced promoters. *J Bacteriol* 186(14), 4813-4817.

Benz, R. & Bauer, K. (1988). Permeation of hydrophilic molecules through the outer membrane of Gram-negative bacteria. *Eur J Biochem* 176, 1-19.

Benz, R. (1994). Solute uptake through the bacterial outer membrane. In *Bacterial cell wall*, pp. 397-423. Edited by J. M. Ghuysen & R. Hakenbeck. Amsterdam: Elsevier Science B.V.

Benz, R. (2001). Porins - structure and function. In *Microbial Transport Systems*, pp. 227-246. Edited by G. Winkelmann. Weinheim: Wiley-VCH.

Benz, R. (2003). Investigation of substrate-specific porin channels in lipid bilayer membranes. In *Planar Lipid Bilayers (BLMs) and Their Applications*, pp. 347-369. Edited by H. T. Tien & A. Ottova-Leitmannova. Amsterdam: Elsevier Science B.V.

Benz, R., Hardie, K. R. & Hughes, C. (1994). Pore formation in artificial membranes by the secreted hemolysins of *Proteus vulgaris* and *Morganella morganii*. *Eur J Biochem* 220, 339-347.

Benz, R., Janko, K. & Läuger, P. (1979). Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. *Biochim Biophys Acta* 551, 238-247.

Benz, R., Janko, K., Boos, W. & Läuger, P. (1978). Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim Biophys Acta* 511, 305-319.

Benz, R., Schmid, A., Nakae, T. & Vos-Scheperkeuter, G. H. (1986). Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *J Bacteriol* 165, 978-986.

Benz, R., Schmid, A., Wagner, W. & Goebel, W. (1989). Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. *Infect Immun* 57, 887-95.

Blattner, F. R., Plunkett, G. 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. & Shao, Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* 277(5331), 1453-1474.

Blum, B., Beier, H. & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8, 93-99.

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

Brock, T. D., Madigan, M. T., Martinko, J. M. & Parker, J. (2002). Biology of microorganisms 10th edition. New Jersey (USA): Prentice Hall.

Cardini, G. & Jurtshuk, P. (1970). The enzymatic hydroxylation of n-octane by *Corynebacterium sp*. Strain 7EC1C. *J Biol Chem* 245, 2789-2796.

Chami, M., Bayan, N., Dediu, J.-C., Leblon, G., Shechter, E. & Gulik-Krzywicki, T. (1995). Organisation of the outer layers of the cell envelope of *Corynebacterium glutamicum*: a combined freeze-tech electron microscopy and biochemical study. *Biol Cell* 83: 219-229.

Chami, M., Bayan, N., Peyret, J. L., Gulik-krzywicki, T., Leblon, G. & Schlechter, E. (1997). The S-layer protein of *Corynebacterium glutamicum* is anchored to the cell wall by its C-terminal hydrophobic domain. *Mol Microbiol* 23, 483-492.

Collins, M. D. & Cummins, C. S. (1986). Genus *Corynebacteria*. In : *Bergey's Manual of Systematic Bacteriology*. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore (USA): Williams and Wilkins.

Collins, M., Goodfellow, M. & Minnikin, D. E. (1981). A survey of the structures of mycolic acids in *Corynebacterium* and related taxa. *J Gen Microbiol* 128, 129-149.

Constantinides, S. (1980). Steroid transformation at high substrate concentrations using immobilized *Corynebacterium simplex* cells. *Biotechnol Bioeng* 22, 119-36.

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, 4779-4786.

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, 1295-1308.

Crick D. C., Mahapatra, S. & Brennan, P. J. (2001). Biosynthesis of the arabinogalactan-peptidoglycan complex of *Mycobacterium tuberculosis*. *Glycobiology* 11, 107R-118R.

Cullis, P. R. & Hope, M. J. (1985). Physical properties and functional roles of lipids in membranes. In: *Biochemistry of Lipids and Membranes*, pp. 25-72. Edited by D. E. Vance & J. E. Vance. New York (USA): Benjamin/Cummings.

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

Daffé, M., Brennan, P. J. & McNeil, M. (1990). Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosyl alditol fragments by gas chromatography/mass spectrometry and by 1H and 13C NMR analyses. *J Biol Chem* 265, 6734-6743.

Demain, A. L. & Birnbaum, J. (1968). Alteration of permeability for the release of metabolites from the microbial cell. *Curr Top Microbiol Immunol* 46, 1-25.

