Comparisons and contrasts illuminated by random mutagenesis

Functional Design of Bacterial Mechanosensitive Channels

MscS and MscL are mechanosensitive channels found in bacterial plasma membranes that open large pores in response to membrane tension. These channels function to alleviate excess cell turgor invoked by rapid osmotic downshock. Although much is known of the structure and molecular mechanisms underlying MscL, genes correlating with MscS activity have only recently been identified. Previously, it was shown that eliminating the expression of Escherichia coli ηggB removed a major portion of MscS activity. ηggB is distinct from MscL by having no obvious structural similarity. Here we have reconstituted purified ηggB in proteoliposomes and have successfully detected MscS channel activity, confirming that purified ηggB protein encodes MscS activity. Additionally, to define functional regions of the channel protein, we have randomly mutagenized the structural gene and isolated a mutant that evokes a gain-of-function phenotype. Physiological experiments demonstrate that the mutated channel allows leakage of solutes from the cell, suggesting inappropriate channel opening. Interestingly, this mutation is analogous in position and character to mutations yielding a similar phenotype in MscL. Hence, although MscS and MscL mechanosensitive channels are structurally quite distinct, there may be analogies in their gating mechanisms.

Essentially all living organisms detect mechanical forces. The senses of balance, touch, and hearing are but a few of the systems within humans that are dependent upon this ability. Microorganisms, too, must detect forces resulting from osmotic gradients across their cellular envelope. Electrophysiological studies in essentially all of these systems have implicated mechanosensitive (MS) channels as one of the major mechanisms by which these forces are sensed (see Ref. 1 for review of MS channels). Despite their importance to human life and health, little is known of how eukaryotic MS channels function in vivo, and functional reconstitution of such a channel into a defined lipid system has not yet been reported. In contrast, prokaryotic and archaeal MS channels have been well characterized and serve as model systems where general mechanisms of gating may be studied.

The first gene shown to encode an MS channel activity was mscL (for mechanosensitive channel of large conductance), which was originally isolated from Escherichia coli in 1994 (2) (see Refs. 3–9 for recent reviews). Functional reconstitution of this purified protein in proteoliposomes demonstrated that the mscL gene, with no other auxiliary proteins, was all that was necessary for channel activity (10, 11). It is now clear that this channel plays a vital role in osmoregulation by opening a large conductance pore in response to acute decreases in osmotic environment, referred to as osmotic downshock, thus playing the role of an in vivo “emergency release valve.” Because of its tractability and parsimonious design, this channel is emerging as a paradigm for mechanically induced channel gating. Mutagenesis studies (10–13) combined with structural data for the closed conformation, derived from x-ray crystallography (14), suggest a model for gating (reviewed in Ref. 4). Recent predictions of a second gate and projections of one possible open state (15, 16) and dynamic simulations (17) have further refined our understanding of this channel.

From an early study of bacterial mechanosensitive channel activities, it became clear that there was at least one additional major E. coli MS activity, MscS (mechanosensitive channel of smaller conductance), that could be reconstituted in azolectin proteoliposomes and remain electrophysiologically active (18). However, the molecular entity(s) responsible for MscS activity remained undefined. Although homologues of MscL are observed throughout the bacterial kingdom (19, 20), and in all cases studied have been shown to encode MS channels (20, 21), no homologues were found within E. coli that could encode the MscS activity, thus implying it belonged to a new gene family. Recently, Levin et al. (22) described a gain-of-function (GOF) mutant of the E. coli kefA (also in the data base as aefA) gene, which subsequently led to the discovery of a large family of genes. In this study, the authors demonstrated that a double null mutant of kefA and a homologue, ηggB, led to an E. coli strain lacking detectable MscS activity. Although the ΔkefA strain contained MscS activity essentially indistinguishable from the wild-type strain, the ΔηggB strain expressed an MscS activity in only a minority of patches, and it did not desensitize as readily as most activities observed in the parental strain. In addition, although the Δηgg, ΔmscL, but not the ΔkefA, ΔmscL, strain exhibited cell lysis upon acute osmotic downshock, there was no apparent increase in this phenotype in the triple null mutant. Although ηggB and kefA are apparently functionally related, and have homologues in essentially all bacterial species, they bear no obvious relation to MscL, suggesting a distinct family. Although it remained unclear whether kefA and ηggB normally formed a heteromultimer,
formed two distinct homomultimers (or monomers), or were simply regulators, it was clear that YggB played a critical role in sustaining the MscS activity and in osmoregulation.

