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

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Corynebacterium callunae and Corynebacterium efficiens are close relatives of the glutamate-producing mycolata species Corynebacterium glutamicum. The properties of the por-forming proteins, extracted by organic solvents, were studied. 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 a 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. A multiple sequence alignment search within the known chromosome of C. efficiens demonstrated that it contains a gene that ﬁts the partial amino acid sequence of PorH C.eff. PorH C.call shows high homology to PorH C.eff. PorH C.eff is encoded in the bacterial chromosome by a gene that is localized within the vicinity of the porA 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.

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, named Corynebacterium efficiens, 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 and 37 °C, with the exception of C. efficiens (Fudou et al., 2002) which 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 of mycolata contains a second layer consisting of covalently bound mycolic acids and extractable lipids (Barksdale, 1981; Goodfellow et al., 1976; Minnikin, 1987; Ochi, 1995) in addition to the thick peptidoglycan layer. The mycolic acids are 2-branched, 3-hydroxylated fatty acids and 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; Daffe et al., 1990; Holt et al., 1994; Brennan & Nikaido, 1995). The mycolic acid layer represents a permeability barrier (Liu et al., 1995, 1996; Nikaido et al., 1993), similar to the outer membrane of Gram-negative bacteria. To overcome this barrier, channel-forming proteins, so-called porins, are necessary to allow the passage of hydrophilic solutes. With respect to the 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

Abbreviations: LDAO, N,N-dimethyldodecylamine-N-oxide; PC, diphytanoyl phosphatidylcholine.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ871585 and AJ871586.
mycolata consisted of a 59 kDa cell-wall protein with a mean single-channel conductance of 2.7 nS in 1 M KCl 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 pores 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 due to negative 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 the 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 aa polypeptide that is encoded without a leader sequence (Lichtinger *et al.*, 1998, 2001). Another porin, PorB, of 99 aa, was found after deleting the porA gene from the *C. glutamicum* chromosome (Costa-Riu *et al.*, 2003a, b). PorB forms anion-selective channels with a single-channel conductance of about 700 pS in 1 M KCl for channel-forming proteins. Two homologous cell-wall channel proteins, PorH*$_{C.call}$* and PorH*$_{C.eff}$* were identified in the two species. The former is highly cation-selective, whereas the latter is slightly anion-selective. Both are voltage-dependent and their single-channel conductances are similar in 1 M KCl. The channel-forming proteins were purified to homogeneity and their biophysical properties were studied in detail. The proteins were partially sequenced and a sequence alignment search within the known chromosome of *C. efficiens* demonstrated that it contained a gene that matched the partial amino acid sequence of PorH*$_{C.eff}$*.

**METHODS**

**Bacterial strains and growth conditions.** *C. efficiens* AI 12310 (obtained from DSMZ, Braunschweig, Germany) was routinely grown in BHI medium (Difco) and *C. callunae* ATCC 15991 (obtained from DSMZ) was grown in double yeast tryptone (2 $\times$ YT; BIO 101) medium, both at 30 $^\circ$C.

**Isolation and purification of channel-forming proteins.** For the isolation of the channel-forming proteins from *C. efficiens* AI 12310 and *C. callunae* ATCC 15991, a method was used that has been described previously for the isolation and purification of PorA from *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 was grown to an OD$_{600}$ of 10 and harvested by centrifugation (10 000 r.p.m. for 10 min in a Beckman J2-21M/E centrifuge). The cells were washed twice in 10 mM Tris/HCl (pH 8). The washed and centrifuged cells were extracted twice with organic solvent, a 1:2 mixture of chloroform/methanol in a proportion of 1 part cells and 5–8 parts chloroform/methanol. The duration of the extraction was about 3 h at room temperature with stirring in a closed tube to avoid loss of chloroform. Cells and the chloroform/methanol solution were centrifuged for 15 min (10 000 r.p.m. in a Beckman J2-21M/E centrifuge). The pellet of 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 overnight at $-20^\circ$C. The precipitated protein was dissolved in a solution containing 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). The column was washed first with a buffer containing 0.4% LDAO and 10 mM Tris/HCl (pH 8), and 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.

