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

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Abstract

The cell wall of *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 also 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 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 on the resulting amino acid sequence, the corresponding gene, designated as $porH_{C.glut}$, was identified in the published genome sequence of *C. glutamicum* ATCC13032. $PorH_{C.glut}$ contains only the inducer methionine but no N-terminal extension, which suggests that the export and assembly of the protein follow a yet unknown pathway. $PorH_{C.glut}$ is coded in the bacterial chromosome by a gene that is localized in the vicinity of $porA_{C.glut}$, within a putative operon of 13 genes. RT-PCR revealed that both porins are cotranscribed. They coexist according to immunological detection experiments in the cell wall of *C. glutamicum* together with $PorB_{C.glut}$ and $PorC_{C.glut}$.

Keywords: Cell wall channel; Mycolic acid; Porin; Lipid bilayer membrane; *Corynebacterium glutamicum*

1. Introduction

In 1957, a bacterium was isolated, which was shown to export large quantities of L-glutamic acid into the culture medium [1]. This bacterium, *Corynebacterium glutamicum*, was described as an aerobic, nonsporulating, Gram-positive rod, capable of growing on a variety of sugars or organic acids [2]. *C. glutamicum* is widely used for industrial production of L-glutamate, L-lysine, and other amino acids through fermentation processes [3–7]. During the year 2002, about 1×10^6 ton of L-glutamate as a flavoring agent in food and 5.5×10^5 ton of L-lysine as animal feed supplements were produced with *C. glutamicum* [8,9]. Little is known about the amino acid efflux properties of corynebacteria [7,10]. Recently, it has been shown that two genes are involved in the export of glutamate in *C. glutamicum* [11,12], but their exact functions are still unknown. *C. glutamicum* is a member of the mycolic-acid-containing actinomycetes, belonging to the mycolata. It was shown in 16 S rRNA and in *rpoB* gene analysis that it is closely related to *C. efficiens* and *C. callunae* [13,14].

Members of the *Corynebacterium–Nocardia–Mycobacterium* complex (i.e. mycolic acid containing members of the Corynebacterineae suborder of Actinomycelates, also known as mycolata) have, in addition to a thick peptidoglycan layer, a second surface layer consisting of covalently

Abbreviations: LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; Genapol, oligoethylenglycol-monoalkylether; PC, diphytanoyl phosphatidylcholine; nS, nanosiemens

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bound mycolic acids and extractable lipids [2,15,16]. Ester bonds link the mycolic acids to the arabinogalactan, which is covalently attached to the murein of the cell wall. The chain length of the mycolic acids varies considerably in different taxa of the *Corynebacterium–Nocardia–Mycobacterium* complex. In Corynebacteria, they consist of 22–38 carbon atoms [17–21]. The outer layer of the *Corynebacterium–Nocardia–Mycobacterium* complex functions as a permeability barrier [22,23], it provides, for example, resistance to cell lysis by egg white lysozyme [24]. This suggests that their cell wall 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. 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 [25–27]. Concerning the transport of amino acids over this barrier, it is of particular importance to understand the hydrophilic pathways present in the cell wall of Corynebacteria [7].

Several channel-forming proteins have been identified and characterized in the cell wall of members of the *Corynebacterium–Nocardia–Mycobacterium* complex [28–31]. Similar to the porins of Gram-negative bacteria, these wide and water-filled channels allow the permeation of hydrophilic solutes through the cell wall [25,26]. Their study is of considerable interest because the Corynebacterineae family comprises microorganisms that cause worldwide dangerous infections such as *M. tuberculosis*, *Mycobacterium leprae*, and *Corynebacterium diphtheriae*. $PorA_{C.glut}$ from *C. glutamicum* (a small 45-amino-acid-long polypeptide) was the first pore forming protein characterized in corynebacteria. The cation-selective channel is formed by a $PorA_{C.glut}$ oligomer with a single-channel conductance of about 5.5 nS in 1 M KCl [29]. By deleting the $porA_{C.glut}$ gene from the *C. glutamicum* chromosome, an anion-selective pore forming protein was discovered, named $PorB_{C.glut}$ [31]. It is a 99-amino-acid-long protein that has a single-channel conductance of about 700 pS in 1 M KCl. The $PorB_{C.glut}$ channel can be blocked by citrate. A search for homologous genes showed that the chromosome of *C. glutamicum* contained also the gene for a PorB-like protein 138 bp downstream from $porB_{C.glut}$, called $PorC_{C.glut}$. 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 both genes are cotranscribed [31].