Here, we have utilized the previous observation that this channel can survive biochemical purification and subsequent reconstitution to confirm that, similar to MscL, no other proteins are necessary for activity. We have also utilized a random mutagenesis approach to isolate a mutant yielding a GOF phenotype. Whole-cell physiological data from the isolated mutant are consistent with the hypothesis that the mutation within YggB leads to a channel that leaks solutes, including potassium and protons. The location of the lesion within the protein is analogous in position and character to mutations previously noted in MscL that were isolated in an identical screen and that yield a similar phenotype.

EXPERIMENTAL PROCEDURES

Strains, Cell Growth, and Viability Determination—The E. coli strain MJF451 (yggB) (22) was used to host expression constructs and for growth studies. Many experiments were repeated in PB111, a recA variant of this strain, generated as previously described for PB104 (2, 23); similar results were obtained for expression within MJF451 or PB111. The E. coli strain MJF455 (ΔmscL.Cm, yggB) (22) is a derivative of the former strain, and was utilized in experiments to determine whether wild-type and mutated YggB mediate the osmotic downshock-induced GOF phenotype. All cultures were grown at 37 °C with shaking at 250 rpm in one of the three following media (as indicated): Lennox Broth (LB), K10 (25) (46 mM NaNO3, 23 mM NaH2PO4, 8 mM (NH4)2SO4, 0.4 mM MgSO4, 6 μM FeSO4, 1 μM of thiamine, 0.2% glucose), or McIlvaine’s medium (pH 7.0) (26) (60 mM NaH2PO4, 5 mM citric acid, 8 mM (NH4)2SO4, 0.4 mM MgSO4, 6 μM FeSO4, 1 μM of thiamine, 0.2% glucose). For plasmid-bearing strains ampicillin (100 μg/ml) was added; expression was induced where indicated by addition of isopropyl-β-d-thiogalactopyranoside (IPTG) (1 mM) to the growth medium. Expression was induced for 20 min to 1 h for in vivo experiments, and 2 h for electrophysiological experiments.

Growth curves were generated from cultures of E. coli PB111 expressing the YggB protein in trans and treated (or not) with 1 mM IPTG to induce expression at the times indicated. Cell viability was determined by diluting cultures into medium or buffer in 20-fold serial dilutions, plating onto LB ampicillin agar plates, and counting the resulting colony-forming units (CFU) per OD600 unit after incubation at 37 °C for 15 h.

Resinatton and Electrophysiological Characterization—The polyhistidine-tagged wild-type YggB protein and V40D mutant YggB were purified and reconstituted as previously described (21). Briefly, cells were grown in LB and expression induced by 1 mM IPTG (1 mg/ml) for 1 h. The cells were washed twice with the following buffer: 50 mM potassium phosphate buffer (pH 7.4), 5 mM MgSO4, and 1 mM dithiothreitol. The cells were then treated with 1 mg/ml lysozyme, 0.5 μg/ml DNase, and 1 mM phenylmethylsulfonyl fluoride for 15 min at room temperature; additional lysozyme (1 mg/ml) was then added and the sample incubated for an additional 15 min. The cells were disrupted by French press twice at 16,000 p.s.i. Unbroken cells and cell debris were pelleted by 6,000 × g for 10 min, and membrane vesicles were isolated from the supernatant by ultracentrifugation (Beckman Ultra TLA100.3 rotor) at 265,000 × g for 30 min, and stored at −80 °C until use. To extract the proteins, the membrane pellet (−0.5 g) was resuspended in 8 ml of extraction buffer (300 mM KCl, 50 mM potassium phosphate buffer (pH 8.0), 20 mM imidazole, and 3% octyl glucoside (Anatrace)) and sheared to homogeneity in a glass Dounce homogenizer. Insoluble particles were removed by ultracentrifugation (Beckman Ultra TLA100.3 rotor) at 346,000 × g for 20 min. The supernatant was incubated at 4 °C for 30 min with 0.5 ml, washed, equilibrated with nickel-nitrotriacetic acid agarose (Qiagen), and packed in a Poly-prep column (Bio-Rad). The column was washed with 10 bed volumes of extraction buffer and then eluted by an elution buffer: 300 mM NaCl, 50 mM potassium phosphate buffer (pH 8.0), 1% octyl glucoside, and a one-bed volume step gradient of 50, 75, and 200 mM imidazole. The eluted fractions were resolved in a 12% SDS-PAGE minigel (Bio-Rad) and stained with Coo massie Blue. An apparently homogeneous fraction bearing a 30-kDa band of purified YggB was selected for reconstitution, at a 1.500 (w/w) protein/lipid ratio, into defined membranes consisting of dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-diacyl-sn-glycero-3-phosphoethanolamine (DOPE) in a 1:1 mass ratio using techniques described previously (21).