Dmitriev, B. A., Ehlers, S. & Rietschel, E. T. (1999). Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function. *Med Microbiol Immunol* 187, 173-181.

Dmitriev, B. A., Ehlers, S., Rietschel, E. T. & Brennan, P. J. (2000). Molecular mechanics of the mycobacterial cell wall: from horizontal layers to vertical scaffolds. *Int J Med Microbiol* 290, 251-258.

Dörner U., Maier E. & Benz R. (2004). Identification of a cation-specific channel (TipA) in the cell wall of the gram-positive mycolata *Tsukamurella inchonensis*: the gene of the channel-forming protein is identical to *mspA* of *Mycobacterium smegmatis* and *mppA* of *Mycobacterium phlei*. *Biochim Biophys Acta* 17, 1667(1), 47-55.

Draper, P. (1982). The anatomy of *Mycobacteria*. In: *The Biology of Mycobacteria*, vol. 1., pp.9-49. Edited by C. Ratledge & J. L. Standford. London (UK): Academic Press.

Eggeling, L. & Sahm, H. (1999). Glutamate and l-lysine: traditional products with impetuous developments. *Appl Microbiol Biotechnol* 52, 146-153.

Eggeling, L. & Sahm, H. (2001). The cell wall barrier of *Corynebacterium glutamicum* and amino acid efflux. *J Biosci Bioeng* 92, 102-213

Engelhardt, H., Heinz, C. & Niederweis, M. (2002). A tetrameric porin limits the cell wall permeability of *Mycobacterium smegmatis*. *J Biol Chem* 277, 37567-37572.

Faller, M., Niederweis, M. & Schulz, G. E. (2004). The structure of a mycobacterial outer-membrane channel. *Science* 303, 1189-1192.

Freudl, R. (1992). Protein secretion in gram-positive bacteria. *J Biotechnol* 23, 231-240.

Fudou, R., Jojima, Y., Seto, A., Yamada, K., Kimura, E., Nakamatsu, T., Hirashi, A. & Yamanaka, S. (2002). *Corynebacterium efficiens sp. nov.*, a glutamic-acid-producing species from soil and vegetables. *Int J Syst Bacteriol* 52, 1127-1131.

Galanos, C., Roppel, J., Weckesser, J., Rietschel, E. T. & Mayer, H. (1977). Biological activities of lipopolysaccharides and lipid A from *Rhodospirillaceae*. *Infect Immun* 6, 407-412.

Gey Van Pittius, N. C., Gamieldien, J., Hide, W., Brown, G. D., Siezen, R. J. & Beyers, A. D. (2001). The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol* 2(10), 44.1-44.18.

Ghuysen, J. M. (1968). Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. *Bacteriol Rev* 32, 425-464.

Glauert, A. M. & Thornley, M. J. (1969). The topography of the bacterial cell wall. *Annu Rev Microbiol* 3, 159-198.

Glazer, A. N. & Nikaido, H. (1995). Microbial Biotechnology: Fundamentals of Applied Microbiology. New York: W. H. Freeman and Company.

Goodfellow, M. & Cross, T. (1983). Classification. In: *The biology of Actinomycetes*, pp. 7-164. Edited by M. Goodfellow, M. Mordaski, & S. Williams. London (UK): Academic Press.

Goodfellow, M., Collins, M. D. & Minnikin, D. E. (1976). Thin-layer chromatographic analysis of mycolic acid and other long-chain components in whole-organism methanolysates of coryneform and related taxa. *J Gen Microbiol* 96, 351-358.

Gross, E. (1967). The cyanogen bromide reaction. *Meth Enzymol* 11, 238-255.

Gutmann, M., Hoischen, C. & Krämer, R. (1992). Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. *Biochim Biophys Acta* 1112, 115-123.

Hancock, R. E. W. (1987). Role of porins in outer membrane permeability. *J Bacteriol* 169, 929-933.

Heinz, C., Karosi, S. & Niederweis, M. (2003). High-level expression of the mycobacterial porin MspA in *Escherichia coli* and purification of the recombinant protein. *J Chromatogr B Analyt Technol Biomed Life Sci* 790(1-2), 337-348.

Hermann, T. (2003). Industrial production of amino acids by coryneform bacteria. *J Biotechnol* 104(1-3), 155-172.