Here, we describe the search for an additional cell wall channel of *C. glutamicum* ATCC13032. The channel-forming protein was purified to homogeneity and named $PorH_{C.glut}$. Interestingly, its gene $porH_{C.glut}$ can be found in the *C. glutamicum* chromosome next to $porA_{C.glut}$. RT-PCR experiments from overlapping regions between $porA_{C.glut}$ and $porH_{C.glut}$ demonstrated that they are also cotranscribed. Electron microscopic analyses of whole *C. glutamicum* cells suggested that the four porins, $PorA_{C.glut}$, $PorH_{C.glut}$, $PorB_{C.glut}$, and $PorC_{C.glut}$, of *C. glutamicum* are all present in its cell wall.

2. Materials and methods

2.1. 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 $CaCl_2{\cdot}2H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeSO_4$, 10 mg $MnSO_4{\cdot}2H_2O$, 1 mg $ZnSO_4{\cdot}7H_2O$, 0.2 mg $CuSO_4{\cdot}5H_2O$, 0.02 mg $NiCl{\cdot}6H_2O$, and 0.2 mg biotin/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 a BHI medium (brain heart infusion, Difco Laboratories).

2.2. 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 the isolation and purification of $PorA_{C.glut}$ of *C. glutamicum* [29]. 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 J2-21 M/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 h at room temperature under stirring in a closed tube to avoid loss of chloroform. Cells and chloroform/methanol solution were centrifuged for 15 min (10,000 rpm in Beckman J2-21 M/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 (*N*,*N*-dimethyldodecylamine-*N*-oxide) and 10 mM Tris–HCl (pH 8) and inspected for channel-forming activity. The protein was subjected to fast protein 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.

2.3. SDS-PAGE

SDS-PAGE was performed with tricine-containing gels [32]. The gels were stained with colloidal Coomassie [33] or silver stain [34]. Before separation, the samples were all incubated for 5 min at 100 °C with loading buffer. Preparative SDS-PAGE was used for the identification and purification of the channel-forming activity from the organic solvent extracts of whole *C. glutamicum* cells. For this, different molecular mass bands were excised and eluted overnight at 4 °C in a solution containing 10 mM Tris–HCl (pH 8) supplemented with 1% Genapol.

2.4. 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 [35]. 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 × 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, besides small subsequences.

2.5. RT-PCR

Total mRNA was isolated from disrupted cells grown until the 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 2 U of DNase I (Ambion, Huntingdon, Cambridgeshire, UK) in 0.1 volumes of 10× 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, 23 S and 16 S) 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 [36], with all primers used for the 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 2,886,331 and 2,907,251 of the chromosomal DNA of *C. glutamicum* (NCBI Reference Sequence accession number NC_003450). The minus strand encodes for all genes inside this 20,920-bp-long fragment, which suggests 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 °C. For negative control, 5 μl 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.

2.6. Lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously [37,38]. The experimental setup 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 min after five-fold salt gradients were established across the membranes [39].

2.7. 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 [29].