**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 3 min at 100 $^\circ$C with loading buffer (containing 4% LDAO (N,N-dimethyldodecylamine-N-oxide) and 0.1 M Tris/HCl (pH 8), and inspected for channel-forming activity. The protein was subjected 2 to fast protein liquid chromatography (FPLC) across a Hitrap-Q column (Amersham Pharmacia Biotech). The column was washed first with a buffer containing 0.4% LDAO and 10 mM Tris/HCl (pH 8), and 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.

**Peptide sequencing.** The precipitated protein pellet resulting from the active FPLC fractions or preparative SDS-PAGE was dissolved in 100 $\mu$L 70% (v/v) formic acid containing 10% (w/v) CNBr (Merck) and incubated in the dark at room temperature for 14 h (Gross, 1967). After lyophilization, the CNBr peptides were dissolved in 20% (v/v) formic acid and separated by reversed-phase-HPLC (SYCAM), using a Luna C-18 column, 150 $\times$ 1 mm, with a flow rate of 40 $\mu$L min$^{-1}$ and a 120 min gradient from 100% A [0-1% (v/v) trifluoroacetic acid in water] to 80% B [0-1% (v/v) trifluoroacetic acid in acetonitrile]. Collected fractions were subjected to amino acid sequence analysis on a 492 protein sequencer (Applied Biosystems) using the conditions recommended by the manufacturer. The major sequences for the precipitated protein pellet of *C. callunae* were DLSLLADNLDDYSTFGKNIGTAL and DPDLKGIKAFFENFGDLAETT; the main sequence for the *C. efficiens* protein was DLSLLKDSLDFATLGN.

**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) dissolved in n-decanes. The temperature was maintained at 20 $^\circ$C during all experiments. All salts were obtained from Merck (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 described previously (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-
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 charges on the single-channel conductance may be given by the following considerations. The first one is based on the Debye–Hückel theory describing the effect of charges in an aqueous environment. The second treatment was proposed by Nelson & McQuarrie (1975) and describes the effect of 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 charges are localized at the PorH channel. In case of a negative charge, q, in an aqueous environment, a potential \( \Phi \) is created that is dependent on the distance, \( r \), from the charge:

\[
\Phi = \frac{q \epsilon e^r}{4 \pi \epsilon_0 \epsilon_r r}
\]

where \( \epsilon_0 \) (=8.85 x 10\(^{-12}\) F m\(^{-1}\) and \( \epsilon \) (=80) are the absolute dielectric constant of vacuum and the relative constant of water, respectively, and \( l_0 \) is the so-called Debye length that controls the decay of the positive potential (and that of the accumulated positively charged ions) in the aqueous phase:

\[
\frac{\epsilon_D}{\epsilon} = \frac{\epsilon_0 \epsilon_r R T}{2 \epsilon_0 e^2}
\]

where \( \epsilon \) is the bulk aqueous salt concentration, \( R \) is 8.3 J mol\(^{-1}\) K\(^{-1}\), \( T \) is 293 K and \( F \) is 96485.3 As mol\(^{-1}\) (\( R T F^{-1} = 25.2 \) mV at 20 °C). The potential \( \Phi \) created by a negative charge on the surface of a membrane is twice that of equation (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 charge increases because of the negative potential. Their concentration is in both cases (Debye–Hückel or Nelson–McQuarrie) dependent on the potential \( \Phi \) and given by:

\[
c^+_0 = c e^{-\Phi\epsilon_0}/\epsilon
\]

Similarly, the anion concentration \( c^-_0 \), near the charge decreases according to:

\[
c^-_0 = c e^{\Phi\epsilon_0}/\epsilon
\]

In the following situation we assume that the negative 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 (equation 3) at the mouth of the pore can now be used for calculation of the effective conductance–concentration curve:

\[
G(c) = G_0 c^+\Phi
\]

where \( G_0 \) is the concentration-independent conductance of the channel.

