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Identification of a cation-specific channel (TipA) in the cell wall of the gram-positive mycolata *Tsukamurella inchonensis*: the gene of the channel-forming protein is identical to *msp*A of *Mycobacterium smegmatis* and *mpp*A of *Mycobacterium phlei*

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Abstract

Detergent extracts of whole cells of the Gram-positive bacterium *Tsukamurella inchonensis* ATCC 700082, which belongs to the mycolata, were studied for the presence of ion-permeable channels using lipid bilayer experiments. One channel with a conductance of about 4.5 nS in 1 M KCl was identified in the extracts. The channel-forming protein was purified to homogeneity by preparative SDS-PAGE. The protein responsible for channel-forming activity had an apparent molecular mass of about 33 kDa as judged by SDS-PAGE. Interestingly, the protein showed cross-reactivity with polyclonal antibodies raised against a polypeptide derived from MspA of *Mycobacterium smegmatis* similarly as the cell wall channel of *Mycobacterium phlei*. Primers derived from *msp*A were used to clone and sequence the gene of the cell wall channels of *T. inchonensis* (named *tip*A for *T. inchonensis* porin A) and *M. phlei* (named *mpp*A for *M. phlei* porin A). Surprisingly, both genes, *tip*A and *mpp*A, were found to be identical to *msp*A of *M. smegmatis*, indicating that the genomes of *T. inchonensis*, *M. phlei* and *M. smegmatis* contain the same genes for the major cell wall channel. RT-PCR revealed that *tip*A is transcribed in *T. inchonensis* and *mpp*A in *M. phlei*. The results suggest that despite a certain distance between the three organisms, their genomes contain the same gene coding for the major cell wall channel, with a molecular mass of 22 kDa for the monomer.

Keywords: Porin; Mycolic acid; MspA; TipA; MppA cation-selective channel; *Tsukamurella inchonensis*; Lipid bilayer membrane

1. Introduction

Tsukamurella inchonensis is a member of the genus *Tsukamurellae* that belongs to the mycolata, a broad and diverse group of mycolic-acid containing actinomycetes [1–5]. Common to all these bacteria is the mycolic acid layer on the surface of the cells. The mycolic acids are covalently bound to the peptidoglycan–arabinogalactan skeleton of the cell wall, which contains also extractable lipids [6–8]. Especially long mycolic acids have been found in mycobacteria (60–90 carbon atoms) and tsukamurellae (64–74 carbon atoms); they are medium-sized in gordonae (52–66 carbon atoms), nocardiae (46–58 carbon atoms), and rhodococci (36–52 carbon atoms), and small in corynebacteria (22–38 carbon atoms) [9,10,6,7,11–18]. Besides the mycolic acids, the cell wall of mycolata contains also free lipids, such as trehalose dimycolates, glycosyl monomycolates and peptidolipids [11,19,14,20,21]. The mycolic acids and free lipids are arranged

Abbreviations: LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; Genapol (oligoethylenglycol-monoalkylether); FPLC, fast protein liquid chromatography; PC, diphytanoyl phosphatidylcholine; PS, phosphatidylserine; pS, picosiemens; SDS, sodium dodecylsulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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perpendicular to the cell surface [11], which suggests that they could form a membrane-like structure, which has also been observed in freeze-fracture samples of mycobacteria [22]. At least in mycobacteria the mycolic acid layer clearly forms a considerable permeability barrier for the diffusion of hydrophilic solutes [23,24,19,15].

Tsukamurellae are slow-growing mycolata mostly found in the soil [25]. Some of them are either animal or human pathogens such as *T. inchonensis* [1] or *Tsukamurella tyrosinosolvens* [18]. Several infections with *Tsukamurellae* were observed in immunosuppressed patients [26,27]. *T. inchonensis* was isolated from blood of a patient who had ingested hydrochloric acid [28] and is resistant to streptomycin, isoniazid, ethambutol, rifampin, *p*-aminosalicylic acid, protionamide, capreomycin, and cycloserine [1]. When the idea of the cell wall of *Tsukamurellae* being an outer lipid permeability barrier for hydrophilic compounds is accepted, the question arises how these molecules cross the cell wall. This means that they must contain cell wall channels similar to other members of the group of mycolic acid-containing actinomycetes, such as *Nocardia* [29,30], *Mycobacteria* [31–33] and *Corynebacteria* [34]. Channels have been identified in the cell wall of all these bacteria, which do not form similar structures but have a similar function as their gram-negative counterparts [35,36].

In this study we identified the permeability pathway in the mycolic acid layer of *T. inchonensis* and characterized its channel properties. The genes of this cell wall channel and that of *Mycobacterium phlei* were cloned and sequenced. The somewhat surprising result of the study was that the two genes, *tip*A and *mpp*A, coding for the cell wall channels were found to be identical to *msp*A of *Mycobacterium smegmatis*. This means that the major cell wall channel of *T. inchonensis* and *M. phlei* are identical to MspA, which has previously been identified in *M. smegmatis* [33,37]. This means that the *msp*A gene appears to be specific for mycolata with long mycolic acids as has already been discussed by Niederweis et al. [37] and Riess et al. [38]. This has already been suggested by the observation that chromosomal DNA of other mycolata, such as *T. inchonensis* or *Nocardia farcinica*, contains genes that are very closely related to *msp*A of *M. smegmatis* [38]. Results of electrophysiological measurement presented here indeed revealed that the cell wall channel of *T. inchonensis* is wide and water-filled and has similar properties as MspA, which are caused by the presence of negatively charged groups at the channel mouth.