The reconstituted proteoliposomes were assayed for functional MS channel activity as described previously (21). Briefly, rehydrated proteoliposomes were patch-clamped under symmetrical buffer conditions; 150 mM KCl, 30 mM MgCl2, 0.1 mM EDTA, 10 μM GTP, 5 mM HEPES (pH 7.2). Recordings were performed at +20 mV (electrode). Data were acquired at a sampling rate of 20 kHz with a 5-kHz filtration using an AxoPatch 200B amplifier in conjunction with Axoscope software (Axon). A piezoelectric pressure transducer (World Precision Instruments) was used to follow the pressure response of the channels.

Pharmacology and Genetic Techniques—The wild-type yggB gene (P11666) was amplified from a pET21 vector containing this gene as an insert (kindly supplied by Dr. I. R. Booth) by PCR using Vent DNA polymerase and standard reaction conditions (27). Linkers, Sall (5′) and Nol (3′), were added to facilitate the directional cloning of the fragment into pBlb10, and a hexahistidine tag was inserted at the carboxyl terminus. The oligonucleotide primers used were 5′ linker GTC GAC GTC GAC CCC TCT AGA AAT TTT GTT T and 3′ linkers CGC TTA GTG GTG ATG ATG ATG GCC ACG TTT GTC TTC TTT and GCG GCC GCG CCG CGG CTT ATG GTG GTG GTG GTG CAT GCG CAG C, which were used in two successive amplifications. For molecular manipulation, the yggB gene was initially ligated into the polylinker of the vector pBluescript II (Stratagene). The sequence was verified, the plasmid amplified, and the insert subsequently transferred to the expression vector pBlb10 (12, 20, 22) for inducible expression in trans.

Random mutagenesis was performed by two methods. First, the mutator strain E. coli XL1-Red (Stratagene) was transformed with the pBl10yggB plasmid DNA, grown, and isolated as recommended by the manufacturer. Mutated yggB was subsequently subcloned into pBlb10 for expression screening. Second, error-prone PCR using Taq polymerase and varying MnCl2 concentrations was used as outlined in established protocols (28, 29). Reaction products were gel-purified and ligated into pBlb10.

The yggB E. coli strain, PB111, was used to host the mutated yggB expression plasmid for screening purposes. As described previously (12), newly transformed cells were initially cultured on LB-ampicillin plates at 37 °C for 15 h. Subsequently, colonies were replica plated onto LB-ampicillin with 1 mM IPTG and grown at 37 °C for 15 h. GOF mutants were scored for suppressed growth in the presence of IPTG-induced expression. Colonies showing such depressed growth were streak-purified and re-screened. Persistence of phenotype received a positive score, and plasmid DNA was isolated, PB111 transformed, and the resulting colony-purified and re-screened. The two clones that maintained the phenotype under these conditions were sequenced on both strands, by automated sequencing (PerkinElmer Life Sciences), and analyzed using GENETYX-MAC/Version 8.0 (Software Development Co., Tokyo, Japan) and DNASTAR (Madison, WI). Such analysis demonstrated that both clones, resulting from a single error-prone PCR reaction, were identical in the mutated residues.