2.8. Immunological techniques

Synthetic polypeptides of part of the $PorB_{C.glut}$ sequence (KGEGFWTTQFPQIGDW-NEDQ), part of the $PorC_{C.glut}$ sequence (AHENSTRSELAANLRNSA), and part of the $PorH_{C.glut}$ sequence (DLSLLKETLGNYETFGGNIG-TALQSIPTLL+SILNFFDNFGDL-ADTIGENLDNFSS) were synthesized using solid-phase synthesis, and polyclonal antibodies against these 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.*

Table 1
Oligonucleotides used in this study

Oligonucleotide	Position in the *C. glutamicum* genome	Sequence 5′- -3′
Operon0-5′	2,886,331–2,886,351	AGCATGCTCGACGTCTTGCTC
Operon0-3′	2,887,096–2,887,116	GCGCTAAGGAAGAGCAGTTCC
Operon1-5′	2,887,601–2,887,621	CTTCAGCAGCTCGATCTGGAG
Operon1-3′	2,887,999–2,888,020	CGGCTACGTCTTCGACTTCCTC
Operon2-5′	2,888,040–2,888,060	GGACATCAAGGTTTCCAAGGA
Operon2-3′	2,888,289–2,888,311	CCTCGGCAACTACGAGACCTTC
Operon3-5′	2,888,260–2,888,280	CTCTGAAGAGCGGTACCGATG
Operon3-3′	2,888,950–2,888,970	TGCTGACAAGCCACAGCCTGC
Operon4-5′	2,890,501–2,890,521	CCACGACGTGCTTCCTCATCA
Operon4-3′	2,890,801–2,890,821	CTGGATCGGCACTGGCATTGC
Operon5-5′	2,890,890–2,890,910	AACCGCATCAAGCCTCACGCC
Operon5-3′	2,892,041–2,892,061	GATCCAGAAGCGACCTCATCA
Operon6-5′	2,892,117–2,892,137	GGGTAAACATCAGGAGCGGTC
Operon6-3′	2,893,079–2,893,099	GAACGTGATTCGACGGGATTG
Operon7-5′	2,892,971–2,892,991	CGGTTGGTTTCTGTGGAAGGA
Operon7-3′	2,894,000–2,894,020	CAGCACCTAAGGTGGAGCCTG
Operon8-5′	2,894,909–2,894,929	GGGTCACCGTTTCAATATGAG
Operon8-3′	2,896,201–2,896,221	CAGCACCGCGGCCGGGAGTAC
Operon9-5′	2,897,400–2,897,379	GTAGTCGCCGCCAGGTTTGAG
Operon9-3′	2,898,381–2,898,401	CAGCTCCGCATTCAAGTGGGC
Operon10-5′	2,900,301–2,900,321	GGTGTCCTGCGAATAGGCGGC
Operon10-3′	2,901,390–2,901,410	CTTTACGGCGATGAAGTCCGC
Operon11-5′	2,903,881–2,903,901	GCACCTGAACCACCACAGCCG
Operon11-3′	2,904,850–2,904,870	GTGCTCGGACTGGATAGCAG
Operon12-5′	2,906,491–2,906,511	TGACGCTCCGGCCTCAACTGC
Operon12-3′	2,907,088–2,907,108	GAATGGCTCGTTCGGCGGTTC

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 2,886,331 and 2,907,251 of the chromosomal DNA of *C. glutamicum* (NCBI Sequence accession number NC_003450).

glutamicum. The antiserum was used for Western blots of the extracted protein samples using standard procedures [40]. The blotting time was 5 min 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 s. Control experiments showed that the antibodies were highly specific for either $PorB_{C.glut}$, $PorC_{C.glut}$, or $PorH_{C.glut}$ of *C. glutamicum* (see Fig. 1). The used $PorA_{C.glut}$ antibody was already described earlier [30].

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 $PorB_{C.glut}$, $PorC_{C.glut}$, or $PorH_{C.glut}$ antibodies. After that, 12 nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG antibodies were used in order to localize the porins.

