YbdG in *Escherichia coli* is a threshold-setting mechanosensitive channel with MscM activity

Ulrike Schumann1,2, Michelle D. Edwards2, Tim Rasmusson, Wendy Bartlett, Pieter van West, and Ian R. Booth3

School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

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We describe a mechanosensitive (MS) channel that has mechanosensitive channel of miniconductance (MscM) activity, and displays unique properties with respect to gating. Mechanosensitive channels respond to membrane tension, are ubiquitous from bacteria to man, and exhibit a great diversity in structure and function. These channels protect *Bacteria and Archaea* against hypoosmotic shock and are critical determinants of shape in chloroplasts. Given the dominant roles played in bacteria by the mechanosensitive channel of small conductance (MscS) and the mechanosensitive channel of large conductance (MscL), the role of the multiple MS channel homologs observed in most organisms remains obscure. Here we demonstrate that a MscS homolog, YbdG, extends the range of hypoosmotic shock that *Escherichia coli* cells can survive, but its expression level is insufficient to protect against severe shocks. Overexpression of the YbdG protein provides complete protection. Transcription and translation of the ybdG gene are enhanced by osmotic stress consistent with a role for the protein in survival of hypoosmotic shock. Measurement of the conductance of the native channel by standard patch clamp methods was not possible. However, a fully functional YbdG mutant channel, V229A, exhibits a conductance in membrane patches consistent with MscM activity. We find that MscM activities arise from more than one gene product because ybdG deletion mutants still exhibit an occasional MscM-like conductance. We propose that ybdG encodes a low-abundance MscM-type MS channel, which in cells relieves low levels of membrane tension, obviating the need to activate the major MS channels, MscS and MscL.

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1Present address: Commonwealth Scientific and Industrial Research Organisation Plant Industry, Canberra, Australian Capital Territory 2601, Australia.

2U.S. and M.D.E. contributed equally to this work.

3To whom correspondence should be addressed. E-mail: i.r.booth@abdn.ac.uk.

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YbdG Expression Is Dependent on Growth Phase and Osmolarity. The ybdG gene lies 108 bp downstream of nfnB (also called nfsB), the structural gene for dihydropteroate reductase (20). Immediately 5′ (51 bp) to ybdG is the phoP gene and its terminator lies proximal to the predicted 3′ end of the ybdG ORF (Fig. S4). There are no obvious transcriptional regulator binding sites 5′ to ybdG (Discussion), but nfnB is known to be regulated by MarA (21, 22). There is no classical transcription terminator between nfnB and ybdG. However, the ybdG gene has a putative promoter (a −10 sequence centered ~30 nucleotides 5′ to the ATG) and a potential Shine–Dalgarno sequence (centered ~8 nucleotides 5′ to ATG), indicating that the protein could be expressed independently of nfnB. We performed quantitative real-time PCR (qRT-PCR) to determine if ybdG expression occurs and to investigate coregulation of ybdG and nfnB expression. Expression was normalized against rpoB levels, which are expected to remain relatively constant (23). Both nfnB and ybdG were transcribed during exponential phase growth in LB medium, but only ybdG mRNA levels increased as cells entered stationary phase (Fig. L4). Expression of mscS and mscL has been shown to be stimulated by growth at high osmolarity in an RpoS-dependent manner (24). Growth into exponential phase in high osmolarity similarly stimulated ybdG mRNA synthesis. In contrast to mscS and mscL genes, elimination of RpoS increased ybdG mRNA abundance. Expression of nfnB was not significantly changed by an rpoS mutation. The effect of rpoS mutation was most marked at high osmolarity where it resulted in a further 10-fold increase in pools of ybdG mRNA (Fig. L4). These data can be rationalized by the known stabilization of RpoS protein at high osmolarity and in stationary phase (25). Previously, superinduction of genes in RpoS null mutants has been associated with relief of the competition between σ70 and σE (26).

Protein abundance during different growth conditions was investigated using peptide-specific antisera to YbdG (Fig. 1B). In the presence of 0.5 M NaCl, protein expression matched the pattern of mRNA production (Fig. 1A and B), confirming the role of high osmolarity in YbdG abundance in the cell. In the absence of salt, protein expression declined as the cells entered stationary phase (Fig. 1B) and this pattern was also observed in an RpoS mutant despite the increased mRNA levels. Surprisingly, in cells grown at low osmolarity there was no significant correlation between mRNA production and YbdG protein production. We infer from these data that ybdG mRNA is subject to translational control that is overcome when the cells are exposed to hyperosmotic stress.