Amino acid mutagenesis of the Val-40 mutant at the Val-40 position was mediated by utilizing a QuikChange™ site-directed mutagenesis kit (Stratagene) following the manufacturer’s recommendations. The Val-40 codon, GCT, was changed to as follows: V40D (GAT), V40N (AAT), V40G (GGT), V40C (TGT), and V40K (AAA). Similarly, the Gly-41 codon GGT was changed to GAT to generate the single-site G41S mutation.

Bacterial Whole-cell Physiology—Steady state intracellular potassium was assayed as previously described (12, 13). Briefly, cells were grown in K10 medium; at OD600 of 0.4, the cells were diluted 1:2 with the same medium plus salts to obtain a final NaCl concentration of 350 mM. IPTG (1 mM) was added at this time to the culture, and growth was continued for 1 h. To harvest, 1 ml of the culture was filtered onto a 25-mm diameter 0.45-μm nitrocellulose filter (Millipore) and washed with 3 ml of 37 °C K0 buffer (46 mM NaH2PO4, 35 mM Na2HPO4, and 8 mM (NH4)2SO4) with 350 mM NaCl. The filters were then placed in a 50-ml plastic beaker and dried at 80–95 °C overnight; solutes within the dried filters were then resuspended in 3–4 ml of double-distilled water. The resulting solution was then assayed for potassium by flame photometry (Buck Scientific, PFP10).

Functional Design of Mechanosensitive Channels

For functional design of osmotic and acid shock experiments, cells were grown in McIlvaine’s medium (pH 7.0). At early log phase, the culture was diluted into the same medium to a final concentration of 0.6× NaCl. At an OD600 of ~0.2, the cells were induced with IPTG (1 mM) and growth continued for 20 min. The cells were diluted 1:20 in McIlvaine’s medium with the NaCl concentration and pH indicated in text and legend of Fig. 4. After incubation for 30 min, the cultures were diluted in isotonic buffer and viability determined as described above.
FIG. 1. yggB encodes an MscS activity. Polyhistidine-tagged YggB was purified to near homogeneity by metal-chelation chromatography (A) as evidenced by a predominant 30-kDa band in a Coomassie-stained SDS-PAGE gel; a fainter band at 60 kDa, consistent in size with a nondissociated dimer, is also seen; upon gross overloading, a band at 90 kDa, assumed to be a trimer, was also observed. This fraction was reconstituted at a 1:500 (w/w) protein to lipid ratio in DOPC:DOPE (1:1) proteoliposomes. Mechanosensitive channel activity, similar to that observed in intact E. coli spheroplasts (data not shown), was observed by patch clamp analysis (B). The proteoliposomes were studied under symmetrical buffer conditions at $+20 \text{ mV}$ pipette potential. Channel openings are upward (O$_1$–O$_3$), and pressure is indicated in the lower trace with a pressure maximum at $-40 \text{ mm Hg}$.

A previously published approach (21) was utilized to determine whether the wild-type or mutated YggB could reconstitute the osmotic downshock-induced lysis phenotype of the yggB,ΔmscL double-null strains (MJF455 and PB112; see above). Briefly, cells were grown to early log phase in LB, diluted 1:2 with the same medium plus salt to achieve a 0.5 mM NaCl final concentration. At this time protein expression was induced by addition of 1 mM IPTG. After 1 h of growth, the cells were diluted 1:20 in sterile water (or isometric medium as the control) and incubated for 20 min prior to viability determination.

