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Study of the Properties of a Channel-forming Protein of the Cell Wall of the Gram-positive Bacterium *Mycobacterium phlei*

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Abstract. The gram-positive bacterium *Mycobacterium phlei* was treated with detergents. Reconstitution experiments using lipid bilayers suggested that the detergent extracts contain a channel forming protein. The protein was purified to homogeneity by preparative SDS-PAGE and identified as a protein with an apparent molecular mass of about 135 kDa. The channel-forming unit dissociated into subunits with a molecular mass of about 22 kDa when it was boiled in 80% dimethylsulfoxid (DMSO). The channel has on average a single channel conductance of 4.5 nS in 1 M KCl and is highly voltage-dependent in an asymmetric fashion when the protein is added to only one side of the membrane. Zero-current membrane potential measurements with different salts implied that the channel is highly cation-selective because of negative point charges in or near the channel mouth. Analysis of the single-channel conductance as a function of the hydrated cation radii using the Renkin correction factor and the effect of the negative point charges on the single-channel conductance suggest that the diameter of the cell wall channel is about 1.8 to 2.0 nm. The channel properties were compared with those of other members of the mycolata and suggest that these channels share common features. Southern blots demonstrated that the chromosome of *M. phlei* and other mycolata tested contain homologous sequences to *mspA* (gene of the cell wall porin of *Mycobacterium smegmatis*).

Key words: Cell wall channel — Channel size — Mycolic acid — Porin — *Mycobacterium phlei* — Lipid bilayer membrane

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Introduction

The mycobacterial cell wall defines the permeability properties of the cell envelope. Besides extractable lipids it consists of long-chain mycolic acids, which form an asymmetric bilayer and a capsule-like coat of polysaccharide and protein (Draper, 1998). Mycolic acids and their homologues are β-hydroxyl-α-branched fatty acids, which are mainly present in different genera of the suborder *Corynebacterineae*, which is also known as mycolata (Stackebrandt, Rainey & Ward-Rainey, 1997). They are linked through ester bonds to the arabinogalactan, which is attached to the murein of the cell wall (Minnikin, 1982; 1987; Brennan & Nikaido, 1995). Mycolic acids are especially long in the genus mycobacteria (60–90 carbon atoms) and tsukamurellae (64–74), medium-sized in gordonae (52–66) and nocardiae (46–58), and short in corynebacteria (22–38) (Yano & Saito, 1972; Minnikin, Patel & Goodfellow, 1974; Minnikin, 1982; 1987; 1991; Daffe, Brennan & McNeil, 1990; Holt et al., 1994; Ochi, 1995; Brennan & Nikaido, 1995; Liu, Rosenberg & Nikaido, 1995; Liu et al., 1996; Yassin et al., 1997). The mycobacterial cell envelope is extremely hydrophobic and forms a strong permeability barrier resulting in a high resistance to most common antibiotics and chemotherapeutic agents (Liu et al., 1996; Brennan & Nikaido, 1995). Small hydrophilic solutes diffuse through pore-forming proteins that resemble the function of gram-negative bacterial porins with much lower efficiency compared to *E. coli* (Jarlier & Nikaido, 1990; Trias, Jarlier & Benz, 1992; Chambers et al. 1995). Cell wall porins of different mycolata have been characterized in recent years (Trias et al., 1992; Trias & Benz, 1993, 1994; Lichtinger et al., 1999; 2000; Niederweis et al., 1999) but the knowledge about the structure, function and other features of these porins is still meager at present.

The gram-positive bacterium *M. phlei* ATCC 356 is a saprophytic rapid growing nontuberculous microorganism and belongs, together with *M. fortuitum, M. chelonae, M. abscessus* and *M. smegmatis* (Gray et al., 1975; Tomioka, 1998), to the "atypical mycobacteria", which are known to cause opportunistic infections in immune-compromised patients (Brennan & Nikaido, 1995). It may play an important role in veterinary medicine, because *M. phlei*-absorbed ELISA is used as a test for the diagnosis and control of bovine paratuberculosis in cattle (Milner et al., 1990) and in dairy cows (Bech-Nielson et al., 1992a; 1992b; 1993). In the immunotherapy of cancer, *M. phlei* cell wall preparations are used instead of *Mycobacterium bovis* BCG to prepare stable, nonliving immunologic adjuvants with minimal residual malignant disease (Gray et al, 1975). Furthermore, the *M. phlei* mycobacterial cell wall complex (MCC) directly induces apoptosis in human bladder cancer leading to the inhibition of cancer growth (Filion et al., 1999). It is noteworthy that two cases of human infection by *M. phlei* have been reported. The first case describes a septic arthritis due to *M. phlei* reported as infantile Reiter's syndrome (Aguilar et al., 1989) and the second case describes an infection of the foot (Spiegl & Feiner, 1994); both cases were probably caused mainly by immunodeficiency of the patients. In this study we investigated the channel-forming properties of the purified cell wall channel of *M. phlei* and compare them to those of other mycolata studied to date.

Materials and Methods

Bacterial Strain and Growth Conditions

M. phlei (ATCC 356) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was grown in 500-ml Erlenmeyer flasks containing 250 ml Bacto-Tryptic-Soy-Medium at 37 ± 1°C using a New Brunswick shaker at 120 rpm for three to four days. The cell culture was checked for purity and the cells were harvested by centrifugation at 12,000 rpm for 10 min at 4°C.