Hirasawa, T., Wachi, M. & Nagai, K. (2000). A mutation in the *Corynebacterium glutamicum ltsA* gene causes susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production. *J Bacteriol* 182(10), 2696-2701.

Hirasawa, T., Wachi, M. & Nagai, K. (2001). L-glutamate production by lysozyme-sensitive *Corynebacterium glutamicum ltsA* mutant strains. *BMC Biotechnol* 1(1), 1-9.

Hirose, Y. & Okada, H. (1979). Microbial production of amino acids. In: *Microbial Technology* 1. pp. 211-240. New York (USA): Academic Press.

Hoischen, C. & Kramer, R. (1990). Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. *J Bacteriol* 172, 3409-3416.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). Nocardioform actinomycetes. In *Bergey's Manual of Determinative Biology, 9th edn.*, pp. 625–650. Baltimore: The Williams and Wilkins Co.

Imoto, T., Johnson, L., North, A., Phillips, D. & Rupley, J. (1972). Vertebrate Lysozymes. In: The Enzymes VII. Edited by P. Boyer. New York (USA): Academic Press.

Ioneda, T. (1993). Chromatographic and mass spectrometric characterization of 3-O-benzoyl methyl ester derivatives of mycolic acid fractions from *Corynebacterium pseudotuberculosis, C. diphtheriae and Rhodococcus rhodochrous*. *Chem Phys Lipids* 65(2), 93-101.

Jarlier, V. & Nikaido, H. (1990). Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. *J Bacteriol* 172, 1418-1423.

Kamio, Y. & Nikaido, H. (1976). Outer membrane of *Salmonella typhimurium*; accessibility of phospholipid head groups to phospholipase C and cyanogens bromide activated dextran in the external medium. *Biochemistry* 15, 2561-2570.

Katsumata, R., Ozaki, A., Oka, T. & Furuya, A. (1984). Protoplast transformation of glutamate-producing bacteria with plasmid DNA. *J Bacteriol* 159, 306-311.

Keddie, R. M. & Cure, G. J. (1977). The cell wall composition and distribution of the free mycolic acids in named strains of coryneform bacteria and in isolates from various natural sources. *J Appl Bacteriol* 42, 229-252.

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

Khamis, A., Raoult, D. & La Scola, B. (2004). *rpoB* gene sequencing for identification of *Corynebacterium* species. *J Clin Microbiol* 42(9), 3925-3931.

Kimura, E., Yaghoshi, C., Kawahara, Y., Ohsumi, T., Nakamatsu, T. & Tokuda, H. (1999). Glutamate overproduction in *Corynebacterium glutamicum* triggered by a decrease in the level of a complex comprising DtsR and a biotin-containing subunit. *Biosc Biotechnol Biochem* 63, 1274-1278.

Kinoshita, S. & Tanaka K. (1972). Glutamic acid. In: *The Microbial Production of Amino Acids* pp. 263-324. Edited by K. Yamada, S. Kinoshita, T. Tsunoda & K. Aida. New York (USA): John Wiley and Sons.

Kinoshita, S., Udaka S. & Shimono M. (1957). Studies on the amino acid fermentation. Production of L-glutamate by various microorganisms. *J Gen Appl Microbiol* 3, 193-205.

Kohler, T., Aautrost, D., Laner, A. K., Rost, A. K., Thamm, B., Pustowoi & Labner, D. (1995). Quantisation of mRNA by Polymerase Chain Reaction: Nonradioactive PCR Methods. Berlin: Editorial Springer.

Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157(1), 105-132.

Lakey, D. L, Voladri, R. K., Edwards, K. M., Hager, C., Samten, B., Wallis, R. S., Barnes, P. F. & Kernodle, D. S. (2000). Enhanced production of recombinant *Mycobacterium tuberculosis* antigens in *Escherichia coli* by replacement of low-usage codons. *Infect Immun* 68(1), 233-238.

Lambert, P.A. (2002). Cellular impermeability and uptake of biocides and antibiotics in gram-positive bacteria and mycobacteria. *J Appl Microbiol* 92, 46S-54S.

Lee, C. W., Lucas, S. & Desmazeaud, M. J. (1985). Phenylalanine and tyrosine catabolism in some cheese coryneform bacteria. *FEMS Microbiology Letters* 26, 201-205.

Lehmann, K. & Neumann, R. (1896). Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik. Edited by J.F. Lehman. München (Germany).

