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Ion channel activities in the *Escherichia coli* outer membrane

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The electrical properties of *Escherichia coli* cells were examined by the patch-clamp technique. Giant cells or giant spheroplasts were generated by five different methods. By electron micrographic and other criteria we determined that the patches are most likely from the outer membrane. We regularly observed currents through at least two types of channels in this membrane. The first current is mechanosensitive and voltage-dependent, and can be observed in single gene mutants of the known major porins (*ompF, ompC, phoE, lamB*); this channel may represent a minor porin or a new class of outer membrane protein. The possible identity of the second, voltage-sensitive channel with one of the known outer membrane proteins is being explored. The high-resistance seals consistently formed on these patches and the presence of gated ion channels suggest that most of the pores of the outer membrane are not statically open, as commonly held, but are closed at rest and may be openable by physiological stimuli.

Introduction

Gram-negative bacteria are enclosed by an outer membrane and an inner (cytoplasmic) membrane, separated by a peptidoglycan cell wall and a periplasmic space [1]. The outer membrane is considered to be a barrier for macromolecules, but not for small hydrophilic solutes. In *Escherichia coli*, solutes smaller than 600 Da diffuse through the two major porins, OmpF and OmpC, proteins which form nonspecific channels across the outer membrane [2–6]. Different porins and other proteins also exist (LamB, PhoE, NmpC, Tsx, Fhu, Fep) for transport of specific solutes [6–9]. Each bacterium is estimated to have 10^5 porins in its outer membrane [10].

The inner membrane acts as a selectively permeable barrier to allow passage only of compounds such as amino acids, sugars, and ions, for which there are transport mechanisms [11]. Several membrane proteins exist for conversion of the protonmotive force into sodium and potassium gradients, as well as for transport of organic molecules, synthesis of ATP, and for driving the flagellar rotary motor. It is expected that any small-conductance bacterial ion channels, such as might be used for sensory transduction, would also be located in this membrane.

We have explored the use of the patch-clamp technique [12] to study the electrophysiological properties of ion channels of native bacterial membranes [13]. This method is applicable to *E. coli*, although we must use giant cells or giant spheroplasts (cells in which the peptidoglycan is partially dissolved). We found activities of ion channels that are clearly gated by mechanical forces and/or by voltage, in manners similar to those of eucaryotic ion channels. Here we have tried to determine whether our patches are formed on the outer or inner membrane by variations in the method of spheroplast and cell preparation, electron microscopic observation, partial purification of channel activities, and treatment of the patches with lysozyme.

Materials and Methods

Chemicals and growth media

Growth media and chemicals were obtained from standard sources as reported previously [13]. Luria-Bertani medium contains 1% Bacto-Tryptone, 0.5% yeast extract, and 1% NaCl. Modified Luria-Bertani medium is the same except that only 0.5% NaCl is used. Patch-clamp solutions were buffered by dilution of a 1 M solution of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) brought to pH 7.2 with concentrated

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aqueous KOH and passed through a 0.2 μm filter before use. Spurr's resin was formed from 10 g vinyl cyclohexene dioxide, 4 g diglycidyl ether of polyethylene glycol-736, and 26 g nonenyl succinic anhydride mixed together, to which was mixed 0.4 g 2-(dimethylamino)ethanol [14]. Agarose Type VIII was obtained from Sigma Chemical Co. (St. Louis, MO), $KMnO_4$ was from Mallinkrodt Chemical Company (St. Louis, MO) and glutaraldehyde was from Electron Microscopy Sciences (Fort Washington, PA). Other electron microscopic solutions were supplied by Ladd Research Industries (Burlington, VT).

Bacterial strains

The genotype and source of the *Escherichia coli* strains used in this paper are listed in Table I. Strains AW694 and AW695 were isolated as spontaneous mutants of AW405 resistant to phage SS4 and to colicin A, respectively. The Omp^- phenotype of AW694 and AW695 was confirmed by absence of the appropriate protein band by 8M urea-9% polyacrylamide-sodium dodecylsulfate gel electrophoresis [9] of purified cell envelopes [15].

Strain AW693 was created by ethylmethanesulfonate-induced mutagenesis of AW405. The mutant grows poorly on modified Luria-Bertani medium plates, unless they are enriched with 400 mM KCl or NaCl. In addition, its shape is aberrant. Round and misshapen cells of various sizes form spontaneously, many of which stick together in large 'rafts'. Strain AW693 reverts at a very high rate to forms with less stringent osmotic requirements; modified Luria-Bertani medium without salt enrichment (= 5% NaCl) permits growth, although modified Luria-Bertani medium made with no NaCl does not. The high reversion rate made mapping difficult; preliminary experiments suggest a mutation between 10 and 25 min on the standard *E. coli* map. This map location and the round phenotype suggest that the mutation may be in the *pbpA* or *rodA* genes, mutations of which cause defects in cell wall synthesis and a round shape [16,17].

Preparation of giant spheroplasts and giant cells

Method 1: Giant spheroplasts from cephalexin-treated cells. E. coli K12 cells were cultured in modified Luria-Bertani medium and grown with cephalexin to 50–150 μm long unseptate filaments, which were then treated with Tris-EDTA and lysozyme in the presence of sucrose to generate giant spheroplasts (6 μm average diameter), as reported previously [13,18]. Lysozyme apparently did not completely digest the peptidoglycan in our treatment, but clipped the polymers and weakened the wall, thereby allowing swelling into spheroplasts [19] (see Results).