Isolation of the Channel-forming Activity from Whole Cells Using Detergents

The channel-forming protein was extracted from about 1g cell material (wet weight) as has been described previously for *Nocardia farcinica* (Rieß et al., 1998). The cells were treated with a number of different washing steps that remove most of the soluble cell wall components. The first two steps (I and II) consisted in washes of the cell walls for 30 min with 10 ml of a buffer containing 10 mM Tris-HCl, pH 8.0 followed by centrifugation at 11,000 rpm in a Beckman J2-21M/E centrifuge (rotor JA20). The pellet was washed twice (steps III and IV) with 10 ml of a solution containing 0.2% SDS followed by centrifugation (11,000 rpm in a Beckman JA20 for 10 min). The resulting pellet was then washed (step V) with 10 ml of a solution containing 1% Genapol and 10 mM EDTA followed by centrifugation (11,000 rpm in a Beckman J2-21M/E centrifuge (rotor JA20) for 10 min). In step VI we shook the pellets with 10 ml of a solution containing 1% Genapol and 10 mM NaP_i, pH 6.0, for 20 hr at 50°C followed by centrifugation (11,000 rpm in a Beckman J2-21M/E centrifuge (rotor JA20) for 10 min). The final pellet was homogenized (step VII) in 3 ml 0.4% LDAO and 10 mM Tris-HCl, pH 8.0, and then incubated for 20 hr at 50°C under agitation. The channel-forming activity was present in the final supernatant.

SDS-PAGE

Analytical and preparative SDS-PAGE was performed according to Laemmli (1970). The gels were stained with Colloidal Coomassie blue (Erhardt et al, 1988), which yields an improved staining of proteins with clear background and has a sensitivity similar to silver stain, utilizing the colloidal properties of Coomassie Brilliant Blue G-250.

Lipid Bilayer Experiments

The methods used for the lipid bilayer experiments have been described previously in detail (Benz et al., 1978). Black lipid bilayer membranes were obtained from a 1% solution of a mixture (molar ratio 4:1) of diphytanoyl phosphatidylcholine (PC) and phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL) in n-decane. Membranes were also formed from PC/mycolic acid (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. Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100 to 1,000 channels as it has been described earlier (Benz, Janko & Läuger, 1979).

Estimation of the Channel Diameter Using the Renkin Correction Factor

Calculation of the channel size is possible from the single-channel conductance data when the channel is wide and water-filled and when only cations or anions can permeate through the channel. Under these conditions the entry of the ions to the channel is the rate-limiting step and the Renkin correction factor (Renkin, 1954) times the aqueous diffusion coefficient of the hydrated ions may be used to estimate the channel radius *a* from the relative permeability of the different ions through the channel. The relative permeability of the ions is calculated from their single-channel conductance relative to that of Rb^+ and is plotted as a function of the hydrated ion radius, *r* (Trias & Benz, 1994). It is noteworthy that a similar approach has been used previously to calculate the radii of porin channels from the relative rate of permeation of different solutes in the liposome swelling assay (Nikaido & Rosenberg, 1981).

Effect of Negatively-charged Groups Attached to the Channel Mouth

Negative charges at the pore mouth result in a substantial ionic strength-dependent surface potential at the pore mouth that attracts cations and repels anions. Accordingly, they influence both single-channel conductance and zero-current membrane potential. A quantitative description of the effect of the point charges on the single-channel conductance has been given in previous studies (Nelson & McQuarrie, 1975; Menestrina & Antolini, 1981; Trias & Benz, 1993; Benz, 1994). The same formalism was also used here for the calculation of the effective channel radius and the number of negatively-charged point charges attached to the channel mouth. This method

depends only on the relative conductance at different ion concentrations and not on the absolute one, which is only a factor. Therefore the calculated parameters (channel radius and net charge) are independent no matter whether the conductance steps are caused by the simultaneous reconstitution of a bundle of channels or by a single channel.

Southern Blot Analysis

Chromosomal DNA from various mycolata was isolated as described previously (van Soolingen et al., 1991). 10 μg of chromosomal DNA was digested overnight with *Bam*HI at 37°C, applied to a 0.7% TAE-agarose gel and transferred onto a nylon membrane (Boehringer, Mannheim, Germany) as described in Southern (1975). For Southern blots we used the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) according to the instructions of the manufacturer. The DNA was UV-crosslinked to the membrane and we used a 3′Digoxigenin-labeled 30 bp-fragment of the *mspA*-gene (sequence position 166–196) as probe (*MspA1*). High-stringency conditions were achieved by hybridizing blots at 60°C, low-stringency hybridization was performed at 45°C using 0.1 × SSC (3 M NaCl, 0.3 M sodium citrate), containing 0.1% SDS as primary washing buffer.

Results

Isolation and Purification of Channel-forming Protein of the Cell Wall of *M. phlei*

Whole *M. phlei* cells were treated with different detergent-free and detergent-containing buffers (*see* Materials and Methods). This method allowed the removal of most of the soluble cell wall components and to obtain large amounts of the porin. Addition of the extract to a planar lipid bilayer resulted in a very fast reconstitution of channels and showed that the final supernatant (step VII) of the detergent treatment contained the highest channel-forming activity. Identification and further purification of the channel-forming protein were achieved by excision of different molecular-mass bands from preparative SDS-PAGE and their extraction with 1% Genapol. Addition of the different fractions to the black lipid bilayer demonstrated that the main channel-forming activity was present in the first fraction, which had an apparent molecular mass of about 135 kDa on SDS-PAGE (Fig. 1*A*). After boiling of the pure protein for 15 min in sample buffer containing 80% DMSO the protein dissociated into subunits of an apparent molecular mass of about 22 kDa on SDS-PAGE (Fig. 1*B*).