Leuchtenberger, W. (1996). Amino acids – Technical production and use. Products of primary metabolism. In *Biotechnology vol VI*., pp. 465-502. Edited by H. J. Rehm, A. Pühler, G. Reed & P. J. W. Stadler. Weinheim: Wiley-VCH.

Lichtinger, T., Burkovski, A., Niederweis, M., Krämer, R. & Benz, R. (1998). Biochemical and biophysical characterization of the cell wall channel of *Corynebacterium*

glutamicum: the channel is formed by a low molecular mass subunit. *Biochemistry* 37, 15024-15032.

Lichtinger, T., Heym, B., Maier, E., Eichner, H., Cole, S. T. & Benz, R. (1999). Evidence for a small anion-selective channel in the cell wall of *Mycobacterium bovis* BCG besides a wide cation-selective pore. *FEBS Lett* 454, 349–355.

Lichtinger, T., Riess, F. G. & Benz, R. (2000). Biochemical identification and biophysical characterization of a channel-forming protein from *Rhodococcus erythropolis*. *J Bacteriol* 182, 764-770.

Lichtinger, T., Riess, F. G., Burkovski, A., Engelbrecht, F., Hesse, D., Kratzin, H. D., Krämer, R. & R. Benz, R. (2001). The low-molecular-mass subunit of the cell wall channel of the Gram-positive *Corynebacterium glutamicum.* Immunological localization, cloning and sequencing of its gene *porA*. *Eur J Biochem* 268, 462-469.

Liu, J., Barry III, C. E. & Nikaido, H. (1999). Cell wall: physical structure and permeability. In: *Mycobacteria: Molecular biology and virulence*, pp.220-239. Edited by Ratlege, C. & J. Dale. Oxford (UK): Blackwell Science Ldt.

Liu, J., Barry III, C. E., Besra, G. S. & Nikaido, H. (1996). Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *J Biol Chem* 271, 29545-29551.

Liu, J., Rosenberg, E. Y. & Nikaido, H. (1995). Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proc Natl Acad Sci USA* 92, 11254-11258.

Luckey, M. & Nikaido, H. (1980). Specificity of diffusion channels produced by lambda phage receptor protein of *Escherichia coli*. *Proc Natl Acad Sci USA* 77, 167-171.

Ludwig, O., De Pinto, V., Palmieri, F. & Benz, R. (1986). Pore formation by the mitochondrial porin of rat brain in lipid bilayer membranes. *Biochim Biophys Acta.* 860, 268-276.

Marienfeld, S., Uhlemann, E. M., Schmid, R., Krämer, R. & Burkovski, A. (1997). Ultrastructure of the *Corynebacterium glutamicum* cell wall. *Antonie Van Leeuwenhoek* 72, 291-297.

Martin, J. F., Santamaria, R., Sandoval, H., del Real, G., Mateos, L. M., Gil, J. A. & Aguilar, A. (1987). Cloning systems in amino acid-producing corynebacteria. *Bio/Technol* 5, 137-146.

McNeil, M. R. & Brennan, J. P. (1991). Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. *Res Microbiol* 142, 451-463.

Menestrina, G. & Antolini, R. (1981). Ion transport through hemocyanin channels in oxidized cholesterol artificial bilayer membranes. *Biochim Biophys Acta*. 643, 616-625.

Minnikin, D. E. & Goodfellow, M. (1980). Lipid composition in the classification and identification of acid-fast bacteria. *Soc Appl Bacteriol Symp Ser* 8, 189-256.

Minnikin, D. E. (1987). Chemical targets in cell envelopes. In *Chemotherapy of Tropical Diseases*, pp. 19–43. Edited by M. Hopper. Chichester: John Wiley & Sons Ltd.

Minnikin, D. E. (1991). Chemical principles in the organization of lipid components in the mycobacterial cell envelope. *Res Microbiol* 142, 423-427.

Minnikin, D. E., Goodfellow, M. & Collins, M. D. (1978). Lipid composition in the classification and identification of coryneform and related taxa. In: *Coryneform bacteria*, pp. 885-159. Edited by: Bousfield I. J. & A. G. Calley. London (UK): Academic Press.

Minnikin, D. E., Minnikin, S. M., Goodfellow, M. & Stanford, J. L. (1982). The mycolic acids of *Mycobacterium chelonei. J Gen Microbiol* 128, 817-822.

