FEBS Letters 434 (1998) 160–164 FEBS 20737

Inhibitory effect of acidic pH on OmpC porin: wild-type and mutant studies

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Received 15 June 1998; revised version received 14 July 1998

Abstract By use of the patch clamp technique, we have compared the electrophysiological signature of OmpC porin channels at neutral and acidic pH. The perfusion of pH 5.4 buffer to the periplasmic side of excised patches promoted the closure or block of ~20% of the open porins present in the patch without changes in their single channel conductance. Besides this effect on the main, long-lived open state, lowering the pH also suppressed the spontaneous transitions of channels to another distinct short-lived open state. The inhibitory effect on the opening kinetics was particularly visible in two mutants (K16Q and E109Q) in which transitions to the short-lived open state are enhanced by the mutations themselves at pH 7.2. On the other hand, the R124Q mutant responded to acidic pH by an increased gating to the short-lived open state. The results suggest that acidic pH stabilizes a closed state of OmpC porin, and that the pH sensitivity might be conferred in part by R124, but not by K16 or E109.

Key words: Channel; Closing; Modulation; Outer membrane; *Escherichia coli*

1. Introduction

Porins are pore-forming proteins of the outer membrane of *Escherichia coli* and other Gram-negative bacteria [1]. They are largely responsible for the overall permeability of this membrane to nutrients, metabolites and many antibiotics. The structure of the trimeric general diffusion porins of *E. coli*, OmpF and PhoE, has been determined at atomic resolution [2]. Each monomer is a transmembrane β-barrel of 16 β-strands connected by short periplasmic turns and long extracellular loops. An open pore is clearly defined within each subunit. The size of the pore is limited to ~7×11 Å by the 3rd extracellular loop folding back inside the mouth of the channel to form a constriction zone called 'eyelet'. Thus, the structural data agrees with the commonly accepted view of porins as permanently open pores.

This picture of porins as static entities is, however, challenged by the observation of a highly dynamic behavior in electrophysiological experiments. Although its physiological relevance is not established, the transition of channels to a closed state at high membrane potentials has been well documented [3–7]. In addition to this type of inactivation, spontaneous oscillations between ion conducting (open) and non-ion conducting (closed) states occur frequently [4,6,8,9]. The kinetics of this spontaneous gating activity is greatly affected by modulators such as membrane-derived oligosaccharides and polyamines [10,11], or in channels mutated at specific residues of the eyelet [12,13].

Here we present data that demonstrate that some components of this spontaneous activity are also modified by acidic pH. The effect of pH on porin properties has been examined by others, but these studies emphasized mainly modifications in conductance and voltage dependence [5,14–16]. Xu and colleagues [14] reported more frequent closing events, but only when the pH was dropped to 3.5, two pH units lower than used here. In this work, we show that the stabilization of closed states of porin occurs through modulation of two distinct open states and in less drastic conditions than previously reported, namely at less acidic pH and lower transmembrane potentials. In addition, we have identified one pore residue that might participate in the pH response. Along with previous studies, our results support the notion that porins can occupy a dynamic range of functional states.

2. Materials and methods

2.1. Strains, growth media and chemicals

OmpC was chosen for this work because of the vast knowledge we have acquired so far on this protein and its mutants [13,17,18]. Because of the high level of homology between porins, we can use the structural information from OmpF for the design of OmpC mutants. The location of the residues that were mutated for this work is indicated in Fig. 1. The expression of wild-type and mutant OmpC porin was driven from a plasmid introduced into an *E. coli* K12 derivative deleted for OmpC and OmpF from the chromosome [18]. Single site-directed mutations were generated with the unique site elimination method (USE kit, Pharmacia Biotech) and the mutations were confirmed by DNA sequencing [18]. Cells were grown in tryptone growth medium (T-broth: 1% tryptone (Difco Laboratories) and 0.5% NaCl) or Luria-Bertani broth (Difco Laboratories). Azolectin (phosphatidylcholine) was from Sigma, and all other chemicals were from Fisher Scientific.