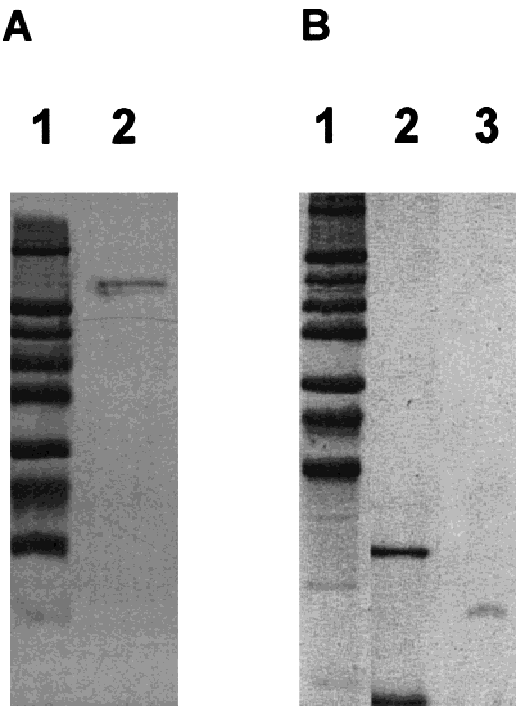

Fig. 1. 10% SDS-PAGE of pure cell wall channel protein of *M. phlei* obtained by elution of the 135 kDa band from preparative SDS-PAGE. (*A*): Lane 1: Molecular mass markers 205 kDa, 116 kDa, 97 kDa, 84 kDa, 66 kDa, 55 kDa, 45 kDa and 36 kDa. Lane 2: 3 μg of the pure protein was solubilized at 40°C for 30 min in 10 μl sample buffer and 10 μl distilled water. Colloidal Coomassie blue staining. (*B*) Lane 1: Molecular mass markers 205 kDa, 116 kDa, 97 kDa, 84 kDa, 66 kDa, 55 kDa, 45 kDa and 36 kDa. Lane 2: Molecular mass markers 26.6 kDa and 17 kDa. Lane 3: 3 μg of the pure protein was solubilized at 100°C for 15 min in 20 μl sample buffer containing 80% DMSO. Colloidal Coomassie blue staining.

Effect of the Cell Wall Protein on the Conductance of Lipid Bilayer Membranes

We performed conductance measurements with lipid bilayer membranes from PC/PS mixtures (molar ratio 4:1) to study the interaction of the cell wall porin with artificial membranes. The addition of the pure 135 kDa cell wall protein in low 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 or after the blackening of the membranes if the protein was present in the aqueous phase before membrane formation. During about 30 min the membrane conductance increased by several orders of magnitude above that of the membranes without the protein (from about 0.05 μS/cm^2 to 150 μS/cm^2). Only a small further increase (as compared with the initial one) occurred after that time. Control experiments with Genapol 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.

Single-Channel Analysis

The addition of lower concentrations (10 ng/ml) of the cell wall protein to lipid bilayers made of PC/PS allowed

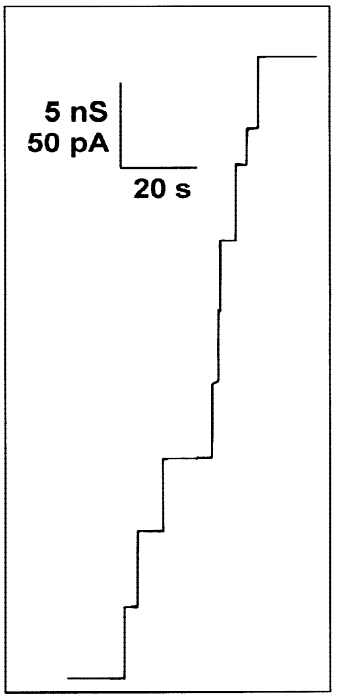

Fig. 2. Single-channel recording of a PC/PS (molar ratio 4:1)/n-decane membrane in the presence of the 135 kDa protein from the cell wall of *M. phlei.* The aqueous phase contained 1 M KCl, 10 mM NaP_i, pH 6.0 and 10 ng/ml cell wall protein. The applied membrane potential was 10 mV; $T = 20$°C.

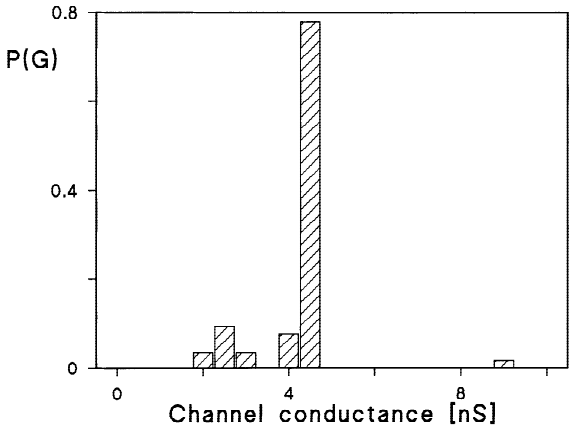

Fig. 3. Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed with membranes formed of PC/PS (molar ratio 4:1)/n-decane in the presence of the cell wall protein of *M. phlei. P*(*G*) is the probability that a given conductance increment *G* is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl, 10 mM NaP_i, pH6. The applied membrane potential was 10 mV; $T = 20$°C. The average single-channel conductance was 4.5 nS for 118 single-channel events (right-hand, maximum).

the resolution of stepwise conductance increases. Fig. 2 shows a single-channel recording in the presence of the cell wall porin added 5 min after the membrane had turned black. Shortly after the addition of the protein the current started to increase in a step-wise fashion; each step corresponded to the incorporation of one channel-forming unit into the membrane. The channels had a long lifetime (mean lifetime more than 5 min). Figure 3 represents a histogram of the conductance fluctuations observed under the conditions of Fig. 2 (10 mV membrane potential; 1 M KCl, 10 mM NaP_i, pH 6.0). Most of the steps had a conductance of 4.5 nS, and only a few steps with other single-channel conductances were observed. Under the low-voltage conditions of Fig. 3, all the steps were directed upwards, which indicated that the channels were always in the open state. The exchange of PC/PS membranes against membranes made of other lipid mixtures such as PC alone, 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.

