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9. Full-length CFTR cDNA (derived from plasmid pBQ4.7) was cloned into plasmid SP64T to generate pSP CFTR. To maximize expression efficiency, the CFTR 5′ untranslated region was removed by digestion of pSP CFTR with Ava I and Nhe I. Into this vector was ligated an Ava I–Nhe I–digested PCR fragment prepared with oligonucleotides (sense: AGGATCTGGCTAGCGATGACC; antisense: TGTCCTCGGGGATCTGCCAAAGTTGAGCGTTTATT) that converted the Bgl II site of the *Xenopus* globin 5′ untranslated region to an Ava I site. The resulting plasmid, pSP CFTR^{HP}, contained the *Xenopus* globin 5′ untranslated region located 10 bp upstream of the CFTR ATG initiation codon, which enhanced CFTR translation efficiency. MDR1 cDNA (plasmid 2000XS) was digested with Bst UI and Eco RI. A 1200-bp fragment was ligated into SP64T following sequential digestion with Bgl II, Klenow, and Eco RI to generate plasmid MDRA2. A 3060-bp Eco RI fragment from plasmid 2000XS was then ligated into MDRA2 to reconstruct the full-length coding region of MDR1. The mRNA encoding CFTR or MDR1 was generated with 4 μg of linearized plasmid per 10 μl of buffer containing 5 mM magnesium acetate, 0.5 mM diguanosine triphosphate, 10 mM dithiothreitol, calf liver tRNA (0.2 mg/ml), ribonuclease inhibitor (0.8 U/μl), SP6 polymerase (0.4 U/μl), and 40 mM tris (pH 7.5). After transcription for 1 hour at 40°C, the mixture was stored at −70°C. Mock-transcription mixture was prepared in the same manner but lacked plasmid DNA.
10. Electrophysiological measurements were made with a Dagen Instruments TEV-200 two-electrode voltage clamp amplifier. De-folliculated oocytes were prepared as for water permeability measurements and impaled with glass microelectrodes filled with 3 M KCl and having resistances of 0.5 to 5 megaohms. Oocytes were superfused continuously with Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and penicillin (10 μg/ml) and streptomycin) at room temperature. The cAMP-activating mixture contained 50 μM forskolin, 0.5 mM 8-chlorophenylthio-cAMP (CPT-cAMP), and 0.5 mM isobutylmethylxanthine (IBMX). In anion substitution experiments in which solution Cl^- was decreased to 5 mM (replaced by gluconate), the reversal potential moved in the positive direction by 40 to 60 mV.
11. NPPB was synthesized by a modification of the published procedure [Wangemann *et al.*, *Pfluegers Arch.* **407**, 128 (1986)]. 2-Chloro-5-nitrobenzoic acid was reacted with excess 3-phenylpropyl-1-amine at 100°C for 2 hours, and the product was purified.
12. Stage V and VI oocytes were microinjected with 50-nl mock transcription mixture or in vitro transcribed mRNA encoding CFTR or MDR1 (~0.2 μg/μl). Oocytes were incubated at 18°C for 48 hours in Barth's buffer with a buffer change at 24 hours. Oocytes were then de-folliculated with collagenase (2 mg/ml, 2 hours, 20°C) and incubated for 3 to 6 hours before measurements were made. P_f was measured from the time course of oocyte swelling in response to a threefold dilution of Barth's buffer with distilled water. Oocyte volume was estimated from cross-sectional area measured by transmission light microscopy and image analysis. P_f was calculated from oocyte surface to volume ratio (S/V_o, 50 cm^{-1}), the initial rate of oocyte swelling [$d(V/V_o)/dt$], and the osmotic gradient according to the equation, $P_f = [d(V/V_o)/dt]/[S/V_o)V_w\Delta Osm]$, where V_w = 18 cm^3/mol (*7*).
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14. Oocyte Cl^- activity was estimated by incubation of de-folliculated oocytes for 24 hours in Barth's buffer containing $Na^{36}Cl$ (1 μCi/ml) at 18°C. Oocytes were washed in ice-cold buffer and oocyte-associated radioactivity was determined; Cl^- activity was calculated from the specific activity of extracellular Cl^- and oocyte aqueous volume.
15. Oocyte plasma membrane–associated fluorescence of trimethylammonium-diphenylhexatriene (TMA-DPH) was used as a measure of plasma membrane surface area [D. Illinger *et al.*, *Cell Biophys.* **14**, 17 (1989)]. De-folliculated oocytes (mock-injected or CFTR-expressing) were incubated for 5 min with (*S*)p-cAMPS (0 or 0.1 mM) in the presence of TMA-DPH (1 μM). Membrane-associated fluorescence was quantified with a Leitz epifluorescence microscope with ultraviolet filter set and Photometrics cooled CCD camera detector.
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18. We measured uptake of [^{14}C]urea, [^{3}H]*O*-methylglucose, and [^{14}C]sucrose by incubating oocytes with each isotope (2 μCi/ml) in Barth's buffer for 10 min at 10°C. Influx was terminated by washing oocytes three times with ice-cold Barth's buffer; individual oocytes were dissolved in 1 ml of 2% SDS, and total uptake was measured by scintillation spectrometry. Uptake was expressed as the oocyte radioactivity divided by the radioactivity contained in the oocyte aqueous volume (~450 nl) at equilibrium.
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Porins in the Cell Wall of Mycobacteria