RESULTS

Polyhistidine-tagged and Highly Enriched YggB Is Functional when Reconstituted in Liposomes—Six histidine codons were fused in frame proximal to the stop codon of the yggB gene. This allowed purification to near homogeneity by a nickel-nitritotriacetic acid column (Fig. 1). When this fraction was reconstituted in synthetic lipids (DOPC:DOPE, 1:1), and examined by patch clamp analysis, MscS activity essentially indistinguishable from that observed in native membranes was observed (Fig. 1). The conductance at positive and negative potentials, the absence of rectification, and the reversibility of channel desensitization for the channels were similar to those previously found in spheroplast preparations (12, 22, 30). In addition, expression of the tagged protein reconstituted normal activity in E. coli strains deleted of the endogenous yggB gene (MJF451 and PB111; data not shown). Hence, the carboxyl-terminal histidine tag does not appear to alter channel activity. In addition, the data indicate that, similar to MscL, YggB directly senses and responds to membrane tension; no other components are necessary.

Random Mutagenesis and Screening for a GOF Phenotype Leads to Identification of YggB Functional Mutations—Two independent strategies were utilized to randomly mutagenize the yggB gene: passage through the Stratagene XL-1 Red mutagenic E. coli strain, and error-prone PCR, as described under “Experimental Procedures.” Because the polyhistidine-tagged protein functioned normally (above and Fig. 1), this modified protein was used for these mutagenesis studies and all subsequent experiments. Cells exhibiting a GOF phenotype of no, or slowed, growth upon expression of mutated YggB were isolated by using a replica plate strategy previously described for MscL (12), and outlined under “Experimental Procedures.” Of $\sim 750,000$ colonies screened, all expressing YggB with various degrees of mutation, only two clones were isolated from a single high mutation frequency error-prone PCR protocol. Upon sequencing the mutated yggB genes, three identical mutations were found within both clones: a T to A mutation, yielding a valine to aspartate transition at position 40; a G to A mutation, leading to a glycine to serine transition at position 41; and a T to A, which is a silent mutation maintaining the residue at position 67 as an alanine.

Expression of YggB V40D Leads to a GOF Phenotype without Loss of Cell Viability—To test if the phenotype was attributable primarily, or exclusively, to one of the missense mutations, each was generated individually by site-directed mutagenesis. Expression of YggB bearing only the V40D mutation reconstituted the full GOF phenotype; no significant perturbation of growth was observed for the G41S mutated YggB (not shown). As with MscL GOF mutants, the phenotype also was observed in liquid culture (11–13). As shown in Fig. 2, uninduced cultures grow normally; however, IPTG-induced expression of the V40D YggB mutated protein, but not wild-type or vector controls, led to a cessation of normal growth.

Previously, in similar studies with MscL, we found that severe mutations can either lead to cell death or retard growth (12, 13). Hence, we tested viability in cultures identical to Fig. 2 in which the cells had been induced (or not) for 1 h. All cultures, including the induced V40D YggB strains, showed normal viability (ranging from 2 to $5 \times 10^8$ CFU/OD$_{600}$ unit) with no significant differences. Hence, expression of the YggB V40D protein leads to a cessation of growth without decreasing viability.

Expression of the YggB V40D Mutated Protein Leads to Increased Cell Permeability to Potassium and Protons—Mutations within the MscL protein that can lead to a GOF phenotype also normally lead to a leakage of solutes, as assayed by steady-state potassium levels or increased efflux of potassium upon osmotic downshock (12, 13). To test whether expression of YggB V40D similarly leads to a leakage of solutes, we assayed steady-state potassium levels in cells expressing YggB V40D and appropriate controls. As seen in Fig. 3, cells that were induced for expression of YggB V40D had approximately two-thirds the steady-state intracellular potassium levels. No similar reduction was observed for uninduced controls, or cells expressing wild-type YggB.

An additional test for increased cellular membrane permeability has recently been developed and utilized for assaying MS channel activity (22, 31). This assay utilizes the observation that a significant drop in intracellular pH will cause the bacterium to lose viability. As seen in Fig. 4, if cells expressing wild-type YggB are placed in a low pH buffer (pH = 3.6), the viability is 20%. A significantly greater decrease in viability is observed upon osmotic downshock, apparently because of the opening of MS channels that will allow equilibration of protons across the membrane, thus decreasing cytosolic pH. In contrast, cells expressing YggB V40D have a profoundly decreased
viability even without osmotic downshock. These data are consistent with increased membrane permeability independent of osmotic changes. Thus, both the steady-state potassium levels and the increased sensitivity to acidic environments strongly suggest that expression of YggB V40D leads to increased membrane permeability.