2. Materials and methods

2.1. Bacterial strains and growth conditions

T. inchonensis ATCC 700082 [1] was routinely grown at 37 °C for 72 h in BHI-medium (3.7% Difco brain-heart infusion). *M. phlei* ATCC 356 [38] was grown at 37 °C for 72 h in DYT medium (Double-Yeast-Trypton). Both strains were grown using a shaker at 150 rpm.

2.2. Isolation and purification of the channel forming proteins

The cells were harvested by centrifugation at 12,000 rpm and 4 °C and washed twice in 10 mM Tris–HCl (pH 8). Two-gram wet cell was treated overnight with a solution containing 10-ml 10 mM Tris–HCl (pH 8) supplemented with 0.4% *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) by shaking at room temperature. Cells and detergent solution were centrifuged for about 15 min at 6000 rpm. The cells (pellet) were discarded. The supernatant contained the channel forming activity. SDS-PAGE indicated that it contained several bands. The protein was loaded and run on preparative SDS-PAGE. The gel was cut in different slices according to different molecular mass bands of the proteins. The slices were eluted overnight in a solution containing 10 mM Tris–HCl (pH 8) and 0.4% LDAO and inspected for channel-forming activity. The cell wall channel protein of *M. phlei* was purified as has been described previously [38].

2.3. SDS-PAGE

SDS-PAGE was performed according to the Laemmli gel system [39] (Fig. 1). The gels were stained with Coomassie brilliant blue or with silver stain [40].

2.4. Lipid bilayer experiments

The methods used for the lipid bilayer experiments have been described previously in detail [41]. Black lipid

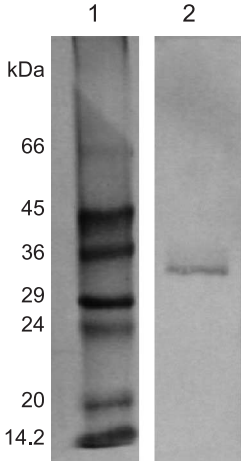

Fig. 1. 12% SDS-PAGE according to Laemmli [39] of the purification procedure of TipA (the 33-kDa cell wall channel protein) of *T. inchonensis*. The gel was stained with silver. Lane 1: Molecular mass marker 66, 45, 36, 29, 24, 20, 14.2 kDa. Lane 2: Purified TipA protein by preparative SDS-PAGE.

bilayer membranes were obtained from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (PC) and diphytanoylphosphatidylserine (PS) (ratio of 4:1) in *n*-decane. The temperature was maintained at 20 °C during all experiments. Zero-current membrane potentials were measured by establishing a salt gradient across membranes containing 100 to 1000 channels as has been described earlier [42].

2.5. Immunological detection of MspA

A synthetic polypeptide of part of the MspA sequence (DRNRLTREWFHSGRA) was synthesized and polyclonal antibodies against this peptide were raised in rabbits. The pre-immune serum was tested giving no reaction against cell extracts of *M. smegmatis*. The antiserum was used for Western blots of the extracted protein samples using standard procedures [43]. The blotting time was 30 min by 350 mA. The ECL Western Detection kit (Amersham Pharmacia Biotech) was used to detect binding of the antibody according to the instructions of the manufacturer. The exposure time was 30 s. The antibodies were highly specific for MspA of *M. smegmatis*.

2.6. PCR analysis

Chromosomal DNA of *T. inchonensis* and *M. phlei* was isolated as described previously [44]. Primers MspAnf and MspK1 (see Table 1) were used for PCR amplification of the *tip*A and *mpp*A genes. Annealing was performed at 60 °C with 50-s extension at 72 °C by using *Taq* polymerase. For this analysis, the PCR MasterTaq Kit (Eppendorf, Hamburg, Germany) was used.

2.7. RT-PCR

Total mRNA was isolated from disrupted cells by using an RNeasy kit according to the instruction of the manufacturer (Qiagen, Hilden, Germany). Purified RNA was treated with 2U of Turbo Dnase (Ambion, Huntingdon, Cambridgeshire, United Kingdom) in 0.1 volume of 10× Turbo DNase buffer for 1 h at 37 °C to remove the DNA. Reverse transcription (RT) was performed in a two-step reaction with an enhanced avian HS RT-PCR kit (Sigma, Deisenhofen, Germany) with random nonamers. The cDNA product was used for a direct PCR with the specific *msp*A primers MspA1, MspN2, MspK1 and MspK3 (see Table 1) and the same PCR program.

3. Results

3.1. LDAO extracts of T. inchonensis cells contain a channel-forming protein

The channel-forming activity was isolated from *T. inchonensis* using a protocol, which has been applied previously to *Nocardia* species [29,30] and also to *M. phlei* [38]. This protocol started from detergent extracts of whole cells. *T. inchonensis* cells were treated with 10 mM Tris–HCl pH 8 supplemented with 0.4% LDAO. The supernatant of the extraction procedure showed channel-forming activity in the lipid bilayer assay. The channels had a single-channel conductance of about 4.5 nS in 1 M KCl. The extracts contained always several molecular mass bands, which included a major band of about 33 kDa. Similar bands have been found in cell extracts of *M. smegmatis* [37] and *M. phlei* [38] when the samples were boiled.