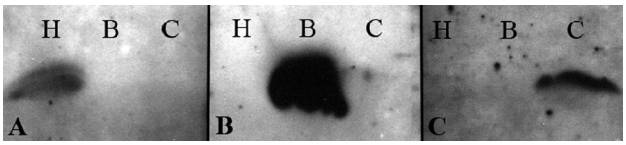

Fig. 1. Western Blot analysis showing the specificity of the anti-$PorB_{C.glut}$, anti-$PorC_{C.glut}$, and anti-$PorH_{C.glut}$ antibodies. The synthetic polypeptide parts of the three proteins were dissolved in 0.4% LDAO, separated on a 12% tricine-containing SDS-PAGE, and blotted on a nitrocellulose membrane. The synthetic polypeptides were visualized using anti-$PorH_{C.glut}$ antibodies (A), anti-$PorB_{C.glut}$ antibodies (B), anti-$PorC_{C.glut}$ antibodies (C), and a chemoluminescence reaction.

3. Results

3.1. 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 $PorA_{C.glut}$, which 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 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 2, Fig. 2) contained the channel-forming activity. The purification of the additional channel-

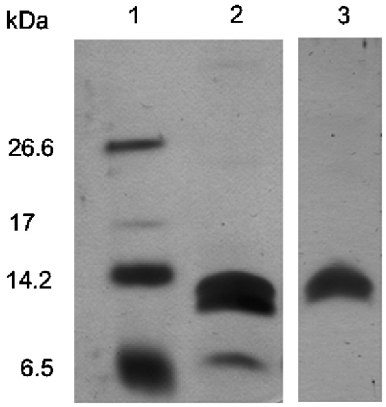

Fig. 2. T12% tricine SDS-PAGE according to Schägger and von Jagow [32] of the purification procedure of PorH$_{C.glut}$ of *C. glutamicum*. The gel was stained with silver. Lane 1: molecular mass marker 26.6 kDa, 17.0 kDa, 14.2 kDa, and 6.5 kDa. Lane 2: 15 μl of ether precipitated extract dissolved in 0.4% LDAO, 10 mM Tris, 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.

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 that 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 from PorA$_{C.glut}$ (see lane 3, Fig. 2). 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 a buffer containing 1% Genapol. Channel formation was found only in the band that corresponded to a molecular mass of 12 kDa, indicating that no other channel-forming impurities were present in the fractions of the HiTrap-Q column, which eluted at 0.3 M NaCl. The channel-forming protein was named PorH$_{C.glut}$.

3.2. PorH$_{C.glut}$ increases the 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 2 min after the addition of the protein, the membrane conductance started to rise and increased by several orders of magnitude in approximately 20 min (see Fig. 3A). 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. Fig. 3A 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. 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 of Fig. 3B). 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* [29,31], *Mycobacterium*