Expression of YggB V40D Remediated the Osmotic Downshock-induced Lysis Phenotype of ΔyggB,ΔmscL Double-null Strains—Previous studies have demonstrated that ΔyggB,ΔmscL double-null strains are acutely sensitive to osmotic downshock. This osmotic downshock lysis phenotype is remediated by expression, in trans, of either yggB or mscL (21, 22). It was therefore of interest to determine whether YggB V40D remediated this phenotype. As in a previous study (21), a severe osmotic shock, which kills a significant proportion of wild-type cells, was utilized. As shown in Fig. 5, cells expressing YggB V40D were as resistant to osmotic downshock as cells expressing wild-type YggB.

Only Highly Hydrophilic Substitutions at the YggB Val-40 Residue Lead to the GOF Phenotype—Aspartate is both larger and more hydrophilic than valine. Under most physiological conditions, the former will, in fact, be negatively charged. Hence, it could be the increased size of the residue, the addition of a negative charge, or substitution of a highly hydrophilic residue at this position that yields the GOF phenotype. To test these possibilities, we mutated Val-40 of YggB to asparagine and lysine by site-directed mutagenesis. As shown in Fig. 6, expression of YggB V40K yielded a GOF phenotype indistinguishable from the V40D mutant. In contrast, expression of YggB V40N yielded a strain indistinguishable from wild-type YggB. Hence, it does not appear to be size (Asn is similar size to Asp) or addition of a negative charge (because Lys is positively charged), but the dramatic increase in hydrophilicity that leads to a channel evoking a GOF phenotype. Consistent with this hypothesis, we also found that mutation of this site to glycine or cysteine also did not evoke a GOF phenotype (data not shown).

**DISCUSSION**

Previous work demonstrated a correlation between a channel activity and yggB expression and predicted that it may be the pore-forming unit; however, a regulatory role could not be
ruled out (22). Here we demonstrate that _yggB_ does indeed encode an MS channel activity indistinguishable from that characterized previously in _E. coli_ spheroplasts. Our data are consistent with previous studies suggesting that distant members of this family from the archaea encode MS channel activities (32, 33). Our data are also consistent with the observation that removal of _YggB_ and _MscL_ from _E. coli_ is sufficient to evoke a lysis phenotype upon osmotic downshock (22). Other homologues within _E. coli_ or _Erwinia chrysanthemi_, where they have been best characterized, appear to be difficult or as yet impossible to observe in patch clamp (22, 31, 34). Many of these homologues contain the core three to four transmembrane domains predicted in _YggB_ (below), but often contain additional hydrophobic and hydrophilic regions; presumably these additional components add functionality and regulation of channel gating, explaining the difficulty of both, determining conditions in which these proteins function, and observing their activities in patch clamp. The alternative that cannot yet be formally discounted is that not all members of this family encode MS channels.

Similar to a previous study with _MscL_ (12), we have utilized a random mutagenesis approach for studying functional regions of the _YggB_ protein. However, in contrast to _MscL_, GOF mutants were not easily obtained. Although we use identical protocols, screening over 10-fold more colonies and varying the mutagenic rate, we only isolated a single GOF mutant. Of course, random mutagenesis is never strictly random. Some changes, even using error-prone PCR, are seen frequently, whereas others are quite rare. Even in the _MscL_ random mutagenesis study, above, some mutations were discovered 10 times, and others only once. The _V40D_ mutation is evidently quite rare. Even using identical mutation conditions on independent occasions, we were unable to isolate more than the two isolates of the _V40D_ mutation mentioned here. This is despite the observation that three mutations were found in the original mutated gene, suggesting a very high mutation rate. If other single-site _yggB_ mutations exist that evoke a GOF phenotype, we suspect that they must be equally rare. One interpretation of the lack of mutations that evoke a GOF phenotype may be that there is more than one lock, or gate; minimally, _YggB_ gating may be more complex than _MscL_.