3.2. Purification of the channel-forming protein

Further purification of the channel-forming protein was achieved by preparative SDS-PAGE. Several molecular mass bands were excised from the gel and were extracted overnight with a buffer containing 0.4% LDAO. Addition of the extract of the 33-kDa band to a planar lipid bilayer resulted in a very fast reconstitution of channels. When different molecular mass regions were excised from the same SDS-PAGE, highest channel-forming activity was always observed for the 33-kDa band (despite the fact that the nucleotide sequence predicts a 22-kDa protein; see below). It is noteworthy, however, that some small channel-forming activity was also observed, when gel slices were extracted in the region between 30 and 80 kDa. This result indicated that the 33-kDa band may represent one subunit of the channel-forming oligomer. It is noteworthy that high molecular mass complexes of the cell wall channels have been observed for *M. smegmatis* [37,45] and *M. phlei* [38]. The monomer of about 22 kDa predicted by the nucleotide sequence could not be observed when the protein was boiled in sample buffer.

Table 1
Oligonucleotides used in this study

Oligonucleotides	Position	Sequence
MspAnf	1–20 downstream	5′ -GGGGCCGCCGGCGATACAGT-3′
MspK1	700–681 upstream	5′ -AAGCGGTCTCAGCGACCGAA-3′
MspK3	512–491 upstream	5′ -TCGACCGAGAACGTTGCGACTT-3′
MspN2	329–350 downstream	5′ -CGCTGGGTGTGGGCATCAACTT-3′

The sequences of the primers were derived from *msp*A [37].

3.3. Analysis of the channels formed by the 33-kDa protein

Fig. 2 shows a single-channel recording of a PC/PS membrane in the presence of the 33-kDa protein, which was added to a black membrane in a concentration of about 10 ng/ml. The single-channel recording demonstrates that the protein formed defined channels. The single-channel conductance of most channels formed by the 33-kDa protein was about 4.5 nS in 1 M KCl. Only about 30% of total number of channels with other conductance was observed, which represent presumably substates of the channel (see the histogram of Fig. 3). It is noteworthy that the channels formed by the 33-kDa protein of *T. inchonensis* had a long lifetime similar to those that have been detected previously for porins of gram-negative [36] and gram-positive bacteria [33]. All these porins formed channels in lipid bilayer membranes with a long lifetime at small transmembrane potential (mean lifetime at least 5 min).

Single-channel experiments were also performed with other salts than KCl to obtain some information on the size and selectivity of the channels formed by the 33-kDa protein of *T. inchonensis*. The results are summarized in Table 2. The conductance sequence of the different salts within the channel was KCl>K-acetate>LiCl, which means that the channel could be cation-selective similar to many other channels from the cell wall of mycolata. Table 2 shows also the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase. Similarly, as many cell wall channels of gram-positive bacteria [38,46] the relationship between conductance and

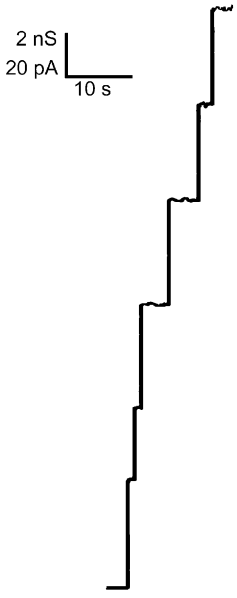

Fig. 2. Single-channel recording of a diphytanoyl-phosphatidylcholine/phosphatidyl-serine (molar ratio 4:1)/*n*-decane membranes in the presence of 20 ng/ml of the TipA protein of *T. inchonensis*. The aqueous phase contained unbuffered 1 M KCl (pH 6). The applied voltage was 20 mV; T=20 °C.

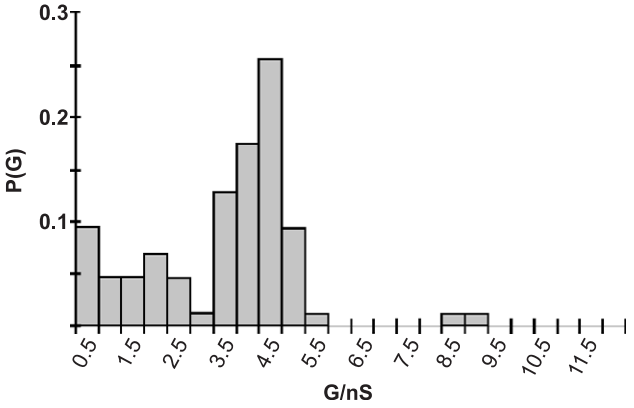

Fig. 3. Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed with membranes formed of PC/PS (molar ratio 4:1)/*n*-decane in the presence of TipA of *T. inchonensis*. $P(G)$ is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T=20 °C. The average single-channel conductance was 4.5 nS for 132 single-channel events.

KCl concentration was not a linear function, which suggests that the cell wall channel of *T. inchonensis* contains point net charges similar to those of *M. smegmatis* [33], *M. chelonae* [32] and *C. glutamicum* [34,46].

