

Full Paper

Identification and characterization of a cell wall porin from *Gordonia jacobaea*

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Guadalupe Jiménez-Galisteo,¹ Ester Fusté,^{1,2} Elisa Muñoz,³ Teresa Vinuesa,¹ Tom G. Villa,⁴
Roland Benz,⁶ Angel Domínguez,⁵ and Miguel Viñas^{1,6,*}

¹ Department of Pathology and Experimental Therapeutics, Medical School, IDIBELL-University of Barcelona, Spain

² Department of Public Health, Mental Health and Perinatal Nursing, IDIBELL-University of Barcelona, Spain

³ Department of Cell Biology and Pathology, University of Salamanca, Salamanca

⁴ Department of Microbiology & Genetics, University of Salamanca, Salamanca

⁵ Department of Microbiology, University of Santiago de Compostela, Spain

⁶ Life Sciences and Chemistry, Jacobs University, Bremen, Germany

Gordonia jacobaea is a bacterium belonging to the mycolata group characterized by its ability to produce carotenoids. Mycolic acids in the cell wall contribute to reducing the permeability of their envelopes requiring the presence of channel-forming proteins to allow the exchange of hydrophilic molecules with the surrounding medium. Identification and purification of the channel-forming proteins was accomplished by SDS-PAGE, Mass spectrometry and Mass peptide fingerprinting and the channel-forming activity was studied by reconstitution in lipid bilayers. Here, we describe for the first time the presence of a cell-wall protein from *G. jacobaea* with channel-forming activity. Our results suggest that this protein bears a low similarity to other hypothetical proteins from the genus *Gordonia* of uncharacterized functions. The channel has an average single-channel conductance of 800 pS in 1 M KCl, is moderately anion-selective, and does not show any voltage dependence for voltages between +100 and –100 mV. The channel characteristics suggest that this protein could be of relevance in the import and export of negatively charged molecules across the cell wall. This could contribute to design treatments for mycobacterial infections, as well as being of interest in biotechnology applications.

Key Words: *Gordonia*; mycolata; porin; single-channel conductance

Abbreviations: CAN, acetonitrile; DTT, dithiothreitol; GjpA, *Gordonia jacobaea* protein A; kDa, kilodaltons; MALDI, Matrix-Assisted Laser Desorption/Ionization; MS/MS, tandem mass spectrometry; mV, millivolts; NCBI, National Center Biotechnology Institute; ng, nanogram; Pfam, protein families database; PMF, peptide mass fingerprinting; pS, picosiemens; PVDF, polyvinylidene difluoride; rpm, revolutions per minute; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, (hydroxymethyl) aminomethane; TSB tryptic soy broth; UAB, Autonomous University of Barcelona; w/v, weight/volumen

Introduction

Gordonia jacobaea strain MV-1 is characterized by its ability to synthesize carotenoids (de Miguel et al., 2000). This bacterium belongs to the broad and diverse group of mycolic acid-containing actinomycetes, known as the mycolata group. Mycolata contains microorganisms of industrial interest such as *Corynebacterium glutamicum*, as well as others of clinical relevance such as *Mycobacterium tuberculosis*, *M. leprae*, and *Nocardia asteroides* complex and *N. brasiliensis*. Mycolata have notoriously impermeable cell walls due to their thick peptidoglycan layer and the covalently linked mycolic acid layer, which contains also other extractable lipids (Daffé et al., 1983; Rangaka et al., 2015). The chain length of these mycolic

*Corresponding author: Miguel Viñas, Molecular Microbiology & Antimicrobials, Campus de Bellvitge, Medical School, University of Barcelona, 08907 Hospitalet (Barcelona), Spain.

E-mail: mvinyas@ub.edu

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acids varies markedly within the taxa, being about 46–66 carbon atoms for the *Gordonia* species (Marrakchi et al., 2014). It has been suggested that long mycolic acids contribute to the low permeability of the cell wall and hinder the passage of hydrophilic compounds (i.e. nutrients and antibiotics). Consequently, the cell wall of *Gordonia* resembles that of the outer membrane of gram-negative bacteria, forming a permeability barrier for these substances (Benz, 1994). Thus, the entrance and exit of hydrophilic solutes should occur via pore-forming proteins.

The first mycobacterial protein with a pore-forming activity was identified in 1992 by Trias et al. (1992). Since then, several porins have been identified in different members of *Nocardia*, *Corynebacterium*, *Mycobacterium*, *Dietzia*, *Rhodococcus* and *Tsukamurella* (Dörner et al., 2004; Hüntgen et al., 2005a, b; Mafakheri et al., 2014; Niederweis, 2008; Rieß et al., 2003; Singh et al., 2015). Some of these channel-forming proteins are related to the two major families MspA and PorA (Faller et al., 2004). The MspA family is present in the genera *Mycobacterium* (Hillmann et al., 2007), *Rhodococcus* (Somalinga and Mohn, 2013), *Nocardia* (Klückta et al., 2011) and *Tsukamurella* (Dörner et al., 2004). PorA-like proteins have exclusively been found in the genus *Corynebacterium* (Costa-Riu et al., 2003).

Material and Methods

Bacterial strain and growth conditions. *G. jacobaea* MV-1 was grown aerobically in tryptic soy broth (TSB) in a rotary shaker at 200 rpm and 30°C for 72 h. The media were purchased in dehydrated form from Scharlau (Spain).