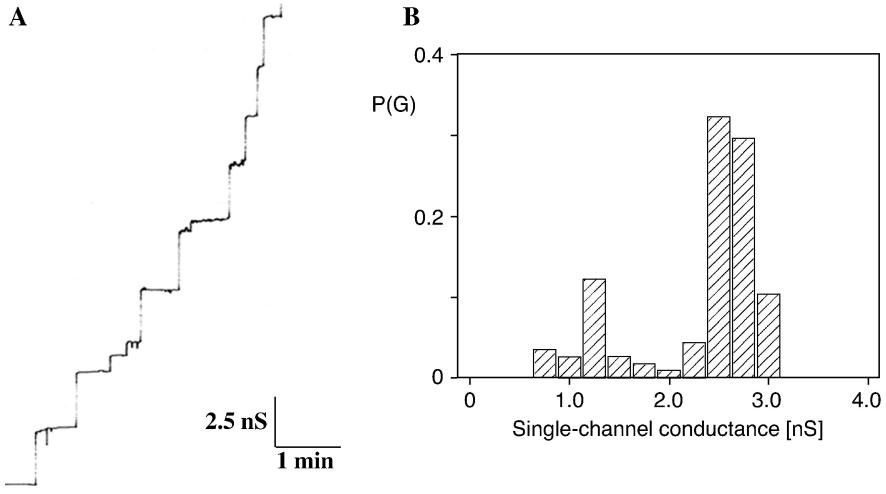

Fig. 3. (A) Single-channel recording of a PC/n-decane membrane in the presence of pure 12 kDa protein (PorH$_{C.glut}$) of the cell wall of *C. glutamicum*. The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml cell wall protein. The applied membrane potential was 20 mV; T=20 °C. (B) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit. $P(G)$ 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 average single-channel conductance was 2.5 nS in 1 M KCl (pH 6) for 115 single-channel events.

chelonae [26], and *Mycobacterium smegmatis* [27]. All these proteins formed channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 min).

Single-channel experiments were 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 conductance was more substantial, whereas the influence of the anion was small. 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 [26,27,29], 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 PorH$_{C.glut}$ channels (see also Discussion and Fig. 9).

3.3. 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 the incorporation of 100 to 1000 channels into a membrane, five-fold KCl gradients were established by the 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 [39] revealed that PorH$_{C.glut}$ was cation selective. On the other hand, it is possible that chloride also has a 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).

Table 2
Average single-channel conductance, G, of PorH$_{C.glut}$ in different salt solutions

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

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. 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 [41, 42] showed that the 13-amino-acid-long stretch of *C. glutamicum* is part of a 57-amino-acid-long hypothetical protein of *C. glutamicum* (see Fig. 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 the translation and assembly of the protein could be very similar to that of PorA$_{C.glut}$ of *C. glutamicum* [30]. 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 Fig. 2) 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$_{C.glut}$.

A search within the chromosome of *C. glutamicum* demonstrated that *porH*$_{C.glut}$ and *porA*$_{C.glut}$ are localized very close to one another (see Fig. 5A). 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*. A comparison of the two 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 Fig. 4).

3.5. RT-RCR

The analysis of the gene region coding for PorH$_{C.glut}$ and PorA$_{C.glut}$ suggested that *porH*$_{C.glut}$ and *porA*$_{C.glut}$ 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 2,886,331 to bp 2,907,251 of the chromo-

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

Fig. 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 after CNBr cleavage 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 homolog 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.

somal DNA of *C. glutamicum*. (GenBank Accession number NC_003450). Interestingly, all genes of this 20,920-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* wild-type 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 Fig. 5B, the mRNA of *C. glutamicum* contained signals that indicated amplification of the overlapping regions between the genes of the putative $porH_{C.glut}$ 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.

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

$PorH_{C.glut}$ is probably too small to account as a monomer for a transmembrane channel. To check whether $PorH_{C.glut}$ forms oligomers, we performed Western blots with different crude cell extracts of *C. glutamicum* cells. One of them was performed with 2% LDAO; the other two consisted of extraction of the cells for 3 h, either with 8 M urea or with a 1:2 mixture of chloroform/methanol (see Materials and methods). After extraction according to the different methods, the cells were centrifuged for 10 min at 14,000 rpm, and the supernatant was subjected to SDS-PAGE followed by Western blot with anti $PorH_{C.glut}$ antibodies. Fig. 6 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