Oligomeric Structure of YbdG. MscS is a homoheptamer (16). With significant variations in pore-lining sequences between MS channel homologs, the potential exists for alternative oligomeric structures. Using five independent preparations of YbdG, a mass range of 346 ± 25 kDa was obtained by blue native gel analysis, which after correction for dye binding (27) indicated a mass for the channel complex at 303 ± 14 kDa, consistent with six to seven 42-kDa subunits per channel (Fig. S5A). Similar analyses with MscS (n = 6) gave a stoichiometry of 6.6 ± 0.4.

We have previously demonstrated the efficacy of cross-linking to estimate the oligomeric state of MscS channel complexes (28). A single native Cys residue (Cys102) is at the periplasmic end of the putative TM1 helix of YbdG (Fig. S1). A single band at ~38 kDa was observed on nonreducing SDS/PAGE, indicating that Cys102 does not spontaneously cross-link subunits. However, a significant proportion of YbdG is driven into cross-linked dimers by oxidation with copper phenanthroline (Cu-Phen) reagent (Fig. 2). Introduction of a Cys residue into the carboxy-terminal domain (YbdG A388C) at a position similar to S267C in MscS that allows detection of heptamers (28) did not significantly increase oligomer formation in the presence of Cu-Phen (Fig. 2). In contrast, introduction of a Cys residue into the predicted periplasmic vestibule (S164C or S158C) led to the formation of higher oligomers up to and including a heptamer (Fig. 2). Together, the blue native and cross-linking data support the formation of heptamer by YbdG.

YbdG Contributes to Cell Viability During Hypoosmotic Shock. Our early work showed that MscS and MscL are the dominant channels providing protection against hypoosmotic shock (1). We extended this analysis to mutants lacking YbdG. A ybdG::apr replacement mutant was constructed (Fig. S4) and transferred to strains MjF429 (MscK−, MscE−) and MjF465 (MscK−, MscE−, MscL−) for analysis (Table S2 and Fig. S5B). As reported previously, MjF465, which retains YbdG, suffers a substantial loss of viability when subjected to a rapid osmotic shock ≥0.25 M NaCl, but cells are able to survive smaller osmotic shifts (Fig. 3A). A quadruple MS channel null strain MjF612 (MjF465 ybdG::apr) exhibits a lowered threshold at which cell death occurs (0.25 M NaCl for MjF612 compared with 0.25 M NaCl for MjF465; Fig. 3A). Cells that retain YbdG (MjF465) exhibit 3-fold better survival than...
MJF612. Thus native levels of expression of YbdG confer protection against mild hypoosmotic shock. Protection could be enhanced by overexpression of YbdG (see Materials and Methods for plasmid construction). Basal expression did not offer protection from a 0.5-M hypoosmotic shock (Fig. 3B). When YbdG expression was increased (0.5 mM IPTG, 30 min), 100% survival was restored (Fig. 3B). Thus, YbdG possesses the capacity to protect cells against hypoosmotic shock, a characteristic expected of a functional MS channel.

Electrophysiological Characterization of YbdG Channels. Previously we noted that mutant cells lacking Mscl, Mscl, and Mscc can display 350–400 pS Mscc activities in patch-clamp assays (1, 29) but openings of this size were infrequent (<5% occurrence) and unpredictable. To determine whether YbdG is a component of the Mscl channel activity, strains MJF429 (YbdG) and MJF611 (ΔybdG), in which Mscl acts as a reference for the presence of MS channels in each patch (30), were compared. No obvious differences were detected between membrane patches of these two strains (n = 15 for each strain) (Fig. 4). Mscl-sized openings were observed only in occasional patches in the presence or absence of chromosomal ybdG. Moreover, overexpression of wild-type YbdG in either MJF429 or MJF611 did not lead to new or more abundant channel openings (n = 15 for each strain).

