Corynebacterium diphtheriae: Identification and characterization of a channel-forming protein in the cell wall

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ABSTRACT

The cell wall fraction of the gram-positive, non-toxic *Corynebacterium diphtheriae* strain C8(-) Tox- (ATCC 11913) contained a channel-forming protein as judged from reconstitution experiments with artificial lipid bilayer experiments. The channel-forming protein was present in detergent treated cell walls and in extracts of whole cells using organic solvents. The protein had an apparent molecular mass of about 66 kDa on tricine-containing SDS-PAGE and consists of subunits of about 5 kDa. Single-channel experiments with the purified protein suggested that the protein formed channels with a single-channel conductance of 2.25 nS in 1M KCl. Further single channel analysis suggested that the cell wall channel is wide and water-filled because it has an only small selectivity for cations over anions and its conductance followed the mobility sequence of cations and anions in the aqueous phase. Antibodies raised against PorA, the subunit of the cell wall channel of *C. glutamicum*, detected both monomers and oligomers of the isolated protein suggesting highly conserved epitopes between the cell wall channel of *C. diphtheriae* and PorA. Localization of the protein on the cell surface was confirmed by ELISA. The prospective homology of PorA with the cell wall channel of *C. diphtheriae* was used to identify its gene *cdporA* within the known genome of *C. diphtheriae*. The gene and its flanking regions were cloned and sequenced. CdporA is a protein, 43 amino acids long, without leader sequence. *cdporA* was expressed in a *C. glutamicum* strain that lacked the major outer membrane channels PorA and PorH. Organic solvent extracts of the transformed cells formed in lipid bilayer membranes the same channels as the purified protein CdporA of *C. diphtheriae* suggesting that the expressed protein is able to complement PorA and PorH deficiency of the *C. glutamicum* strain. The study represents the first report of a cell wall channel from a pathogenic *Corynebacterium*.
INTRODUCTION

The suborder Corynebacteria belong to a distinctive suprageneric actinomycete taxon, the mycolata, which also includes mycobacteria, nocardiae, rhodococci and closely related genera. These bacteria share with corynebacteria the property of having an unusual cell envelope composition and architecture (19). They have a thick peptidoglycan layer, which is covered by lipids in form of mycolic acids and other lipids (5, 27, 54). The mycolic acids are covalently linked through ester bonds to the arabinogalactan attached to the murein of the cell wall (46). The chain length of these 2-branched, 3-hydroxylated fatty acids varies considerably within the mycolic-acid-containing taxa. Long mycolic acids have been found in mycobacteria, but they are short in corynebacteria (22 - 38 carbon atoms) (13, 20, 30, 46, 47, 76). The cell walls of corynebacteria and closely related genera are very similar to that of mycobacteria, especially in terms of ultrastructure and cell wall chemical composition (4, 45, 69). This means that the cell wall of the mycolata forms a permeability barrier and probably has the same function as the outer membrane of gram-negative bacteria, which contains channel-forming proteins, the porins, for the passage of hydrophilic solutes (8, 9, 32, 50, 51, 52, 53). In analogy to the situation in the outer membrane of gram-negative bacteria channels for the passage of hydrophilic compounds are present in the mycolic acid layer of the mycobacterial cell wall and the cell wall of Corynebacterium glutamicum (17, 18, 31, 39, 42, 70, 72). The assumption that the mycolic acids represent a permeability barrier on the surface of the mycolata has been confirmed in recent years by the investigation of porins in different members of the Corynebacterineae (39, 40, 41, 62, 63, 64, 72).

Members of the genus Corynebacterium are of considerable interest because some are potent producers of glutamate, lysine and other amino acids through fermentation processes on an industrial scale. Prominent examples of amino acid producers are C. glutamicum or Corynebacterium callunae (22, 33, 36 38, 65, 73). The genus Corynebacterium contains on
the other hand only a few pathogens. The main pathogen is *C. diphtheriae* (44), well known as the cause of diphtheria which is an acute, communicable respiratory disease. Other possible pathogens are only *Corynebacterium urealyticum* and *Corynebacterium jeikeium* (56). Diphtheria disease is caused by exotoxin-producing *C. diphtheriae* cells that infect the throat or nose, and sometimes the eyes or skin inducing the formation of an inflammatory pseudomembrane. The exceedingly potent toxin is absorbed into the circulation and damages remote organs, potentially resulting in death (21, 29). In 1990 the World Health Organization (WHO) observed a re-emergence of the pathogen, which led to a worldwide launch of immunization programs. 1214 declared cases of diphtheria originated an epidemic which spread through Russia, Ukraine and neighboring countries and even reached a few subjects in Europe and North America (12, 14). Especially disturbing is the fact that non-toxigenic *C. diphtheriae* strains are associated with invasive diseases (28) and non-toxigenic strain convert to toxigenic strains by lysogenic conversion (2, 57). Furthermore it is known that the epidemiological pattern of the disease has changed (26, 59). The current situation clearly demonstrates that the risk of a diphtheria epidemic still exists, even in our Western countries. These results emphasize the importance of further studies of this microorganism to understand the metabolic pathways and to find new mechanisms of prevention and treatment.