Isolation of channel-forming proteins from the cell wall. Channel-forming proteins were extracted as described by Lichtinger et al. (1998) with some modifications. Briefly, bacterial cells were harvested by centrifugation at $9,000 \times g$ at 4°C for 10 min and washed once in 10 mM Tris-HCl (pH 8). Then, about 2 g of centrifuged cells were extracted with 12 ml of a 1:2 mixture of chloroform-methanol for 24 h at room temperature whilst gently stirring in a closed container to prevent the evaporation of chloroform. Afterwards, the chloroform : methanol solution was cooled in an ice bath for 1 h, and then the solution was centrifuged at $9,000 \times g$ for 10 min at 4°C to remove any insoluble material and remaining whole cells. The pellet was discarded and the supernatant mixed with ice-cold ether in a proportion of 9 ml of ice-cold ether to 1 ml of supernatants. The mixture was kept at –20°C for 24 h and precipitated proteins were centrifuged at $3,000 \times g$ for 30 min at 4°C. The pellet was then homogenized in a solution containing 1% Genapol X-80 and 10 mM Tris-HCl (pH 8).

Identification and purification of the channel-forming proteins. Preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for identification and purification of proteins with a channel-forming activity present in the organic solvent extracts. This was carried out by using tricine-containing gels for the observation of low molecular mass proteins, followed by staining with Coomassie blue (Schägger, 2006). This

staining method allows the detection of small quantities of proteins (nanograms) and is compatible with mass spectrometry studies and Edman degradation for the identification of proteins. Protein bands were excised from the polyacrylamide gel and extracted with a solution of 1% Genapol in 10 mM Tris-HCl (pH 8) for 24 h at room temperature for further experiments. The 100 KDa protein was studied in detail.

Mass spectrometry analyses were carried out in SePBioEs, the Proteomics facility from UAB. Samples were destained with 50 mM ammonium bicarbonate in 50% acetonitrile (CAN) during three 30 min periods and reduced by 10 mM Dithiothreitol (DTT) for 30 min at room temperature. Alkylation was achieved with 25 mM iodoacetamide during 30 min at room temperature in darkness. Proteins were digested by trypsin (sequencing grade Promega Corporation (Madison, WI, USA) (50 ng/sample)) for 3 h at 37°C and eluted in water: acetonitrile (1:1) in 0.2% Trifluoroacetic acid (TFA).

For MALDI analysis, 1 μ l of sample was mixed with 1 μ l of a solution of α -cyano-4-hydroxy-cinnamic acid matrix and was spotted onto a MALDI target plate (Bruker). MALDI-mass spectra were recorded in the positive ion mode on an UltrafleXtreme time-of-flight mass spectrometer (Bruker) with an acceleration voltage of 25 kV. All mass spectra were externally calibrated using a standard peptide mixture (Bruker).

For PMF (peptide mass fingerprinting) analysis, the MASCOT search engine (Matrix Science, London UK) was used with the following parameters: two missed cleavages, 25 ppm tolerance, cysteine carbamidomethylation and methionine oxidation were set as variable modifications, and searches were performed using the NCBI nr 20160404 database. Positive identifications were accepted with a Mascot score higher than that corresponding to a *P* value of 0.05 (score greater than 90).

For MS/MS analysis, the MASCOT search engine (Matrix Science) was used with the following parameters: searches were restricted to Actinobacteria taxonomy (10714020 sequences), using two missed cleavages, 100 ppm precursor mass tolerance, a fragment tolerance of 0.6 Da and cysteine carbamidomethylation and methionine oxidation were set as variable modifications. Searches were performed by using the NCBI nr_20160404 database. Positive identifications were accepted with a Mascot score higher than that corresponding to a *P* value of 0.05.

Lipid bilayer experiments. The method used for black lipid bilayer experiments was reported by Benz et al. (1978). Membranes were made by painting a 1% (w/v) lipid diphytanoyl phosphatidylcholine solution in *n*-decane (Avanti Polar Lipids) onto a 0.8 mm² hole in a divider separating two compartments of the Teflon chamber containing 5 mL each of a bathing solution of salt. All salts (analytical grade) were from Sigma Aldrich, Spain. Voltages were applied across the formed membrane through Ag/AgCl electrodes connected by a salt bridge, and the resultant current was boosted 10^9 – 10^{10} fold by a current amplifier, and recorded on a Rikadenki strip chart recorder. The temperature was maintained at 20°C throughout.

The selectivity of the protein was explored by experiments at zero-current membrane potentials by establishing a salt gradient across membranes that had previously incorporated around 100 pores.

Sequence analysis of the 100 kDa protein. The eluted protein extracts were analyzed by electrophoresis in polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Laemmli, 1970; Schagger, 2006). The protein band of 100 kDa was excised to analyze the amino terminal acid sequence with Edman degradation. In parallel, the 100 kDa protein was identified by mass spectrometry MS/MS.

Prediction of signal peptide and secondary structure of the obtained sequence. Prediction of signal peptide of the obtained sequence was made by using a SignalP3.0 algorithm (www.cbs.dtu.dk/services/SignalP; (Bendtsen et al., 2004)).

PHOBIUS (www.ebi.ac.uk/Tools/pfa/phobius) and PROTTER (<http://wlab.ethz.ch/protter/start/programs>) were used to predict hydrophobic transmembrane α -helices (Hofmann and Stoffel, 1993; Krogh et al., 2001; Melen et al., 2003; Omasits et al., 2014). The membrane-spanning regions and their orientation were predicted with TMPred (www.ch.embnet.org/software/TMPRED_form.htm). This algorithm is based on the statistical analysis of TMbase—a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring (Hofmann and Stoffel, 1993).

The secondary structure was predicted from the sequence by using JPred algorithm (www.compbio.dundee.ac.uk/jpred4/index.html) (Drozdetskiy et al., 2015) and the GOR method (www.compbio.dundee.ac.uk) (Feng and Doolittle, 1996). JPred algorithm gives the best performance among secondary structure prediction algorithms and achieved a 76.4% average accuracy on a large test set of proteins (Cuff and Barton, 1999, 2000). It predicts α -helix, β -barrel and random structures by comparing the protein sequence problem with secondary structures of proteins previously characterized and registered RCSB-Protein Data Bank (Cole et al., 2008). The GOR method analyzes sequences to predict α -helix, β -sheet, turn, or a random coil secondary structure at each position based on 17-amino-acid sequence windows.