RESULTS

Purification of PorH\(_{C.cali}\) 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. Fig. 1(a), lane 2 shows the protein composition of the organic solvent extract of \( C. callunae \) that was applied to the column. 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 an NaCl concentration of 0.25 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 Fig. 1(b)).

Single channel analysis of PorH\(_{C.cali}\) and PorH\(_{C.eff}\)

Fig. 2(a) shows a single-channel recording of a PC 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$_{\text{C.call}}$ formed defined channels. The single-channel conductance of most channels was about 3 nS in 1 M KCl. Only a minor fraction of channels with different conductance values was observed (see Fig. 2a). It is noteworthy that the channels formed by PorH$_{\text{C.call}}$ of $C. \text{callunae}$ 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 low transmembrane potential (mean lifetime at least 5 min). However, voltage-dependence closure was observed for PorH$_{\text{C.call}}$ of $C. \text{callunae}$ for voltages higher than about 30–40 mV (see below). Channels formed by PorH$_{\text{C.eff}}$ had a very similar lifetime compared to those formed by PorH$_{\text{C.call}}$, as the single-channel recording of Fig. 2(b) clearly indicates. The only exception was the occurrence of two maxima in the histogram of the single-channel distribution (see Fig. 2b). These two maxima (2±3 and 4±7 nS in 1 M KCl) most probably reflect the reconstitution of two channels at once, because the conductance of the right-side maximum was twice that of the left side.

Single-channel experiments were also performed with salts other than KCl to obtain some information on the size of the channels formed by PorH$_{\text{C.call}}$ and PorH$_{\text{C.eff}}$ and their ion selectivity. The results are summarized in Table 1. The conductance sequence of the different salts within the channel formed by PorH$_{\text{C.call}}$ was RbCl > KCl > K acetate > NaCl > LiCl > N(CH$_3$)$_3$Cl > N(C$_2$H$_5$)$_4$Cl, which means that the single-channel conductance followed approximately the aqueous mobility of the different cations in the aqueous phase. Presumably, this means that the influence of cations on the conductance of the channel in different salt solutions was more substantial than that of anions (Table 1), suggesting a cation selectivity of the channel. Table 1 also shows the mean single-channel conductance, $G_i$ of PorH$_{\text{C.call}}$ 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 net charges in or near the channel (Trias & Benz, 1994; Lichtinger et al., 1999).

Table 1 demonstrates that the single-channel conductance of the channels formed by PorH$_{\text{C.eff}}$ was in the same range as that measured for PorH$_{\text{C.call}}$. However, it seems that the ion selectivity of PorH$_{\text{C.eff}}$ was somewhat different because the conductance of the channels in LiCl was higher than in K-acetate (Table 1). Furthermore, the conductance of salts containing tetraalkylammonium ions was more or less independent of the size of the cation. These results suggested that PorH$_{\text{C.eff}}$ could form anion-selective channels. Zero-current membrane potential measurements were performed in the presence of KCl gradients to check such a possibility. Fivefold KCl gradients (100 versus 500 mM) were established across lipid bilayer membranes which contained about 100–1000 PorH$_{\text{C.eff}}$ or PorH$_{\text{C.call}}$ channels. The measurements with PorH$_{\text{C.eff}}$ resulted in an asymmetry potential of about −6 mV at the more dilute trans side (mean of four measurements). This result indicated some preferential movement of chloride over potassium ions through the PorH$_{\text{C.eff}}$ channel at neutral pH. Similar experiments with PorH$_{\text{C.call}}$ resulted in an asymmetry potential of about 28 mV at the more dilute side. The zero-current membrane potentials were analysed using the Goldman–Hodgkin–Katz equation (Benz et al., 1979). The ratio of the potassium permeability, $P_K$, divided by the chloride permeability, $P_C$, was about 0.7 and 7 for PorH$_{\text{C.eff}}$ and

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**Table 1**

<table>
<thead>
<tr>
<th>Salt</th>
<th>Conductance (nS)</th>
<th>$G_i$ of PorH$_{\text{C.call}}$ (M$^{-1}$ cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RbCl</td>
<td>3.8</td>
<td>7.6</td>
</tr>
<tr>
<td>KCl</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>K acetate</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>LiCl</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>N(CH$_3$)$_3$Cl</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>N(C$_2$H$_5$)$_4$Cl</td>
<td>1.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>
PorH_{C,call} respectively, which indicated a small anion selectivity for PorH_{C,eff} and cation selectivity for PorH_{C,call} (see also Discussion).