In this study, we extended the search for cell wall channels to the *C. diphtheriae* strain C8(-) Tox(+) (ATCC 11913) that is another member of the genus Corynebacterium. It is known that the cell wall of this strain contains a channel-forming protein but it has not been investigated in detail (60). Using lipid bilayer experiments we could demonstrate that the extracts of cell walls and whole *C. diphtheriae* cells contain a protein that forms wide and water-filled channels similar to the porins found in gram-negative bacteria (7, 8, 9). The channel-forming protein, named CdporA, was identified within the accessible genome of *C. diphtheriae* NCTC 13129 (16) by using its homology to PorA of *C. glutamicum*. CdporA was expressed in a
PorA/PorH-deficient strain of *C. glutamicum* (31, 42). We present in this study the characterization of the first channel-forming protein of a pathogenic strain within the genus *Corynebacterium*.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** The *C. diptheriae* strain C8r(-) Tox- (ATCC 11913) (3) was used in all experiments. The strain was routinely grown in 500 ml Erlenmeyer flasks containing 250 ml Brain Heart Infusion medium (BHI, Difco Laboratories) broth at 36 ± 1°C using a New Brunswick shaker at 120 rpm for 24 hours. *C. glutamicum* ATCC 13032 cells were routinely grown in BHI-medium as has been described previously in detail (31). The PorA and PorH deficient *C. glutamicum* strain ATCC 13032ΔporAΔporH (see below) was used to complement for PorA deficiency. Growth rates were determined in triplicate by OD₆₀₀ measurements.

**Construction of *C. glutamicum* ATCC 13032ΔporHΔporA.** The up and downstream region of the target genes were amplified by PCR with the primer pairs FP KO1, RP KO2 (containing EcoRI, ApaI restriction sites) and FP KO3, RP KO4 (carrying ApaI, BamHI restriction sites) (see Table 1). The FailSafe™ PCR System (Biozym Scientific, Oldendorf, Germany) and buffer G were used according to the manufacturer manual. The respective PCR products were separately digested using ApaI (Fermentas, St. Leon-Rot, Germany) and ligated overnight with T4 DNA ligase. The ligation product served as a template for a further PCR with the primers FP KO1 and RP KO4. After double digestion with EcoRI and BamHI the knockout fragment was inserted in the MCS of BamHI-EcoRI cleaved pk18mobsacB resulting
in the plasmid pK18mobsacBΔporHΔporA. This plasmid was transformed by electroporation in competent *C. glutamicum* ATCC 13032 cells.

Integration of the plasmid in the chromosome indicating the first single cross-over event was tested by plating the cells on BHIS plates supplemented with 25µg/ml kanamycin. For deletion of the target genes one of the colonies on the plate was grown overnight in liquid LB and spread on BHIS plates containing 10% sucrose. Cells growing on this plate were tested for kanamycin sensitivity by parallel picking on BHIS plates containing either kanamycin or sucrose. Sucrose-resistant and kanamycin-sensitive cells indicate the second cross-over. The deletion was verified by PCR and by DNA sequencing (data not shown).

**Preparation of the cell wall, plasma membrane and cytosol fractions.** The cell fractions were produced as previously described for mycobacteria (20, 61). Wet cells (5g) were suspended in 20 ml phosphate buffer (50 mM, pH 7.5) and the resulting bacterial suspension was passed through a cell disrupter and then centrifuged at 4000 rpm for 15 min to eliminate unbroken cells; cell walls were recovered from the supernatant by centrifugation at 10,000 rpm (8,300 g) for 60 min. The 10,000 rpm supernatant was centrifuged at 50,000 rpm (170,000 g) for 60 min at 4 °C in an ultracentrifuge (Beckmann Omega 90 XL, rotor 70.1 Ti) to yield the membrane fraction in the pellet; the supernatant was considered as the cytosol fraction (61). The pellets were washed and lyophilized (20) or directly used for the experiments. All fractions were analyzed for protein content by SDS-PAGE and for pore-forming activity by reconstitution experiments in the black lipid bilayer assay following detergent treatment of the different fractions. The correct separation of the different fractions was analyzed by their diverse levels of NADH-oxidase activity. This activity was measured by detecting the decrease of absorbance at 340 nm (55). The reaction followed a first order
kinetics. The specific activity was calculated by dividing the appropriate rate constant $k_1$ by the relative protein concentration of the sample.

**Isolation and purification of the channel-forming protein from the cell wall fraction.** Whole cells of *C. diphtheriae* were extracted with a 1:2 mixture of chloroform-methanol in the proportions 1 part cells and 5-8 parts organic solvent (39). The protein extracted with the chloroform/methanol mixture was precipitated with ice-cold diethyl ether at –20°C for 24 hours and dissolved in 1% Genapol X-80. Further purification was achieved by excision of different molecular mass bands from preparative SDS-PAGE and their extraction with 1% Genapol X-80. Possible oligomers of the channel-forming protein were obtained when ethanol was added to the protein solution in an excess of 2.5 times the volume of the solution. The protein was then precipitated at 4°C for 24 hours. After centrifugation at 4°C the resulting pellet was dried under vacuum to completely remove the remaining ethanol.

**Digestion of the polypeptide.** The purified polypeptide with a molecular mass of about 5 kDa was treated for 5 min with 50 units/ml proteinase K (EC 3.4.21.64; Sigma, St.Louis, MO) in a buffer containing 1% Genapol X-80.

**SDS-PAGE.** Analytical and preparative SDS-PAGE was performed according to (37) or because of the low resolution of this gel system at low molecular mass according to (66) with tricine containing gels. The gels were stained with Coomassie brilliant blue or with Colloidal Coomassie blue (23). Utilizing the colloidal properties of Coomassie Brilliant Blue G-250 the latter resulted in an improved staining of proteins with sensitivity similar to silver stain.
Immunological techniques. In Western-blot (immunoblot) experiments, the proteins separated by 10% tricine-containing SDS-PAGE were transferred onto nitrocellulose sheets (Protran, Schleicher&Schuell, BA83, 0.2µm) in a semi-dry blotting apparatus according to (67). This method is a modification of the procedure described by (34) taking into account the higher ionic strength of tricine-containing SDS gels. The reactive sites were blocked with 5% skimmed milk in TBS-T (20 mM Tris-HCl pH 7.5, 0.01 M NaCl, 0.1% Tween) for 1 h and shortly washed three times with TBS-T. The blots were incubated for 1 h (or overnight) at RT with rabbit polyclonal antibodies against C. glutamicum PorA in a dilution 1:100 (39). After incubation the membrane was washed three times with TBS-T. Bound antibodies were detected by using horseradish-peroxidase-coupled rabbit immunoglobulins (DAKO, Denmark) at a dilution at 1:1,000. Color reaction was obtained by using a mixture of 94% TBS, 6% chloronaphtol (0.3%), 0.075% hydrogenperoxide. After 10 min incubation the bands appeared. For detection of the monomer we used the ECL Western blotting detection system (GE Healthcare; UK) because the chemiluminescent detection provides an extremely sensitive system to detect small amounts of protein of extremely low molecular mass. We followed the manual supplied by the manufacturer. Signal detection was achieved by incubating the membrane in the detection mix for 1 min, draining off the mix and wrapping the blot in Saran Wrap. Next, we placed the blot into a film cassette and on top placed a piece of autoradiography film (Hyperfilm™MP; GE Healthcare; UK) for 15 seconds to 5 minutes as required by the sample. The exposed film was immediately developed by use of the X-omat M35 (Kodak).