The amphipathic helix prediction was performed using the HELIQUEST program (<http://heliquet.ipmc.cnrs.fr>) (Gautier et al., 2008). A predicted model 3D was obtained by using <https://swissmodel.expasy.org/interactive#sequence>.

Results and Discussion

Isolation and purification of the channel-forming proteins from whole *G. jacobaea* cells

We used the method described by Lichtinger et al. (1998), but the extraction of proteins was performed at room temperature instead of 40°C to prevent alterations in their functionality, and the time of precipitation with ether was increased from 12 to 24 h because it was observed that at 12 h the precipitation was incomplete. When

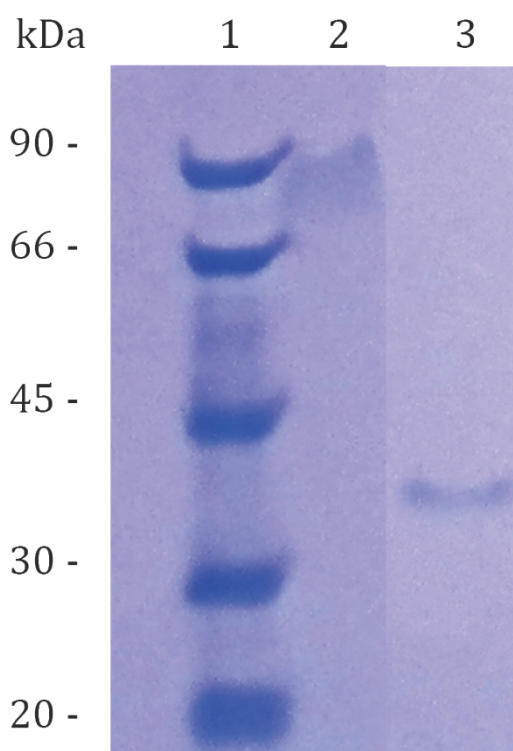


Fig. 1. SDS-Page of boiled GjpA protein (3) with an apparent molecular mass of 40 kDa, and unboiled GjpA protein (2).

the extract of protein was added to lipid bilayer membranes, channels were rapidly reconstituted demonstrating the pore-forming activity of the supernatant.

Non-boiled extracts of cell-wall proteins showed a prominent band of an apparent molecular mass of 100 kDa. To corroborate if the 100 kDa protein consists of more than one monomer, the protein was boiled at 100°C for 10 min, after which SDS-PAGE showed only an apparent 40 kDa band (Fig. 1). SDS-PAGE of the total extracts of *G. jacobaea* revealed numerous bands. Among them, some bands were due to channel forming. The 100 kDa band was studied since its molecular mass was close to that of MspA (the major porin in mycolata, including *Mycobacterium*, *Tsukamurella* and others).

Electrophysiological studies in planar lipid bilayers showed pore-forming activity in gel slices of the 100 kDa protein band. We named such a protein GjpA. Purity was achieved by MALDI spectrometry, and the running of PMF resulted in a match with the expected protein. The score was 87 and, subsequently, MS/MS was performed. Five different peptides were obtained which matched the protein with scores of 93, 99, 103, 134, 214.

Pore formation by GjpA of *G. jacobaea* in lipid bilayer membranes

To explore the pore-forming activity of GjpA in black lipid membranes formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine dissolved in *n*-decane, small amounts of concentrated protein were added to the aqueous phase bathing the artificial membranes. A noticeable increase of the conductance of the lipid bilayers was observed, indicating that this protein had a channel-forming activity. The increase of conductance was immediate

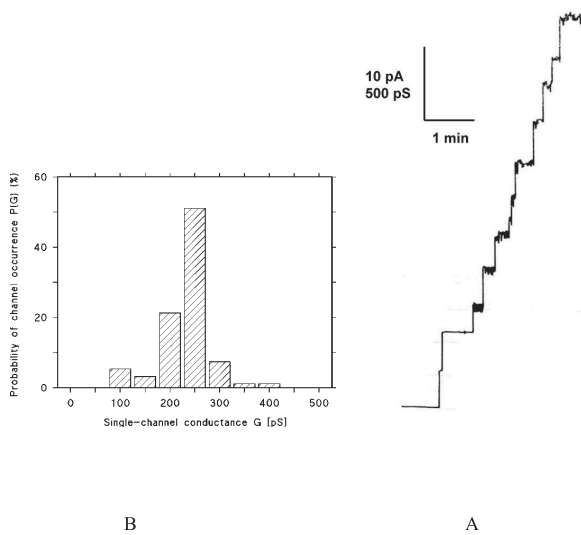


Fig. 2. Electrophysiological measurements.

(A) Single-channel recording of a DiPhPC/*n*-decane membrane in the presence of pure GjpA protein of *G. jacobaea*. The aqueous phase contained 0.1 M KCl, (pH 6.0) and 100 ng/ml GjpA added to the aqueous phase on both sides of the membrane. The applied membrane potential was 20 mV and the temperature was 20°C. (B) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit G in DiPhPC/*n*-decane membranes in the presence of GjpA protein of *G. jacobaea*. $P(G)$ 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 0.1 M KCl, pH 6, and about 100 ng/ml GjpA. The applied membrane potential was 20 mV; $T = 20^\circ\text{C}$. The most frequent single-channel conductance (G in pS) was 250 pS for a total of about 94 single-channel events. A Gaussian function was applied to the histogram and the maximum of the curve was taken as the average single-channel conductance. This was 240 (± 42) pS. The data were collected from different individual membranes.

after the addition of the purified protein to the aqueous phase. Experiments using Genapol alone at the same concentration as with the protein demonstrated that pore-forming activity was only due to the presence of the protein.