**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. The channel-forming protein was added at a concentration of 500 ng ml⁻¹ to one side of a black PC/ 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 and then −60 mV. 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 a symmetric response of PorH_{C,eff} to the voltage applied to the membranes. Similar experiments were performed with PorH_{C,call} and a symmetrical response to the applied voltage was also observed (data not shown).

The voltage-dependence experiment, and similar ones, were analysed 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_p. G was divided by the initial value of the conductance (G_0, a linear function of the voltage) obtained immediately after the onset of the voltage. The data in Fig. 3(a) (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. The results suggest that PorH_{C,eff} exhibited a somewhat higher voltage-dependence than PorH_{C,call}. The voltage-dependence of the data of Fig. 3(a) was analysed 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 semi-logarithmic plot of the ratio N_o/N_c, which is given by:

\[
\frac{N_o}{N_c} = \frac{(G - G_{\text{min}})}{(G_0 - G)}
\]  

where G in this equation is the conductance at a given membrane potential, V_m, and G_0 and G_{\text{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:

\[
N_o/N_c = \exp[-nF(V_m - V_o)/RT]
\]  

where F (Faraday’s constant: 96485·3 C mol⁻¹), R (gas constant: 8·3 J mol⁻¹ K⁻¹) and T (absolute temperature: 293 K) 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_o is the potential at which 50% of the total number of channels are in the closed configuration (i.e. N_o/N_c = 1). Semi-logarithmic plots of the data given in Fig. 3(a) using least-squares fits (see Fig. 3b) show that they could be fitted to straight lines. The slope of the lines was such that an e-fold change of N_o/N_c occurred when the

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration (M)</th>
<th>PorH_{C,call} G (nS)</th>
<th>PorH_{C,eff} G (nS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>1·0</td>
<td>1·25</td>
<td>1·50</td>
</tr>
<tr>
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</tr>
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<td>NM</td>
<td>0·025</td>
</tr>
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<td>0·35</td>
<td>0·075</td>
</tr>
<tr>
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<td>0·1</td>
<td>0·55</td>
<td>0·45</td>
</tr>
<tr>
<td>KCl</td>
<td>0·3</td>
<td>1·10</td>
<td>0·70</td>
</tr>
<tr>
<td>KCl</td>
<td>1·0</td>
<td>3·0</td>
<td>2·3</td>
</tr>
<tr>
<td>KCl</td>
<td>3·0</td>
<td>7·0</td>
<td>6·5</td>
</tr>
<tr>
<td>RbCl</td>
<td>1·0</td>
<td>3·0</td>
<td>NM</td>
</tr>
<tr>
<td>N(CH₃)₄Cl</td>
<td>1·0</td>
<td>1·0</td>
<td>1·8</td>
</tr>
<tr>
<td>N(C₂H₅)₂Cl</td>
<td>1·0</td>
<td>0·70</td>
<td>1·7</td>
</tr>
<tr>
<td>CH₃COO (pH 7)</td>
<td>1·0</td>
<td>2·0</td>
<td>1·0</td>
</tr>
</tbody>
</table>
Voltage was changed by about 16 mV (PorH C.call) or 14 mV (PorH C.eff). This means that the number of gating charges is approximately \( n = 1.6 \) in the first case and \( n = 1.8 \) in the latter case (because \( RT/F = 25 \pm 2 \) mV; see Fig. 3b). The broken numbers could mean that the gating charges do not move through the entire membrane thickness. Whereas the voltage dependence (the slope of the lines of Fig. 3a) was approximately similar for both channels, the midpoint potential \( V_0 \) (i.e. \( N_0/N_c = 1 \)) differed somewhat for PorH C.eff \( (V_0 \approx 50 \text{ mV}) \) and PorH C.call \( (V_0 \approx 80 \text{ mV}) \) (see Fig. 3b).