We determined that YbdG protein is present in membrane samples derived from cephalaxin-treated cells, the immediate precursor of giant protoplasts used for patch clamp (5). YbdG accumulated at levels similar to Mscl protein, which is readily assayed (Fig. S5C). Thus, failure to detect abundant single channels was not due to lack of YbdG protein, but must reflect specific requirements for gating that are lacking in membrane patches. To isolate mutant YbdG channels with altered gating frequencies, we randomly mutagenized the ybdG gene and screened for mutant channels that suppressed the potassium transport deficiency of a strain deleted for the major K⁺ transport systems. This selection operates on the basis that the channel gates frequently enough to open the nonselective MS pore and allow K⁺ ions to move into the cell down the membrane potential; through frequent cycles of transient openings, followed by rapid closure, the cell can acquire the K⁺ needed for growth (31). Strain MJF622 is deficient for both potassium uptake and YbdG protein (Table S2) and requires ≥30 mM NaCl for rapid growth. Gating mutants were identified by growth of the transformants on medium containing only 5 mM K⁺. Among several mutants, V229A was chosen for analysis because it proved functional, protecting cells from hypoosmotic-induced death when overexpressed (Fig. 3B), and it provided strong complementation (Fig. S6), suggesting an increased frequency of gating. This mutation location in YbdG is also sufficiently distant from the pore that the channel conductance should not be affected. In patch clamp, YbdG V229A exhibited a high frequency of Mscl-like channel activity when expressed in MJF611 (ΔybdG): 9/23 patches exhibited one to two long open-dwell openings of 350–400 pS that are indistinguishable from those of Mscl (Fig. 4).

However, no increased channel activity was observed when the same mutant was expressed in MJF429 (YbdG). These data are consistent with competition for a limited supply of a protein, metabolite, or lipid required for activity or with heteromeric channels being formed that are inhibited from gating by integration of wild-type and mutant subunits into the same complex.

Discussion
In this study we demonstrate that one of the previously uncharacterized homologs of Mscl in E. coli, YbdG, is a functional and physiologically relevant MS channel that exhibits conductance properties similar to Mscl. Overexpression of this channel gives excellent protection against hypoosmotic shock, equivalent to that provided by Mscl or Mscl. Because the contribution of chromosomally expressed YbdG to protection is subtle, it is clear that the normal abundance of this channel protein is too low to afford the high levels of protection seen with Mscl and Mscl. This lack of channel abundance occurs despite increased transcription and
In the earliest experiments measuring *E. coli* MS channels using cytoplasmic membranes fused with phosphatidylcholine liposomes (5–7, 43), multiple activities were observed that could be differentiated by their kinetics. Individual patches contained single types of activities rather than the mixtures of channels routinely measured in cells, suggesting that the channels are clustered in the mixed membranes of the reconstituted system (6). These data can be interpreted to suggest that some channel activities require either specific lipids for their activity or the dilution of inhibitory lipids to allow gating. Microarray data indicate transcription of the MscS homologs under a range of conditions, suggesting that the proteins, like YbdG, are expressed (http://genexpdb.ou.edu/). Therefore the failure to detect frequent channel activities of the other MscS homologs by electrophysiology indicates a further subtlety in their regulation that may involve specific lipid requirements for activity. The ease with which MscS, MscL, and MscK are observed may be due to a combination of their abundance and an inherent lack of constraint in their lipid interactions.

A remarkable observation is that the YbdG mutant V229A activity is apparently suppressed by coexpression with the wild-type subunits. Possible explanations for this phenomenon include formation of heteromeric channels, competition for limiting amounts of either a lipid or an activating molecule, and/or competition during assembly into the membrane. Expression from the chromosome should be substantially lower than from the plasmid. Purification of mutant YbdGH4, after expression in both ΔybdG and YbdG+ cells did not reveal any significant change in protein abundance when wild-type subunits were present, but we have been unable to detect heteromeric complexes. Thus, the available data point to complex regulation of YbdG channel activity by either an unknown protein or specific lipids. The V229A channel exhibits activity similar to MscM, namely a conductance of 350–400 pS, sustained open dwell times, and rarity. Only small numbers of V229A channels were recorded in any one patch despite control experiments that indicated an abundance of protein (Fig. S3C). It appears that the mutation, although facilitating gating, does not completely compensate for the missing factors required to gate YbdG in membrane patches.

Genome analysis across the microbial and plant kingdoms has revealed the presence of multiple MscS homologs, but only rarely multiple MscL proteins (10–12). Thus, it seems plausible that MscS homologs have evolved a wide range of sequences that enable them to gate in response to different tensions and/or lipid and environmental contexts. This multiplicity would allow a diversity of channel characters that introduces subtlety to the hypoosmotic shock response. In contrast, MscL may not be able to be engineered to possess such subtlety and has evolved as the “channel of last resort,” opening when the increase in turgor is too rapid to be countered by the multiple members of the MscS family.