2.2. Membrane preparation and electrophysiology

Outer membrane fractions were purified according to a published protocol [19]. Protein concentrations were determined by the bicinchonninic acid method (Pierce). For electrophysiology, an aliquot of native outer membrane was mixed with sonicated azolectin at a protein-to-lipid ratio of ~1:1800 (w/w), and reconstituted according to a dehydration-rehydration protocol [19]. Standard patch clamp technique [20] was applied to blisters induced from liposomes directly in the recording chamber. Patches were excised and recordings were first made in symmetric conditions of 150 mM KCl, 5 mM HEPES (*N*-[2-hydroxyethyl-3-piperazine-*N*′-[2-ethanesulfonic acid]]), 0.1 mM K-EDTA, 10 μM $CaCl_2$, pH 7.2. For buffer at pH 5.4, 5 mM MES (2-[*N*-morpholino]ethanesulfonic acid) was used instead of HEPES. All solutions were filtered through a 0.2-μm filter before use.

Current measurements were made at clamped membrane potentials with an Axopatch 1D amplifier (Axon Instruments). Throughout the paper, the voltages given refer to those of the pipette with respect to the bath. Pipettes had an initial resistance of 10 MΩ. The data was filtered at 2 kHz with an 8-pole Bessel filter (Frequency Devices), and stored on VCR tapes (Instrutech).

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PII: S0014-5793(98)00975-2

2.3. Data analysis

For data analysis, specific segments of data were re-filtered at 1 kHz and digitized at a 100-μs sampling interval. Data acquisition and analysis were performed with programs written in the laboratory using Axobasic (Axon Instruments). Amplitude histograms were generated by counting the number of sample points in a specific stretch of data that fall within bins of 0.1 pA. Closing transitions OmpC channels are too transient and infrequent to yield sizeable peaks in amplitude histograms. Therefore, the conductance of a single monomer was deduced from the amplitude of individual events [18]. The amplitudes of closing transitions typically cluster around values that are integer multiples of the smallest observed value. Because there is no favored conductance level, we have made the working hypothesis that the transitions of the smallest amplitude represent a single monomer. On the basis of this assumption, we use all-size transitions and scale them appropriately for the calculation of the monomer conductance.

3. Results

When reconstituted OmpC porins are examined by patch clamp electrophysiology, the patches typically contain a large number of open monomers giving rise to an overall current of several tens of pA, referred to as the baseline. The magnitude of this current is decreased upon perfusion of the bath solution with pH 5.4 buffer. The traces of Fig. 2 show the behavior of 3 patches obtained on wild-type (WT) and two mutant porins (K16Q and E109Q) before (leftmost trace) and after (rightmost trace) a drop in pH during the same experiment. The dashed lines indicate the baseline current level in buffer at neutral pH. From the current values indicated on the right-hand side of the figure, one calculates a ~20–30% decrease in the baseline current. This value has been found reproducibly in a large number of experiments (>40) performed on wild-type channels, the K16Q and E109Q mutants, and other mutants (R92Q, D105Q, R174Q, D315Q; not shown). This decrease in current in response to acidic pH is not observed in patches devoid of reconstituted porins.

A pH-induced change in the total current passing through many open pores can be due to a change in the conductance of single channel monomers, or to a shift in the reversal potential due to different concentrations of permeant ions (protons) on each side of the membrane, or to a change in the number of open channels. We can rule out the reversal potential shift because the current at −60 mV would be expected to increase (and not decrease) at low bath pH if proton flux contributed substantially to the current.