Experiments at higher voltages demonstrated that the cell wall channel of *T. inchonensis* was voltage-dependent in an asymmetric manner. The voltage dependence could be demonstrated for single- and multi-channel experiments when the protein was added to the *cis*-side of the membrane. In the latter experiments the membrane current started to decrease already at −50 mV in an exponential fashion whereas it was stationary when the potential was positive at the *cis*-side (data not shown). For −50 mV at the *cis*-side the current showed a stronger decrease but only a minor decrease was observed at +50 mV. This result indicated oriented insertion of the cell wall porin into the

Table 2
Average single-channel conductance, G, of the cell wall channel of *T. inchonensis* in different salt solutions[a]

Salt	Concentration [M]	Single-channel conductance G [nS]
LiCl	0.1	0.3
KAc pH 7	0.1	0.7
KCl	0.003	0.07
	0.01	0.02
	0.03	0.4
	0.1	0.8
	0.3	1.6
	1.0	4.5
	3.0	10

[a] The membranes were formed of PC/PS (molar ratio 4:1) dissolved in *n*-decane. 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.

membranes, when it was added to only one side of the membrane. It is noteworthy that the voltage dependence of TipA was very similar if not identical to that of MppA [38] and that of MspA [33].

Further information about the structure of the channel formed by the 33-kDa protein of *T. inchonensis* was obtained from zero-current membrane potential measurements in presence of KCl gradients. A fivefold KCl gradient (100 versus 500 mM) across a lipid bilayer membrane, in which a sufficient number of channels were reconstituted, resulted in an asymmetry potential of about 20 mV at the more dilute side (mean of five measurements). This result indicated little preferential movement of potassium ions over chloride through the channel at neutral pH. The zero-current membrane potentials were analyzed using the Goldman–Hodgkin–Katz equation [41,47]. The ratio of the potassium permeability, P_K, divided by the chloride permeability, P_{Cl}, was about four, which indicated indeed a certain selectivity of the channel formed by the 33-kDa protein of *T. inchonensis* (see also Discussion).

3.4. Western blots of the cell wall channel proteins of T. inchonensis and M. phlei with antibodies against MspA of M. smegmatis

In a previous study we have demonstrated that a DNA probe derived from the *msp*A gene of *M. smegmatis* (sequence position 166–196) hybridized with 9-, 6- and 4.5-kb bands of *M. phlei* and also with a 2-kb band of *T. inchonensis* as obtained by *Bam*HI digestion of the corresponding chromosome [38]. These hybridizations were observed as clear bands at a high stringency. Here we checked if the cell wall channels of *M. phlei* and *T. inchonensis* cross-reacted with the highly specific polyclonal anti-MspA antibodies (lane 1 and 2). The results are shown in Fig. 4. Pure cell wall channel protein from *T. inchonensis* (lane 1) and from *M. phlei* (lane 2) showed cross-reactivity with the antibodies. Interestingly, three bands corresponding to molecular masses of 33, 45 and 52 kDa were observed for *T. inchonensis* (lane 1) and *M. smegmatis* (lane 4), again indicating the cell wall channel is presumably formed by a protein oligomer as has been found for MspA of *M. smegmatis* [45,35] and *M. phlei* [38]. Faint 22-kDa bands were observed for *M. phlei* and *M. smegmatis*, whereas in the case of the cell wall channel protein of *T. inchonensis* there was no reaction in the range of 20 kDa.

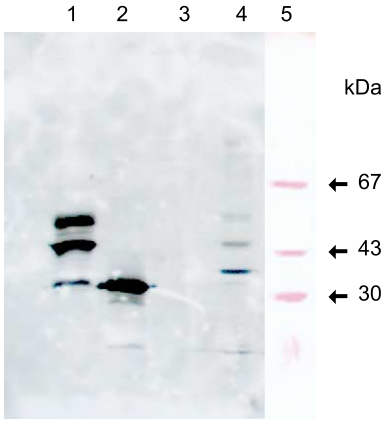

Fig. 4. Western blot of purified cell wall channel proteins of *T. inchonensis* (lane 1), *M. phlei* (lane 2) [lane 3 is free] and MspA of *M. smegmatis* (lane 4). Lane 5 shows molecular mass markers stained with Ponceau S. The proteins were loaded onto a 12% SDS-PAGE and blotted onto a nitrocellulose membrane. Rabbit antiserum obtained against a synthetic polypeptide derived from MspA was used in a dilution of 1:100.

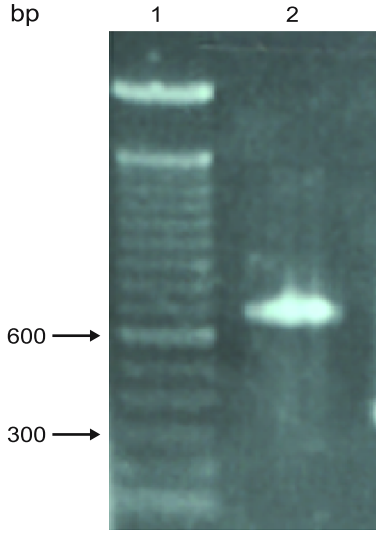

Fig. 5. 1.5% agarose gels from PCR. DNA from *M. phlei* was used as a template for PCR reaction performed with the couple of primers MspAnf and MspK1 (see Table 1) for the 700-bp fragment. The PCR products were run on a 1.5% agarose gel. Lane 1: 100-bp ladder. Lane 2: PCR product using *M. phlei* DNA.