The ELISA (enzym-linked immunosorbent assay) experiments were carried out as described (15). This method allows the performance of a rapid, simple and sensitive enzyme-linked immunosorbent assay, suitable for the detection of bacterial surface proteins. Shortly, different amounts of cells were coupled per well (MaxiSorp immuno plates; Nunc, Roskilde,
Cells of *C. glutamicum* ACCT 13032 were used as positive control, the corresponding preimmuniserum and wells including either only cells of *C. glutamicum* or *C. diphtheriae* ACCT 11913 without any primary antibody were used as negative control. Absorption at 405 nm was measured with a microplate reader (Thermomax, Molecular Devices).

**Polymerase Chain Reaction and construction of the expression plasmid.** Primers Cdiph_XbaI_for and Cdiph_KpnI_rev (see Table 1) were used for PCR amplification of the region, which contained the gene coding for the PorA homolog of *C. diphtheriae*. Chromosomal DNA of the strain C8r(-) Tox- (ATCC 11913) was used as a template for PCR amplification. The program consisted of 30 cycles with the following steps: 1 min denaturation at 95°C, 1 min annealing at 45°C and 2 min extension at 72°C, using FailSafe polymerase (Epicentre Biotechnologies) with buffer E. 50 µl of the reaction were loaded on a 0.8% agarose gel and compared to 1 kb ladder (Gibco-BRL® Life Technologies Ltd., Paisley, Scotland, UK). The PCR-product was cut out from the gel, ligated in a TOPO 2.1 vector and transformed in One Shot® Top10 F’ cells. Plasmid miniprep of the *Escherichia coli* cells was used for sequencing the PCR-product using the primers M13 forward and reverse. The gene coding for the prospective cell wall channel and its flanking regions were cut out of the TOPO 2.1 vector using the restriction enzymes EcoRI and XbaI. The DNA piece was ligated into the Shuttle vector pXMJ19. *C. glutamicum* ATCC 13032ΔporAΔporH cells were transformed with the vector according to a slightly modified standard electrottransformation method (74). The transfected cells were grown until an OD$_{600}$ of 3. Then protein expression was induced with 1 mM IPTG and the culture was grown for another 16 hours. Cells were harvested by centrifugation. Subsequently the cells were extracted with a 1:2 mixture of chloroform-
methanol in the proportions 1 part cells and 5-8 parts organic solvent. The protein was precipitated with ether in the cold.

**Lipid bilayer experiments.** The methods used for the lipid bilayer experiments have been previously described in detail (10). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.3 mm². Black lipid bilayer membranes were obtained by painting a 1% (w/v) solution of diphytanoyl phosphatidylcholine (PC) or phosphatidylserine (PS; Avanti Polar Lipids, Alabaster, AL) in n-decane onto the hole. Membranes were also formed from PC/mycolic acids (MA; Sigma) or PC/PS/MA mixtures to study the effect of mycolic acids on channel formation. The temperature was maintained at 20°C during all experiments and the current recordings were filtered at 300 Hz. All salts were obtained from Merck (Darmstadt, F.R.G., analytical grade). Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100 to 1000 cell wall porins as it has been described earlier (11).

**RESULTS AND DISCUSSION**

**Isolation and purification of the channel-forming protein.** The homogenate of the *C. diphtheriae* ATCC 11913 cells was centrifuged at two different speeds. The pellet of the first centrifugation should contain the cell walls and that of the second one the cytoplasmic membrane. The supernatant of the second centrifugation is the cytosol of the cells. Pellets and supernatant were inspected for protein content, NADH-oxidase activity and channel-forming ability. Highest channel-forming ability was observed in Genapol X-80 extracts of the cell wall fraction. This fraction was essentially free of cytoplasmic membrane as assessed by NADH-oxidase activity. The specific NADH-oxidase activity of the proteins of the cell wall
fraction was relative to the protein concentration 0.10. The corresponding specific activities of
the proteins of the cytosol and the fraction containing the cytoplasmic membrane relative to
their protein concentrations were 0.03 and 0.87, respectively (the relative total activity of
NADH-oxidase was set to 1.0). Proteins of the cytosol and the cytoplasmic membrane showed
an only very weak single-channel activity indicating that the cell wall contained most of the
channel-forming protein.