Size of the Cell Wall Channel

Single-channel experiments were also performed with salts containing ions other than K^+ and Cl^-. These experiments were performed to study the biophysical properties of the cell wall porin of *M. phlei*. The results summarized in Table 1 show that the influence of the cations on the single-channel conductance in various 1 M salt concentrations is more substantial than that of the replacement of chloride by acetate. This result is consistent with the assumption that the cell wall channel is cation selective. The ionic selectivity of cations was $K^+ > Rb^+ \approx NH_4^+ \approx Cs^+ > Na^+ > Li^+ > N(CH_3)_4^+ > N(C_2H_5)_4^+ \approx Tris^+$, which means that the permeability of the cations followed approximately their mobility sequence in the aqueous phase. This means probably that the cell wall porin is a wide and water-filled channel, which has inside only a small field strength and no small selectivity filter (i.e., no binding site) as is suggested by the fact that the large organic $Tris^+$- and $N(C_2H_5)_4^+$-cations could also penetrate the channel.

Table 1 shows also the average single-channel conductance, *G*, as a function of the KCl concentration in the aqueous phase. Measurements were performed down to 0.05 M KCl. As with other cell wall porins (Trias & Benz, 1992, 1993; Lichtinger et al., 1998), we did not observe a linear function between single-channel conductance and KCl-concentration, which would be expected for wide water-filled channels similar to those formed by gram-negative bacterial porins (Benz, 1988; Weiss et al., 1991). Instead, the single-channel conductance, *G*, was proportional to the square root of the concentration, which indicated point charge effects on the electrical properties of the channel (*see also* Discussion). It is noteworthy that the charge effect was caused by

Table 1. Average single-channel conductance, *G*, of the cell wall channel of *M. phlei* in different salt solutions.

Salt	Concentration M	Single-channel conductance *G* nS	Cation permeability relative to Rb^+
LiCl	1.0	1.5	0.375
NaCl	1.0	2.5	0.625
KCl	0.05	1.0	
	0.10	1.4	
	0.30	2.0	
	1.0	4.5	1.125
	3.0	10.0	
RbCl	1.0	4.0	1.0
$N(CH_3)_4Cl$	1.0	0.9	0.225
$N(C_2H_5)_4Cl$	1.0	0.15	0.0375
TrisCl	1.0	0.20	0.05
KAc	1.0	4.0	
NH_4Cl	1.0	4.0	1.0
CsCl	1.0	4.0	1.0

The membranes were formed of PC/PS (molar ratio 4:1) dissolved in n-decane. The aqueous solutions were buffered with 10 mM NaP_i, pH 6.0. The applied voltage was 10 mV, and the temperature was 20°C. The average single-channel conductance, *G*, was calculated from at least 80 single events. The permeability of the cations relative to Rb^+ was calculated assuming that the cell wall channel is highly cation selective.

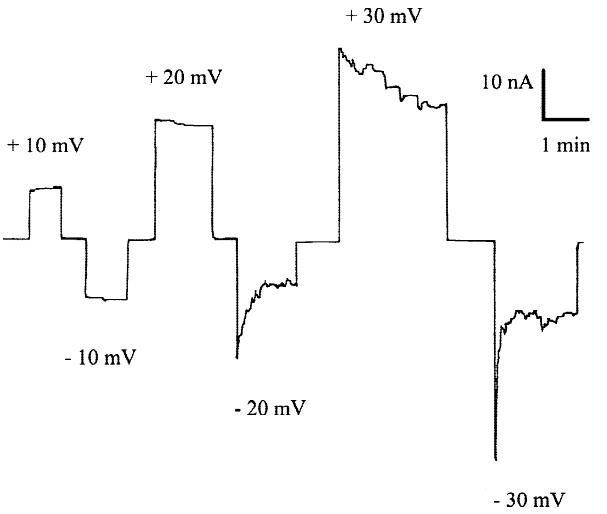

Fig. 4. Study of the voltage-dependence of *M. phlei* porin. 500 ng/ml of the cell wall channel protein was added to the cis-side of a PC/PS (molar ratio 4:1)/n-decane membrane and the reconstitution of the channels was followed for about 30 min. When about 90 channels were reconstituted into the membrane, increasingly positive (upper traces) and negative voltages (lower traces) were applied to the cis-side of the membrane and the membrane current was measured as a function of the time. The aqueous phase contained 1 M KCl, 10 mM NaP_i, pH 6.0; T = 20°C.

charges attached to the channel and not by the negatively charged lipids present in the membrane. This result was also confirmed by the observation that the cell wall porin had the same conductance in neutral (PC) and in negatively charged (PC/PS, PC/MA, PC/MA/PS) membranes.

Selectivity of the Cell Wall Channel

Zero-current membrane potential measurements were performed to measure the selectivity of the *M. phlei* cell wall porin. After the incorporation of 100 to 1,000 channels into the PC/PS membranes, the salt KCl-concentration on one side of the membranes was raised fivefold beginning from 100 mM by the addition of concentrated KCl. The more dilute side of the membrane (100 mM) became always positive, which indicated preferential movement of potassium ions through the channel, i.e., the cell wall porin is cation-selective, as was already suggested from the single-channel data. The zero-current membrane potential for KCl was on average about 40 mV under these conditions (fivefold gradient). Analysis of the potential using the Goldman-Hodgkin-Katz equation (Benz et al., 1979) suggested that anions also could have a certain permeability through the channel because the ratio of the permeabilities P_K divided by P_{Cl} was about 15 for KCl.

