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Identification of Channel-Forming Activity in the Cell Wall of *Corynebacterium glutamicum*

MICHAEL NIEDERWEIS,[1] ELKE MAIER,[1] THOMAS LICHTINGER,[1] ROLAND BENZ,[1]* AND REINHARD KRÄMER[2]

Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, D-97074 Würzburg,[1] and Institut für Biotechnologie I, Forschungszentrum Jülich, D-52425 Jülich,[2] Germany

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The cell wall of the gram-positive *Corynebacterium glutamicum* was prepared. It contained an ion-permeable channel with a single-channel conductance of about 6 nS in 1 M KCl. The mobility sequence of the ions in the channel is similar to that in the aqueous phase, suggesting that it is a water-filled channel wide enough to allow unhindered diffusion of ions. The results indicate that we have identified the hydrophilic pathway through the mycolic acid layer of *C. glutamicum*.

Cell wall-containing eubacteria have traditionally been divided into two main groups, gram-negative and -positive bacteria, on the basis of the structure of the cell wall. The first group has a cell wall with a thin peptidoglycan layer and an asymmetric outer membrane. Small hydrophilic compounds can diffuse through the outer membrane by the porin pathway (2, 4, 13). Gram-positive bacteria have a thick peptidoglycan layer which is rather porous and does not form a permeability barrier on the surface. Therefore, it has been widely accepted that gram-positive bacteria do not possess pore-forming proteins. In contrast to this belief, channels in the cell walls of *Mycobacterium chelonae* (16, 18) and *Mycobacterium smegmatis* (17) have recently been identified. Mycobacteria are special because they contain, in addition to the thick peptidoglycan layer, a large amount of lipids in the form of mycolic acids in the cell wall. The permeability of the cell wall of mycobacteria is unusually low (10), presumably because the mycolic acids are part of a second bilayer surrounding the peptidoglycan (12).

Corynebacteria belong to the same group of bacteria, namely, the actinomycetes. Like mycobacteria, they have a thick peptidoglycan layer covalently bound to arabinogalactan and they possess mycolic acids linked to the polysaccharides (1, 8). Little is known about the assembly of the cell wall of *Corynebacterium glutamicum*. Thus, it is not clear whether it acts in the same way as a permeability barrier in mycobacteria (12).

It is interesting to note that coryneform bacteria are widely used in biotechnology for the production of amino acids. Methods to improve the excretion of particular amino acids have been developed, which may influence the integrity of the bacterial cell wall and/or membrane, such as treatment with penicillin or with amine surfactants. Thus, in addition to the presence of specific excretion carriers (11), the permeability properties of the cell wall may be important for the particular properties of these bacteria.

C. glutamicum MH 20-22B, which is a leucine auxotroph derivative of the wild-type strain ATCC 13032 (15) was grown in continuous fermentation at 30°C and pH 7.0 at a dilution rate of 0.05 h^{-1} in a medium containing the following additions (in grams per liter): ammonium sulfate (38.9), urea (2.0), corn protein hydrolysate (83.5, which corresponds to 1.2 g of leucine per liter), K_2HPO_4 (0.5), KH_2PO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.285), KCl (1.0), $CaCl_2 \cdot 2H_2O$ (0.05), citric acid (0.1), EDTA (0.1), glucose (125), and biotin (0.00085). The following trace elements (in micrograms per liter) were added: $FeSO_4 \cdot 7H_2O$ (28.5), $MnSO_4 \cdot H_2O$ (16.5), $CuSO_4 \cdot 5H_2O$ (0.76), $ZnSO_4 \cdot 7H_2O$ (6.3), $CoCl_2 \cdot 6H_2O$ (0.13), $NiCl_2 \cdot 6H_2O$ (0.043), $Na_2MoO_4 \cdot 2H_2O$ (0.065), $KAl(SO_4)_2 \cdot 12H_2O$ (0.028), $Na_2SeO_3 \cdot 5H_2O$ (0.019), H_3BO_3 (0.05), $SrCl_2 \cdot 6H_2O$ (0.05), and $BaCl_2 \cdot 2H_2O$ (0.05). The cells were harvested by centrifugation, washed once in 10 mM Tris HCl (pH 8), and passed three times through a French pressure cell at 900 lb/in^2. Unbroken cells were removed by centrifugation at 5,000 × *g* for 15 min. The cell envelopes (cytoplasmic membrane and cell wall) were obtained by centrifugation of the supernatant at 170,000 × *g* for 90 min (rotor 70.1 Ti). The pellet containing the cell envelope was resuspended in 2 ml of 10 mM Tris HCl (pH 8) and applied to a sucrose-step gradient of 30 (3 ml), 40 (4 ml), and 70% (3 ml) sucrose similar to that used previously to separate the cytoplasmic membrane and cell wall of *M. chelonae* (9, 16, 18). The gradient was centrifuged at 170,000 × *g* for 16 h in a Beckman Optima 90 XL ultracentrifuge (rotor SW40Ti). Eight fractions of the gradient were collected (Fig. 1) and analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, for the presence of cytoplasmic membrane by NADH oxidase activity (14), and for pore-forming activity by reconstitution experiments in lipid bilayers (5). Fraction F3 was light brown and accounted for about 40% of the NADH oxidase activity, indicating a major fraction of the cytoplasmic membrane.