The detergent-solubilized material from the cell wall fraction contained too many bands in
tricine containing SDS-PAGE that it was impossible to relate one single band to the channel-
forming activity although it showed a strong band in the low molecular mass region (see Fig. 1). As a further step whole cells were treated with organic solvents. This method provided, in
the case of the cell wall channel of C. glutamicum a simple purification procedure of the
channel-forming protein (39). Tricine-containing SDS-PAGE of the ether precipitate
following chloroform/methanol extraction showed that a protein with a molecular mass of
about 5 kDa was enriched in this fraction (data not shown). Further purification of the
channel-forming protein was achieved by excision of this band from preparative SDS-PAGE
and its extraction with 1% Genapol X-80 (see Fig. 1). The addition of the 5 kDa band to
planar lipid bilayers resulted in a very fast reconstitution of channels. When regions of
different molecular mass were excised from the same SDS-PAGE, highest channel-forming
activity was always observed for the 5 kDa band. However, we noticed also that small
channel-forming activity was smeared across the molecular mass region between about 5 and
70 kDa of the SDS-PAGE. This result indicated that the 5 kDa band may represent a channel-
forming monomer as it is the case for PorA of C. glutamicum (42). This was confirmed by
tricine-containing SDS-PAGE of the ethanol precipitate of the 5 kDa protein eluted from the
preparative SDS-PAGE. The corresponding gel showed that the oligomer of the channel-
forming protein has an apparent molecular mass of about 66 kDa (data not shown; see also below). This is consistent with the situation in C. glutamicum where PorA or PorH form also oligomers (31, 42). In this respect it is interesting to note that the 20 kDa molecular mass protein MspA of Mycobacterium smegmatis forms an octamer in the cell wall with a molecular mass of 160 kDa (24). The smaller size of the CdPorA oligomer as compared with that of the MspA octamer may be explained by the smaller thickness of the cell wall of corynebacteria. This presumably has to do with the length of the corynomycolic acids, which are considerably shorter (22-38 carbon atoms) than the mycolic acids of other members of the mycolata. 43 amino acids are presumably sufficient to cross the mycolic acid layer of bacteria of the genus Corynebacterium whereas longer polypeptides are necessary to cross the cell wall of other members of the taxon mycolata.

The mycolic acid layer of the suprageneric actinomycete taxon, the mycolata, acts as a permeability barrier towards hydrophilic compounds (32, 52, 53). Our results indicate that the cell wall fraction of C. diphtheriae also contains a channel-forming protein similar as the mycolic acid layer of different members of the genus Corynebacterineae (13, 39, 49, 62, 71, 71). The channel-forming activity of the cell wall was rather high with respect to the protein concentration. In addition, NADH-oxidase activity of this fraction that is a marker of cytoplasmic membrane was rather low. This result rules out the possibility that we are dealing with a contaminant protein responsible for channel-formation. Furthermore, it is clear that these channels can only be present in the cell wall of C. diphtheriae and not in the cytoplasmic membrane. Otherwise, the presence of these high-conducting channels would result in cell death.

**Interaction of the cell wall protein with lipid bilayer membranes.** Conductance measurements were performed with lipid bilayer membranes to study the interaction of the
cell wall protein with artificial membranes. Membranes were formed from 1% PC or PC-PS mixtures (molar ratio 4:1) dissolved in n-decane. The addition of the 5 kDa cell wall protein in small concentration (100 ng/ml) to one or both sides of the lipid membranes resulted in a strong increase of the conductance. The conductance increase was not sudden but it was a function of time after the addition of the protein to membranes in the black state. Within about 20 to 30 min the membrane conductance increased by several orders of magnitude above that of membranes without the protein (from about 0.05 µS/cm² to 150 µS/cm²). Only a small further increase (as compared with the initial one) occurred after that time. Similar results were obtained when the membranes were formed from PC/PS/MA mixtures to study the effect of mycolic acids on channel formation suggesting that mycolic acid did not influence channel formation. Control experiments with Genapol X-80 alone at the same concentration as in the experiments with protein demonstrated that the membrane activity was caused by the presence of the cell wall protein and not by the detergent. Similarly, proteolytic degradation of the purified 5 kDa protein using proteinase K for 5 min completely destroyed its channel-forming ability.

Single Channel Analysis. The addition of smaller concentrations of the cell wall porin (10 ng/ml) to PC/n-decane membranes allowed the resolution of stepwise conductance increases. Fig. 2 shows a single channel recording in the presence of the 5 kDa protein. It was added 5 min after the membrane was in the black state. A few minutes after the addition of the protein the current increased in step-like fashion because of the reconstitution of long-lasting channels, which led to a superposition of the steps. The current steps had a long lifetime (mean lifetime more than 5 min). Fig. 3 shows a histogram of the conductance fluctuations observed under the conditions of Fig. 2 (20 mV membrane potential; 1 M KCl, 10 mM Tris-HCl, pH 7). Besides a major conductance step of about 2.25 nS (more than 30% of all
conductance fluctuations) we observed also channels with a higher single-channel conductance, in particular channels with a single-channel conductance of about 4.5 nS. The latter ones are presumably dimers of the 2.25 nS channel that could not be separated within the time resolution of our experimental set up. Under the low voltage conditions of Fig. 2, all the steps were directed upwards, which indicated that the channels were always in the open state. The exchange of PC membranes against membranes made of other lipid mixtures such as PC/PS (molar ratio 4:1), PC/MA (molar ratio 4:1) or PC/MA/PS (molar ratio 4:4:1) did not influence the single channel conductance of the porin.

Single-channel experiments were also performed with salts containing ions other than K⁺ and Cl⁻. These experiments were done to get some insight in the biophysical properties of the cell wall porin of *C. diphtheriae*. The results summarized in Table 2 show that the channel is only moderately selective. This conclusion can be derived from experiments in which KCl was replaced by LiCl or KCH₃COO. The exchange of the mobile ions K⁺ and Cl⁻ by the less mobile ions Li⁺ and acetate indicates that cations and anions have certain permeability through the channel of *C. diphtheriae*. The permeability of the cations through the channels followed approximately their mobility sequence in the aqueous phase. This probably means that the cell wall porin is a wide channel, which has only a small field strength inside and no small selectivity filter (i.e. no binding site) as is suggested by the fact that the large organic Tris⁺ cation could also penetrate the channel.