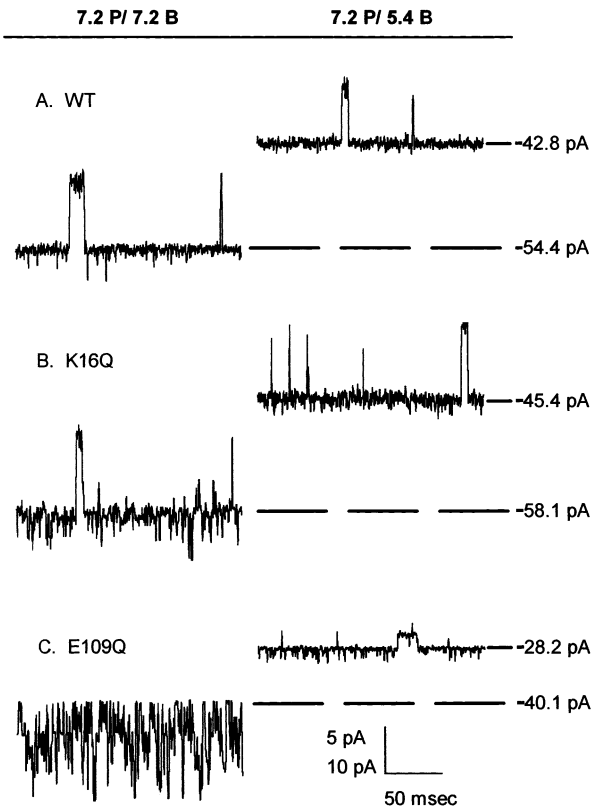

Fig. 2. Electrophysiological signature of wild-type (WT) and mutant OmpC porins at neutral and acidic periplasmic pH. Traces in the left column were obtained at neutral pH in the pipette ('P') and the bath ('B'). Traces in the right column were recorded after the bath pH was switched to 5.4 during the same experiment. The dashed line indicates the original current value for the baseline before the pH switch. Current values given on the right-hand side are negative to indicate that positive charges are flowing in the pipette (pipette voltage is −60 mV). The *Y* axis of the scale bar is 5 pA for panels A and B, and 10 pA for panel C.

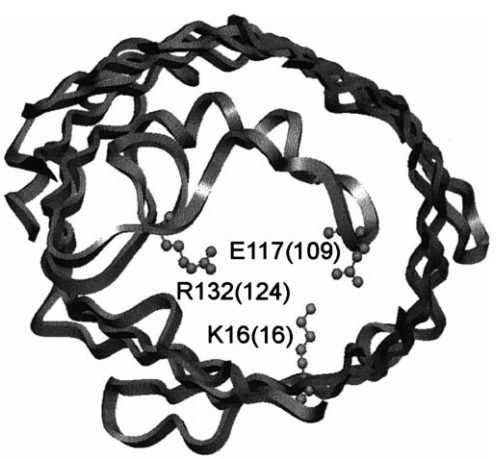

Fig. 1. Molecular model of the constriction zone of the OmpF porin. The parentheses give the number for the homologous residues in OmpC. The image was obtained from the published crystal structure [2], and the Insight II Molecular Modeling System (Biosym Technologies). To obtain a better view of the L3 loop spanning across the pore, most of the other extracellular loops have been clipped.

We have measured the single monomer conductance of wild-type channels as described in Section 2. We found the values to be similar at neutral and low pH, as shown in the current-voltage relationships of Fig. 3. The linear regressions to each set of points gave conductance values for a single monomer of 28.8 and 28.1 pS for neutral and acidic pH, respectively. The large-size transitions observed in Fig. 2A and B originate from the simultaneous closure of many monomers, as documented by us and others [4–7,9]. For example, the trace of wild-type OmpC at bath pH 7.2 shows a closure of 4 monomers (7.0 pA), but only 3 monomers (5.0 pA) at pH 5.4 in this particular stretch of data (Fig. 2A). The two recordings of Fig. 2B show well-defined closures of the same size (8.4 pA), corresponding to the closures of 5 monomers; some more transient closures of single monomers are also observed in the left-most trace of Fig. 2B. The size of the well-defined closing event seen at pH 5.4 in the E109Q mutant trace (Fig. 2C) is also identical to the smallest conductance

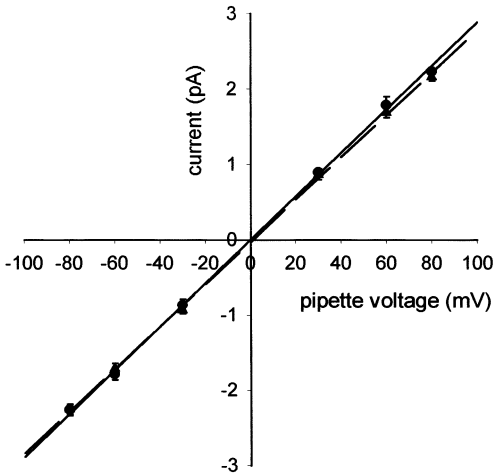

Fig. 3. Current-voltage relationships of wild-type OmpC channels obtained at pH 7.2 (circles, solid line) or pH 5.4 (triangles, dashed line). The data is averaged from 3 experiments. Error bars are S.D. and may lie within the thickness of the symbols. Regression analysis was performed with Sigma Plot software.

level observed at neutral pH (~3 pA at −60 mV, for this mutant).