TABLE I

E. coli strains tested for presence of the mechanosensitive ion channel

Strain	Relevant genotype	Missing phenotypes	Reference/Source [a]
AW405	*fhuA31 tsx-78*	ferric hydroxamate uptake, phage T6 receptor	42
AW693		growth at low osmolarity, normal size, rod shape	This study
AW694	*ompF fhuA31 tsx-78*	OmpF major porin, ferric hydroxamate uptake, phage T6 receptor	This study
AW695	*ompCfhuA31 tsx-78*	OmpC major porin, ferric hydroxamate uptake, phage T6 receptor	This study
JE5505	*lpp*	outer membrane lipoprotein	43/CGSC
lpp-omp	*lpp ompA*	outer membrane lipoprotein, OmpA protein	21/U. Henning
W2961	*Δ(gpt-proA)62 (= Δ phoE)*	phosphorin	44/CGSC
pop3208	*lamB204 flhD5301* [b]	maltoporin, flagella	45/CGSC
CS483	*nmpC103 ompF254 ompC171 tsx-63*	OmpF and OmpC major porins, NmpC minor porin, phage T6 receptor	9/CGSC
χ2844	*tsx-462::Tn10*	phage T6 receptor	Roy Curtiss via CGSC
AN102	*fep-401 tonA23 tsx-67*	iron transport protein Fep, phages T1 and T6 receptors	46/CGSC
SG480	*Δ(envZ-malT)76flhD5301* [b]	osmotic control of porin synthesis, flagella	47/J. Slauch
GC7378	*pbpA::kan*	cell wall synthesis, normal size, rod shape	22/A. Jaffé

[a] CGSC refers to strains supplied by the Coli Genetic Stock Center, New Haven, CT.
[b] The *flbB* gene has been renamed *flhD* [48].

Method 2: Giant spheroplasts from UV-treated cells. Wild-type *E. coli* cells irradiated with ultraviolet light also form unseptate filaments [20], albeit shorter than those generated by Method 1. Cells were grown with shaking in modified Luria-Bertani medium at 35°C to an A_{590} of 0.2. Two 1.5-ml aliquots were each diluted with modified Luria-Bertani medium to 10 ml in plastic Petri dishes, and shaken at 40 rpm under UV light (254 nm, 124 erg · $mm^{-2} \cdot s^{-1}$) for 3 min. The cultures were pooled and modified Luria-Bertani medium added to 30 ml in a 250-ml flask. Cells were grown with shaking at 42°C until 80–100-μm-long filaments developed, in about two hours. Spheroplasts from these filaments (about 5 to 10 μm in diameter) were prepared as for Method 1.

Method 3: Magnesium-induced giant cells. Cells were grown with shaking in modified Luria-Bertani medium plus 50 mM $MgCl_2$ at 35°C to an A_{590} of 0.5, then diluted 1 : 10 into modified Luria-Bertani medium plus 50 mM $MgCl_2$ containing 60 μg/ml cephalexin, and grown for 2 h by shaking at 42°C. This culture yielded a mixture of giant round cells (about 5 to 10 μm in diameter) and long filaments, some with bulges on the sides. These bulges and giant cells were used for patch-clamp studies.

Method 4: lpp ompA giant cells. Cells of a mutant which lacks both Lpp and OmpA proteins, two major components of the outer membrane, round up in the presence of 30 mM $MgCl_2$ [21]. This mutant was cultured in Luria-Bertani medium plus 30 mM $MgCl_2$ at 35°C to an A_{590} of 0.5, then diluted 1 : 10 in Luria-Bertani medium plus 30 mM $MgCl_2$ containing 60 μg/ml cephalexin, and grown with shaking for 4 h. It formed giant round cells with an average size of 5–10 μm in diameter, comparable to that of the giant spheroplasts. Prior to electrical recording, the giant cells were usually washed twice with 0.8 M NaCl. We found an increase in membrane-pipette seal resistance after this salt wash, although no changes in ion channel activity were observed, and no structural alterations could be discerned by thin-section electron microscopy.

Method 5: Giant cells of an osmotic-sensitive mutant. AW693 cells were grown in modified Luria-Bertani medium plus 400 mM KCl to an A_{590} of about 0.6 and incubated overnight at 4°C without shaking. A few of the cells were quite large, a few large enough (4 to 8 μm in diameter) to be patch-clamped directly. The number of these large cells increased to about 10% of the total by the inclusion of 10 mM $MgCl_2$ in the growth medium. The traces of channel activities presented in this paper are from AW693 cells grown without added $MgCl_2$. As patch-clamp experiments are recorded from single cells, we cannot be sure whether the recordings were from AW693 or from a partial revertant. We therefore obtained mutant GC7378 [22], which is deficient in penicillin-binding protein 2 (PbpA), and also forms giant round cells similar in appearance to those of AW693; GC7378 exhibits no growth inhibition at low osmolarity, however. Giant GC7378 cells also formed seals, though at a much lower rate than AW693, and exhibited similar channel activities to those of spheroplasts prepared by method 1.