Table 2 shows also the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase. The values for G always corresponded to that of the left-hand maximum of the histograms, i.e. to the 2.25 nS peak in the case of 1 M KCl. Measurements were performed down to 0.03 M KCl. In contrast to other cell wall channels of the mycolata (39, 41, 64, 70), we observed a linear relationship between single-channel conductance and KCl-concentration, which would be expected for wide water-filled channels
that do not contain point charges similar to those formed by gram-negative bacterial porins (7, 8, 9, 75). The CdPorA channel sorts mainly according to the molecular mass of the solutes similar to the function of general diffusion pores in gram-negative bacteria (8, 9). It represents definitely the major permeability pathway of the cell wall similar to the situation in C. glutamicum (18). This result is very surprising because up to date only cell wall channels within the taxon mycolata were identified that contained charges in or near the channel opening (see Table 4). The channel presented in this study is the first channel within the Corynebacterineae that does not contain point charges. This means also that the single channel analysis does not allow estimation of the channel size as was the case for other cell wall channels investigated to date (39, 41, 62, 63, 64). On the other hand, the size of CdporA channel could be very similar to that formed by PorA of C. glutamicum because of the sequence homology between CdPorA and PorA (see below). PorA and PorH have diameters of about 2.2 nm and it is possible that the CdPorA channel has a similar size (see Table 4).

Selectivity of the cell wall channel of C. diphtheriae. Zero-current membrane potential measurements were performed to obtain further information on the molecular structure of the C. diphtheriae cell wall channel. The experiments were performed in the following way. After the incorporation of 100 to 1000 channels into the PC membranes, the salt concentration on one side of the membranes was raised fivefold beginning from 100 mM and the zero-current potential was measured 5 min after every increase of the salt gradient across the membrane. For KCl and KCH₃COO the more diluted side of the membrane (100 mM) always became positive, whereas negative membrane potentials were observed for LiCl (Table 3). This result indicates that the channel functions as a general diffusion pore, which simply filters the solutes as already known for general diffusion pores of gram-negative bacteria (9). Analysis of the membrane potential using the Goldman-Hodgkin-Katz equation (11) confirmed the
assumption that anions and cations are permeable through the channel. The ratios of the
permeability $P_{\text{cation}}$ and $P_{\text{anion}}$ were 0.72 (LiCl), 1.26 (KCl), and 3.26 (potassium acetate).
This means that the selectivity of the CdPorA channel was dependent on the mobility of the
ions in the aqueous phase.

The cell wall channel of *C. diphtheriae* is voltage-dependent. In single-channel recordings
the cell wall porin exhibited some flickering at higher voltages, i.e. it showed rapid transitions
between open and closed configurations. This could be caused by voltage-dependent closing
of the cell wall porin. This was studied in separate experiments. The channel-forming protein
was added in a concentration of 100 ng/ml to one side of a black PC/n-decane membrane (the
cis-side). After 30 min the conductance had increased considerably. At this point different
positive and negative potentials were applied to the cis-side of the membrane. For negative
and for positive potentials at the cis-side of the membrane the current decreased in an
exponential fashion (data not shown). This result indicated symmetrical voltage-dependence
of the cell wall channel. The addition of the protein to the trans-side of the membrane or to
both sides of the membrane also resulted in a symmetric response to the applied voltage (data
not shown).

The data of the experiments were analyzed in the following way: the membrane conductance
($G$) as a function of voltage, $V_m$, was measured when the opening and 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
Fig. 4 correspond to the symmetric voltage-dependence of the cell wall porin (mean of four
membranes) when the protein was added to the cis-side (closed circles). To study the voltage-
dependence in more detail the data of Fig. 4 were analyzed assuming a Boltzmann distribution between the number of open and closed channels, \( N_0 \) and \( N_C \), respectively (43):

\[
\frac{N_0}{N_C} = \exp \left( \frac{nF(V_m - V_o)}{RT} \right)
\]  

(1)

\( F \), \( R \) and \( T \) are standard symbols (Faraday constant, gas constant and absolute temperature, respectively), \( n \) is the number of charges moving through the entire transmembrane potential gradient for channel gating and \( V_m = V_o \) is the potential at which 50% of the total number of channels are in the closed configuration. The open-to-closed ratio of the channels, \( N_0/N_C \), may be calculated from the data in Fig. 4 according to

\[
\frac{N_0}{N_C} = \frac{G - G_{\text{min}}}{G_0 - G}
\]  

(2)

\( G \) is in this equation the conductance at a given membrane potential \( V_m \), \( G_0 \) and \( G_{\text{min}} \) is the conductance at 10 mV (conductance of the open state) and at very high potentials, respectively. The data of Fig. 4 could be fitted with combination of eqs. (1) and (2). The fit (see the solid line in Fig. 4) 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 numbers of open and closed channels are identical). The midpoint potential for the addition of the protein to the cis-side was for applied positive voltages 46 mV and for applied negative voltages -48 mV. The gating charge was in both cases close to 1.