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The cell wall of mycobacteria is an efficient permeability barrier that makes mycobacteria naturally resistant to most antibiotics. Liposome swelling assays and planar bilayer experiments were used to investigate the diffusion process of hydrophilic molecules through the cell wall of *Mycobacterium chelonae* and identify the main hydrophilic pathway. A 59-kilodalton cell wall protein formed a water-filled channel with a diameter of 2.2 nanometers and an average single-channel conductance equal to 2.7 nanosiemens in 1 M potassium chloride. These results suggest that porins can be found in the cell wall of a Gram-positive bacterium. A better knowledge of the hydrophilic pathways should help in the design of more effective antimycobacterial agents.

The outer membrane of Gram-negative bacteria plays an important role in the intrinsic resistance of these organisms to antibiotics by decreasing the flow of antimicrobial agents into the cell. In contrast, Gram-positive bacteria do not contain an outer membrane in their cell wall, and consequently such organisms are, in general, more sensitive to antibiotics. Mycobacteria are Gram-positive bacteria that are highly resistant to a wide range of antibiotics and are major human pathogens. Tuberculosis, caused by *Mycobacterium tuberculosis*, kills 3 million people yearly worldwide (*1*). Leprosy, caused by *M. leprae*, is a major health problem in developing countries (*2*). Mycobacterial infections caused by *M. avium-intracellulare* complex and *M. tuberculosis* are among the most frequent opportunistic infections in patients with acquired immunodeficiency syndrome (AIDS) (*3, 4*). The mycobacteria have a distinct cell wall that contains a thick conventional peptidoglycan covalently linked to arabinogalactan polysaccharides that are esterified to mycolic acids (branched, long-chain fatty acids). It also includes complex lipids, lipopolysaccharides anchored in the cell wall, and proteins.

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The lipid layer of the mycobacterial cell wall acts as a strong permeability barrier in a manner analogous to that of the outer membrane of the Gram-negative bacteria and so is a major factor governing the resistance of pathogenic mycobacteria to otherwise potent antibiotics (*5*, *6*). The organization of the mycobacterial cell wall is not well understood, and the way hydrophilic molecules (nutrients, waste, and antibiotics) diffuse into the cell is not known. On the basis of the orientation of mycolic acids, two possible cell wall structures have been proposed. If mycolic acids were oriented parallel to the peptidoglycan plane (*7*), hydrophilic molecules could diffuse through the lipid layer along hydrophilic gaps around molecules such as polysaccharides that would span the cell wall. Instead, if mycolic acids were perpendicular to the plane (*8*), an organized membrane-like lipid layer would exist and the diffusion could take place through pore-forming molecules.