Materials and Methods

**Strains and Media.** All strains used are derivatives of *E. coli* K12 and are listed in Table S2. JM109 and JM110 were used for transformation of newly generated mutants. Frag1 derivative strains were grown at 37 °C in either LB medium ([220 mOsmol; 8.58 g Na2HPO4, 0.87 g K2HPO4, 1.34 g citric acid, 1.0 g NH4SO4, 0.001 g thiamine, 0.1 g MgSO4·7H2O, and 0.002 g (NH4)2SO4·FeSO4·6H2O per liter], Strain DY330 was grown in LB at 32 °C. All strains were stored at 4 °C on agar plates containing selective antibiotic, where appropriate. Overnight cultures contained 25 μg/ml ampicillin, where appropriate, to ensure maintenance of a plasmid. Solid medium contained 14 g/l agar.

**Primers.** All primers used in this study were manufactured by Sigma-Genosys and are listed in Table S3.

**Bioinformatic Methods.** All programs used are available online. Protein sequence alignments were performed using either BLASTP (19) or MAFFT (44). Conservation analysis was performed using the Weblogo server (45). Protein hydrophobicity and mutant sequencing results were analyzed using the
DNASTAR Lasergene program suite. All E. coli protein and DNA sequences were obtained from the Colibri Web Server (http://genolist.pasteur.fr/colibri). Reference was made to RegulonDB (33) and OU Gene expression databases (46).

Creation of ybdG Deletion Strains. Deletions of the ybdG gene by homologous recombination was essentially as described (47), using the strategy depicted in Fig. 54 for primer design. See SI Materials and Methods for full details.

Cloning of the ybdG ORF. The full ORF was amplified by standard PCR using primers YbdG-F and YbdG-R (Table 53). The obtained product was cloned into the pTrcHis2-TOPO vector (Invitrogen) and verified by sequencing. The cloned gene contained a number of nucleotide changes compared with the published sequence (Colibri Web Server); however, all were silent mutations leaving the protein sequence unaffected. Restriction sites NcoI and XhoI were introduced in frame at the start and stop of the gene, respectively, by site-directed mutagenesis using primers YbdGXhoI-1, YbdGXhoI-2, YbdGCNcoI-1, and YbdGCNcoI-2. To create a HIS-tagged protein, the plasmid pTrcMcsFH was used for subcloning (28). Successful creation of pTrcYbdGH6 was checked by PCR amplification and restriction analysis and confirmed by sequencing.

Generation of YbdG Mutants. Mutants were created either by QuikChange site-directed mutagenesis or by selection from XL1-generated mutated plasmids as specified in SI Materials and Methods.

YbdG Antiprotein Antibody Purification. The peptide sequence 276–FLD–DEMQRLNKAHL–290, in the C-terminal region of YbdG, was selected for the antibody antigen. Further details are given in SI Materials and Methods.

Membrane Preparations and Western Blot Analysis. Protein expression and detection were carried out essentially as described previously (28) with the modifications outlined in SI Materials and Methods.

Cross-Linking of Cysteine Residues. Cysteine cross-linking using copper phe- nanthroline was carried out on membrane samples as reported previously (28). Reactions were run on SDS gels and Western blot analysis was carried out as described above.

qRT-PCR Analysis. Cells were grown to the desired optical density (exponential phase, OD600 = 0.5; early stationary, OD600 = 1; late stationary, OD600 = 5), total RNA was isolated, and cDNA was obtained by reverse transcription and treated as described in SI Materials and Methods. Primers for qRT-PCR were designed within the ORF of nfnB and ybdG; rp08 was used as internal standard.

Electrophysiology. Patch-clamp recordings were conducted as previously published (18), with amendments as in SI Materials and Methods.

Materials. All media components were obtained from Oxoid and all general chemicals purchased from Sigma. Restriction enzymes and polycondes were from Roche or Promega and Novex precast SDS-polyacrylamide gels were from Invitrogen. All qRT-PCR materials and chemicals were from Roche. Kits for DNA or RNA isolation and purification were from Qiagen.

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Supporting Information

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SI Materials and Methods

Strains and Primers. All strain genotypes are given in Table S2 and all primer sequences are listed in Table S3.