Patch-clamp recording

The method of electrical recording from giant cells or giant spheroplasts was essentially as published [13]. Pipettes (Boralex, Rochester Scientific, Rochester, NY) had a tip opening of approximately 2 μm in diameter as measured directly under a light microscope, and were filled with a solution of 200 mM KCl, 40 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes. Prior to recording, the pipettes were coated with transparent nail enamel (Hard as Nails, Sally Hansen) to reduce pipette capacitance. Suction was monitored with an in-line pressure transducer (differential type, ±5 psi, Omega Engineering) calibrated to a Hg manometer. Excised patches were formed by lifting the patch-pipette and cell out of the bath as briefly as possible. The bath solution was initially 250 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes. All experiments were done at room temperature by use of standard patch-clamp procedures [12] with a List Medical EPC7 amplifier. A 10 kHz output signal was digitized through a 1 kHz 8-pole lowpass Bessel filter (Frequency Devices), and analysed on an Indec Systems computer by use of a program developed by Dr. Yoshiro Saimi.

Electron microscopy

Cultures of AW693 cells (25 ml) and *lpp ompA* cells (60 ml after treatment with cephalexin) were prepared as for patch-clamping above, centrifuged for 10 min at 3000 × *g*, washed in 20 ml of a solution of 0.4 M KCl, 5 mM Hepes, and harvested into 2 ml of the same buffer. The samples were fixed in 2% glutaraldehyde in 0.3 M KCl, 5 mM Hepes, then embedded in 2% Agarose Type VIII in 0.4 M KCl for further fixation and staining in 1% $KMnO_4$, 0.4 M KCl and 0.5% uranyl acetate. The samples were dehydrated and embedded in Spurr's Resin, and 70 mm slices placed on copper grids, which were restained in 0.5% uranyl acetate and lead citrate. The samples were observed in a Philips EM-410 electron microscope.

Outer membrane enrichment

Membrane vesicles enriched for outer membrane components were prepared as reported [23]. Enrichment of outer membrane components was confirmed by scanning densitometry with a Zeineh SL-504-XL scanning densitometer of the porin bands from starting and enriched materials electrophoresed on an 8 M urea-9% polyacrylamide-sodium dodecylsulfate gel [9].

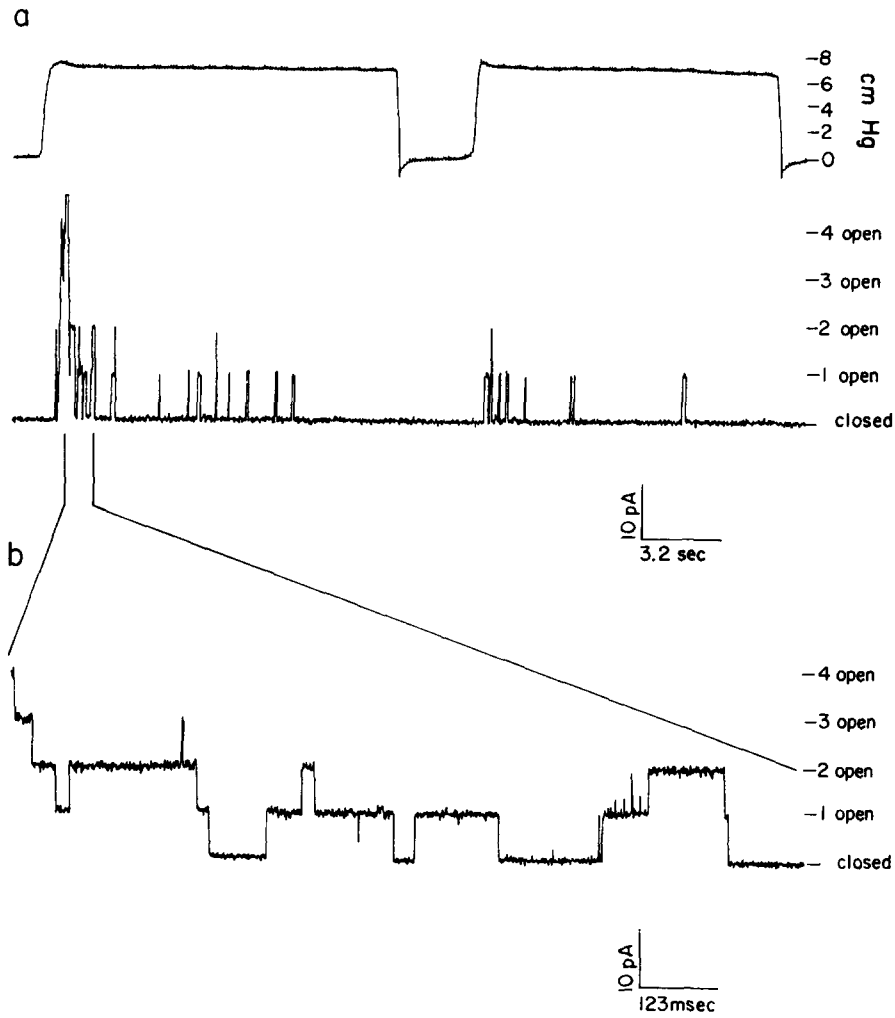

Fig. 1. Patch-clamp recording of mechanosensitive channels in a round mutant cell of *E. coli* strain AW693 (method 5, Table II). (a) The top trace indicates pressure applied to an excised patch in symmetric solutions. The bottom trace records current through the patch at +21.7 mV. Upon suction, currents corresponding to the openings of at least four channels can be seen, followed by a decrease in activity. This behavior may indicate that intact peptidoglycan restrains deformation of the outer membrane so as to lower the tension detected by the channel. Upon release of pressure followed by reapplication, the channels again opened. These openings could be repeated indefinitely. (b) An expansion of the early part of the current trace of (a) shows openings at higher time resolution.