The Cell Wall Channel (Porin) of *M. phlei* is Voltage-dependent in an Asymmetric Manner

The single-channel recordings of the cell wall porin exhibited some flickering at voltages higher than 10 mV, i.e., it showed rapid transitions between the open and the closed configuration. This could be caused by voltage-dependent closing of the cell wall porin, which was studied in detail in single- and multi-channel experiments at higher voltages. Figure 4 shows an experiment of the latter type. The channel-forming protein was added in a concentration of 500 ng/ml to one side of a black PC/PS-n-decane membrane (the cis-side). After 30 min the conductance had increased considerably. At this point we applied different potentials to the membrane. We applied first +10 mV (upper trace of Fig. 4) and then −10 mV to the cis-side of the membrane (lower trace of Fig. 4). Then we performed experiments with ±20 and ±30 mV. For negative potentials at the cis-side the membrane current started to decrease already at −20 mV in an exponential fashion and decreased even more at −30 mV. For positive potentials at the cis-side only a minor decrease was observed at +20 mV. At 30 mV the current showed a stronger decrease. This result indicated oriented insertion of the cell wall porin into the membranes when it was added to only one side of the membrane. The addition of the protein to both sides of the membrane resulted in a symmetric response to the applied voltage

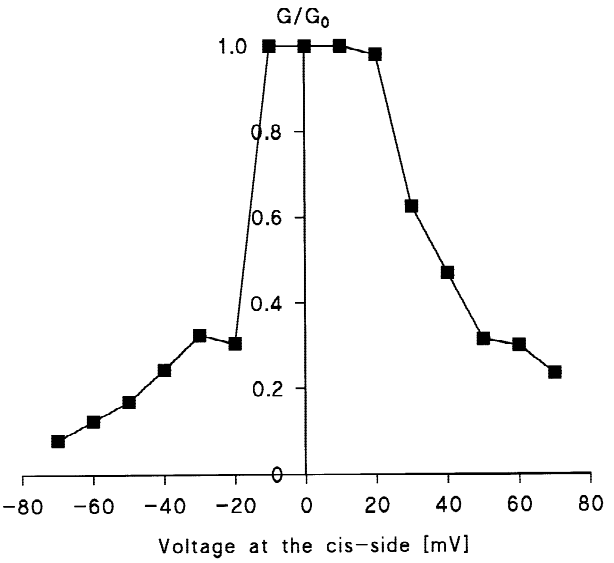

Fig. 5. Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance G_0 at 10 mV as a function of the membrane potential V_m. *M. phlei* porin was added to the cis-side of membranes. The aqueous phase contained 1 M KCl, 10 mM NaP_i, pH 6.0 and 100 ng/ml porin. The membranes were formed from PC/PS (molar ratio 4:1) dissolved in n-decane. $T = 20$°C.

(*data not shown*). The data of the experiment of Fig. 4 and similar 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_0, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The data of Fig. 5 shows the asymmetric voltage-dependence of the cell wall porin (mean of four membranes) when the protein was added only to the cis-side.

In additional experiments we studied the voltage dependence at the single-channel level. Figure 6 shows a measurement, in which one porin channel was reconstituted in a PC/PS membrane. To the cis-side (the side of the addition of the protein to the membrane) and to the trans-side of the membrane positive and negative voltages were applied ranging from ±10 mV to ±30 mV. Again, the channel closed for negative polarity at the cis-side at lower potential as compared to positive polarity. The conductance of the closing events was not defined. This result suggested that the porin of *M. phlei* is not a bundle of channels, otherwise we should have observed defined closing events as has been observed in the case of gram-negative bacterial porins (i.e., one third or two thirds of the opening of the channels (Schindler & Rosenbusch, 1981).

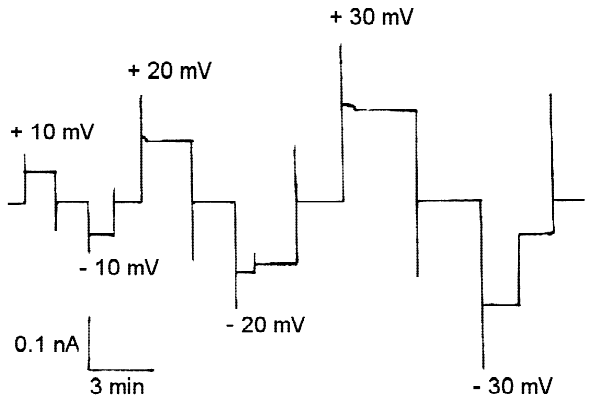

Fig. 6. Study of the voltage-dependence of *M. phlei* porin at the single-channel level. 20 ng/ml of the cell wall channel protein was added to the cis-side of a PC/PS (molar ratio 4:1)/n-decane membrane and channel reconstitution was followed for about 20 min. When one channel was reconstituted into the membrane, increasingly positive (upper trace) and negative (lower trace) voltages were applied to the cis-side of the membrane and the membrane current was measured as a function of the time. The aqueous phase contained 1 M KCl, 10 mM NaP_i, pH 6.0; $T = 20$°C.

Southern Hybridization of the *msp*A Gene with DNA from other Mycolata

The *msp*A gene encoding the cell wall channel of *M. smegmatis* has recently been cloned and sequenced. To study its relation to the cell wall channel protein of *M. phlei* and other members of the mycolata we performed Southern blots with *Bam*HI digested chromosomal DNA of different bacteria. A DNA probe derived from the *msp*A gene (sequence position 166–196) was used for these experiments. At high-stringency conditions (60°C) we observed hybridization with 9 kb, 6 kb and 4.5 kb bands of both *M. smegmatis* and *M. phlei* and additionally with a 2 kb band of *T. inchonensis* (Fig. 7*A*). These hybridizations were observed as clear bands at a stringency of 45°C up to 60°C. This result means probably that the chromosome of *M. smegmatis* contained more than one gene for cell wall channels because there is no *Bam*HI restriction site in the sequenced DNA fragment containing the *msp*A gene. Interestingly, the chromosome of *M. phlei* showed an identical behavior. At medium stringency (50°C) additional hybridization bands of *N. farcinica* occurred and at low stringency conditions (45°C) bands for all tested *Corynebacterineae* were observed, suggesting that their chromosome may contain conserved sequences for porins (Fig. 7*B*).