**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 aa (*C. callunae*) and 18 aa (*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 aa stretch of *C. efficiens* is part of a 57 aa hypothetical protein of *C. efficiens* (DDBJ/EMBL/GenBank accession no. AJ871586; see Fig. 4a), 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 of *C. glutamicum* (Lichtinger et al., 2001). The gene porH C.eff consists of 174 bp and encodes a 57 aa acidic polypeptide (6 aspartic and glutamic acids compared to 2 lysines) without a leader sequence. 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 are (Freudl, 1992; Lichtinger et al., 2001). A search within the chromosome of *C. efficiens* demonstrates that porH and the gene encoding PorA of *C. efficiens* are localized very close to one another (see Fig. 4b). The genes encoding both proteins are only separated by 77 bp and there is no indication of a transcription terminator between them. Thus it seems very likely that both proteins share a common mode of export to the cell wall in *C. efficiens* and presumably also in *C. glutamicum* because the chromosome of the latter also contains the porH C.glut gene that has a high degree of homology to porH C.eff. Comparison of the two amino acid stretches (22 and 23 aa) 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 Fig. 4a). PorH C.call is also an acidic protein (8 aspartic and glutamic acids compared to 2 lysines of the partial sequence). The interesting feature of the channels formed by the two homologous proteins is the observation that one protein forms slightly anion-selective channels (PorH C.eff) and the other forms highly cation-selective channels (PorH C.call). This means presumably that their
were named PorHC.eff and PorHC.call, respectively. Partial efficiencies and resistance of this bacterium to neutral or positively charged antibiotics, which indicates a lower permeability of the cell wall in the deletion mutant (Costa-Riu et al., 2003). In this study, we identified different cell-wall channels in C. glutamicum (Lichtinger et al., 1998, 2001; Costa-Riu et al., 2003a, b). PorAC.glut forms a highly conductive cation-selective channel. Its deletion resulted in a much higher resistance of this bacterium to neutral or positively charged antibiotics, which indicates a lower permeability of the cell wall in the deletion mutant (Costa-Riu et al., 2003a). Nevertheless, growth of the mutant strain was only slightly impaired, in particular in rich medium. A search for another cell-wall channel revealed the existence of the anion-selective PorB.glut channel (Costa-Riu et al., 2003a, b). 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 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.glut channels. The channel-forming proteins of C. efficiens and C. callunae were purified to homogeneity and were named PorH.eff and PorH.call, respectively. Partial N-terminal sequencing of the proteins after CNBr cleavage resulted in three amino acid stretches that allowed the identification of the porH gene within the chromosome of C. efficiens. This gene encodes a 57 aa polypeptide without a leader extension, but it starts with the inducer methionine, which could be cleaved either during maturation or by CNBr. PorH.call is highly homologous to PorH.eff and the chromosome of C. glutamicum also contains a homologue gene of a similar protein PorH.glut (see Fig. 4; DDBJ/EMBL/GenBank accession no. AJ871585), which will be described in detail in a future study. The lack of an N-terminal leader extension suggested that PorH.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.glut and considering the genes within the flanking regions of porA and porH it seems likely that their gene products share the same mode of translation, export and assembly, which represents a yet unknown secretion mechanism (Lichtinger et al., 2001).

### Effects of negative charges on the channel properties of PorH.call

The channels formed by PorH.call and by PorH.eff differ somewhat in their ionic selectivity. PorH.eff forms slightly anion-selective channels despite the fact that the protein is overall acidic (6 negative charges 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.glut. The data in Table 1 demonstrate that the single-channel conductance of the channels formed by PorH.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.call is not related to the presence of a binding site because saturation would be expected, and (ii) that negative charges are involved in ion selectivity as we and others have demonstrated previously for a variety of membrane channels (Menestrina & Antolini, 2001).