We obtained the cell wall of M. *chelonae* PS4770, a fast-growing mycobacterium, by step-gradient centrifugation. Cell wall proteins were solubilized with a detergent, Zwittergent 3-12, and EDTA [see methods in (*16*)]. To find hydrophilic channels in the cell wall of mycobacteria, we reconstituted liposomes with the detergent and EDTA extracts, and we measured the permeability of the liposome by studying the diffusion of hydrophilic compounds in a liposome swelling assay (*9*). The cell wall extract caused 90% of the pore-forming activity of the cell envelope extract (cell wall and cytoplasmic membrane, see Table 1). The action of proteases inhibited the pore-forming activity of reconstituted liposomes (Table 1), which indicates the involvement of one or more proteins in the formation of diffusion channels in the cell wall.

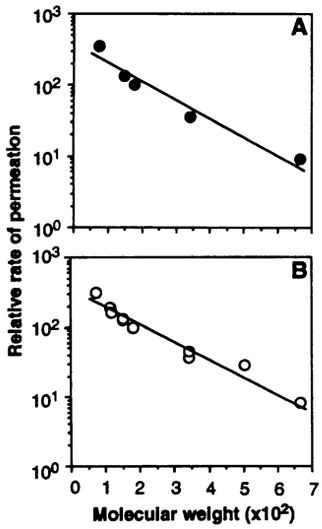

Fig. 1. Diffusion rates of liposomes reconstituted with (**A**) cell wall extract and (**B**) purified 59-kD protein from *M. chelonae* PS4770. The rates are expressed as percent of the rate of permeation of D-glucose. The following compounds were used: glycine, threonine, arabinose, xylose, galactose, glucose, mannose, maltose, sucrose, raffinose, and stachyose. Liposomes were reconstituted with 2.4 μmol of egg phosphatidylcholine and 0.1 μmol of dicetylphosphate according to the methods of Nikaido *et al.* (*9*), and 5 or 30 μg of protein were used for proteoliposome reconstitution. When necessary, detergent was removed by application of the sample to an Extracti-Gel D Detergent Removing Gel column (Pierce) according to the manufacturer's instructions. In all cases, the sample was extensively dialyzed against distilled water. Specific activity of the channel is defined as the change in optical density ×1000 min^{-1} (microgram protein)$^{-1}$.

Table 1. Relative rates of proteoliposomes reconstituted with protease-treated and untreated cell wall extracts (*16*).

Substrate	Swelling rate (%)		
	Extract	Extract + protease K	Extract + Pronase
Stachyose	100	28 [1.7]	32 [1.4]
Glucose	100	15 [0.8]	21 [1.7]
Glycine	100	26 [0.8]	31 [1.4]

We examined the diffusion of various zwitterionic and noncharged molecules through the channels taken from the cell wall of M. *chelonae* that were reconstituted into liposomes and found that the rate of diffusion was inversely related to the size of the molecules tested (Fig. 1A). The exclusion limit of the channels was high, as shown by the significant rate of the relatively large molecule stachyose to diffuse into the liposomes. Temperature had only a minor effect on the rate of diffusion of solutes and Q_{10} (multiplication factor for an increase of 10°C) for the swelling rate in glycine was 1.3. This result indicates that the cell wall contained a water-filled pathway because a much higher temperature sensitivity would have been expected if diffusion took place through the hydrocarbon core of a membrane (*10*). The activity for the solute diffusion into the proteoliposomes copurified with a fraction that contained a 59-kD protein (Fig. 2); fractions that did not contain the 59-kD protein did not show any significant swelling. This protein was not modifiable by heat because the apparent electrophoretic mobility in SDS–polyacrylamide gel electrophoresis of the 59-kD protein was not dependent on the heat applied to the sample (*11*). The diameter of the channel formed by the pure protein, as calculated from the Renkin equation (*10*), was 2 nm, which is similar to the large pore formed by protein F of *Pseudomonas aeruginosa* (*12*). The specific activity for arabinose diffusion into the proteoliposomes for the