The highest pore-forming activity was measured for fraction F7. This fraction was essentially free of cytoplasmic membrane, as assessed by NADH oxidase activity (Fig. 1). Fraction F2 also showed significant but less pore-forming activity than that of F7. Channels could also be detected in fraction F3, whereas fractions F1, F4, and F5 exhibited no pore-forming activity. Minor channel-forming activity in fractions F6 and F8 was presumably due to contamination from the high-activity fraction F7. All single-channel recordings were similar to that shown in Fig. 2.

For further characterization of the channel, 1 volume of fraction F7 was diluted in 10 volumes of 1% Genapol–10 mM Tris HCl (pH 8). Small amounts of the protein solution (about 1 ng/ml) were added to the aqueous phase on one or both sides of the membrane. After a delay of 1 to 2 min, which was probably caused by slow aqueous diffusion of the protein, the

* Corresponding author. Mailing address: Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany. Phone: 49-(0)931-888-4501. Fax: 49-(0)931-888-4509.

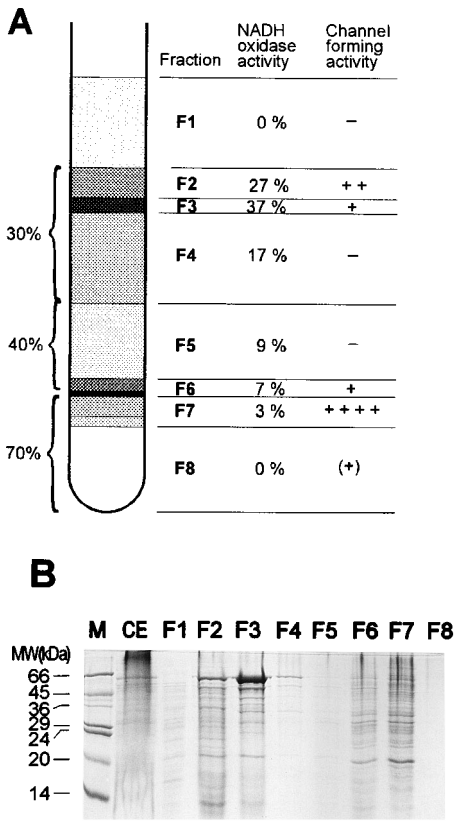

FIG. 1. (A) Fractions formed in a sucrose-step gradient of the cell envelope from *C. glutamicum*. The sucrose concentration before centrifugation is indicated to the left of the tube. The different zones formed in the tube after centrifugation could be clearly distinguished by their colors. The sizes of the zones correspond to their actual sizes in the centrifugation tube. Eight fractions were collected as marked to the right of the tube. NADH oxidase activity was measured by detecting the decrease of A_{340} (14). The reaction followed a first-order kinetic. The specific activity was calculated from the rate constants by normalization to the protein concentration and is indicated on the right side of the figure. Channel-forming activity was estimated from bilayer reconstitution experiments by using the same volume of each fraction. Symbols: –, no activity; (+), minor activity; +, mediate activity; ++, high activity; ++++, very high activity. (B) Protein composition of the fractions of the sucrose-step gradient. The lanes are denoted by the fraction number. Lane MW contains the molecular mass standards, and lane CE contains all proteins of the cell envelope. The proteins were separated on a denaturing 12% polyacrylamide gel and stained with Coomassie blue.