Results

We have previously described patch-clamp electrical recording from giant spheroplasts of *E. coli* [13]. It was possible that the channels we reported could have been an artifact of spheroplast preparation with lysozyme or EDTA, both of which have strong effects on the bacterial envelope. In addition, cephalexin has been reported to cause the formation of large pores in bacterial membranes [24]. We have since found other means to prepare giant cells and giant spheroplasts which obviate the use of one or both of these chemicals. These procedures (see Materials and Methods) and over 100 recordings of high seal resistances are summarized in Table II. For all five methods, we could readily form seals in the range of 1 gigaOhm or greater between the pipette and surface of giant spheroplasts and cells of *E. coli*; this resistance is needed to detect ion channel activities reliably.

Mechanosensitive channel

We described the behavior of this channel in giant spheroplasts generated by method 1 previously [13]. We have now observed its activity in all five preparations. This includes preparations made with Tris-EDTA-lysozyme alone, cephalexin alone, or neither, so channel activity cannot be attributed to these agents. The channel activities in Fig. 1 were recorded without any such treatment, from a cell of mutant strain AW693, which formed giant cells in culture without the addition of any drugs (method 5). These channels had the characteristic large conductance, pressure-activation, and voltage-activation described in the previous study [13], but higher pressure, equivalent to 10 mosM, was needed for

TABLE II

Electrical resistance of membrane patches from five different preparations of E. coli giant cells or giant spheroplasts

See Materials and Methods for detailed descriptions of each method.

Method	Use of cephalexin	Use of lysozyme	Resistance (gigaOhm)			Rate of seal formation (%)
			mean ± S.D.	range	*N*	
1. Giant spheroplasts from cephalexin-induced filaments	+	+	3.0 ± 2.3	1.0–10.0	46	80
2. Giant spheroplasts from UV-induced filaments	−	+	2.5 ± 2.1	0.7–6.4	6	10
3. Magnesium-induced giant cells	+	−	0.7 ± 0.5	0.2–1.2	3	10
4. *lpp ompA* giant cells	+	−	5.8 ± 3.9	1.0–20.0	49	80
5. Osmotic-mutant giant cells	−	−	0.8 ± 0.4	0.3–1.2	12	10

opening. Stock et al. reported that osmotic pressures in this range and higher are normally sustained across the cell envelope [25]. The open times were on the order of tens to hundreds of milliseconds, whereas in spheroplasts generated by method 1 the open times were on the order of seconds. After several openings, the activity of

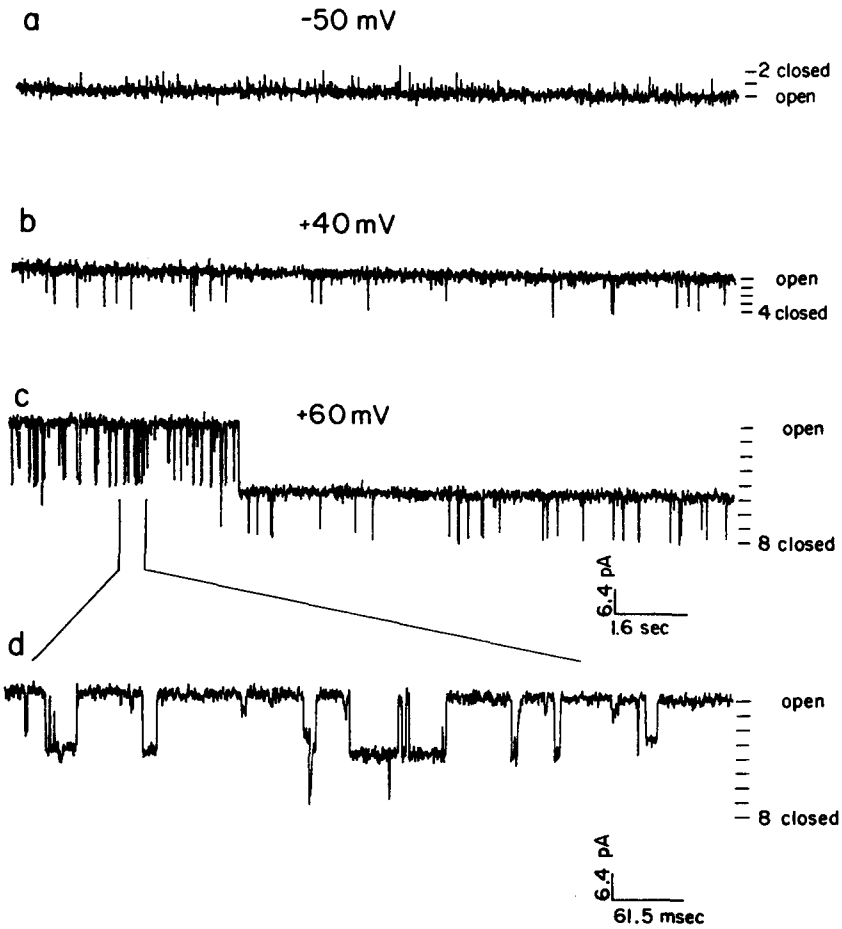

Fig. 2. Patch-clamp recording of the voltage-sensitive channel (method 4, Table II). An excised patch shows channel activity at (a) −50 mV, (b) +40 mV, and (c) +60 mV in symmetric solutions. Only the open and maximally closed levels at each voltage are labeled; other levels are indicated by tick marks. The difference in current amplitude from one level to the next corresponds to a unit conductance of 60 pS. The traces are presented at low time resolution to show the increased number of closed levels and frequency of transitions at +40 mV and +60 mV, which indicates voltage-dependent gating. (d) An expansion of an early part of trace (c) shows closures not evident at lower time resolution. The same expansion of traces (a) and (b) also shows well-resolved closures (data not shown).