Discussion

The Cell Wall of *M. phlei* Contains an Ion-permeable Channel

In this study we have investigated *M. phlei* cells for the presence of channel-forming activity in detergent ex-

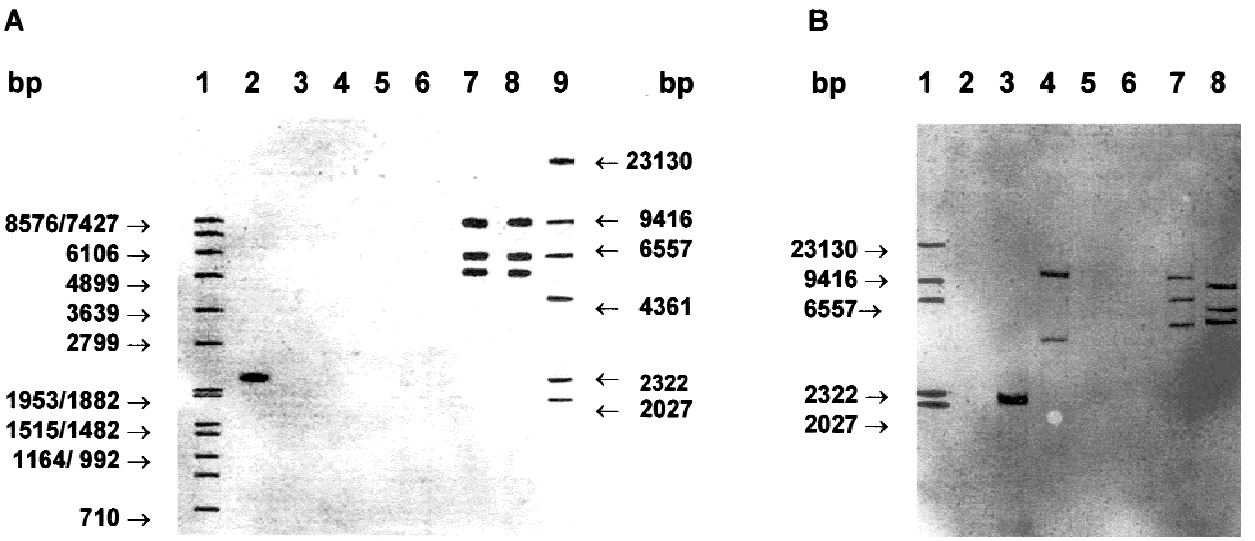

Fig. 7. Southern blot analysis concerning the presence of the *msp*A gene in different species of *Corynebacterineae.* Chromosomal DNA of each strain was cut with *Bam*HI, separated on a 0.7% TAE agarose gel, and nonradioactive Southern blotting (DIG; Boehringer) was performed with Dig-labeled *Msp*A1. The primary washing buffer always consisted of 0.1 × SSC containing 0.1% SDS. Fragment sizes are indicated. (*A*) High stringency (60°C): DNA Molecular Weight Marker; Boehringer (Lanes 1, 9); *Tsukamurella inchonensis* (Lane 2), *Nocardia farcinica* (Lane 3); free (Lanes 4, 5); *Rhodococcus equi* (Lane 6); *M. phlei* (Lane 7); *Mycobacterium smegmatis* (Lane 8). (*B*) Low stringency (45°C): DNA Molecular Weight Marker; Boehringer (Lane 1); free (Lane 2); *Tsukamurella inchonensis* (Lane 3), *Nocardia farcinica* (Lane 4); free (Lanes 5, 6); *Rhodococcus equi* (Lane 7); *M. phlei* (Lane 8).

tracts of whole cells. The cells were treated with different detergent-free and detergent-containing buffers. The highest channel-forming activity was observed in extraction step VII using 0.4% LDAO and 10 mM Tris-HCl, pH 8.0. The cell wall porin of *M. phlei* was purified to homogeneity using preparative SDS-PAGE and has a molecular mass of about 135 kDa. Upon heating in sample buffer containing 80% DMSO the protein dissociated into subunits of molecular masses of about 22 kDa on SDS-PAGE. This means that the cell wall channel of *M. phlei* is formed by a single subunit, as has been found for *Nocardia corynebacteroides* (Rieß & Benz, 2000), *Mycobacterium smegmatis* (Niederweis et al., 1999; Mukhopadhyay, Basu & Chakrabarti, 1997), *Mycobacterium tuberculosis* (Kartmann, Stengler & Niederweis, 1999) and *Rhodococcus erythropolis* (Lichtinger, Reiss & Benz, 2000). The data presented in this paper are consistent with the role of the cell wall of *M. phlei* as a permeability barrier toward hydrophilic compounds and that an aqueous channel is required to overcome this permeability barrier (Jarlier & Nikaido, 1990).