The _V40D_ mutated _YggB_ isolated in this study evokes a GOF phenotype that has several similarities to those observed previously with mutated _MscL_. First, both _YggB_ and _MscL_ mutant-induced phenotypes are observed on plates, in liquid culture, and in both minimal and rich media (Fig. 2 and data not shown). Second, phenotypically, upon expression of the mutated _YggB_, the cell growth is drastically decreased without a substantial decrease in viability. This too has been observed with mutated _MscL_ protein expression (12, 13). Finally, the mutation leads to a “leak” of solutes. In this study, we demonstrate that steady-state levels of potassium are decreased upon expression of the _YggB_ mutant (Fig. 3). In addition, the decreased viability because of acid shock and acid shock combined with osmotic downshock strongly suggests that protons can also permeate the cell envelope (Fig. 4). This latter finding strongly suggests that the proton-motive force across the inner membrane is no longer intact. Because many cellular functions are dependent upon this transmembrane potential, it is likely that this loss is ultimately what leads to inhibition of cell growth. Analogously, our previous studies demonstrated that low steady-state potassium levels were common among _MscL_ GOF mutants (12, 13); although proton permeability was not determined in this study, given the lack of ion preference of this channel, it is likely that the _MscL_ mutations also induce proton leakage.

One of the contrasts between mutants of _MscL_ and _YggB_ is that of channel activity. Using standardized protocols (23), no channel activity has yet been detected for _YggB_ _V40D_ in either spheroplasts or the reconstituted liposome system, even though the mutant protein was as easily purified as wild-type _YggB_ (data not shown). Note, however, that GOF mutants of the _Mycobacterium tuberculosis_ _MscL_ homologue have been generated that are extremely difficult to detect by patch clamp because of their tendency to gate only at very high pressures (21); to date no one has explained this apparent paradox. In addition, a recent study (35) suggests gating of an _E. coli_ _L19C_ mutant _in vivo_, although channel activity has not yet been observed in patch clamp. Similarly, we have recently generated _MscL_ mutants that evoke a GOF phenotype but cannot be assayed by normal patch clamp procedures. The finding here that expression of _YggB_ _V40D_ rescues the Δ_oggB_Δ_mscL_ cell from lysis upon osmotic downshock could be interpreted that this mutated channel still gates a channel _in vivo_ in response to membrane tension, and therefore must need some factor or environment only found in the cell. Alternatively, the suppression of the downshock-induced lysis phenotype may be secondary to the solute leakage, which decreases the cell turgor and consequently makes the cell less sensitive to osmotic downshock. If true, the data suggest the channel may be “locked” into a partially open conformational state not detectable by patch clamp. Alternatively, the channel may be gating, but the open dwell times are too short to be resolved by our patch clamp equipment. The observation that many _MscL_ mutants have significantly decreased open dwell times sets precedent for the latter possibility (10–12). Finally, another possibility is that an auxiliary protein or other factor (e.g., steep membrane potential) not found in our patch clamp conditions may be required for gating of the _YggB_ _V40D_ mutated protein. Unfortunately, the possibilities listed above are not easily distinguished at this time.

__Fig. 6. Cells expressing V40K, but not V40N, demonstrate a GOF phenotype. Cells were grown in LB with ampicillin. At the time indicated, half of the culture was stimulated to express either V40N or V40K YggB. Shown are typical experiments.__