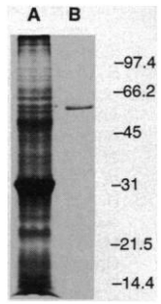

Fig. 2. Images of (**A**) cell wall fraction and (**B**) purified 59-kD protein by SDS–polyacrylamide gel electrophoresis. The 59-kD protein was purified from the detergent and EDTA extract of the cell wall (*16*) by gel filtration (Superose 6 column, Pharmacia) and anion-exchange chromatography (MonoQ anion exchange column, Pharmacia) (*11*). The eluted fractions were tested for pore-forming activity by the liposome swelling assay as described (Fig. 1). Molecular size markers are given at the right in kilodaltons.

59-kD protein was 27 (as defined in Fig. 1). This value was significantly lower than that of other porins such as OmpF from *Escherichia coli* B [approximately 380, as calculated from (*10*)] or protein F of *P. aeruginosa* H103 [approximately 97, according to (*9*)]. There is no contradiction between low specific activity and large pore size because these two parameters represent different channel properties. The channel diameter defines the exclusion limit at the narrowest part of the channel, whereas the specific activity represents a velocity that depends on many different parameters including channel length, channel size, interaction between the solutes and channel interior, and, especially, number of open pores.

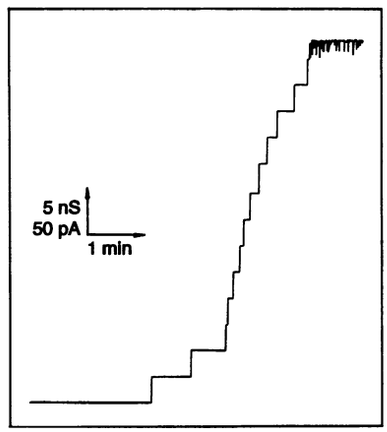

Fig. 3. Single-channel recording of an artificial lipid bilayer membrane in the presence of the 59-kD protein (2 ng/ml) from the cell wall of *M. chelonae*. The membrane was formed from a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) dissolved in *n*-decane. It was spread across a circular hole (area 0.6 mm^2) in a Teflon divider between two compartments containing a 1 M KCl solution (*13*). The applied membrane potential was 10 mV at 20°C. The current was measured with a current-to-voltage converter whose design was based on a Burr Brown operational amplifier. The bandwidth of the instrumentation was 300 Hz. The amplified signal was monitored with a digital oscilloscope and recorded with a strip-chart recorder.

The 59-kD protein was a minor protein in the cell wall (Fig. 2). The low protein number and the low specific activity of the channel were in good agreement with the low permeability of the cell wall of *M. chelonae*, as measured by the liposome swelling assay (the specific activity of the cell wall was 0.54 for arabinose) or measured in intact cells (5). The permeability of the cell wall of *M. chelonae* was significantly lower than that of the outer membranes of the Gram-negative bacteria *P. aeruginosa* and *Escherichia coli* (5).

To test whether the crude cell wall extracts and the purified 59-kD protein of the cell wall formed defined channels or simply represented an undefined leakage pathway, we performed single-channel experiments with lipid bilayer membranes (13). Addition of small amounts of the cell wall or of the pure 59-kD protein (final concentrations, 1 to 10 ng/ml) to the aqueous solution in contact with a lipid bilayer membrane caused the formation of ion-permeable channels (Fig. 3). These channels could only have been present in the cell wall because their presence in the cytoplasmic membrane would have resulted in the death of the cell. The channels were mostly stable and occasionally showed a fast flickering between a closed and an open state (Fig. 3). The average single-channel conductance was 2.7 nS in 1 M KCl, which is larger than those found with porins of enteric bacteria (14). The single-channel conductance could also be used to generate a rough estimate of the channel size (13). If we assume that the lipid layer has a thickness of 12 nm (15), the channel size would be approximately 1.9 nm. This value has to be considered an approximation because we do not know the exact length of the lipid layer and because we may be dealing with a specialized channel such as the specific porins of Gram-negative bacteria (14). The results from the liposome swelling assays and from the lipid bilayer experiments suggest that our picture of a defined hydrophilic pathway formed by the 59-kD protein was correct. The 59-kD protein would act as a mycobacterial porin and form the necessary channels for the diffusion of nutrients into the cell and of waste products to the environment. Better knowledge of this system could help improve antibiotic design to overcome the permeability barrier of the cell-wall lipid layer of mycobacteria.