Single-channel experiments

After the addition of the protein to the aqueous phase on one or both sides of the bilayers made by diphytanoyl phosphatidylcholine/*n*-decane, the conductance increased in a stepwise manner similar to that observed for cell-wall channels of mycolata (Lichtinger et al., 1998) and for porins from gram-negative bacteria (Benz et al., 1978). Each step indicated the insertion of a channel-forming protein into the artificial planar lipid membrane. Figure 2 shows a single-channel recording of GjpA in 0.1 M KCl. The life-time of the channels was long and all of the conductance fluctuations were directed upward (open state of the channel) and observed over several minutes. Non-terminating events were observed.

The histogram of all conductance events in reconstitution experiments with GjpA in 0.1 M KCl applying a voltage of 20 mV is also shown in Fig. 2. The average single-channel conductance was about 250 pS and only a small number of channels with other conductance values were observed.

Table 1. Average single-channel conductance (G) of GjpA.

Salt	Concentration (M)	G (pS)
KCl	0.03	125 \pm 19
	0.05	125 \pm 22
	0.1	240 \pm 42
	0.3	400 \pm 44
	1	800 \pm 38
3	1400 \pm 89	
LiCl	0.1	250 \pm 19
CH ₃ COOK	0.1	40 \pm 3

Table 2. Selectivity of GjpA channels.

Electrolyte	Permeability ratio ($P_{\text{cations}}/P_{\text{anions}}$)	V_m (mV)
KCl	0.56	-10 \pm 2.3
LiCl	0.15	-25 \pm 1.2
KCH ₃ COO	1.22	+1.1 \pm 2.1

Zero-current membrane potentials V_m of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of GjpA measured for a fivefold gradient of different salts. V_m is defined as the difference between the potential at the dilute side and the potential at the concentrated side. $T = 20^\circ\text{C}$. The permeability ratio $P_{\text{cation}}/P_{\text{anion}}$ was calculated using the Goldman-Hodgkin-Katz equation as the mean of at least three individual experiments.

Single-channel properties of GjpA

The biophysical properties of the channel-forming protein of *G. jacobaea* were explored by single-channel experiments with several concentrations of KCl and salts containing ions other than K⁺ and Cl⁻ (Table 1). At 1 M KCl (pH 7) most of the insertions had an average single-channel conductance of 800 pS. At 0.1 M KCl, most of the insertions had a single-channel conductance of 250 pS, and at 3 M KCl most of the pores had a single-channel conductance of 1400 pS and a few of 2200 pS. The latter conductance seemed to be too low to account for double insertions of the protein; moreover, the solubility and properties of the proteins (and then, pores) at a high ionic strength were shown to be nonlinear and this may explain the higher value.

Similarly to results obtained for many cell-wall channels of gram-positive bacteria (Costa-Riu et al., 2003; Kläckta et al., 2011), a linear relationship between single-channel conductance and KCl concentration was not observed, suggesting that the channels contained point net charges, or could have a specific binding site for ions (cations or anions).

Selectivity measurements of GjpA

Zero-current membrane potential measurements were performed in three independent experiments to study the selectivity of a GjpA channel. After incorporation of a large number of channels (100–1000) in membranes bathed in 0.1 M KCl, 0.1 M LiCl and 0.1 M CH₃COOK, salt gradients were established across the membranes by the addition of small amounts of concentrated KCl, LiCl and CH₃COOK solution to one side of the membrane.

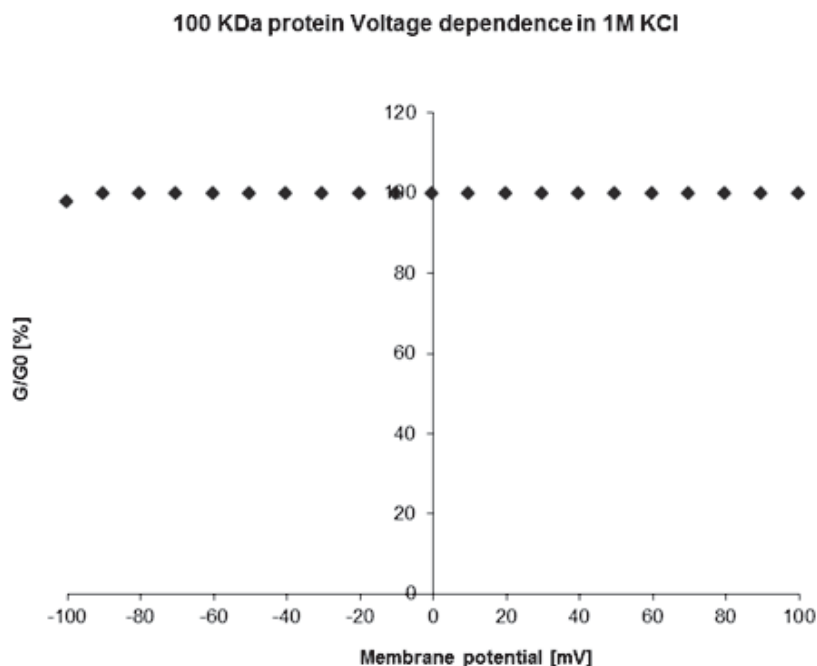


Fig. 3. Ratio of conductance G at a given membrane potential (V_m) divided by the conductance G_0 at 10 mV as a function of V_m .

The membrane potential always refers to the cis side of the membrane. The aqueous phase contained 1 M KCl. The membranes were formed of diphtanoyl phosphatidylcholine/*n*-decane. $T = 20^\circ\text{C}$. The means of values obtained for 3 different membranes are shown.