**Immunological detection of the channel-forming protein of *C. diphtheriae*:** A channel-forming low molecular mass peptide is present in the cell wall of *C. glutamicum* (39, 42). To check its possible relationship with the 5 kDa channel-forming protein of *C. diphtheriae* Western blot analysis was performed using a polyclonal antibody directed against the porin of *C. glutamicum*. The channel-forming protein of *C. glutamicum* was purified as described previously (39) and used as control. We observed strong cross reactivity of the antibodies with
the 5 kDa channel-forming protein of *C. diphtheriae* and its oligomer (Fig. 5, lanes 1 and 2) indicating the presence of highly conserved immunodominant epitopes. It has to be noted, however, that it was not possible to show monomers and oligomers in one slot of the Western blot because of the highly different blotting times of monomers and oligomers. In a next step an ELISA test was performed with whole cells to confirm the localization of the 5 kDa protein on the surface of *C. diphtheriae* cells. Different amounts of cells were coupled per well. Experiments with immobilized cells of *C. glutamicum* and *C. diphtheriae* resulted in a very low signal using the preimmunserum similar to the signals that were detected using immobilized cells treated without any primary antibody (see Fig. 6). The anti-porA antibody detected both, immobilized cells of *C. glutamicum* and of *C. diphtheriae*. The results of the ELISA experiments (Fig. 6) demonstrated that the antigenic structures of PorA and the 5 kDa protein are localized on the surface of the cells, which is in agreement with their function as cell wall channels. This result suggests that CdPorA is accessible from the surface by anti-PorA antibodies, which means that it could be used as an antigenic structure also in vaccination. Approximately the same concentration of protein on the surface was observed on *C. glutamicum* and on *C. diphtheriae* cells indicating that the cell wall of both species contains the same number of channels. Control experiments with pre-immune serum demonstrated that the antibodies were highly specific for both cell surfaces (see Fig. 6). In addition, the antibodies did not react with cell wall proteins of other mycolata than those of *C. diphtheriae*, *C. glutamicum*, *Corynebacterium efficiens* and *Corynebacterium callunae* (data not shown). In particular, they did also not react with cell wall extracts of *Corynebacterium xerosis* and *Corynebacterium amycolatum* (60) suggesting that the immune reaction described here was not a non-specific interaction. It also suggested that these corynebacteria do not contain PorA-like proteins.
Identification of the gene coding for the cell wall channel of *C. diphtheriae* ATCC 11913.

The immunological cross reactivity between PorA and the cell wall channel of *C. diphtheriae* ATCC 11913 suggested an interesting homology between both proteins. Similarly, we observed previously Southern hybridization of the *porA* gene with DNA from *C. diphtheriae* (42) suggesting that this organism contains a *porA*-like gene. A NCBI BLAST-translation tool search (1, 77) using *porA* of *C. glutamicum* in the known genome of *C. diphtheriae* NCTC 13129 (16) suggested that the genome contained an open reading frame (ORF) between the genes coding for GroEL2 (DIP2020) (6) and a putative secreted protein (DiP2017) that could code for a low molecular mass cell wall protein similar to PorA (see Fig. 7). This means that the ORF is localized within a region homologous to that of the *C. glutamicum* genome containing *porA*. Primers were designed to clone the whole region between the two genes DIP2020 (*GROEL2*) and DIP2017 using DNA of *C. diphtheriae* ATCC 11913 as a template (see Table 1). The PCR-product was cloned into the TOPO 2.1 vector and was sequenced. It contained the ORF (132 bp) coding for a PorA-like protein that showed only some minor amino acid exchanges (11 residues) as compared with the corresponding protein of *C. diphtheriae* NCTC 13129 (see Fig. 7). The protein was named CdPorA (for *C. diphtheriae* pore-forming protein A). CdPorA has a length of 43 amino acids (with the inducer methionine) and a (calculated) molecular mass of 4,640 Da. The CdporA studied here has one more negatively charged amino acid compared to strain NCTC 13129 (in total five glutamic and aspartic acids as compared to four lysines), which agrees with its small cation selectivity. This is in some contrast to the highly cation selective PorA channel of *C. glutamicum* (5 negatively charged amino acids as compared with one single lysine) and possibly also to the hypothetical PorA of *C. efficiens* (4 negatively charged amino acids as compared with two lysines; see Fig. 7). Positive and negative amino acids are balanced for CdporA of strain NCTC 13129.
A 5' AAAGG-3' sequence was found eight nucleotides upstream of the start codon of cdporA, which could act as ribosome binding site. Comparative analysis with the accessible genome of *C. diphtheriae* NCTC 13129 revealed the same result. A stem-loop structure suitable to be a putative termination signal of mRNA transcription could be identified downstream from the stop codon (TAG) with the tool TransTermHP (http://tranterm.cbcb.umd.edu). TransTermHP is an algorithm to find rho-independent terminators in bacterial genomes (35). The palindromic sequence of *C. diphtheriae* ATCC 11913 (5' AAAAGGGCCCGCATCTAAAAGCGGGTCCTTTT-3') and of *C. diphtheriae* NCTC 13129 (5' ATAAAGGGCCCGCATCTAAAAGCGGGCCCTTTT-3') has a free energy level at -15.5 and -17.8 kcal/mol, respectively (http://www.genebee.msu.su).

CdporA of both strains also does not contain any C-terminal sorting signal for targeting to the cell wall similar to PorA and PorH of *C. glutamicum* (31, 42). On the other hand, as demonstrated here CdporA is clearly a protein localized in the cell wall of *C. diphtheriae*. The absence of any obvious signal peptide suggests that its translocation through the cytoplasmic membrane uses an export system different from the *Sec* system normally responsible for protein sorting and export in Gram-positive bacteria (25, 48, 58, 68).

Expression of cdporA in *C. glutamicum* ATCC 13032ΔporAΔporH and study of its channel-forming ability. The results of the search for the channel-forming protein within the genome of *C. diphtheriae* NCTC 13129 suggested that CdporA could be the channel-forming protein in its cell wall. cdporA was expressed in *C. glutamicum* ATCC 13032ΔporAΔporH and whole cells were extracted with organic solvents. Proteins were precipitated with ether in the cold. The precipitate was dissolved in 1% Genapol X-80 and investigated for channel formation in the lipid bilayer assay using membranes from PC/n-decane. The precipitate had a
high channel-forming activity with a single-channel conductance of 2.25 nS the same as that of purified CdporA under the same conditions (see Fig. 8A, upper trace). Control experiments with extracts from PorA/PorH deficient C. glutamicum cells showed only a limited number of small channels that are presumably caused by PorB/PorC (see Fig. 8A, lower trace), which forms small channels with a conductance of 700 pS in 1 M KCl (17, 18). This result revealed the close structural and functional relationship between PorA and CdporA. It is noteworthy that the cell wall porin of C. diphtheriae had no properties similar to those found in the cell walls of other distantly related actinomycetes, which have molecular masses of about 20 kDa (49, 63) (see Table 4 for a comparison of cell wall channels from a variety of different cell wall channels from actinomycetes).