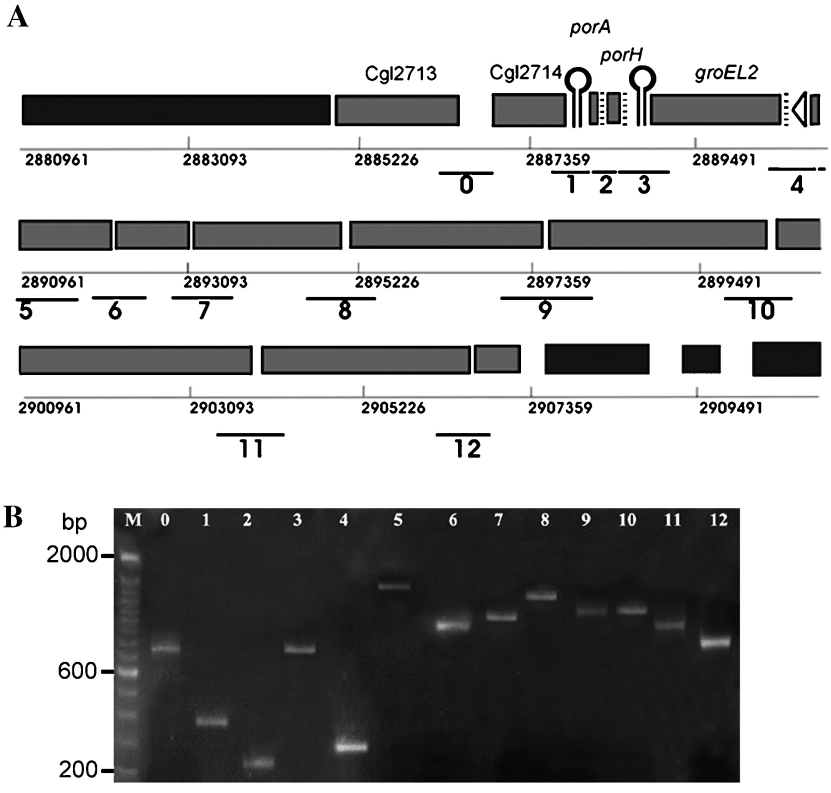

Fig. 5. (A) Overview of the $porH_{C.glut}$ gene locus and its flanking regions within the *C. glutamicum* genome. Putative transcriptional terminators are shown by stem loop structures; potential ribosome binding sites with the sequence AGGAG are shaded, and a putative promoter is presented by a triangle.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

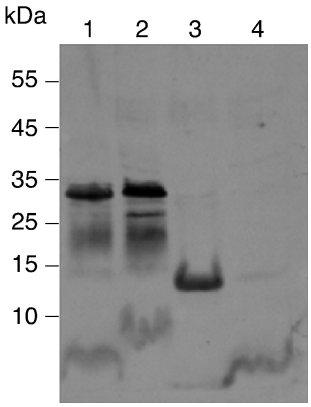

Fig. 6. Western Blot analysis of PorH$_{C.glut}$. The samples were separated on a 10% tricine-containing SDS-PAGE and blotted on a nitrocellulose membrane. Proteins were visualized using anti-PorH$_{C.glut}$ antibodies and a chemoluminescence reaction. Lane 1: 15 μl supernatant of 2% LDAO extraction was solubilized in 5 μl sample buffer without mercaptoethanol. Lane 2: 15 μl supernatant of 2% LDAO extraction, treated for 5 min at 100 °C with 5 μl sample buffer. 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 dissolved pellet after ether precipitation of organic solvent extraction, in 0.4% LDAO, treated for 5 min at 100 °C with 5 μl sample buffer.

LDAO and solubilized at room temperature. Interestingly, the oligomers seem to resist boiling for 5 min in the sample buffer (lane 2, Fig. 6). The 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).

3.7. 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 the 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. Fig. 7A 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 immunogold 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 immunogold 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 immunogold particles were visible in the case of PorB$_{C.glut}$ (C) and PorC$_{C.glut}$ (D). Possibly, the antigenic determinants, chosen for the generation of the corresponding antibodies, were not well accessible from the surface of the cells. SDS-PAGE of total cell extracts using organic solvent, followed by immunodecoration with antibodies against all four channel-forming proteins, demonstrated that they were all present in the cells (see Fig. 7B). 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 [31].