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1  mqrspialq  gdievpkksp  rawvaagvai  agasvmavap  gvhsevptay  katteatvrl
61  aadwkplepy  idafneaskn  aatlahnyfl  apglplqqii  snqskyln dv  lndpsqaddv
121 lkkmranlea  vitgvtgiga  sdetlaqtkd  htvdglhglv  lqllpqflpe  ggpdpapatit
181 aimnvlasp  sgvliglvgp  vispavavln  sgiaiaaaic  agdpdavfsd  vfsapanvvg
241 avfngatldl  taltpiinet  gllgsttlns  ldiafgglft  pgsvgrgsyd  gpngtsitap
301 ggsilnsgm  nvttdalgfp  ltdipggav  gpigamegls  qtvgvllgdg  wdgkngkpvv
361 plagvefpti  sdkaadnwaa  aigkladtll  gkkdsettps  pvtaktaaav  attsvalnsv
421 esesakaesa  ktevaqtess  stpagsstet  rtssaaeest  sasptaqpss  sqpsssatap
481 assstdapsd  ssdstqgsas  sgsstsnagt  dadttssadt  kgsagagtss  ssdtssksse
541 ssgaasssd  stssastd

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Fig. 4. Amino acid sequence of the hypothetical protein c6/294 of *G. jacobaea*.

Translation of the genomic sequence was performed with the automatic annotation program PGAAP (Angiuoli et al., 2008). Partial sequences obtained by mass spectrometry are given in light grey, and partial sequences obtained by Edman degradation are given in dark grey. Bold underline indicate the sequences corresponding to the amphipathic α -helices predicted.

For KCl, the more diluted side of the membrane became more negative when the salt gradient across the membrane was increased. The zero-current membrane potential for the KCl gradient was about -10 ± 1.4 mV (Table 2). This result could be reasonably well fitted to the Goldman-Hodkin-Katz equation, revealing a ratio of 0.56 for the permeability ratio P_K divided by P_{Cl} , which indicated a preferential movement of chloride through the channel, i.e. it is slightly anion selective.

Similar results were obtained with other salts, such as lithium chloride, where the more diluted side of the membrane became more negative when the salt gradient across the membrane was increased. The gradient resulted in an asymmetry potential of -25 ± 1.2 mV at the more diluted side of the membrane, indicating a higher preference for anions over cations at practically neutral pH. The perme-

ability of GjpA was at least 6.5-times higher for Cl^- than for Li^+ ($P_{Li}/P_{Cl} = 0.15$).

The zero-current membrane potential for the CH_3COOK (pH 7) gradient was initially positive in the more diluted side and became negative with increasing salt gradient. The ratio of permeability P_K/P_{CH_3COO} using the Goldman-Hodgkin-Katz equation was 1.22, indicating that potassium ions could have a certain permeability through GjpA. The Table 1 data shows that the conductance sequence of the different salts within the channel was $KCl \sim LiCl > CH_3COOK$, indicating again that the channel formed by GjpA from the cell wall of *G. jacobaea* is moderately anion selective. This can be derived from the experiments where KCl was replaced by LiCl or CH_3COOK , i.e. the mobile ions K^+ and Cl^- were replaced by the less mobile ions Li^+ and CH_3COO^- .

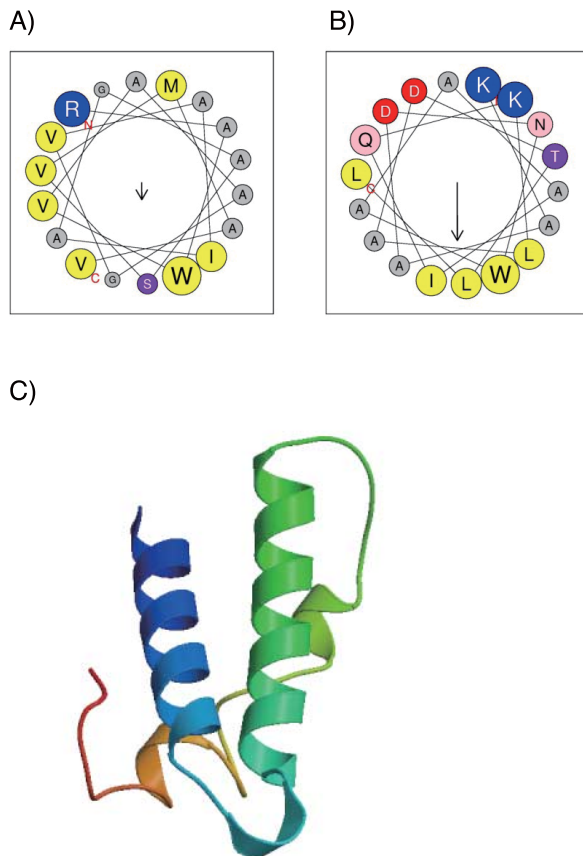


Fig. 5. Amphipathic α -helices of 18 amino acids predicted with the HELIQUEST program.

Letters indicate the amino acid and colours their characteristics: red = negatively charged; blue = positively charged; purple = polar; grey = glycine/alanine and yellow = hydrophobic residues A) α -helices at positions 20–38 of the sequence c6/294. B) α -helices at positions 372–390 of the sequence 6/294.

Up to now, most of the porins described in the actinomycetes family are selective for cations. Anion selective channel-forming proteins have only been found in *C. glutamicum*, *C. efficiens*, *M. bovis* and *R. equi*. (Hüntner et al., 2005a, b; Lichtinger et al., 1998, 2000; Rieß et al., 2003).

The channel formed by the GjpA protein is not voltage gated

Our channel-forming protein GjpA did not show transitions between open and closed configurations (no flickering) at higher voltages when it was added to one or both sides of black diphytanoyl phosphatidylcholine/*n*-decane membranes. This result indicated that the single-channel conductance of GjpA is independent of the applied voltage within a range of +100 to –100 mV (Fig. 3) and shows a symmetric response in these voltage ranges.

Partial sequencing of GjpA protein and identification of the gene

The predominant protein band with an apparent molecular mass of 100 kDa (GjpA) was excised to determine the N-terminal amino-acid sequence with automated Edman degradation. A sequence of 8 amino acids (AD-KPLE-YI)

was obtained.