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2 G. Levin and P. Blount, manuscript in preparation.
Hydropathy plots of YggB and MscL proteins appear quite distinct (Fig. 7). Members of the MscL family of channels are found throughout the bacterial kingdom, consistently contain only two transmembrane domains, and preserve highly conserved motifs throughout the protein. Members of the YggB family of channels are also found throughout the bacterial kingdom (22, 36), but they are also found in the archaea, yeast, and plants (36), and all members preserve a few highly conserved motifs, invariably at the carboxyl end of the protein.3 YggB is one of the most streamlined members of the family, probably containing only three transmembrane domains as suggested from studies of KefA (31); recent results suggest this topology is also true of the YggB protein4 (see Fig. 7). Other members of this family, including KefA, have additional transmembrane domains and hydrophilic elements that are presumably involved in regulatory roles. Although a common evolutionary origin for YggB and MscL has been proposed (32), there are problems with this hypothesis.5 First, there is no obvious sequence homology conserved between motifs preserved within each of the two families. Second, the observation that highly homologous members of the YggB family from Methanococcus jannaschii, which share 44% identity (33), are plotted as distant relations within the original evolutionary tree (32), suggesting that the alignment upon which the hypothesis is based is questionable. Finally, the recent proposal of the inversion of the membrane topology of the TM1s (Fig. 7 and below) makes structural homology of the aligned regions highly unlikely. On the other hand, utilizing HMMER searches, MscL (but not YggB) has been shown to share conserved motifs with the sensor module of voltage-gated and transient receptor potential channel families; the confidence level of this latter finding was $E < 0.01$, making a common evolutionary origin highly probable; here the location of the motifs is conserved relative to the proposed membrane topologies.6 Hence, it seems likely that MscL and YggB are structurally and evolutionarily distinct.

The YggB V40D mutation that evokes the GOF phenotype is in the first predicted transmembrane domain (Fig. 7). Although this is not the most conserved region of this family of proteins, there is a large degree of diversity between family members and it remains a question whether all members encode MS channels (22, 31, 34). The substitution is valine to aspartate. The addition of a charge appears to be the salient feature because mutation to the neutral cysteine, glycine, or asparagine (the latter being similar in size to aspartate) did not lead to a GOF phenotype; however, substitution to the oppositely charged lysine did. Random mutagenesis of MscL led to the isolation of 18 independent mutants that evoked a GOF phenotype (12). Here, too, the addition of a charge within the first transmembrane domain seemed to be a common trait. Sequencing confirmed that 9 of the 18 mutations were to charged residues; 14 of the 18 substitutions were in or near the first transmembrane domain, with 7 of these 14 being substitutions to charged amino acids; all but one of the 14 changes were to more hydrophilic residues (12). Interestingly, one of the most severe mutations was also a valine to aspartate substitution at position 23 in E. coli MscL. Subsequent studies demonstrated that, at one of the mutated sites, glycine 22, the increased hydrophilicity of the substitution correlated with the severity of GOF phenotype and aberrant channel properties (10, 11). These findings, combined with the channel kinetics of the mutants and the structure of the M. tuberculosis MscL (14), has led to the “hydrophobic lock” hypothesis (4). From the structure, it was noted that the most constricted portion of the closed channel pore contains some small polar (glycine) and several hydrophobic (e.g. valine) residues that form a hydrophobic lock (4, 14). The hypothesis contends that it is the “breaking” of this lock and the exposure of these residues to an aqueous environment, perhaps the lumen of the opening channel, that is the primary event and major energy barrier to full channel opening (4, 11). Under normal circumstances, this lock breaks before the membrane ruptures. One possible interpretation of the parallels between the mutations in YggB and MscL is that a similar hydrophobic lock mechanism may be at play for both

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3 A. Kumánovics and P. Blount, unpublished data.
4 I. R. Booth, personal communication.
5 A. Kumánovics, personal communication.
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**Fig. 7.** Comparison and contrasts of the functional design of MscL and YggB as illuminated by random mutagenesis studies. The topology (left) and hydropathy plot (right) for MscS (top) and MscL (bottom) are shown. DNASTAR PROTEAN software, using Kyte-Doolittle values and a window of 9 residues averaged, was used to generate the hydroplicity plots shown. The scale bar between plots shows the relative position of the residues within the protein. “Hot spots” found to yield GOF mutants via random mutagenesis are shown as hatched boxes within the topology sketch and above the hydroplicity plot. The location of the valines that generated the most severe GOF phenotype in MscL, and the only GOF phenotype in YggB are indicated in the hydroplicity plot with an arrow. Note that, although the membrane topology of YggB has not been determined, the topology presented is consistent with a study of the KefA homologue, which shares homology throughout the yggB gene (31); recent results suggest this topology is also true of the YggB protein (see Footnote 4).
mechanosensors; could this be a general mechanism, even among structurally and evolutionarily distinct families of membrane-tension-gated channels?

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