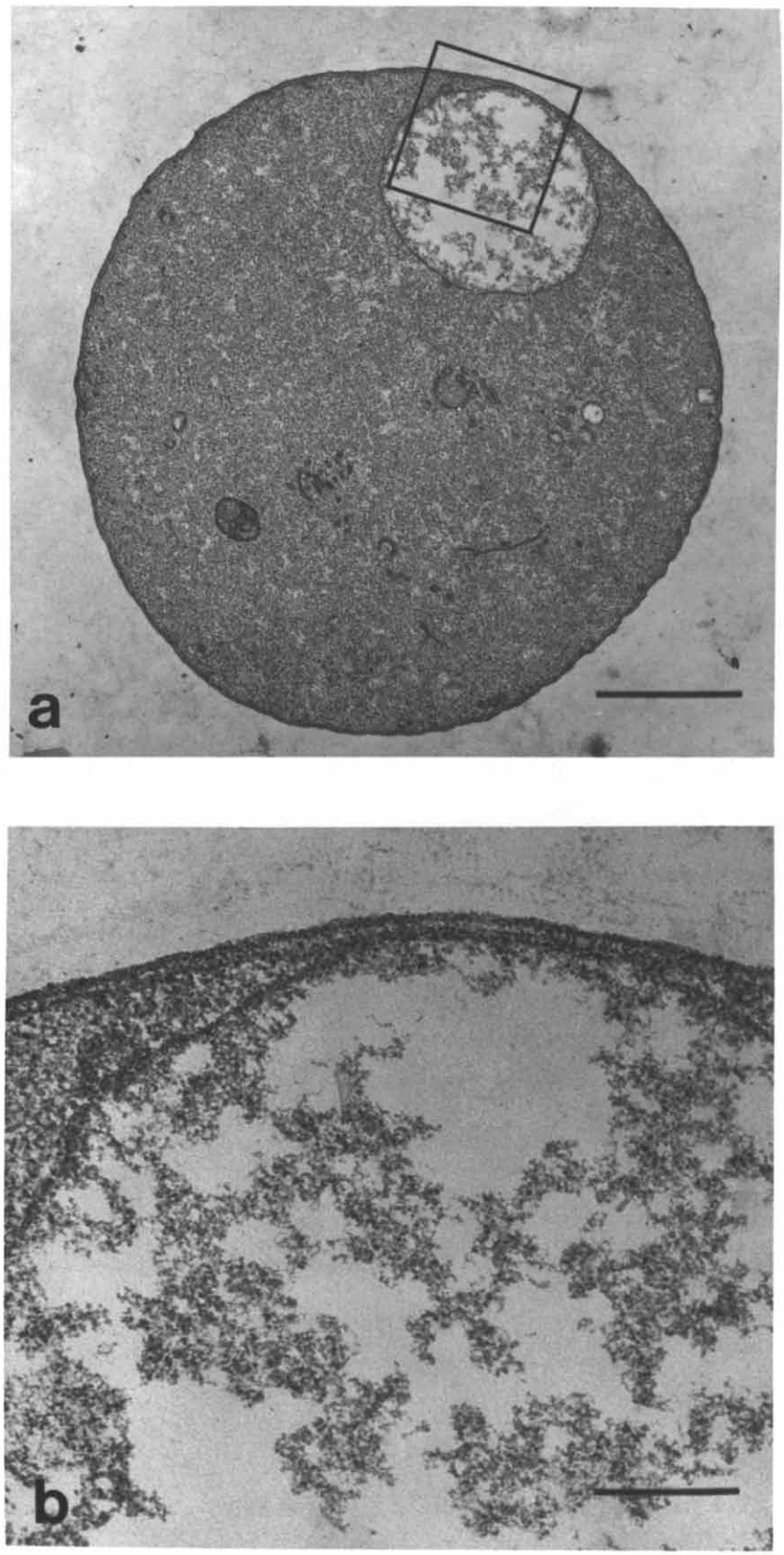

Fig. 3. Thin-section electron micrographs of a giant cell prepared by method 5 of Table II. Samples were prepared for microscopy as specified in Materials and Methods. (b) is an enlargement of (a) to show the two cellular membranes more clearly in contrast to the single vacuolar membrane. The bar in (a) represents 1 μm and in (b) represents 0.22 μm.

the channels decayed in the continued presence of suction, but could be reinstated by release and reapplication of pressure. As these patches were formed on viable cells with relatively intact cell envelopes, this activity may better represent the actual behavior of this channel in vivo.