In parallel, the GjpA protein excised from the gel was identified by mass spectrometry MS/MS. Twelve peptides were identified, five with scores greater than 90. The sequences were YLNDVLNDPSQADDVLK, AADNWAAAIQK, YLNDVLNDPSQADDVLKK, YLNDVLNDPSQADDVLKK and ANLEAVITGVTGIGASDETLAQTK with scores of 93, 99, 103, 134 and 214, respectively. After protein identification, similarities were found with a putative protein from *G. jacobaea* (NCBI gi1902981106).

All the sequences obtained by Edman degradation and mass spectrometry were searched in the complete genome sequence of *G. jacobaea* (Angiuoli et al., 2008; Jiménez-Galisteo et al., 2015).

All the sequences are part of a 558-acid-long hypothetical protein (NCBI Reference Sequence: WP_049699721.1) (Fig. 4). This hypothetical protein was called c6/294, because it was located in the contig 6, position 294 of the complete sequenced genome of *G. jacobaea* (Fig. 4).

The estimated molecular weight of the hypothetical protein c6/294 was 55 kDa. This result suggests that this could correspond to a GjpA monomer. It is well known that apparent molecular mass in SDS-PAGE strongly depends upon structure and that, fully denatured, run faster. This could be the explanation for the differences in the GJA Mw in gels and that determined based on amino acid sequences.

By using Pfam for the amino-acid-sequence alignment of the protein, c6/294 revealed low sequence similarities to other proteins from the genus *Gordonia* of uncharacterized functions.

Analysis of the Hypothetical Protein c6/294

Signal peptide prediction

Most of the bacterial outer membrane proteins (OMPs) have a canonical N-terminal signal sequence (hydrophobic α -helix signal, peptidase cleave site or amino acids with positive charges), which targets proteins to the Sec system for translocation across the inner membrane (Costa-Riu et al., 2003; Freudl, 1992). Proteins that have at least one hydrophobic α -helix after signal peptide cleavage are considered to be inner membrane proteins, because the hydrophobic α -helix acts as a stop-transfer sequence and anchor the protein in the membrane (Pugsley, 1993). In our work, we were not able to identify any signal peptide in the c6/294 protein by using the SignalP algorithm. Other porin-like proteins lacking signal peptides have been described in the gram-positive bacterial group mycolata, such as the small pore-forming peptides PorA and PorH of *C. glutamicum*, which means that these peptides are exported to the cell wall by a yet unknown mechanism (Hüntner et al., 2005a, b; Lichtinger et al., 2000). Similarly, the pore-forming protein OmpAtb from *M. tuberculosis* does not show a signal peptide, probably because its N-terminus does not adequately fit the definition of classical signal peptides (Alahari et al., 2007; Senaratne et al., 1998).

Trans-membrane region prediction

A 21-amino-acid-long transmembrane α -helix domain

oriented from inside to outside the bacterial membrane was predicted by TMpred (total score of 1960). This result was also confirmed by a PHOBIUS algorithm and PROTTTER, predicting a transmembrane region spanning from amino acid 21–42.

Secondary structure predictions

All known integral OMPs of gram-negative bacteria, and, also, the major porin of mycobacteria, the MspA, have a β -barrel structure with a hydrophobic surface (Koebnik et al., 2000; Mahfoud et al., 2006; Schulz, 2002; Wimley, 2003). The presence of this structure in both gram-negative (Bagos et al., 2004, 2005; Zhai and Saier, 2002) and gram-positive (Heinz et al., 2003) bacteria can be predicted by several types of algorithms. In our sequence c6/294, JPred algorithm and the GOR program predicted the presence of several α -helical regions located in the amino-terminal region and a low proportion of β -sheet, mainly located in the intermediate region.

Most bacterial porins, including MspA from *M. smegmatis*, have antiparallel β -sheet structures conferring stability inside the bacterial wall (Fairman et al., 2011; Heinz et al., 2003). However, there are some microbial pore-forming proteins where α -helix structures are relevant for their stability, such as PorH from *Corynebacterium glutamicum*, *C. efficiens*, *C. callunae* and PorACj from *C. jeikeium* (Abdali et al., 2013; Hüntten et al., 2005a, b). Other proteins, like PorA from *M. tuberculosis*, have an N-terminal domain with both α -helix and β -sheet secondary structures (Teriete et al., 2010; Yang et al., 2011).

The analysis of the obtained α -helix predicted two 18-amino-acid amphipathic α -helices (positions 20–38 and 372–390 of the amino-terminal) (Fig. 5) having one side highly hydrophobic and another side hydrophilic and charged. This organization is presumably sufficient for the stabilization and anchorage in the mycolic acid layer as has been observed in PorH from *C. efficiens*, PorA from *C. callunae* and PorACj from *C. jeikeium* (Abdali et al., 2013; Hüntten et al., 2005a, b).

Our results suggest that the insertion of the protein GjpA to the *Gordonia*'s wall and its ability to form stable pores is due to the presence of the α -helix structures identified in c6/294.

Conclusion

Knowledge concerning the penetration of solutes through the wall of mycolata can contribute to design treatments for mycobacterial infections. Our results suggest that the insertion of the protein GjpA to the *Gordonia*'s wall and its ability to form stable pores is due to the presence of the α -helix structures identified in c6/294. Mycobacteria are intrinsically resistant to many antimicrobials due to their low cell-wall permeability that prevents the entry of agents. Also, members of the mycolata can produce metabolites having a high commercial value. Here, we describe for the first time the presence of a cell-wall protein from *Gordonia jacobaea* with a channel-forming activity. This protein shares some similarity to other hypothetical proteins from the genus *Gordonia* of yet uncharacterized functions. Channel characteristics (800 pS

in 1 M KCl, moderately anion-selective, and voltage-independent) suggest that this protein could be of relevance in the import and export of negatively charged molecules across the cell wall.