Minnikin, D. E., Minnikin, S. M., Parlett, J. H., Goodfellow, M. & Magnusson, M. (1984). Mycolic acid patterns of some species of *Mycobacterium*. *Arch Microbiol* 139, 225-231.

Minnikin, D. E., Patel, P. V. & Goodfellow, M. (1974). Mycolic acids of representative strains of *Nocardia* and the "rhodochrous" complex. *FEBS Lett* 39, 322-324.

Montilla, R., Williams, R. P., Lóren, J. G. & Viñas, M. (1991). Lipopolysaccharide is the receptor for kappa phage in *Serratia marcescens*. *Antonie Van Leeuwenhoek* 59, 15-18.

Nelson, A. P. & McQuarrie, D. A. (1975). The effect of discrete charges on the electrical properties of the membrane. *J Theor Biol* 55, 13-27.

Neuhoff, V., Arold, N., Taube, D. & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9, 255-262.

Niederweis, M., Ehrt, S., Heinz, C., Klöcker, U. Karosi, S., Swiderek, K. M., Riley, L. W. & Benz, R. (1999). Cloning of the *mspA* gene encoding a porin from *Mycobacterium smegmatis*. *Mol Microbiol* 33, 933–945.

Niederweis, M., Maier, E., Lichtinger, T., Benz, R. & Krämer, R. (1995). Identification of channel-forming activity in the cell wall of *Corynebacterium glutamicum*. *J Bacteriol* 177, 5716-5718.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* 49, 1-32.

Nikaido, H. (1994). Porins and specific diffusion channels in bacterial outer membranes. *J Biol Chem* 269, 3905-3908.

Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. Microbiol. *Mol Biol Rev* 67(4), 593–656.

Nikaido, H., Kim, S. H. & Rosenberg, E. Y. (1993). Physical organization of lipids in the cell wall of *Mycobacterium chelonae*. *Mol Microbiol* 8, 1025-1030.

Nishio, Y., Nakamura, Y., Kawarabayasi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K. & Gojobori, T. (2003). Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. *Genome Res*13(7), 1572-1579.

Nunheimer, T. D., Birnbaum, J., Ihnen, E. D. & Demain, A. L. (1970). Product inhibition of the fermentative formation of glutamic acid. *Appl Microbiol* 20, 215-217.

Ochi, K. (1995). Phylogenetic analysis of mycolic acid-containing wall-chemotype IV actinomycetes and allied taxa by partial sequencing of ribosomal protein AT-L30. *Int J Syst Bacteriol* 45, 653-660.

Ogata, K., Kinoshita, S., Tsunoda, T. & Aida, T. (1976). Microbial production of nucleic acid related substances. New York (USA): John Wiley & Sons Inc.

Palomar, J., Lenaroz, S. & Viñas, M. (1995). *Serratia marcescens* adherence: the effect of O-antigen presence. *Microbios* 81, 107-113.

Paul, T. R. & Beveridge, T. J. (1992). Re-evaluation of envelope profiles and cytoplasmic ultrastructure of mycobacteria processed by conventional embedding and freeze-substitution protocols. *J Bacteriol* 174, 6508-6517.

Peyret, J. L., Bayan, N., Joliff, G., Gulik-Krzywicki, T., Mathieu, L., Schechter, E. & Leblon, G. (1993). Characterization of the *cspB* gene encoding PS2, an ordered surface-layer protein in *Corynebacterium glutamicum*. *Mol Microbiol* 9, 97-109.

Plesiat, P. & Nikaido, H. (1992). Outer membranes of Gram-negative bacteria are permeable to steroid probes. *Mol Microbiol* 6, 1323-1333.

Prager, E., Wilson, A. & Arnheim, N. (1974). Widespread Distribution of Lysozyme in Egg White of Birds. *J Biol Chem* 249, 7295-7297.

Pratt, L. A., Hsing, W., Gibson, K. E. & Silhavy, T. J. (1996). From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Mol Microbiol* 20(5), 911-917.

Puech, V., Chami, M., Lemassu, A., Laneelle, M. A., Schiffler, B., Gounon, P., Bayan, N., Benz, R. & Daffe, 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.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K. E., Marchal, G., Leclerc, C. & Cole, S. T. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis, *Nat Med* 9(5), 533-539.

Rastogi, N. (1991). Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug-resistance. *Res Microbiol* 142, 464-476.