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**Table 1.** Oligonucleotides used in this study. The sequences of the primers were derived from the prospective gene of the cell wall channel and its flanking regions taken from the genome of *C. diphtheriae* NCTC 13129 (11). The accession number for the genome of *C. diphtheriae* NCTC 13129 is NC_002935.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdiph_XbaI_for</td>
<td>2073825-2073796</td>
<td>GCTTTTGCTATTTCTAGAGGGAGGTATTGAC</td>
</tr>
<tr>
<td>Cdiph_KpnI_rev</td>
<td>2073038-2073067</td>
<td>CCTAGCCAGCTAGGTACCAAGCCAAAAAC</td>
</tr>
<tr>
<td>FP KO 1</td>
<td>2862508-2862538</td>
<td>GACGAGGCAACCGGAATTCGCATCGTCCGCG</td>
</tr>
<tr>
<td>RP KO 2</td>
<td>2861687-2861717</td>
<td>GTTGCCAGTTTGCTGGGCTCAGGACGTC</td>
</tr>
<tr>
<td>FP KO 3</td>
<td>2861216-2861246</td>
<td>AACTTCGCCCACGGGCCCAGTTTTCAAAAAC</td>
</tr>
<tr>
<td>RP KO 4</td>
<td>2860356-2860385</td>
<td>ATTCGACTTGATGGGATCCACGGGACTC</td>
</tr>
</tbody>
</table>
Table 2. Average single-channel conductance, G, of the cell wall channel of *C. diphtheriae* ATCC 11913 in different salt solutions.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration [M]</th>
<th>G [nS]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
<td>1.60</td>
</tr>
<tr>
<td>KCl</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>6.00</td>
</tr>
<tr>
<td>KCH(_3)COO (pH6)</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>CsCl</td>
<td>1.0</td>
<td>2.50</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>1.0</td>
<td>2.25</td>
</tr>
<tr>
<td>N(CH(_3))(_4)Cl</td>
<td>1.0</td>
<td>0.80</td>
</tr>
<tr>
<td>TrisCl (pH6)</td>
<td>1.0</td>
<td>0.90</td>
</tr>
</tbody>
</table>

The membranes were formed of 1% PC dissolved in n-decane. The aqueous solutions were buffered with 10 mM Tris-HCl and had a pH of 7 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 derived from measurements of at least four individual membranes.
Table 3. Zero-current membrane potentials, $V_m$, of PC/n-decane membranes in the presence of the cell wall channel of *C. diphtheriae* ATCC 11913 measured for a 5-fold gradient of different salts$^a$.

<table>
<thead>
<tr>
<th>Salt</th>
<th>$P_{\text{cation}}/P_{\text{anion}}$</th>
<th>$V_m$/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>1.26</td>
<td>+ 5.4</td>
</tr>
<tr>
<td>LiCl</td>
<td>0.72</td>
<td>- 1.2</td>
</tr>
<tr>
<td>KCH$_3$COO</td>
<td>3.26</td>
<td>+ 17.5</td>
</tr>
</tbody>
</table>

$^aV_m$ is defined as the difference between the potential at the dilute side and the potential at the concentrated side. The aqueous salt solutions were buffered with 10 mM Tris-HCl, pH 7; $T = 20^\circ$C. The permeability ratio $P_{\text{cation}}/P_{\text{anion}}$ was calculated using the Goldman-Hodgkin-Katz equation (11) from at least 3 individual experiments.
Table 4. Comparison of the cell wall channel properties of *C. diphtheriae*, *C. glutamicum*, *Mycobacterium chelonae*, and *Nocardia farcinica*.

<table>
<thead>
<tr>
<th>Cell wall porin</th>
<th>G [nS] in 1 M KCl</th>
<th>Selectivity P_{e}/P_{a} in KCl</th>
<th>Point charges at the channel mouth</th>
<th>Channel Diameter [nm]</th>
<th>Reference(s) or source.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. diphtheriae</em> ATCC 11913</td>
<td>2.25</td>
<td>1.26</td>
<td>No point charges</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td><em>C. glutamicum</em> ATTC 13092</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PorA</td>
<td>5.5</td>
<td>8.1</td>
<td>-2.0</td>
<td>2.2 nm^{2,3}</td>
<td>39</td>
</tr>
<tr>
<td>PorH</td>
<td>2.5</td>
<td>5.1</td>
<td>-2.0</td>
<td>2.2 nm^{2,3}</td>
<td>31</td>
</tr>
<tr>
<td>PorB</td>
<td>0.70</td>
<td>0.12</td>
<td>+1.5</td>
<td>1.4 nm^{3}</td>
<td>18</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>6.0</td>
<td>11.8</td>
<td>2.7</td>
<td>2 nm^{2,3}</td>
<td>62</td>
</tr>
<tr>
<td><em>Rhodococcus equi</em> ReqPorA</td>
<td>4.0</td>
<td>9.0</td>
<td>-1.5</td>
<td>1.8^{2} 2.0^{3}</td>
<td>62</td>
</tr>
<tr>
<td>ReqPorB</td>
<td>0.30</td>
<td>0.16</td>
<td>+1.5</td>
<td>1.4^{2,3}</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium chelonae</em></td>
<td>2.7</td>
<td>14</td>
<td>2.5</td>
<td>2.2 nm^{1}; 2.0 nm^{3}</td>
<td>70, 72</td>
</tr>
<tr>
<td><em>Nocardia farcinica</em></td>
<td>3.0</td>
<td>8.2</td>
<td>1.3</td>
<td>1.4 nm^{2}, 1.6 nm^{3}</td>
<td>62</td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>0.85</td>
<td>1.3</td>
<td>No point charges</td>
<td>-</td>
<td>31</td>
</tr>
</tbody>
</table>

The channel diameters were estimated from the liposome swelling assay\(^1\), the single-channel conductance as a function of the hydrated ion radii\(^2\) or the effect of negative point charges on single channel conductance\(^3\).
Fig. 1. 10% tricine-containing SDS-PAGE according to (66) of the cell wall fraction and the cytoplasmic membrane and the cytosol of *C. diphtheriae* ATCC 11913. Coomassie blue staining (lanes 1 to 3); Colloidal Coomassie blue staining (lane 4).