3.5. Cloning and sequencing of the cell wall channel protein of T. inchonensis and M. phlei

Western and Southern blots suggested a high homology between MspA of *M. smegmatis* and the cell wall channel proteins of *T. inchonensis* and *M. phlei*. Primers were derived from the DNA-sequence of *msp*A because of this high homology to identify and clone the genes of the cell wall channels from *T. inchonensis* and *M. phlei*. These primers (MspAnf and MspK1) were used to clone the putative gene of the cell wall channels of these two bacteria using PCR. The result of the PCR is shown in Fig. 5. The PCR products were cloned into TOPO 2.1 from Invitrogen and amplified for sequencing. Primer M13 reverse and forward from Invitrogen were used for sequencing the PCR product of both *T. inchonensis* and *M. phlei*. The interesting result of the sequencing of the genes for the cell wall channels of both bacteria [named *tip*A (EMBL Nucleotide Sequence Database accession

number AJ812029) and *mpp*A (EMBL Nucleotide Sequence Database accession number AJ812030)] was that their whole nucleotide sequences were identical to *msp*A. The genes code in all three cases for 22-kDa proteins.

3.6. RT-PCR

To see whether the *tip*A and *mpp*A genes are translated in *T. inchonensis* and *M. phlei*, total RNA was isolated from both organisms. No PCR products were detected in both cases after 20 cycles of PCR for specific primers using the DNase-treated RNA, indicating that the genomic DNA from the strains present in the samples was completely digested (data not shown). Via reverse transcription, the total mRNA of *T. inchonensis* and *M. phlei* was converted into cDNA with random nonamers and afterwards amplified with the *msp*A-specific couple of primers (MspAnf, MspK1, MspK3, MspN2). The mRNA of *T. inchonensis* and *M. phlei* contained signals that indicated amplification with the primers listed above. Fig. 6 shows the amplification product of total cDNA of *T. inchonensis* (data for *M. phlei* not shown). This result suggested that both bacterial strains contained the mRNA for MspA-like protein, which means that *tip*A and *mpp*A are both transcribed and the proteins are expressed as suggested by the Western blot.

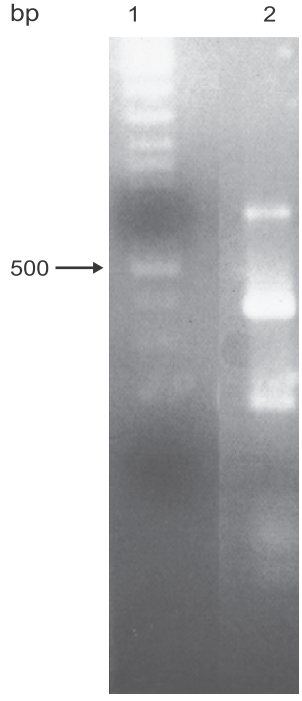

Fig. 6. Reverse transcription of total mRNA from *T. inchonensis*. Total mRNA was converted into cDNA and amplified with the primers MspAnf, MspN2, MspK3, and MspK1, respectively (Table 1). Lane 1: 100-bp ladder. Lane 2: result of RT-PCR amplification of *T. inchonensis* with the *tipA*(*msp*A)-specific primers.

4. Discussion

4.1. The cell wall of T. inchonensis contains an ion-permeable channel

Cell wall proteins of gram-positive bacteria, such as *M. chelonae* [31,32], *M. smegmatis* [33,37] and *C. glutamicum* [34], have been shown in recent years to form channels in lipid bilayer membranes and liposomes. Common to all these bacteria is that they contain besides the peptidoglycan layer a large amount of lipids in form of mycolic acids in their cell wall, which forms a permeability barrier [23,15]. *T. inchonensis* belongs also to the broad and diverse group of mycolic acid containing actinomycetes [1]. In agreement with the possible barrier function of the cell wall, we were able to identify a channel-forming protein (called TipA) with a molecular mass of about 33 kDa for the channel-forming activity. Western blots suggested that several bands showed cross-reactivity with the antibodies, which indicated that the protein forms aggregates. The monomers of TipA and MppA have a molecular mass of 22 kDa according to their genes although a 33-kDa protein was identified to be responsible for channel formation. The channels are presumably formed by oligomers and the smear of channel-forming activity was observed across a considerable molecular mass range. This agrees well with observations that were made previously with MspA and MppA, which both form aggregates [37,38]. This has to do with the close relationship to MspA of *M. smegmatis*, which has recently been crystallized and forms octamers [35]. It is noteworthy that the formation of channels by the cell wall protein of *T. inchonensis* was not a rare event. The addition of 100 ng/ml of this protein to the aqueous phase bathing lipid bilayer membranes was able to increase their conductance considerably and more than 1000 channels were formed under these conditions. Higher protein concentration led to the formation of even more channels. The single-channel conductance of the cell wall channel from *T. inchonensis* was fairly homogeneous (see Fig. 3). More than 70% of all observed channels had a single-channel conductance within the range from 3.5 to 5.5 nS. The broad range may be caused by change of the numbers of monomers in an oligomer (normally eight [35]) Only about 30% of the channels had a smaller single-channel conductance and represent presumably substates.