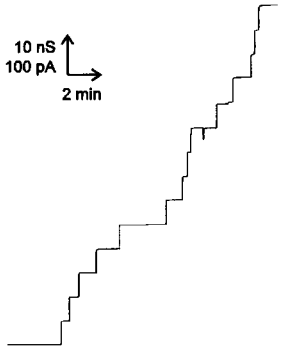

FIG. 2. Single-channel recording of fraction F7 of the sucrose-step gradient containing the cell wall from *C. glutamicum*. Channel-forming activity was measured with black lipid bilayer membranes from a solution of 1% diphytanoyl phosphatidylcholine in *n*-decane (Avanti Polar Lipids, Alabster, Ala.) with a surface area of about 0.4 mm^2 between two aqueous compartments (5) containing 1 M KCl and protein (about 1 ng/ml) of fraction F7 from the sucrose-step gradient. The applied membrane potential was 10 mV, and the temperature was 20°C. The current through the membrane was measured with a pair of calomel electrodes switched in series with a current amplifier (Keithley 427). The amplified voltage output signal was monitored with a storage oscilloscope and recorded with a strip-chart recorder.

current increased in a stepwise fashion similar to that observed for gram-negative bacterial porins (7). Figure 2 demonstrates that the channels had a lifetime of at least several minutes. The average single-channel conductance was about 6 nS in 1 M KCl. The histogram of the single-channel distribution shows that we also observed a small number of channels with an average conductance of 3.4 nS (Fig. 3), which did not show up in the recordings of Fig. 2. So far it is not clear whether this smaller channel results from a protein composed of about half the number of monomers, a different configuration of the same channel-forming protein, or another channel from the cell wall of *C. glutamicum.*

The channel from the cell wall of *C. glutamicum* is moderately cation selective. This can be derived from single-channel experiments, in which KCl was replaced by LiCl or potassium acetate, i.e., the mobile ions K^+ and Cl^- were replaced by the less-mobile ions Li^+ and acetyl (minus charge). The single-channel conductances in 1 M LiCl and 1 M potassium acetate were about 3.5 and 4.6 nS, respectively, compared with 6 nS in 1 M KCl, which indicates that anions have a smaller influence on the movement of the ions through the channel. This result is in agreement with zero-current membrane potential measurements in the presence of salt gradients. After incorporation of a large number of channels in membranes bathed in 50 mM KCl, 10-fold salt gradients were established across the membranes by the addition of small amounts of concentrated KCl solution to one side of the membrane. In all cases, the more-diluted side of the membrane became positive, which indicates preferential movement of cations through the channel. The zero-current membrane potential for a 10-fold KCl gradient was about 40 mV. Analysis of the zero-current membrane potentials with the Goldman-Hodgkin-Katz equation (6) revealed a ratio of the permeabilities of K^+ and Cl^- between 9 and 11, which means that anions also have a certain perme-

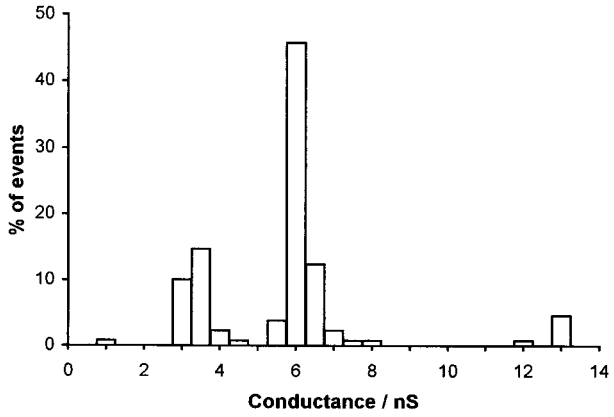

FIG. 3. Histogram of the probability of the occurrence of conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine–*n*-decane in the presence of 1 ng of fraction F7 per ml from the sucrose-step gradient centrifugation of the cell envelope of *C. glutamicum*. The aqueous phase contained 1 M KCl. The applied membrane potential was 10 mV, and the temperature was 20°C. The average single-channel conductance was 6 nS for 85 single-channel events (right-hand maximum) and 3.4 nS for 36 events (left-hand maximum). The data were collected from nine different membranes.

ability. This result is in agreement with the assumption that the channel from the cell wall of *C. glutamicum* is an aqueous channel with little or no interactions between the channel wall and ions.

It should be noted that several procedures widely used in biotechnology to improve amino acid excretion by *C. glutamicum* are likely to affect the state of the corynebacterial cell wall (11). It thus remains to be elucidated whether the ability of coryneform bacteria to excrete amino acids effectively under certain conditions is related to the channel-forming activity identified in this study or to other properties of the cell wall.

The channel-forming activity of fraction F7 with respect to the protein concentration was rather high, similar to that of gram-negative bacterial porins. This rules out the possibility that the observed channel formation results from a contaminant protein. Furthermore, it is clear that these channels can be present only in the cell wall of *C. glutamicum* and not in the cytoplasmic membrane. Otherwise the presence of these high-conducting channels would result in cell death. It is noteworthy that their single-channel conductance was considerably larger than those of most gram-negative bacterial porins under otherwise identical conditions (4). This means probably that it is also larger. The diameter of the channel in the cell wall of *C. glutamicum* may be even larger than 2 nm when its conductance is compared with those of mitochondrial porins (3).

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