Lane 1: Molecular mass markers 97 kDa, 66 kDa, 45 kDa, 30 kDa, 20.1 kDa and 14.4 kDa.

Lane 2: 15 µl of the cell wall fraction (8,300 g pellet) was solubilized at 40°C for 30 min in 1% Genapol X-80 and 5µl sample buffer.

Lane 3: 15 µl of the 8,300 g supernatant containing the cytoplasmic membrane and cytosol was solubilized at 40°C for 30 min in 5µl sample buffer.

Lane 4: 3 µg of the pure cell wall channel protein of *C. diphtheriae* ATCC 11913 were incubated at 100°C for 30 min in 5µl sample buffer.

Fig. 2. Single-channel recording of a PC/n-decane membrane in the presence of the channel-forming 5 kDa protein from the cell wall of *C. diphtheriae* ATCC 11913 (trace I). The aqueous phase contained 1 M KCl and 10 mM Tris-HCl, pH 7 and 10 ng/ml cell wall protein added 2 minutes before the start of the recording. The applied membrane potential was 20 mV; T = 20°C. Trace II shows a control without the 5 kDa protein.

Fig. 3. Histogram of the probability of the occurrence of a given conductivity unit observed with membranes formed of PC/n-decane in the presence of the cell wall protein of *C. diphtheriae* ATCC 11913. The aqueous phase contained 1 M KCl and 10 mM Tris-HCl, pH 7. The applied membrane potential was 20 mV; T = 20°C. The average single-channel conductance was 2.25 nS for 148 single-channel events.
Fig. 4.

Ratio of the conductance $G$ at a given membrane potential ($V_m$) divided by the conductance $G_0$ at 10 mV as a function of the membrane potential $V_m$. The open squares represent the mean of 4 measurements, in which 5 kDa protein from *C. diphtheriae* ATCC 11913 was added to the cis-side of membranes. The solid line represents the fit of the experimental data using eqs. (1) and (2) using the parameters $n = 1; V_0 = -48$ mV (left branch of the curve) and $V_0 = 46$ mV (right branch of the curve). The aqueous phase contained 1M KCl, 10 mM Tris-HCl, pH 7.0 and 100 ng/ml porin. The membranes were formed from PC dissolved in n-decane. $T = 20^\circ$C.

Fig. 5.

Western-blot analysis of CdporA of *C. diphtheriae* ATCC 11913. SDS-PAGE of CdPorA oligomers and CdporA monomers were blotted onto nitrocellulose membrane, probed with anti-porA polyclonal antibodies. Bands were detected using HRP-conjugated antibodies and chloronaphtol/hydrogenperoxide as substrate. Lane 1: *C. diphtheriae* porin oligomers, Lane 2: CdporA monomers. Note that monomers and oligomers cannot be shown on one slot because of the highly different blotting times of monomers and oligomers because of their different molecular masses.

Fig. 6.

Detection of CdporA of *C. diphtheriae* on the cell wall surface using ELISA (enzym-linked immunosorbent assay). Intact cells of *C. diphtheriae* ATCC 11913 and *C. glutamicum* ATCC 13032 were immobilized and incubated with anti-porA (dilution 1:100), preimmunserum
(dilution 1:1000) and buffer. The maximum binding was set to 100% and the cell number per well is indicated. The bars show the result ±SD of at least 8 experiments.

**Fig. 7.**

Amino acid sequence of CdporA of *C. diphtheriae* NCTC 13129 and its comparison with the amino acid sequences of CdporA of *C. diphtheriae* ATCC 11913, PorA of *C. glutamicum* ATCC 13032 and hypothetical PorA of *C. efficiens* AJ12310 using Pole Bioinformatique Lyonnaise Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr). The charged residues of the proteins (+/−) are specified on the top line. Conserved residues in the four homolog proteins are shown in bold. The sequences of CdporA from NCTC 13129 and ATCC 11913 have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers AM689937 and AM690207, respectively.

**Fig. 8.**

**A:** Single-channel recording of a PC/n-decane membrane in the presence of organic solvent extracts of *C. glutamicum* ATCC 13032ΔporAΔporH cells transfected with shuttle vector pXMJ19 dissolved in Genapol X-80. Expression of CdporA was induced with 1 mM IPTG. The aqueous phase contained 1 M KCl and 10 mM Tris-HCl, pH 7 and 20 ng/ml of the organic solvent extract added about 2 minutes before the start of the recording. The applied membrane potential was 20 mV; T = 20°C. Trace II shows a control performed with 20 ng/ml organic solvent extract of *C. glutamicum* ATCC 13032ΔporAΔporH cells added about 2 minutes before the start of the recording.

**B:** Histogram of the probability of the occurrence of a given conductivity unit observed with membranes formed of PC/n-decane in the presence of 20 ng/ml of the organic solvent extract of *C. glutamicum* ATCC 13032ΔporAΔporH cells transfected with shuttle vector pXMJ19.
dissolved in Genapol X-80. The aqueous phase contained 1 M KCl and 10 mM Tris-HCl, pH 7. The applied membrane potential was 20 mV; T = 20°C. The average single-channel conductance was 2.25 nS for 101 single-channel events.
2.5 nS
50 pA
10 s

Fig. 2
Fig. 3

Fig. 4
Fig. 6
C. diphtheriae

hyp_porA_NCTC13129

C. diphtheriae

hyp_porA_ATCC11913

C. glutamicum

porA_ATCC13032

C. efficiens

hyp_porA_AJ12310

Fig. 7
Fig. 8A

Fig. 8B