4.2. The cell wall channel of T. inchonensis is wide and water-filled

The *T. inchonensis* cell wall channel has a high conductance, similar to those of the cell wall channels from *M. smegmatis* [33], *M. phlei* [38], *C. glutamicum* [34], and *N. farcinica* [29] (see Table 3). These channels share some common features. All are wide and water-filled and have

Table 3
Comparison of the cell wall channel properties of different actinomycetes

Cell wall protein of	*G* (nS) in 1 M KCl	Channel diameter (nS)
T. inchonensis	4.5	2.0[a,b]
M. smegmatis	4.1	1.8[a], 3.0[b]
M. phlei	4.5	1.8[a], 2.0[b]
N. farcinica	3.0	1.4[a], 1.6[b]
C. glutamicum	5.5	2.2[a,b]

[a] The channel diameter was estimated from the single-channel conductance as a function of the hydrated ion radii.
[b] The channel diameter was estimated from the effect of negative point charges on single-channel conductance.

diameters of more than 1.6 nm. Using the Renkin correction factor [48,49], it is possible to calculate the diameter of the *T. inchonenis* cell wall channel from the conductance of the channel in different salts [33]. For this the Renkin equation (Eq. (1) of Trias and Benz [33]) times the aqueous diffusion coefficient of the ions normalized to the one of rubidium ions (i.e., relative permeability of rubidium ions=1) was plotted as a function of the hydrated ion radii (Table 4 of Trias and Benz [33]). The best fit of the relative permeability was obtained with r=1.0 nm (diameter 2.0 nm). The properties of the cell wall channel of *T. inchonensis* resemble closely those of MspA of *M. smegmatis* [33] and MppA of *M. phlei* [38].

It is noteworthy that there does not exist an apparent contradiction between low cell wall permeability and large pore size because the meanings of the two parameters are different and not comparable. The pore size defines the exclusion limit at the narrowest part of the channel (see also the structure of the MspA channel [35]) and the cell wall permeability represents a velocity that is dependent on many characteristics of the channel such as length, pore size, and number of open channels. The latter may be controlled by cell wall properties such as asymmetric distribution of lipids and charges because of the asymmetric voltage dependence of the cell wall channel. The asymmetric voltage dependence agrees with the 3D structure of MspA of *M. smegmatis*, which should be very similar if not identical to that of TipA of *T. inchonensis*. The channel is formed by a hydrophobic beta-barrel cylinder (length about 3.7 nm) and a big more hydrophilic protrusion (length about 6 nm). Thus, it is very likely that the beta-barrel cylinder is inserted from the *cis*-side (the side of addition of TipA) into the membrane and that protrusion faces the *cis*-side because it is not very likely that the hydrophilic protrusion is able to cross the hydrophobic interior of a lipid bilayer membrane.

4.3. 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 [50]. This is not the case for the channel investigated in this study, since the single-channel conductance for KCl increased only about fivefold for an increase of the KCl concentration by a factor of 20 (see Table 1). A quantitative description of the effect of the point charges on the single channel conductance may be given with the considerations of Nelson and McQuarrie [51] by using Eqs. (1) to (3) of Trias and Benz [32], assuming that the channel has a diameter of 2.0 nm and that 2.2 negative point charges (=$-3.52\ 10^{-19}$ As) are attached to the channel mouth (data not shown). 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 *T. inchonensis* because the concentration of cations is increased at the channel mouth, while that of anions is decreased [32]. This means that the channel, under physiological conditions, conducts cations considerably better than anions of the same aqueous mobility without being really selective due to the presence of a selectivity filter. Negative point charges have also been observed for the cell wall channel of other actinomycetes, in particular for MspA of *M. smegmatis* and for MppA of *M. phlei* with almost identical parameters (diameter and number of point charges) as derived here for TipA of *T. inchonensis* [32,33,29,38].

4.4. The genes coding for the major cell wall channels of M. smegmatis, M. phlei and T. inchonensis are identical

The somewhat unexpected and interesting result of cloning and sequencing of the genes of the cell wall channel proteins *tip*A and *mpp*A was that these genes are identical to *msp*A, the gene of the subunit of the cell wall channel of *M. smegmatis* [37]. Southern blots have demonstrated already previously that an *msp*A-derived probe hybridized with three identical bands of the *Bam*HI-digested chromosomal DNA of *M. phlei* and *T. inchonensis* at high stringency conditions (60 °C) [38]. This result indicated already that the chromosomes of *M. smegmatis*, *M. phlei* and *T. inchonensis* contain highly conserved regions in the genes of their different porin subunits. Here, we could show that the genes are identical, despite some phylogenetic distance between both organisms. It is noteworthy that the results of the 16S rRNA sequencing suggest that a sequence similarity of 98.2% exists between the chromosomes of *M. smegmatis* and *M. phlei* [52]. Similar data are not available for a comparison between *T. inchonensis* and *M. smegmatis*, but the phylogenetic distance between these bacteria is presumably even higher. The results of this study suggest that the genes coding for the major cell wall channels of fast-growing members of the mycolata related to *M. smegmatis* are very closely related or even identical to *msp*A of *M. smegmatis*.