Rastogi, N., Fréhel, C. & David, H. L. (1986). Triple-layered structure of mycobacterial cell wall: evidence for the existence of a polysaccharide-rich outer layer in 18 mycobacterial species. *Curr Microbiol* 13, 237-242.

Rieß, F. G., Dörner, U., Schiffler, B. & Benz, R. (2001). Study of the properties of a channel forming protein of the cell wall of the gram-positive bacterium *Mycobacterium phlei*. *J Membr Biol* 182, 147-157.

Riess, F. G., Elflein, M., Benk, M., Schiffler, B., Benz, R., Garton, N. & Suttcliffe, I. (2003). The cell wall of the pathogenic bacterium *Rhodococcus equi* contains two channel-forming proteins of different properties. *J Bacteriol* 145, 2952-2960.

Riess, F. G., Lichtinger, T., Cseh, R.,Yassin, A. F., Schaal, K. P. & Benz, R. (1998). The cell wall channel of *Nocardia farcinica*: biochemical identification of the channel-forming protein and biophysical characterization of the channel properties. *Mol Microbiol* 29, 139–150.

Rowlands, R. T. (1984). Industrial strain improvement: mutagenesis and random screening procedures. *Enzyme Microb Technol* 6, 3-10.

Sahm, H., Eggeling, L. & de Graaf, A. A. (2000). Pathway analysis and metabolic engineering in *Corynebacterium glutamicum. Biol Chem* 381, 899-910.

Sahm, H., Eggeling, L., Eikmanns, B. & Krämer, R. (1995). Metabolic design in amino acid producing bacterium *Corynebacterium glutamicum*. *FEMS Microbiol Rev* 16, 243-252.

Sahm, H., Eggeling, L., Eikmanns, B. & Krämer, R. (1996). Construction of L-lysine-, L-threonine-, and L-isoleucine-overproducing strains of *Corynebacterium glutamicum*. *Ann NY Acad Sci* 782, 25–39.

Santamaria, R., Gil, J. A., Mesas, J. M. & Martin, J. F. (1984). Characterization of an endogenous plasmid and development of cloning vectors and transformation system in *Brevibacterium lactofermentum*. *J Gen Microbiol* 130, 2237-2246.

Schägger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166, 368-379.

Schlegel, H. G. (1993). General microbiology, 7th Edition. New York (USA): Cambridge University Press.

Schleifer, K. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36, 407-477.

Schmid, K., Ebner, K., Jahreis, K., Lengeler, J. W. & Titgemeyer, F. (1991). A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. *Mol Microbiol* 5, 941-950.

Schülein, K., Schmid K. & Benz R. (1991). The sugar specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substrate-specific porins. *Mol Microbiol* 5, 2233-2241.

Schulz, G.E. (2002). The structure of bacterial outer membrane proteins. *Biochim Biophys Acta* 1565(2), 308-317.

Senaratne R. H., Mobasheri H., Papavinasasundaram K. G., Jenner P., Lea E. J. & Draper, P. (1998). Expression of a gene for a porin-like protein of the OmpA family from *Mycobacterium tuberculosis* H37Rv. *J Bacteriol* 180(14), 3541-3547.

Sleytr, U. B., Messner, P., Pum, D. & Sára, M. (1996). Occurrence, location, ultrastructure and morphogenesis of S-layers. In: *Crystalline Bacterial Cell Surface Proteins*, pp. 5-33. Edited by U. B. Sleytr, P. Messner, D. Pum & M. Sára. Austin (USA): R. G. Landes/Academic Press.

Soual-Hoebeke, E., de Sousa-D'Auria, C., Chami, M., Baucher, M. F., Guyonvarch, A., Bayan N., Salim, K. & Leblon, G. (1999). S-layer protein production by *Corynebacterium* strains is dependent on the carbon source. *Microbiol* 145, 3399-3408.

Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Inter J Syst Bact* 47, 479-491.

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, 451-464.

Sutcliffe, I. (1997). Macroamphiphilic cell envelope components of *Rhodococcus equi* and closely related bacteria. *Vet Microbiol* 56, 287-299.

Suzuki, T., Honda, H. & Katsumata, R. (1972). Production of antibacterial compounds analogous to chloramphenicol by n-paraffin-grown bacteria. *Agric Biol Chem* 36, 2223-2228.