Declarations

As corresponding author, MV declares that no ethics approval and consent to participate is applicable to this article.

All authors agree and have specifically given their consent for publication.

All material is at the entire disposal of the scientific community. All relevant data are included in the main paper.

Competing interests: The authors declare that they have no competing interests.

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Authors' contribution: All authors have significantly contributed to the paper. GJG, EF, EM and TV performed experimental laboratory work. RB supervised the single-channel recording and did the interpretation of the electrophysiological data. GJG and AD did the proteomics. TV significantly contributed to the interpretation of the data. MV and RB wrote the paper.

Availability of data and materials: Genome sequence of *G. jacobaea* is in the gene bank (LDTZ00000000). Results of the amino-acid sequences and identification are in this paper.

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References

- Abdali, N., Barth, E., Norouzy, A., Schulz, R., Nau, W. M. et al. (2013) *Corynebacterium jeikeium* jk0268 constitutes for the 40 amino acid long PorACj, which forms a homooligomeric and anion-selective cell wall channel. *PLoS One*, **8**, 5651–5668.
- Alahari, A., Saint, N., Campagna, S., Molle, V., Molle, G. et al. (2007) The N-terminal domain of OmpATb is required for membrane translocation and pore-forming activity in mycobacteria. *J. Bacteriol.*, **189**, 6351–6358.
- Angiuoli, S. V., Gussman, A., Klimke, W., Cochrane, G., Field, D. et al. (2008) Toward an online repository of Standard Operating Procedures (SOPs) for (meta)genomic annotation. *OMICS*, **12**, 137–141.
- Bagos, P. G., Liakopoulos, T. D., Spyropoulos, I. C., and Hamodrakas, S. J. (2004) A Hidden Markov Model method, capable of predicting and discriminating beta-barrel outer membrane proteins. *BMC Bioinformatics*, **5**, 29.
- Bagos, P. G., Liakopoulos, T. D., and Hamodrakas, S. J. (2005) Evaluation of methods for predicting the topology of beta-barrel outer membrane proteins and a consensus prediction method. *BMC Bioinformatics*, **6**, 7.
- Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.*, **340**, 783–795.
- Benz, R. (1994) *In Bacterial Cell Wall*, ed. by Hackenbeck, R. and Ghuysen, J. M., Elsevier, Amsterdam, pp. 397–423.
- Benz, R., Janko, K., Boos, W., and Lauger, P. (1978) Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim. Biophys. Acta*, **511**, 305–319.
- Cole, C., Barber, J. D., and Barton, G. J. (2008) The JPred 3 secondary structure prediction server. *Nucl. Acids Res.*, **36**, 197–201 (web server issue).
- Costa-Riu, N., Burkovski, A., Krämer, R., and Benz, R. (2003) PorA represents the major cell wall channel of the Gram-positive bacterium *Corynebacterium glutamicum*. *J. Bacteriol.*, **185**, 4779–4786.
- Cuff, J. A. and Barton, G. J. (1999) Evaluation and improvement of multiple sequence methods for protein secondary structure prediction. *Proteins*, **34**, 508–519.
- Cuff, J. A. and Barton, G. J. (2000) Application of multiple sequence alignment profiles to improve protein secondary structure predic-