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References

[1] A.F. Yassin, F.A. Rainey, H. Brzezinka, J. Burghardt, H.J. Lee, K.P. Schaal, *Tsukamurella inchonensis* sp. Nov., Int. J. Syst. Bacteriol. 45 (1995) 522–527.
[2] J. Chun, S.-O. Kang, Y.C. Hah, M. Goodfellow, Phylogeny of mycolic acid-containing actinomycetes, J. Ind. Microbiol. 17 (1996) 205–213.
[3] M. Goodfellow, D.E. Minnikin, Identification of *Mycobacterium chelonei* by thin-layer chromatographic analysis of whole-organism methanolysates, Tubercle 62 (1981) 285–287.
[4] M. Goodfellow, The family Nocardiaceae, in: A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer (Eds.), The Prokaryotes, Springer Verlag, New York, 1992, pp. 1188–1213.
[5] E. Stackebrandt, F.A. Rainey, N.L. Ward-Rainey, Proposal for a new hierarchic classification system, *Actinobacteria classis* nov., Int. J. Syst. Bacteriol. 47 (1997) 479–491.
[6] D.E. Minnikin, Lipids: Complex lipids, their chemistry, biosynthesis and roles, in: C. Ratledge, J.C. Stanford (Eds.), The Biology of the Mycobacteria, Academic Press, New York, 1982, pp. 95–184.
[7] D.E. Minnikin, Chemical targets in cell envelopes, in: M. Hopper (Ed.), Chemotherapy of Tropical Diseases, John Wiley and Sons, Chichester, 1987, pp. 19–43.
[8] D. Chatterjee, C.M. Bozic, M. McNeil, P.J. Brennan, Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis*, J. Biol. Chem. 266 (1991) 9652–9660.
[9] I. Yano, K. Saito, Gas chromatographic and mass spectrometric analysis of molecular species of corynomycolic acids from *Corynebacterium ulcerans*, FEBS Lett. 23 (1972) 352–356.
[10] D.E. Minnikin, P.V. Patel, M. Goodfellow, Mycolic acids of representative strains of *Nocardia* and the "rhodochrous" complex, FEBS Lett. 39 (1974) 322–324.
[11] D.E. Minnikin, Chemical principles in the organization of lipid components in the mycobacterial cell envelope, Res. Microbiol. 142 (1991) 423–427.
[12] M. Daffe, P.J. Brennan, M. McNeil, Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycolsyl alditol fragments by gas chromatography/mass spectrometry and by 1H and 13C NMR analyses, J. Biol. Chem. 265 (1990) 6734–6743.
[13] J.G. Holt, N.R. Krieg, P.H.A. Sneath, J.T. Staley, S.T. Williams (Eds.), Nocardioform Actinomycetes, Bergey's Manual of Determiniative Biology, Williams & Wilkins, Baltimore, USA, 1994, pp. 625–650.
[14] K. Ochi, Phylogenetic analysis of mycolic acid-containing wall-chemotype IV actinomycetes and allied taxa by partial sequencing of ribosomal protein AT-L30, Int. J. Syst. Bacteriol. 45 (1995) 653–660.
[15] P.J. Brennan, H. Nikaido, The envelope of mycobacteria, Annu. Rev. Biochem. 64 (1995) 29–63.
[16] J. Liu, E.Y. Rosenberg, H. Nikaido, Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 11254–11258.
[17] J. Liu, C.E. Barry III, G.S. Besra, H. Nikaido, Mycolic acid structure determines the fluidity of the mycobacterial cell wall, J. Biol. Chem. 271 (1996) 29545–29551.
[18] A.F. Yassin, F.A. Rainey, H. Brzezinka, J. Burghardt, S. Schmitt, P. Seifert, O. Zimmermann, H. Mauch, D. Gierth, I. Lux, K.P. Schaal, *Tsukamurella tyrosinosolvens* sp. Nov., Int. J. Syst. Bacteriol. 47 (1997) 607–614.
[19] H. Nikaido, S.H. Kim, E.Y. Rosenberg, Physical organisation of lipids in the cell wall of *Mycobacterium chelonae*, Mol. Microbiol. 8 (1993) 1025–1030.
[20] I.C. Sutcliffe, Cell envelope components of *Rhodococcus equi* and closely related bacteria, Vet. Microbiol. 56 (1997) 287–299.
[21] I.C. Sutcliffe, Cell envelope composition and organisation in the genus *Rhodococcus*, Antonie Van Leeuwenhoek 74 (1998) 49–58.
[22] L. Barksdale, K.-S. Kim, Mycobacterium, Bacteriol. Rev. 41 (1977) 217–372.
[23] V. Jarlier, H. Nikaido, Permeability barrier to hydrophilic solutes in *Mycobacterium chelonae*, J. Bacteriol. 172 (1990) 1418–1423.
[24] H. Nikaido, V. Jarlier, Permeability of the mycobacterial cell wall, Res. Microbiol. 142 (1991) 437–443.
[25] M.M. McNeil, J.M. Brown, The medically important aerobic actinomycetes: epidemiology and microbiology, Clin. Microbiol. Rev. 7 (1994) 357–417.
[26] D. Rey, D. De Briel, R. Heller, P. Fraisse, M. Partisani, M. Leiva-Mena, J.M. Lang, *Tsukamurella* and HIV infection, AIDS 9 (1995) 1379.
[27] M.A. Schwartz, S.R. Tabet, A.C. Collier, C.K. Wallis, L.C. Carlson, T.T. Ngyyen, M.M. Kattar, M.B. Coyle, Central venous catheter-related bacteremia due to *Tsukamurella* species in the immunocompromised host: a case series and review of the literature, Clin. Infect. Dis. 35 (2002) 72–77.