Estimation of the Diameter of the Cell Wall Channel Protein

The *M. phlei* cell wall channel has a high single-channel conductance, similar to those of the cell wall channels from *M. smegmatis* (Trias & Benz, 1994), *C. glutamicum* (Lichtinger et al., 1998), and *N. farcinica* (Rieß et al., 1998) (*see* Table 2). These channels share some common features. All are wide and water-filled and have diameters between 1.6 to 3.0 nm. When we consider the observation that the large $Tris^+$ cation could also permeate through the channel it is obvious that the cell wall channel of *M. phlei* could have a similarly large diameter. Using the Renkin correction factor (Renkin, 1954; Nikaido & Rosenberg, 1981) it is possible to calculate the diameter of the *M. phlei* cell wall channel from the conductance of the channel in different salts (Trias & Benz, 1994). Fig. 8 shows the fit of the single-channel conductance of the porin channel from *M. phlei* with the Renkin equation (eqn. (*1*) of Trias & Benz (1994)) times the aqueous diffusion coefficient of the ions. The data are given relative to the data for Rb^+ (*see* Table 1), as a function of the hydrated ion radii (Table *4* of Trias & Benz (1994)). The best fit of the relative permeabilities was obtained with $r = 0.9$ nm (diameter 1.8 nm). It is noteworthy, that there is no apparent contradiction between low cell wall permeability and large pore size since the meaning of the two parameters is different and not comparable. The pore size defines the exclusion limit at the narrowest part of the channel and the cell wall permeability represents a velocity that is dependent on many characteristics of the channel such as length, pore size, and the number of open channels. The latter may be controlled by cell wall properties such as asymmetric distribution of lipids and charges because of the voltage dependence of the cell wall channel (*see* below).

Table 2. Comparison of the cell wall channel properties of *M. phlei, M. smegmatis, C. glutamicum, R. erythropolis* and *N. farcinica.*

Cell wall porin	*G* in 1 M KCl (nS)	Selectivity P_c/P_a in KCl	Negative point charges at the channel mouth	Channel diameter nm	Reference
M. phlei	4.5	14.9	2.2	1.8[b]; 2.0[c]	This study
M. smegmatis	4.1	9.7	4.0	1.8[b]; 3.0[c]	Trias & Benz, 1994
C. glutamicum	5.5	8.1	2.0	2.2[b,c]	Lichtinger et al., 1998
R. erythropolis	6.0	11.8	2.7	2.0[c]	Lichtinger et al., 2000
N. farcinica	3.0	8.2	1.3	1.4[b]; 1.6[c]	Rieß et al., 1998

The channel diameters were estimated from the liposome swelling assay[a], the single-channel conductance as a function of the hydrated ion radii[b] or the effect of negative point charges on single channel conductance[c]. P_c and P_a are cation and anion permeability, respectively.

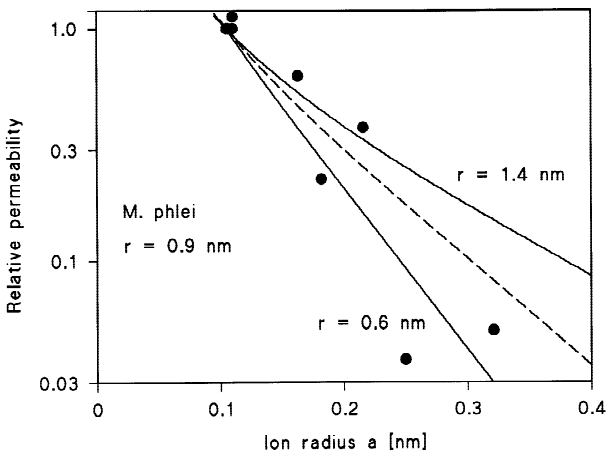

Fig. 8. Fit of the single-channel conductance data of the *M. phlei* cell wall channel by using the Renkin correction factor times the aqueous diffusion coefficients of the different cations (Trias & Benz, 1994). The product of both numbers was normalized to 1 for $a = 0.105$ nm (Rb^+). Single-channel conductances were normalized to the ones of Rb^+ and plotted versus the hydrated ion radii taken from Table 1. The data points correspond to Li^+, Na^+, K^+, NH_4^+, Cs^+, $N(CH_3)_4^+$, $N(C_2H_5)_4^+$ and $Tris^+$, which were all used for the pore diameter estimation (*see* Discussion). The fit (solid lines) is shown for $r = 1.4$ nm (upper line) and $r = 0.6$ nm (lower line). The best fit was achieved with $r = 0.9$ nm (diameter = 1.8 nm), which corresponds to the broken line.

Effect of Point Charges at the Channel Mouth

General diffusion porins of gram-negative bacteria form channels in which ions move in a fashion similar to the way they move through the aqueous phase and which show a linear relationship between single-channel conductance and bulk aqueous conductivity (Benz, 1994). This is not the case for the channel investigated in this study, since the single-channel conductance for KCl increased only about 5-fold for an increase of the KCl concentration by a factor of 20 (*see* Table 1). A quantitive description of the effect of the point charges on the single-channel conductance may be given with the considerations of Nelson and McQuarrie (1975) by using eqs. (*1*) to (*3*) of Trias and Benz (1993), assuming that the channel has a diameter of 2.0 nm and that 2.2 negative point charges ($= -3.52\ 10^{-19}$ Asec) are attached to the channel mouth. The results of this fit are enclosed in Fig. 9. It is noteworthy that the negative potential at the mouth of the channel has important implications on the function of the cell wall channel of *M. phlei* because the concentration of cations is increased at the channel mouth, while that of anions is decreased (Trias & Benz, 1993). This means that the channel conducts under physiological conditions cations considerably better than anions of the same aqueous mobility without being really selective by means of a selectivity filter. Negative point charges have also been observed for the cell wall channel of other actinomycetes (Trias & Benz, 1993; 1994; Rieß et al., 1998).