Voltage-regulated channel

We also found a second channel which could not be activated by pressure. This channel (Fig. 2) is mostly open when the membrane is hyperpolarized (a) and at depolarizing voltages near 0 mV (b), but tends to close more frequently upon stronger depolarization (c). It can be completely closed beyond +60 mV (data not shown). The expanded trace at +60 mV shows transitions which correspond to multiples of a unit conductance of 60 pS. This value was measured as a chord conductance, and may be an underestimate of the true conductance, as the channel rectifies at voltages greater than +40 mV [26]. The early part of trace (c) shows current flowing through at least 8 open channels, with closures mostly of 4 channels, but also of 1, 2, and 3 channels as well. After about 5 s, 5 channels closed for the remainder of the recording, and further transitions were observed which correspond to closures of 1, 2, and 3 channels. A detailed analysis of the voltage-dependent channel is presented elsewhere [26]; the similar reversal potentials, as well as the cooperativity of gating, and uniform size between conductance levels, suggest that these are truly simultaneous closures of identical channels.

Location of the ion channels

To determine if these bacterial ion channels were in the inner or the outer membrane, we next tried to determine if the outer membrane were intact in our preparations and if perturbations to the outer membrane affected channel activities.

First, as reported above, giant cells generated by methods 3, 4, and 5 were not treated with EDTA or lysozyme, and therefore probably have an intact peptidoglycan layer. It is unlikely that the patch-clamp pipette, which must remain clean to form a high-resistance seal, could bond to the inner membrane, passing through both the outer membrane and this peptidoglycan.

The integrity of the two membranes was confirmed by thin-section electron micrographs of preparation 5 stained with permanganate to enhance observation of membranes (Fig. 3). A typical cell is shown in (a), with an enlargement of a section in (b) which shows the two membranes and the periplasmic space between them more clearly. The outer membrane was observed to be continuous, intact, and closely apposed to the inner membrane in these cells. An example is shown of a cell

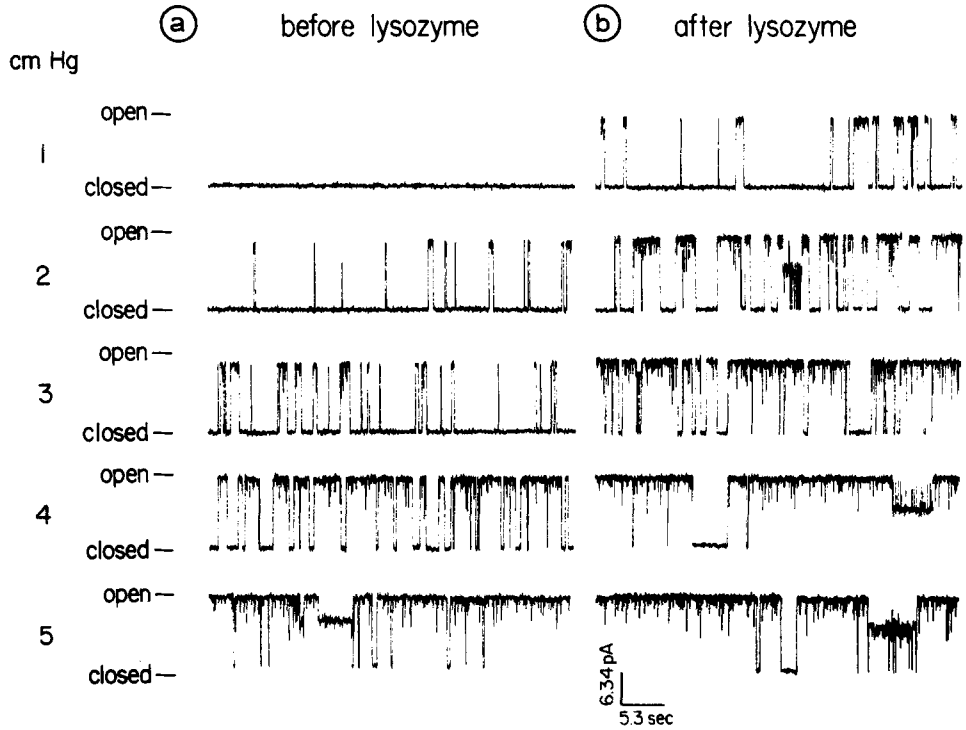

Fig. 4. Effect of lysozyme on the mechanosensitive channel. (a) Pressure-sensitivity of a single mechanosensitive channel in a spheroplast of strain AN102 (Table I), as recorded from an inside-out excised patch. The spheroplast was prepared by method 1, Table II, to allow long-term recording of opening probability without interference from the decreases in activity as seen in Fig. 1. The traces were recorded at +30 mV. The suction applied is listed to the left of each trace. As suction was increased, the channel opened more frequently, and the openings were of longer duration. (b) 5 mg/ml lysozyme was added to the bath solution for 30 min, then replaced with bath solution without lysozyme. These traces were recorded directly after removal of lysozyme. The level of applied suction for each trace is the same as for the equivalent trace in (a).

with a vacuole to demonstrate the difference in appearance between single and double membranes. While vacuoles were very rare in the osmotic-mutant giant cells, the vacuoles were always surrounded by a single membrane.