[28] Y. Chong, K. Lee, C.Y. Chon, M.J. Kim, O.H. Kwon, H.J. Lee, *Tsukamurella inchonensis* bacteremia in a patient who ingested hydrochloric acid, Clin. Infect. Dis. 24 (1997) 1267–1268.
[29] F.G. Riess, T. Lichtinger, R. Cseh, A.F. Yassin, K.P. Schaal, R. Benz, The cell wall porin of *Nocardia farcinica*: biochemical identification of the channel-forming protein and biophysical characterisation of the channel properties, Mol. Microbiol. 29 (1998) 139–150.
[30] F.G. Riess, T. Lichtinger, A.F. Yassin, K.P. Schaal, R. Benz, The cell wall porin of the gram-positive bacterium *Nocardia asteroides* forms cation-selective channels that exhibit asymmetric voltage-dependence, Arch. Microbiol. 171 (1999) 173–182.
[31] J. Trias, V. Jarlier, R. Benz, Porins in the cell wall of mycobacteria, Science 258 (1992) 1479–1481.
[32] J. Trias, R. Benz, Characterization of the channel formed by the mycobacterial porin of *Mycobacterium chelonae* in lipid–bilayer membranes: demonstration of voltage dependent regulation and the presence of negative point charges at the channel mouth, J. Biol. Chem. 268 (1993) 6234–6240.
[33] J. Trias, R. Benz, Permeability of the cell wall of *Mycobacterium smegmatis*, Mol. Microbiol. 14 (1994) 283–290.
[34] T. Lichtinger, A. Burkovski, M. Niederweis, R. Krämer, R. Benz, Biochemical and biophysical characterization of the cell wall channel of *Corynebacterium glutamicum*: the channel is formed by a low molecular mass subunit, Biochemistry 37 (1998) 15024–15032.
[35] M. Faller, M. Niederweis, G.E. Schulz, The structure of a mycobacterial outer-membrane channel, Science 303 (2004) 1189–1192.
[36] R. Benz, Porins—structure and function, in: G. Winkelmann (Ed.), Microbial Transport Systems, WILEY-VCH Verlag, Weinheim, Germany, 2001, pp. 227–246.
[37] M. Niederweis, S. Ehrt, C. Heinz, U. Klöcker, S. Karosi, K.M. Swiderek, L.W. Riley, R. Benz, Cloning of the *msp*A gene encoding a porin from *Mycobacterium smegmatis*, Mol. Microbiol. 33 (1999) 933–945.
[38] F.G. Riess, U. Dörner, B. Schiffler, R. Benz, Study of the properties of a channel-forming protein of the cell wall of the gram-positive bacterium *Mycobacterium phlei*, J. Membr. Biol. 182 (2001) 147–157.
[39] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[40] H. Blum, H. Beier, H.J. Gross, Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, Electophoresis 8 (1987) 93–99.
[41] R. Benz, K. Janko, W. Boos, P. Läuger, Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*, Biochim. Biophys. Acta 511 (1978) 305–319.
[42] R. Benz, K. Janko, P. Läuger, Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*, Biochim. Biophys. Acta 551 (1979) 238–247.
[43] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[44] D. van Soolingen, P.W. Hermans, P.E. de Haas, D.R. Soll, J.D. van Embden, Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis, J. Clin. Microbiol. 29 (1991) 2578–2586.
[45] C. Stahl, S. Kubetzko, I. Kaps, S. Seeber, H. Engelhardt, M. Niederweis, MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*, Mol. Microbiol. 40 (2001) 451–464.
[46] N. Costa-Riu, E. Maier, A. Burkovski, R. Krämer, F. Lottspeich, R. Benz, Identification of an anion-specific channel in the cell wall of the gram-positive bacterium *Corynebacterium glutamicum*, Mol. Microbiol. 50 (2003) 1295–1308.
[47] R. Benz, A. Schmid, R.E.W. Hancock, Ion selectivity of Gram-negative bacterial porins, J. Bacteriol. 162 (1985) 722–727.
[48] E.M. Renkin, Filtration, diffusion, and molecular sieving through porous cellulose membranes, J. Gen. Physiol. 38 (1954) 225–243.
[49] H. Nikaido, E.Y. Rosenberg, Effect on solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*, J. Gen. Physiol. 77 (1981) 121–135.
[50] R. Benz, Solute uptake through bacterial outer membranes, in: R. Hackenbek, J.-M. Ghuysen (Eds.), Bacterial Cell Wall, Elsevier, Amsterdam, 1994, pp. 397–423.
[51] A.P. Nelson, D.A. McQuarrie, The effect of discrete charges on the electrical properties of a membrane, J. Theor. Biol. 55 (1975) 13–27.
[52] A. Roth, M. Fischer, M.E. Hamid, S. Michalke, W. Ludwig, H. Mauch, Differentiation of phylogenetically related slowly growing Mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences, J. Clin. Microbiol. 36 (1998) 139–147.