**DISCUSSION**

**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, b). This gene encodes a 57 aa polypeptide without a leader extension, but it starts with the inducer methionine, which could be cleaved either during maturation or by CNBr. PorH.call is highly homologous to PorH.eff and the chromosome of C. glutamicum also contains a homologue gene of a similar protein PorH.glut (see Fig. 4; DDBJ/EMBL/GenBank accession no. AJ871585), which will be described in detail in a future study. The lack of an N-terminal leader extension suggested that PorH.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.glut and considering the genes within the flanking regions of porA and porH it seems likely that their gene products share the same mode of translation, export and assembly, which represents a yet unknown secretion mechanism (Lichtinger et al., 2001).

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1981; Benz et al., 1989; Benz, 1994), which also includes mycobacterial porins (Trias & Benz, 1993, 1994; Lichtinger et al., 1998). When we apply equations (1) to (5) to the conductance of PorH_C_call we receive a reasonable fit of the data in Table 1 if the channel has a diameter of about 2·2 nm and 1·6 negative charges \((q = -2·4 \times 10^{-19} \text{ As})\) are attached to the channel mouth. The results of this fit are shown in Fig. 5. The solid line represents the fit of the single-channel conductance versus concentration by using the Nelson & McQuarrie (1975) treatment and the parameters mentioned above together with a single-channel conductance, \(G_0\), of 2·8 nS at 1 M KCl. The broken line corresponds to the single-channel conductance of PorH_C_call without 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 charges (2 negative charges; \(q = -3·2 \times 10^{-19} \text{ As}\)) using the same treatment. Interestingly, the diameter of channels formed by PorA_C_glut is very similar to those of PorH_C_call. The precise number of charges involved in the charge effect is somewhat difficult to determine because of the use of either Nelson–McQuarrie or Debye–Hückel methods, which results in a difference in the potential by a factor of two. In one case the charge is in an aqueous environment (i.e. the charge is \(q\)) and in the other the charge on the surface of the membrane creates an image charge on the other side (i.e. the charge is \(2q\)). The decay of the potential in the aqueous phase is, on the other hand, independent from the localization of the charge, which means that the diameter of the channels is precise.

**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 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 a \(\beta\)-sheet structure similar to that of Gram-negative bacterial porins. However, such an arrangement is rather unlikely for the PorH_C_call and PorH_C_eff oligomers. Secondary structure predictions suggest that a stretch of about 28 aa (see Fig. 4a) of both proteins form amphipathic \(\alpha\)-helices with about eight windings and a length of 4·2 nm in the mycolic acid layer (see Fig. 6). The arrangement is such that all hydrophilic amino acids are localized on one side of the helix and all hydrophobic ones are on the other side. Comparison of the helical wheels from the two organisms indicates 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 well with the selectivity of both channels (see above). Secondary structure predictions for

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**Fig. 5.** Single-channel conductance of PorH_C_call as a function the KCl concentration in the aqueous phase (■). The solid line represents the fit of the single-channel conductance data with the Nelson & McQuarrie (1975) formula [equations (1)–(5)], assuming the presence of negative point charges (1·6 negative charges; \(q = -2·85 \times 10^{-19} \text{ As}\)) at the channel mouth and assuming a channel diameter of 2·2 nm. \(G\), mean single-channel conductance. 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.

**Fig. 6.** Schematic prediction of the PorH_C_call and PorH_C_eff secondary structures. Both molecules can form \(\alpha\)-helices with eight windings corresponding to an overall length of 4·2 nm based on secondary structure predictions of the primary sequence shown in Fig. 4a between the two vertical bars. Residues of the heptameric repeats are labelled 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 oval rings). The hydrophilic residues are localized at the positions b, f, c and g and may face the channel lumen. Created with the help of Helical Wheel Java Applet (www.site.uottawa.ca/~turtcote/resources/HelixWheel/).
the homologous porin PorH$_{\text{C,glat}}$ of C. glutamicum likewise point to amphipathic $\alpha$-helices (data not shown).

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$_{\text{C,cell}}$ and PorH$_{\text{C,eff}}$ is associated 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, nocardiaceae 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 $\alpha$-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.

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