That these two kinds of ion channels appear to be located in the outer membrane was suggested by its relative integrity. In an inside-out excised patch of outer membrane, the cell wall is exposed to the bath. We tried to perturb ion channel activity in such patches by addition to the patch-clamp bath of lysozyme, which degrades the cell wall to which the outer membrane is attached. Fig. 4 shows the irreversible effect of lysozyme on the activity of a single mechanosensitive channel in such a patch. In panel a, the channel activity is shown at a voltage (+30 mV) at which no channel openings are observed unless tension is applied. Upon exposure to lysozyme (panel b), the frequency and duration of openings increased at all applied tensions, and channel activity was present even when no tension was applied. This irreversible increase in sensitivity to tension is most easily explained if these channels are in the outer membrane, whose deformation is resisted by attachment to the cell wall, and if the cell wall is directly accessible to lysozyme in the bath.

The activities of both types of ion channels reported here were observed in membrane vesicles reconstituted into liposomes [23]. Artificial liposomes fused with vesicles enriched for outer membrane contained the voltage-sensitive channel about three times as frequently (1 : 600 dilution of vesicle protein : added lipids to achieve about 25% success rate of observing channel activities) as did liposomes fused to vesicles enriched for inner membrane (1 : 200 dilution). This roughly corresponds to the enrichment of outer membrane porins as measured by scanning densitometry of a urea-polyacrylamide-sodium dodecylsulfate gel after electrophoresis (data not shown). The porins were present in enriched outer membrane vesicles at 2.4-times the amount present in vesicles enriched for inner membranes, relative to the total amount of protein loaded per lane.

These data all suggest that both these kinds of ion channels are in fact in the outer membrane. To test whether the mechanosensitive channel is one of the known outer membrane proteins, we recorded from giant spheroplasts (method 1) of mutants each missing one or more types of porin, as well as from other mutants missing other outer membrane components. For all of the strains listed in Table I, the activities of the mechanosensitive channel were observed; therefore, this channel is unlikely to be one of the major porins. This channel may be a minor porin not tested here, or a new outer membrane protein not previously identified. The possible identity of the voltage-sensitive channel with one of the known outer membrane proteins is being explored by similar techniques.

Discussion

Ion channels in the bacterial outer membrane

In our previous report on channels on the surface of *E. coli* [13], we could not discern whether the patches were formed on the outer or the inner membrane. We have now gathered evidence for our recording from the outer membrane, as shown above by the integrity of the outer membrane, effect of lysozyme on channel activities, and copurification of channel activity with outer membrane material. In addition, we note that the mechanosensitive channel has a conductance similar to those of porins of bacterial outer membranes and of the voltage-dependent anion channel of the outer mitochondrial membrane [27], much larger than those of known channels in the cytoplasmic membranes of other organisms [12]. Even a single such pore in the inner membrane would drain the cellular protonmotive force if opened for only a few milliseconds. It should be noted, however, that in the presumably more intact cells of preparation 5, the mechanosensitive channel was much less active. In normal-size cells, this activity could be even less, which would prevent dissipation of the protonmotive force. In addition, the mechanosensitive channel was present in every one of the outer membrane protein mutants tested. By calculation of the frequency of this channel in our patches and the relative size of the amount of membrane in the patches and in wild-type cells, the mechanosensitive channel should be present in at most a few hundred copies per cell. Although this low occurrence is consistent with an outer membrane channel still being present in cells missing the major outer membrane proteins, an alternative explanation is that this channel is a component of the bridges described by Bayer, several hundred of which cross the periplasmic space to connect the inner and outer membranes [28]. This latter explanation is attractive as it also suggests a way to measure tension by the amount of stretch between the two membranes, and could explain why the mechanosensitive channel is less often observed than the voltage-sensitive channel in liposomes fused with purified membrane vesicles. A mechanosensitive Bayer's bridge could provide the cell a means to respond to sudden drops in osmolarity, which are known to cause the rapid loss of small molecular weight compounds from the cytoplasm [29].

The gigaOhm seal

Suction through the patch-clamp pipette electrode draws a patch of membrane into the interior of the pipette tip. The tight apposition between glass and membrane seals the junction against ion passage. Such passage can be detected as leakage of electric current. When a voltage is applied across the patch, the measured current flows through residual leakage and through open ion channels in the patch. The total resistance of

the seal and the membrane is approx. 10^9 Ohm (= 1 gigaOhm). A high seal resistance is usually taken to mean that there are no open channels in the patch, and establishes the low-noise background against which channel activities can be observed clearly.

We were able to obtain such high-resistance seals at a favorable rate on preparations 1 and 4, comparable to that on eucaryotic surfaces in our and others' hands. As does the surface membrane of yeast [30], the outer membrane of *E. coli* yielded seals only after the application of suction sustained over tens of seconds. A longer time was needed to generate even seals of low resistance on preparations 3 and 5. The small systematic differences in seal resistances on the five different preparations (Table II) may reflect small differences in cell wall or surface structures and concomitant pliability of the envelopes, which must be drawn into the pipettes to make close contact with the interior surface of the electrodes.