Since the conductance of single monomers is the same at neutral and low pH, we propose that acidic pH leads to a reduction in the number of open channels. For this, two mechanisms can be envisioned: either a physical block of some open pores by impermeant protons, or a proton-dependent stabilization of closed non-conducting states such that pore opening is less likely (an allosteric mechanism). As discussed [21], it is difficult to distinguish between these two possibilities. Proton block is not uncommon in eukaryotic channels, where the protonation of key residues at the narrowest part of the channel can inhibit the flux of permeant ions [21]. In porin, a blocking mechanism may be unlikely because of the large size of the pore and the wide separation between titrable groups [2,22], although it cannot be ruled out with the available data. The allosteric hypothesis, however, is supported by the observation that the probability of channel openings is also decreased by pH (see below).

Traces of wild-type activity are shown in Fig. 4B on an expanded time scale to highlight downward deflections from the baseline (labeled 'BL'). These deflections are clearly distinct from noise, illustrated in the top trace, and correspond to the openings of additional channels. Although porins are not purified, we know that these openings are made by porin channels because their kinetics are altered in OmpC mutants (Fig. 2; see also [13] and [18]). Our working hypothesis is that channels can occupy at least two open states: a short-lived open state that yields the transient, ill-resolved, downward spikes from the baseline, and a long-lived stable state that gives rise to the prolonged dwell times at the baseline level [9,13,18]. In some mutants, such as K16Q, the frequency of transitions to the short-lived open state are greatly increased, as seen in Fig. 2.

Fig. 4B and C show that there is a drastic decrease in the frequency of downward deflections (openings), but no change in the upward deflections (closures), when the bath solution is switched to pH 5.4 buffer. The suppression of these openings from the baseline level – with no concomitant increase in the frequency of upward deflections – is consistent with a stabilization of a closed state, that is distinct from the closed state visited during the upward transitions from the baseline. Similarly, a decreased opening activity from the baseline is also observed in patches where the pipette solution is at pH 5.4 from the onset of the experiment (Fig. 4D). Because porins examined in a reconstituted system and in native cells display the same asymmetric voltage dependence [7,23], we know that the pipette side corresponds to the natural extracellular side. Thus, acidic pH can exert its effect from both sides of the membrane.

In order to quantitate this effect, we have generated amplitude histograms from 30-s stretches of recordings before and after the change in pH of the bath solution. Fig. 4E and F display the data obtained at neutral pH (dotted line) and acidic pH (solid line) for the wild-type (panel E) and the K16Q (panel F) OmpC porins. We arbitrarily assigned a value

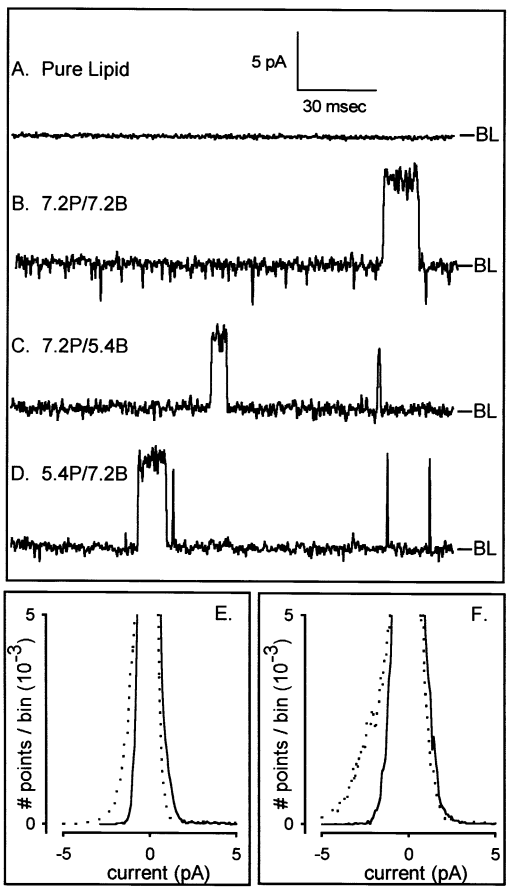

Fig. 4. Acidic pH suppresses short-lived openings in wild-type and K16Q channels. Panels A–D: Current traces were obtained from liposomes containing either no porins (panel A) or wild-type OmpC (panels B–D) at the pHs indicated in the bath ('B') and the pipette ('P'). Downward deflections from the baseline current level ('BL') represent transitions to the short-lived open state and are mostly visible at neutral bath pH (panel B). The pipette voltage was −60 mV. Panels E, F: Amplitude histograms were obtained from current records of wild-type (panel E) and K16Q mutant (panel F) OmpC porin when the bath pH was either 7.2 (dotted line) or 5.4 (solid line). The pipette pH was 7.2 and the voltage was −60 mV. The baseline current is arbitrarily assigned a value of 0 pA, and openings have negative values. Only the foot of the baseline peak is shown for the sake of clarity. A rightward shift of the left shoulder represents a decrease in the occurrence of openings. The bin size was 0.1 pA.