Voltage Control of Cell Wall Channel Permeability

The cell wall channel of *M. phlei* was found to be strongly voltage-dependent in an asymmetric manner. The application of a negative membrane potential as small as −20 mV to the cis-side, the side of the addition of the protein, led to a channel closure by more than 50%. It is noteworthy that also other cell wall channels of the mycolata are voltage-dependent. Most of them close when the side of the addition of the protein has negative polarity (Lichtinger et al., 1999; Trias & Benz, 1994; Rieß et al., 1998). Assuming that the channels have in all these cases the same orientation in vivo and in vitro, voltage-dependent control would require a negative potential at the inner side of the cell wall. A small voltage across the cell wall of *M. phlei* may exist because of an intrinsic membrane potential (asymmetry of lipid distribution) or a Donnan potential (asymmetry of charge distribution). This may explain the extremely small permeability of hydrophilic solutes through the mycobacterial cell wall because the cell wall channel is almost completely closed at small voltages as the reconstitution experiments clearly indicated.

Does the Chromosome of *M. phlei* Contain Several Porin Genes?

The gene, *msp*A, of the subunit of the cell wall channel of *M. smegmatis* has recently been cloned and sequenced

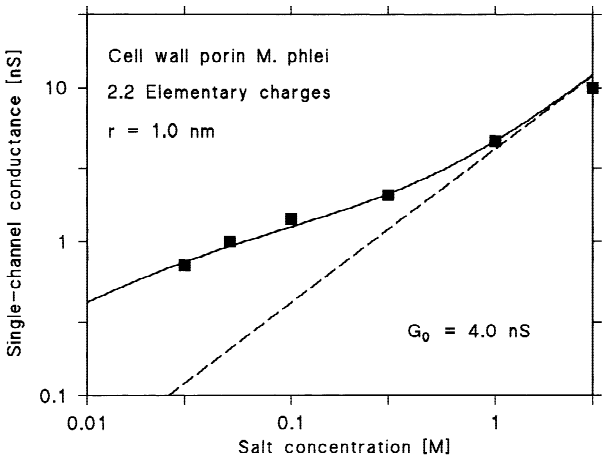

Fig. 9. Single-channel conductance of the cell wall channel of *M. phlei* as a function of the KCl concentration in the aqueous phase. The solid line represents the fit of the single-channel conductance data with the Nelson and McQuarrie formalism (eqs. (*1*) to (*3*) of Trias and Benz, 1993), assuming the presence of negative point charges (2.2 negative charges) at the channel mouth and assuming a channel diameter of 2.0 nm. Abscissa, concentration of the KCl solution; ordinate, average single-channel conductance. The broken (straight) line shows the single-channel conductance of the cell wall channel in the absence of point charges and corresponds to a linear function between channel conductance and bulk aqueous concentration. The single-channel conductance in the absence of negative point charges is 4.0 nS at 1 M KCl.

(Niederweis et al., 1999). Southern blots demonstrated that an *msp*A-derived probe hybridized with three identical bands of the *Bam*HI-digested chromosomal DNA of *M. phlei* and *M. smegmatis* at high stringency conditions (60°C). No additional band occurred when the stringency conditions were decreased. This result indicated that the chromosomes of *M. phlei* and *M. smegmatis* contain highly conserved regions in the genes of their different porin subunits. They seem to contain either two copies of the *msp*A gene or three homologous genes which encode for other cell wall channels. It is noteworthy that the hybridization signals agree with the results of the 16S rRNA sequencing, which suggests that a sequence similarity of 98.2% exists between the chromosomes of *M. smegmatis* and *M. phlei* (Roth et al., 1998) and an average similarity value greater than 95% among the mycobacteria (Stahl & Urbance, 1990). Surprisingly, database searches did not reveal any significant similarity to any other DNA sequence. In particular, there was no homology between *msp*A and the genomes of *M. tuberculosis* (Cole et al., 1998) and *M. leprae* (Cole et al., 2001). This means that the *msp*A gene appears to be specific for fast-growing mycobaceria, as has already been discussed by Niederweis et al. (1999). With *Bam*HI-digested chromosomal DNA of other mycolata we observed hybridization for *Tsukamurella inchonensis* under high stringency conditions, for *Nocardia farcinica* at medium (50°C) and *Rhodococcus equi* at low (45°C) stringency conditions indicating that the genes encoding for the cell wall channels of these organisms are very closely related to *msp*A.

Implications of the Cell Wall Channel Properties for the Antibiotic Resistance of Mycobacterial Cell Walls

The mycobacteria are a closely related coherent group, in which the pathogenic members characteristically show broad natural resistance to many antibiotics currently available (Heym, Philipp & Cole, 1996), especially β-lactams. Insufficient inhibitory activities of antibiotics could be caused by permeability problems (Barry & Mdluli, 1996). The presence of negatively charged groups in or near the cell wall channels explains the small permeability of anionic antibiotics and the high permeability for neutral, zwitterionic and positively charged antibiotics like isoniazid, ethambutol, rifampin, and pyrazinamide, which are known to cure tuberculosis (Barry, 1997). The positively charged isoniazid is particularly effective against biosynthesis of mycolic acids (Mdluli et al., 1998). However, the pyrazinamide (PZA) resistance is variable among the mycobacteria, because the resistance is known to be caused by mutations in the gene encoding pyrazinamidase (PZase). The microorganisms contain their own drug uptake system for PZA and the limiting step for the diffusion of this compound is probably not the hydrophobic mycolate cell wall layer, as has been demonstrated by liposome diffusion experiments (Raynaud et al, 1999). It is noteworthy that porins of the fast growing atypical mycobacteria (*M. smegmatis, M. chelonae, M. phlei*) and those of the pathogenic slow growers (*M. bovis BCG*) both exhibit negative point charges at the channel mouth, despite the fact that the Southern blot suggests two different types of porins within the mycobacteria.

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