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16. *Mycobacterium chelonae* PS4770 was used for all the experiments and grown as described in (5). Cell suspensions were broken by homogenization with microglass beads by a Homogenisator MSK (B. Braun Biotech) according to the manufacturer's instruction. The homogenized sample was then centrifuged at 3,000 rpm for 10 min and the supernatant was collected and pelleted at 20,000 rpm for 30 min to obtain the cell envelopes (cell wall and cytoplasmic membrane). The cell wall was purified by sucrose-gradient centrifugation in a modification of the method of Hirschfield and colleagues (17). The pellet was suspended in 1 ml and applied to a step sucrose gradient of 30, 40, and 70% sucrose (w/v). The fraction containing the cell wall, between 40 and 70%, was centrifuged and the pellet was washed twice and used as cell wall fraction. This fraction was essentially free of cytoplasmic membrane as assessed by reduced nicotinamide adenine dinucleotide (NADH) oxidation; at this point only 0.1% of total NADH activity was associated with this fraction. The cell wall fraction was washed once by centrifugation with a buffer containing 20 mM tris-HCl (pH 8), 1% Zwittergent 3-12 (Calbiochem), and 3 mM NaN_3, and proteins were extracted with a solution containing the same buffer solution supplemented with 40 mM EDTA. After 1 hour of incubation at room temperature, the solution was centrifuged and the supernatant was collected. After dialysis, the cell wall extracts (20 μg) were incubated with 0.1 μg of Pronase (Boehringer Mannheim) or protease K (Sigma) for 5 hours at 37°C according to the manufacturer's instructions. Liposomes were reconstituted with 10 μg of protein after extensive dialysis against distilled water, and swelling rates were determined as in Fig. 2 and normalized to the untreated control rate. Control liposomes with Pronase or protease K were also reconstituted and did not show any significant swelling. Data represent the average of three experiments; standard errors are in brackets.
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Antisense and Antigene Properties of Peptide Nucleic Acids

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Peptide nucleic acids (PNAs) are polyamide oligomers that can strand invade duplex DNA, causing displacement of one DNA strand and formation of a D-loop. Binding of either a T_{10} PNA or a mixed sequence 15-mer PNA to the transcribed strand of a G-free transcription cassette caused 90 to 100 percent site-specific termination of pol II transcription elongation. When a T_{10} PNA was bound on the nontranscribed strand, site-specific inhibition never exceeded 50 percent. Binding of PNAs to RNA resulted in site-specific termination of both reverse transcription and in vitro translation, precisely at the position of the PNA · RNA heteroduplex. Nuclear microinjection of cells constitutively expressing SV40 large T antigen (T Ag) with either a 15-mer or 20-mer PNA targeted to the T Ag messenger RNA suppressed T Ag expression. This effect was specific in that there was no reduction in β-galactosidase expression from a coinjected expression vector and no inhibition of T Ag expression after microinjection of a 10-mer PNA.

Sequence-specific binding of oligodeoxynucleotides (ODNs) to RNA or in the major groove of duplex DNA through triple-helix formation provides a way to modulate gene expression (1, 2). Although the potential of ODNs as antisense or antigene agents is actively being explored, the problems associated with creating in vivo bioefficacy and maintaining binding specificity and affinity are formidable.

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