- tion. *Proteins*, **40**, 502–511.
- Daffé, M., Lanéelle, M. A., Asselineau, C., Lévy-Frébault, V., and David, H. (1983) Taxonomic value of mycobacterial fatty acids: proposal for a method of analysis. *Ann. Inst. Pasteur Microbiol.*, **134B**, 241–256.
- De Miguel, T., Sieiro, C., Poza, M., and Villa, T. G. (2000) Isolation and taxonomic study of a new canthaxanthin-containing bacterium, *Gordonia jacobaea* MV-1 sp. nov. *Int. Microbiol.*, **3**, 107–111.
- Dörner, U., Maier, E., and Benz, R. (2004) Identification of a cation-specific channel (TipA) in the cell wall of the gram-positive mycolata *Tsakumurella inchonensis*: the gene of the channel-forming protein is identical to mspA of *Mycobacterium smegmatis* and mppA of *Mycobacterium phlei*. *Biochim. Biophys. Acta*, **1667**, 47–55.
- Drozdetskiy, A., Cole, C., Procter, J., and Barton, G. J. (2015) JPred4: a protein secondary structure prediction server. *Nucl. Acids Res.*, **43**, 389–394.
- Fairman, J. W., Noinaj, N., and Buchanan, S. K. (2011) The structural biology of β -barrel membrane proteins: a summary of recent reports. *Curr. Opin. Struct. Biol.*, **21**, 523–531.
- Faller, M., Niederweis, M., and Schulz, G. E. (2004) The structure of a mycobacterial outer-membrane channel. *Science*, **303**, 1189–1192.
- Feng, D. F. and Doolittle, R. F. (1996) Progressive alignment of amino acid sequences and construction of phylogenetic trees from them. *Methods Enzymol.*, **266**, 368–382.
- Freudl, R. (1992) Protein secretion in gram-positive bacteria. *J. Biotechnol.*, **23**, 231–240.
- Gautier, R., Douguet, D., Antonny, B., and Drin, G. (2008) HELIQUEST: a web server to screen sequences with specific α -helical properties. *Bioinformatics*, **24**, 2101–2102.
- Heinz, C., Engelhardt, H., and Niederweis, M. (2003) The core of the tetrameric mycobacterial porin MspA is an extremely stable beta-sheet domain. *J. Biol. Chem.*, **278**, 8678–8685.
- Hillmann, D., Eschenbacher, I., Thiel, A., and Niederweis, M. (2007) Expression of the major porin gene mspA is regulated in *Mycobacterium smegmatis*. *J. Bacteriol.*, **189**, 958–967.
- Hofmann, K. and Stoffel, W. (1993) TMBASE—A database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler.*, **374**, 166.
- Hünten, P., Costa-Riu, N., Palm, D., Lottspeich, F., and Benz, R. (2005a) Identification and characterization of PorH, a new cell wall channel of *Corynebacterium glutamicum*. *Biochim. Biophys. Acta*, **1715**, 25–36.
- Hünten, P., Schiffler, B., Lottspeich, F., and Benz, R. (2005b) PorH, a new channel-forming protein present in the cell wall of *Corynebacterium efficiens* and *Corynebacterium callunae*. *Microbiology*, **151**, 2429–2438.
- Jiménez-Galisteo, G., Villa, T. G., Vinuesa, T., Viñas, M., Domínguez, A. et al. (2015) Draft Genome sequence of the bacterium *Gordonia jacobaea*, a new member of the *Gordonia* genus. *Genome Announc.*, **3**, e00995-15.
- Klückta, C., Knörzer, P., Riess, F., and Benz, R. (2011) Hetero-oligomeric cell wall channels (porins) of *Nocardia farcinica*. *Biochim. Biophys. Acta*, **1808**, 1601–1610.
- Koebnik, R., Locher, K. P., and van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol. Microbiol.*, **37**, 239–253.
- Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.*, **305**, 567–580.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lichtinger, T., Burkovski, A., Niederweis, M., Krämer, R., and Benz, R. (1998) Biochemical and biophysical characterization of the cell wall porin of *Corynebacterium glutamicum*: the channel is formed by a low molecular mass polypeptide. *Biochemistry*, **37**, 15024–15032.
- Lichtinger, T., Reiss, G., and Benz, R. (2000) Biochemical identification and biophysical characterization of a channel-forming protein from *Rhodococcus erythropolis*. *J. Bacteriol.*, **182**, 764–770.
- Mafakheri, S., Bárcena-Uribarri, I., Abdali, N., Jones, A. L., Sutcliffe, I. C. et al. (2014) Discovery of a cell wall porin in the mycolic-acid-containing actinomycete *Dietzia maris* DSM 43672. *FEBS J.*, **281**, 2030–2041.
- Mahfoud, M., Sukumaran, S., Hülsmann, P., Grieger, K., and Niederweis, M. (2006) Topology of the porin MspA in the outer membrane of *Mycobacterium smegmatis*. *J. Biol. Chem.*, **281**, 5908–5915.
- Marrakchi, H., Lanéelle, M. A., and Daffé, M. (2014) Mycolic acids: structures, biosynthesis, and beyond. *Chem. Biol.*, **21**, 67–85.
- Melen, K., Krogh, A., and von Heijne, G. (2003) Reliability measures for membrane protein topology prediction algorithms. *J. Mol. Biol.*, **327**, 735–744.
- Niederweis, M. (2008). Nutrient acquisition by mycobacteria. *Microbiology*, **154**, 679–692.
- Omasits, U., Ahrens, C. H., Müller, S., and Wollscheid, B. (2014) Protter: interactive protein feature visualization and integration with experimental proteomic data. *Bioinformatics*, **30**, 884–886.
- Pugsley, A. P. (1993) The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.*, **57**, 50–108.
- Rangaka, M. X., Cavalcante, S. C., Marais, B. J., Thim, S., Martinson, N. A. et al. (2015) Controlling the seedbeds of tuberculosis: diagnosis and treatment of tuberculosis infection. *Lancet*, **386**, 2344–2353.
- Rieß, F. G., Elflein, M., Benk, M., Schiffler, B., Benz, R. et al. (2003) The cell wall of the pathogenic bacterium *Rhodococcus equi* contains two channel-forming proteins with different properties. *J. Bacteriol.*, **185**, 2952–2960.
- Schägger, H. (2006). Tricine-SDS-PAGE. *Nature Protocols*, **1**, 16–22.
- Schulz, G. E. (2002) The structure of bacterial outer membrane proteins. *Biochim. Biophys. Acta*, **1565**, 308–317.
- Senaratne, R. H., Mobasher, H., Papavinasundaram, K. G., Jenner, P., Lea, E. J. et al. (1998) Expression of a gene for a porin-like protein of the OmpA family from *Mycobacterium tuberculosis* H37Rv. *J. Bacteriol.*, **180**, 3541–3547.
- Singh, P. R., Bajaj, H., Benz, R., Winterhalter, M., and Mahendran, K. R. (2015) Transport across the outer membrane porin of mycolic acid containing actinomycetales: *Nocardia farcinica*. *Biochim. Biophys. Acta*, **1848**, 654–661.
- Somalinga, V. and Mohn, W. W. (2013) *Rhodococcus jostii* porin A (RjpA) functions in cholate uptake. *Appl. Environ. Microbiol.*, **79**, 6191–6193.
- Teriete, P., Yao, Y., Kolodzik, A., Yu, J., Song, H. et al. (2010) *Mycobacterium tuberculosis* Rv0899 adopts a mixed alpha/beta-structure and does not form a transmembrane beta-barrel. *Biochemistry*, **49**, 2768–2777.
- Trias, J., Jarlier, V., and Benz, R. (1992) Porins in the cell wall of mycobacteria. *Science*, **258**, 1479–1481.
- Wimley, W. C. (2003) The versatile beta-barrel membrane protein. *Curr. Opin. Struct. Biol.*, **13**, 404–411.
- Yang, Y., Auguin, D., Delbecq, S., Dumas, E., Molle, G. et al. (2011) Structure of the *Mycobacterium tuberculosis* OmpATb protein: a model of an oligomeric channel in the mycobacterial cell wall. *Proteins*, **79**, 645–661.
- Zhai, Y. and Saier, M. H., Jr. (2002) The beta-barrel finder (BBF) program, allowing identification of outer membrane beta-barrel proteins encoded within prokaryotic genomes. *Protein Sci.*, **11**, 2196–2207.