Takinami, K., Yoshii, H., Tusi, H. & Okada, H. (1965). Biochemical effects of fatty acid and its derivates on glutamic acid fermentation. Part III. Biotin-tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentatum*. *Agric Biol Chem* 29, 351-359.

Tomas, J. M., Benedí, V. J., Ciurana, B. & Jofre J. (1986). Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect Immun* 54, 85-89.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* 76, 4350-4354.

Trias, J. & Benz, R. (1993). Characterization of the channel formed by the mycobacterial porin in lipid bilayer membranes. Demonstration of voltage gating and of negative point charges at the channel mouth. *J Biol Chem* 268, 6234-6240.

Trias, J. & Benz, R. (1994). Permeability of the cell wall of *Mycobacterium smegmatis*. *Mol Microbiol* 14, 283-290.

Trias, J., Jarlier V. & Benz, R. (1992). Porins in the cell wall of mycobacteria. *Science* 258, 1479-1481.

Uchida, K. & Aida, K. (1979) Taxonomic significance of cell-wall acyl type in *Corynebacterium-Mycobacterium-Nocardia* group by a glycolate test. *J Gen Appl Microbiol* 25, 169-183.

Udaka, S. (1960). Screening method for microorganisms accumulating metabolites and its use in the isolation of *Micrococcus glutamicus*. *J Bacteriol* 79, 745-755.

Vaara, M. W., Plachy, Z. & Nikaido, H. (1990). Partitioning of hydrophobic probes into lipopolysaccharide bilayers. *Biochim Biophys Acta* 1024, 152-158.

Walthers, D., Go, A. & Kenney, L. J. (2004). Regulation of porin gene expression by the two-component regulatory system EnvZ/OmpR. In: *Bacterial and Eukaryotic Porins, Structure, Function, Mechanism*, pp. 1-24. Edited by R. Benz. Weinheim: Wiley-VCH.

Weiss, M. S., Wacker, T., Weckesser, J., Welte, W. & Schulz, G. E. (1990) The three-dimensional structure of porin from *Rhodobacter capsulatus* at 3 A resolution. *FEBS Lett* 267(2), 268-272.

Wietzerbin, J., Das, B. C., Petit, J. F., Lederer, E., Leyh-Bouille, M. & Ghuysen, J. M. (1974). Occurrence of D-alanyl-(D)-meso-diaminopimelic acid and meso-diaminopimelyl-meso-diaminopimelic acid interpeptide linkages in the peptidoglycan of Mycobacteria. *Biochemistry* 13, 3471-3476.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews* 51, 221-271.

Woese, C. R., Olsen, G. J., Ibba, M. & Söll, D. (2000). Aminoacyl-tRNA synthethases, the genetic code, and the evolutionary process. *Microbiol Mol Biol Rev* 64, 202-236.

Wohlleben, W., Muth, G. & Kalinowski, J. (1993). Genetic engineering of gram-positive bacteria. In: *Biotechnology: Genetics and fundamentals of genetic engineering*, pp.477-505. Edited by H. J. Rehm, G. Reed, A. Puehler & H. Sahm. Weinheim (Germany): VCH Publishers.

Wood, S., Williams, S. T. & White, W. R. (1983). Microbes as a source of earthy flavors in potable water-A Review. *Int Biodet Bull* 19, 83-97.

Yague, G., Segovia, M. & Valero-Guillen P. L. (2000). Detection of mycoloylglycerol by thin-layer chromatography as a tool for the rapid inclusion of corynebacteria of clinical origin in the genus *Corynebacterium*. *J Chromatogr B Biomed Sci Appl* 738(1), 181-185.

Yamada, Y., Won-Seo, C. & Okada H. (1985). Oxidation of acyclic terpenoids by *Corynebacterium sp*. *Appl Environ Microbiol*. 49, 960-963.

Yano, I. & Saito, K. (1972). Gas chromatographic and mass spectrometric analysis of molecular species of corynomycolic acids from *Corynebacterium ulcerans. FEBS Lett* 23, 352-356.

Yoshihama, M., Higashiro, K., Rao E. A., Akedo, M., Shanabruch, W. G., Follettie, M. T., Walker, G. C. & Sinskey, A. J. (1985). Cloning vector system for *Corynebacterium glutamicum*. *J Bacteriol* 162, 591-597.

Zarjic, J. E., Guinard, H. & Gerson, D. F. (1977). Emulsifying and surface active agents from *Corynebacterium hydrocarboclastum*. *Biotechnol Bioeng* 19, 1295-1301.