As the outer membrane is apparently intact in our preparations, the formation of high-resistance seals is puzzling. The outer membrane contains thousands of porin molecules, which allow passage of low-molecular weight compounds through the outer membrane. The conductance of a single porin molecule should be approximately 370 pS in our solutions [31]. The opening of one porin will reduce the resistance of even a high-resistance seal to 2.6 gigaOhms. The 1 gigaOhm seals that we routinely form on preparations 1 and 4 therefore indicate that at most three of the porins in our patches are open, even if we assume that all of the measured current is conducted through permanently-open porins. The patches we used had about the same surface area as that of an *E. coli* cell, about 6 μm^2, which makes unlikely the possibility that all of the open porins are clustered outside the patch. We are therefore forced to conclude that at most three, and probably fewer, of the 10^5 porins in these patches are constantly open at rest. For methods 2, 3, and 5, the lower rate in forming gigaOhm seals may indicate that more porins are open initially, but may be destroyed by the suction used to form the seal. We routinely are able to form seals of about 0.1 gigaOhms in these preparations, though, with application of very little suction (less than 1 cm Hg, equivalent to less than 0.6 mosM pressure difference across the membrane). By the argument above, no more than 27 of the 10^5 porin proteins can be open in these patches. This furthermore implies that these few open porins have different properties from the others, as even at low opening probabilities we would expect many overlapping events if there really were 100 000 independent channels in these patches.

Dynamic nature of the outer membrane

Our results suggesting that only a few porins are open are supported by the observations of Bavoil et al. [32], who showed that mutants having only 1–3% of the amount of porins of the parental wild-type strain nonetheless grew normally in rich medium. The authors concluded that the number of porin molecules present in wild-type cells is as much as four orders of magnitude higher than necessary for uptake of substrates. Similarly, Schindler and Rosenbusch found a ratio of closed to open channels of 2000 : 1 in porin-reconstituted bilayers at physiological pH [33], and in a later study found that the current measured through such bilayers was "orders of magnitude lower than expected if all channels present were open" [34].

Purified porins reconstituted into planar lipid bilayers did behave as static open pores in some studies [2,31]. Permeability measurements of porins by non-electrophysiological techniques in intact cells also suggest a static nature of the outer membrane, in which a large number of porin molecules are open at all times [32,35–37]. Sen et al. further observed no change in permeability as transmembrane voltage was altered [36], unlike what would be expected from the presence of voltage-sensitive channels in the outer membrane. At present, we cannot reconcile these conclusions with our observations. It is possible that the reconstitution studies separated the porins from regulatory components in the cell envelope and periplasm. The flux studies averaged the properties of many channels over the course of several minutes, while the patch-clamp technique resolves the behavior of individual channels on a millisecond time scale. We note that Sen et al. [36] measured electrical potential at various external ionic strengths. The different membrane tensions thus produced might counteract the effect of the transmembrane voltage on the porins, as it does for the mechanosensitive channel [13], such that the overall effect is constant outer membrane permeability.

Some other studies also indicate a more closable nature of reconstituted porins. Closure was demonstrated with reconstituted porins from *E. coli* [7,33,38] and from *Neisseria gonorrhoeae* [39], although the voltage required for gating in these studies may not be realized in vivo. Channel closure at voltages approaching 75 mV has been observed with reconstituted porins purified with non-ionic detergents (Garavito, M., personal communication). Xu et al. [38] showed that detergent solubilization, four trypsin treatments, twenty freeze-thaw cycles, and treatment in high salt, methods similar to those of Benz [2] and Lakey et al. [40], still allowed voltage-dependent closures at membrane potentials above 75 mV in reconstituted bilayers. Both of the outer membrane channels that we found are also gated by relatively small voltages. A difference of tens of millivolts across the outer membrane is expected from fixed negative charges in the periplasm [25,36,41]. Gating of outer membrane channels by low voltages contrasts markedly with the potentials of over 100 mV

required to gate purified reconstituted porins in previous studies [33]. Lakey hypothesized that the contradictory findings on whether porins are gated might stem from the different techniques used to purify porins [4]. Ours should be one of the gentlest methods used, since patches were examined on cells capable of growth (method 5). It remains possible that the suction needed to draw the patch into the pipette, although released after seal formation, somehow closed or destroyed the porins. At present, we know of no method that can be used to test this possibility. As noted above, partial seals could be formed at very low pressure. In addition, all of the eucaryotic ion channels studied by patch-clamp as well as the two types of *E. coli* channels reported here survive the suction needed for seal formation.

The frequency of encountering the activities of the voltage-dependent channel was much higher in reconstituted membranes than in spheroplasts. This may indicate that this channel is regulated in vivo by the presence of periplasmic or outer membrane components, which are lost during membrane purification, and which may in vivo sense internal or external stimuli. The mechanosensitive channel activity, conversely, is less frequently found in reconstituted membranes than in spheroplasts. Mechanosensitivity may depend on the presence of elements in the cell wall, as is suggested by the change in mechanosensitivity by the application of lysozyme.

We therefore favor an alternative view to the model of the outer membrane as a static sieve with permanently open porins. Most of the outer membrane channels are closed, but may be opened by physiologically relevant stimuli, as in other organisms. The channels may be gated by voltage, by turgor (osmotic) pressure, or by specific solutes. Interactions with lipopolysaccharides appear to be important [34], as may be interactions with peptidoglycan, periplasmic proteins, and membrane-derived oligosaccharides. The channels could even be regulated by growth rate. Previous studies which found porins to be continuously open used isolated membrane fragments or isolated proteins and did not include such interactions. Although our recordings are not from untreated wild-type cells and therefore cannot resolve the dilemma conclusively, the cell envelopes here are relatively intact, and should allow complex regulation of outer membrane channels. We have found two such gated channels to date, and further studies may reveal regulation of other outer membrane channels as well.

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