of 0 pA to the favored current level defined as the baseline (BL), and negative current values to opening events from the baseline (downward deflections in Figs. 2 and 4). These openings are so transient that they appear only as a shoulder of the peak at the baseline level. To obtain a clearer distinction of these shoulders between different pH conditions, only the foot of the baseline peak is shown. An assessment of the suppression of openings can be obtained by comparing the negative abscissa of points that have the same ordinate value (500 points per bin) and are taken from the two curves. A shift of this parameter, which we call I_{500} (for current I at the 500-point mark), is an indication of changes in the frequency of short-lived opening transitions. Thus, in Fig. 4E, the shift of I_{500} from −1.96 pA (dotted line) to −1.00 pA (solid line) is due to the disappearance of unresolved openings of wild-type channels in acidic pH conditions. A similar shift is observed for K16Q channels (Fig. 4F) with values of I_{500} of −3.79 pA (dotted line; neutral pH) and −1.64 pA (solid line; acidic pH). The average shifts in I_{500} and standard deviations are 0.76 ± 0.15 ($n = 4$) and 1.85 ± 0.44 ($n = 3$) for wild type and the K16Q mutant, respectively. These values are significantly different from zero ($P < 0.005$ for wild type and $P < 0.01$ for K16Q, paired-sample t-test).

Karshikoff and colleagues proposed the existence of a conserved cluster of positive charges in the β-barrel lining the constriction zone of OmpF and PhoE [22]. They suggested that two members of this cluster (R82 and R132 in OmpF) might have anomalous titration properties such that they behave as a single titrable site of $pK_a \sim 7$. If this site is part of the pH sensor, its enhanced protonation at acidic pH might trigger the observed kinetic changes. In order to test whether this site might participate in pH sensing, we mutagenized the homologous residue of OmpC (R124) to uncharged glutamine. In the R124Q mutant, acidic pH still produced a significant ($P < 0.005$, paired-sample t-test) reduction in the total current through the patch, although milder than in wild type ($12 \pm 5\%$, $n = 7$). In 4 out of 7 experiments, the opening transitions from the baseline, however, appeared enhanced (Fig. 5A and B); the I_{500} values shifted towards the left by an average value of 0.37 ± 0.21 (Fig. 4C), which is significantly different from zero ($P < 0.025$). In the other experiments, the I_{500} value was either unchanged or slightly decreased, although the traces still showed an increase in fluctuations around the baseline. In no case was a strong suppression of openings seen, as in wild type and the E109Q and K16Q mutants. The lack of suppression of short-lived openings despite a decrease in total current through the patch strengthens the proposal that distinct mechanisms exist for the pH-dependent inhibition of the short-lived open state and the reduction of the number of open pores. Because the mutation at the R124 residue alters the response of the channels to acidic pH, these results also suggest that arginine 124 may be part of the pH sensor that controls the kinetics of the short-lived open state.