Zhang, J. & Madden, T. L. (1997). PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. *Genome Res*. 7, 649-656.

Zierhut, G., Piepersberg, W. & Bock, A. (1979). Comparative analysis of amino-glycosides on bacterial protein synthesis in vitro. *Eur J Biochem* 98, 577-583.

6.3 Curriculum vitae

Name: **Peter Hünten**

Address: Scharoldstrasse 7,
97080 Würzburg

Date and Place of birth: 12/05/1974 Würzburg/Germany

Marital status: unmarried

Schooling:

1980-1984	Elementary school Schweinfurt
1984-1993	Alexander-von-Humboldt-Gymnasium Schweinfurt
06/1993	Abitur

Civilian service: 10/1993–11/1994 Franziskus-Schule Schweinfurt

Academical Carreer:

11/1994-12/2000	Study of biology at the University of Würzburg Major subject: Biotechnology, minor subject: human genetics, physiology of animals Diploma thesis: “Studies of channel-forming proteins in the cell wall of actinomycetes“
12/2000	Diploma
01/01/2001	Start of Ph.D.-Thesis in the laboratory of Prof. Dr. R. Benz at the University of Würzburg
01/2002	Research stay and guest speaker at the IPBS Toulouse in the research group of Prof. Mathias Winterhalter

6.4 Acknowledgements

Mein herzlicher Dank gilt...

Prof. Dr. Roland Benz

für die Möglichkeit, meine Doktorarbeit in einem so netten Umfeld durchführen zu können, für das interessante Thema, seine Geduld, seinen Enthusiasmus und nicht zu vergessen, für seine interessanten Ausführungen in die Weinkunde.

Prof. Dr. Joachim Reidl

für seine Bereitschaft, Zweitprüfer dieser Arbeit zu sein.

meinen Eltern

sie haben mich unterstützt wo sie nur konnten, lieben Dank für Alles!

Alex

meine Mitbewohnerin, mi vida

meinen Freunden

im besonderen Vox, der Vogel, Artur, ein alter Weggefährte auf den man sich immer verlassen kann, Frank, ein treuer Freund von der alten Truppe, Stepho, der schlaue Engländer, und allen anderen.....

der derzeitigen Besatzung des Arbeitskreises

Enrico Barth, Martina Egerer, meine opti Diplomanden, Elke Maier, unsere fleissige Imme (hmm), Bettina Schiffler, hat sich inzwischen als opti Schafkopfersatzfrau herausgestellt und war mir während meiner ganzen Zeit immer mit Rat und Tat zur Seite gestanden, Ursel Dörner, unser wandelndes Informationszentrum, Markus Thein, Pamela Bogner, Maja Klug und Katrin Denker, die Kellerkinder, Tobi Neumeyer und Michel Leuber, die Viktorias Spezialisten, Peter Reichling und Bernd Pfaff, die Intech Crew, die man nur selten zu Gesicht bekommt

der AG Andersen

Christian Andersen, er hatte immer ein offenes Ohr für mich, bei ihm begann meine Zeit in der AG Benz, Joschi Stegmeier, der einzige der immer bereit war, eine treue Zockerseele, Standard Begleiter und Fussball-Gucker, Bettina Mühling, das Würstchen mit den kalten Pfoten, Georg Polleichtner, der Tänzer, Nico Brandes, der harte Tabasco-Mann, und Andi Glück, unser Call-Boy

den vielen Ehemaligen

Oli Knapp, mein kurzzeitiger Mitbewohner, für seine ausgefallenen Kochkreationen und die Gespräche über seine interessanten Geschäftsideen (jetzt wird's langsam Zeit eine davon zu verwirklichen), Michi Benk, unser Granitschwabe, Emir Berkane, es war ein Träum mit Dir, cool, Dich kennengelernt zu haben, Noelia Costa, durch die ich die Köstlichkeiten Spaniens kennengelernt habe, Frank Orlik, der geborene Lehrer, Arno Karner, unser Öschi, Christian Hotz und Jorge Rodriguez, für sehr feuchtfröhliche Abende

und den Mitarbeitern des Lehrstuhls für Biotechnologie

für die gute Atmosphäre und die Hilfsbereitschaft, besonders zu erwähnen sind Brunni, der Bausparer und Charles, der Besonnene