4. Discussion

4.1. 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 [29,30]. It is a small 45-amino-acid-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

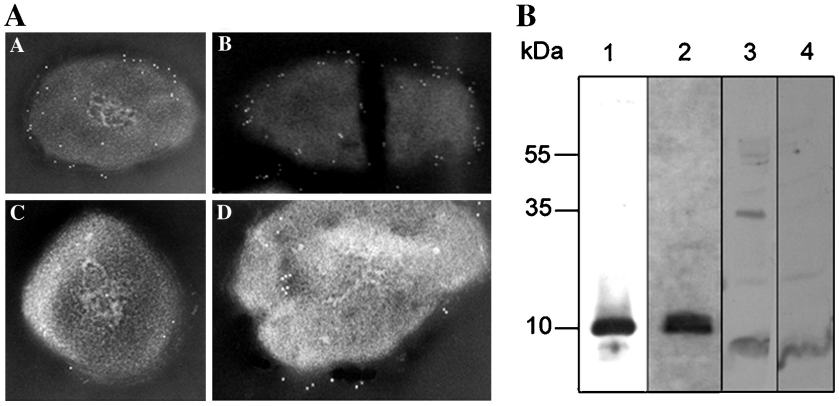

Fig. 7. (A) Electron micrograph of *C. glutamicum* cells, grown in BHI medium, fixed with formaldehyde, treated with anti-PorA$_{C.glut}$ (A), anti-PorH$_{C.glut}$ (B), anti-PorB$_{C.glut}$ (C), and anti-PorC$_{C.glut}$ (D) antibodies and then treated with 12 nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG antibodies. Original magnification: 63,000×. (B) Western Blot analysis of PorA$_{C.glut}$, PorH$_{C.glut}$, PorB$_{C.glut}$, and PorC$_{C.glut}$. The samples (in 0.4% LDAO dissolved pellet resulting from ether precipitation of the organic solvent extract of *C. glutamicum* cells grown in BHI medium) were separated on a 10% tricine 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.

much lower permeability, which is anion selective [31]. 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 [43] and also in the Gram-positive *Rhodococcus equi*, which is likewise a member of the genus *Corynebacterium–Nocardia–Mycobacterium* complex [44]. 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 $PorA_{C.glut}$ [30,45].

The existence of two cation-specific channels of high permeability in the cell wall of *C. glutamicum* seems to be contradictory. So far, the role of $PorH_{C.glut}$ and $PorA_{C.glut}$ is not clear. However, *E. coli* has also two major cation-selective porins, and their expression is regulated through the OmpR–EnvZ system [46,47]. Under high osmotic strength and high temperature conditions, OmpC is expressed and OmpF expression is repressed, whereas under low solute concentration, OmpF is expressed. OmpF produces a slightly larger channel, thus bigger substrates diffuse better through the channel. As a consequence of the presence of OmpF in the outer membrane under low-osmolarity conditions, *E. coli* will benefit from the facilitation of influx of scarce nutrients [48].

The situation in *C. glutamicum* is somewhat different because $PorH_{C.glut}$ and $PorA_{C.glut}$ seem to be transcribed and expressed together, 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 on growth conditions in *C. glutamicum*. 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 (data not shown).

4.2. 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 Fig. 5A). The results of the RT-PCR experiments suggest 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 Fig. 5A). 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. [49] 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. 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.

Another interesting feature of $PorH_{C.glut}$ and $PorA_{C.glut}$ is their export of to the cell wall. Both proteins do not contain a leader sequence, which could mean that their export is similar to that of the ESAT-6/CFP10 gene families of *M. tuberculosis* H37Rv [50,51]. On the other hand, Lichtinger et al. [30] 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 [31]. Interestingly, we found also some evidence for a modification of $PorH_{C.glut}$. $PorH_{C.glut}$ 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 also be modified as part of a yet unknown export pathway.