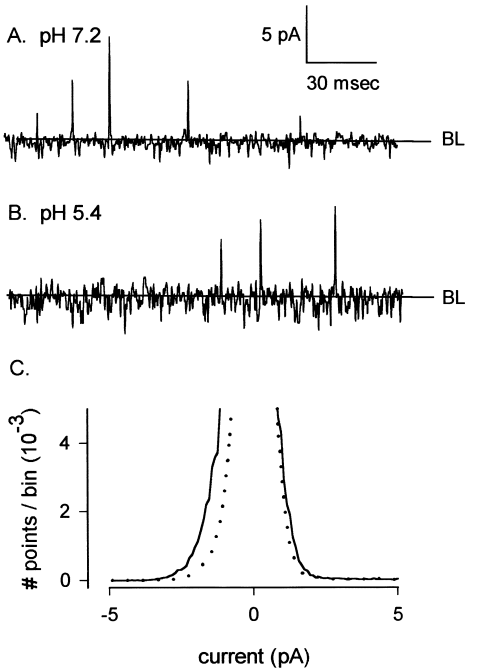

Fig. 5. The R124Q mutation alters pH sensitivity. A, B: Current traces were obtained from liposomes containing the R124Q mutant OmpC porin at the indicated bath pHs. Downward deflections from the baseline current level ('BL') represent transitions to the short-lived open. C: Amplitude histograms obtained from 30-s recordings at a bath pH of either 7.2 (dotted line) or 5.4 (solid line). The baseline current is arbitrarily assigned a value of 0 pA, and openings have negative values. Only the foot of the baseline peak is shown for the sake of clarity. A leftward shift of the left shoulder represents an increase in the occurrence of openings. The I_{500} values were −1.83 pA (pH 7.2) and −2.23 pA (pH 5.4). The bin size was 0.1 pA. In all panels, the pipette pH was 7.2 and the voltage was −60 mV.

4. Discussion

Former studies have documented pH-induced changes in conductance and voltage sensitivity [5,14–16]. It was proposed that multiple states of porins might exist in equilibria that are affected by pH, and thus that porins displayed a certain degree of functional 'plasticity'. Our finding that acidic pH stabilizes closed states of porins agrees with the conclusions presented by others that pH causes the porin protein to switch between various conformations. The effect of acidic pH, however, is more complex than previously described, since it appears to target two distinct open states. It comprises the inhibition of transitions to a short-lived open state, and the permanent closure (inactivation) or block of some channels from a long-lived open state (as reflected in a decrease in the baseline current). This later phenomenon must have occurred rapidly, during perfusion of the bath solution (~2–3 min). It is akin to the voltage-induced porin closure [3,4,6,14] and to some aspects of modulation by polyamines [11]. The pH effects are reversible. Both the original baseline current level and opening transitions are restored upon return to neutral pH. Thus, these effects are specifically promoted by pH and do not reflect protein denaturation or other non-specific alterations in pore function.

The effect of acidic pH on conductance appears more variable. Some studies report an increase [5], or a decrease [14,15], or no change [16]. We were also unable to detect any change in the wild-type and the mutant channels shown here, in agreement with Saint and colleagues [16]. We do not have an explanation for these discrepancies, except that since the channels open and close cooperatively, any modulation of the cooperativity might be interpreted as a change in conductance. Indeed, Xu and colleagues suggested that a pH drop

might promote a less cooperative behavior of the channels [14].

Karshikoff and colleagues calculated the pK_a of all ionizable residues in OmpF and PhoE on the basis of the X-ray structure [22]. They proposed that two arginine residues (R82 and R132) of the β-barrel wall directly opposed to the L3 loop might behave as a single unit with a pK_a of ∼7.0. They suggested that the protonation of this single site at low pH might enhance the attraction of E117 at the tip of L3 towards the opposite barrel wall, and hence be responsible for the functional changes introduced by low pH exposure. Such a scenario implies that the mutation of E117, R82 or R132 to non-polar residues would abolish the pH sensitivity of porin kinetics. Here we show that the removal of charge at position 109 of OmpC (corresponding to E117 in OmpF) does not inhibit pH sensitivity, nor does the K16Q mutation. However, the substitution of arginine for glutamine at position 124 (corresponding to R132 in OmpF) alters the pH response. These results suggest that E117 and K16 of OmpF (E109 and K16 of OmpC) do not play a major role in pH sensing, although it is possible that multiple mutations need to be introduced to completely abolish pH sensitivity. Arginine 132 (R124 in OmpC), however, may be a key determinant, but not via an interaction with E117, as hypothesized [22].

One of the responses of *E. coli* to acidic pH is the down-regulation of *ompF* expression [24]. Presumably, there is an adaptive advantage in reducing proton influx in the periplasm because of the damaging impact it can have on the cell and/or on metabolic processes of the cytoplasmic membrane. It is conceivable that the physiological relevance of the modulation of porin *function* by pH also resides in the context of a response to pH stress. Determining the pH sensitivity of strains harboring porin mutants with pH-insensitive electrophysiological phenotypes should shed some light on this interesting possibility.

Acknowledgements: We thank Linda Guynn for technical assistance, and Ramkumar Iyer for reading the manuscript. Daniel T. Colbert and Richard E. Smalley are acknowledged for the use of the Insight II Molecular Graphics Program. The work was supported by NIH Grant AI34905.

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