4.3. 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 [52]. 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 Fig. 6). 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 Fig. 8). This should be sufficient

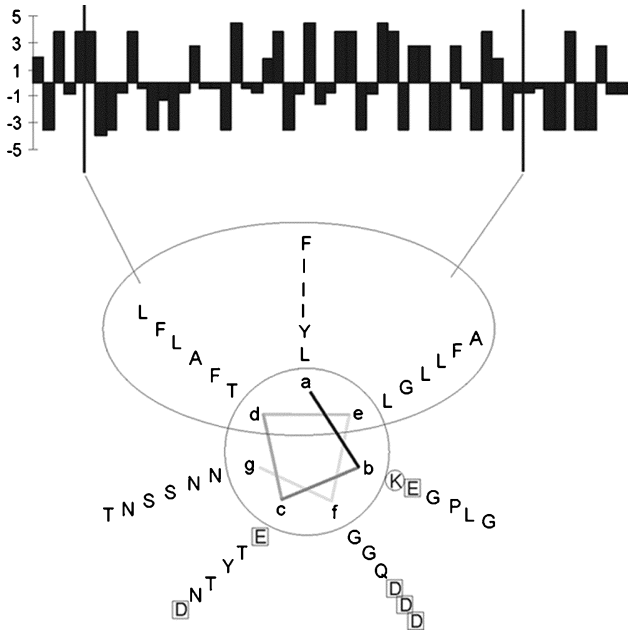

Fig. 8. Schematic prediction of the PorH$_{C.glut}$ secondary structure, according to the method of Kyte and Doolittle [53]. The molecule can form α-helices with 12 windings corresponding to an overall length of 6.3 nm based on secondary structure predictions of the primary sequence. Residues of the heptameric repeats were labeled in the sequence as a–g. The hydrophobic residues are located at positions a, e, and d, indicating that they may be 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.

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).

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 [54]. MspA monomers (molecular mass of about 20 kDa [55]) contain, in the mycolic acid layer, a spanning part β-sheet structure similar to the structure of Gram-negative bacterial porins [43]. 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 Corynebacterineae. Mycobacteria contain rather long mycolic acids (60–90 carbon atoms [16,17,56]), whereas they are short in Corynebacteria (22–38 carbon atoms [19]). 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.

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

A possible arrangement of PorH$_{C.glut}$ in the cell wall channel 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 [57–59], including mycobacterial porins and PorA$_{C.glut}$ of *C. glutamicum* [26,27,29]. A quantitative description of the effect of point charges on the single-channel conductance may be given with the considerations of Nelson and McQuarrie [59], as previously described [26,31]. A best fit of the data of Table 2 was obtained by assuming that 2 negative point charges ($q = -3.2 \times 10^{-19}$ 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 Fig. 9. The solid line represents the fit of the single-channel conductance versus concentration by using the Nelson and McQuarrie [59] 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

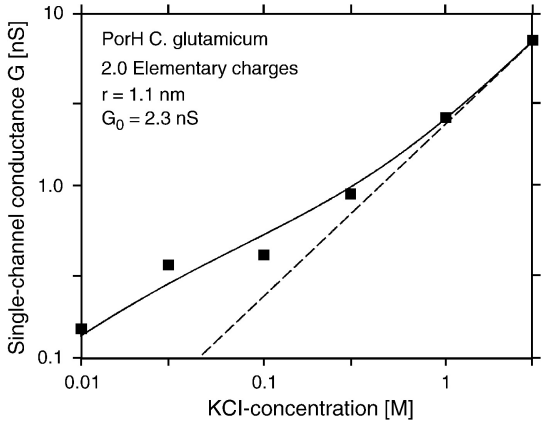

Fig. 9. Single-channel conductance of PorH$_{C.glut}$ of *C. glutamicum* as a function of the KCl concentration in the aqueous phase (full squares). The solid line represents the fit of the single-channel conductance data (see Costa-Riu et al. [31] for equation details) assuming the presence of negative point charges (2.0 negative charges; $q = -3.2 \times 10^{-19}$ 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 (nanosiemens, 10^{-9} S). 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.

concentration in the aqueous phase and single-channel conductance.

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