Creation of ybdG Deletion Strains. To create a linear DNA fragment for homologous recombination, primers contained 50 bp homologous sequence of the region flanking ybdG (forward primer) or 50 bp within the 5′ region of ybdG (reverse primer) followed by 20 bp of the Apramycin resistance cassette sequence (ybdG-F, ΔybdG-R). Both primers carry a BglII recognition site for identification and selection. The Apramycin cassette was PCR amplified and the product purified. The design strategy avoided disruption of the terminator sequence of the phoP gene downstream of ybdG and, to not disrupt any potential terminator sequence of the upstream gene fnIB, 114 nucleotides at the 5′ end of ybdG were left in place and a stop codon was incorporated. This strategy is illustrated in Fig. S4.

Deletion of ybdG was by homologous recombination in strain DY330 (1). The purified linear DNA fragment (100 ng) was transformed by electroporation (Gene Pulser II; Bio-Rad). Cells were recovered overnight at room temperature and then cultured in fresh LB medium containing 50 μg/mL apramycin (32 °C, shaking). Viable cells were serially diluted and recovered on selective plates. Single colonies were purified, PCR tested, and verified by sequencing using primers homologous to flanking regions outside the recombination region (YFL-F, YFL-R).

Transfer of the deletion was achieved by P1 transduction of strains MJF465 (MscK−, MscS−, MscL−) and MJF429 (MscK+, MscS+, MscL−, YbdG−) and MJF611 (MscK−, MscS−, YbdG+), respectively. Strain DY330 ΔybdG::apr was used as donor to produce the P1 lysate. Recipient cells were suspended in P1 salt solution (10 mM MgCl2, 5 mM CaCl2), lysate was added, and the cells were incubated at 37 °C for 30 min and then washed twice with 0.5 mL LB containing 20 mM Na-Citrate. Cultures were incubated for 1 h in 1 mL LB containing 20 mM Na-Citrate at 37 °C before plating on selective agar containing 20 mM Na-Citrate. Positive transductants were purified and confirmed by PCR and restriction digest.

Hypotonic Shock Assay. Either wild-type or mutant YbdG protein was expressed in strain MJF612, under both noninduced and induced (0.3 mM IPTG, 30 min) conditions. Cells were adapted in LB + 0.5 M NaCl and diluted into LB (shock) or LB + 0.5 M NaCl (control), serially diluted, and recovered overnight on agar plates of the same osmolarity. To assess osmotic shock survival of parental strains, cells were cultured in McIlvaine’s buffer, pH 7.0, and then adapted to McIlvaine’s buffer containing 0.5 M NaCl (2). Cultures were diluted into medium containing various salt concentrations to create the desired osmotic shock and recovered as described above. All experiments were repeated a minimum of three times.

YbdG Antipeptide Antibody Purification. Amino acid sequence 276–FLDEDEMRNLKALH–290 of YbdG was highlighted using the Protein program in DNASTAR Lasergene to have a high antigenic index. The peptide was synthesized and the antibodies were affinity purified from rabbit serum by coupling of the peptide to activated maleimidе-Sepharose, using the SulfoLink Kit (Pierce) according to manufacturer’s instructions. Aliquots were stored at −20 °C.

It should be noted that the YbdG antiserum detected bands in addition to that at the expected mass of the YbdG protein. These bands appear to be from contaminating antibodies present in the antipeptide antiserum, evident after prolonged exposure when detecting chromosomal YbdG expression levels. Similar bands, although weaker, were also detected in membrane preparations of E. coli strains that were deleted for ybdG (Fig. S5B) and in membrane preparations derived from cells where YbdG was overexpressed from a plasmid (see control in Fig. S5B).

Membrane Preparations and Western Blot Analysis. For analysis of chromosomal expression profiles, parental strains were grown in LB or LB + 0.5 M NaCl medium to midexponential (OD650 = 0.5), early stationary (OD650 = 1), or late stationary (OD650 = 3) phase. For plasmids, expression was analyzed after growth in LB and induction with 0.3 mM IPTG for 30 min. All membrane samples were obtained as described previously by ultracentrifugation after cell breakage at 18,000 psi using a French Pressure cell and the protein concentrations were assayed (3). A total of 15 μg of membrane proteins (unless otherwise stated) was separated on a 4–12% Bis-Tris SDS/PAGE gel and Western blot analysis was performed as previously reported (4), using either Penta-His antibody (Qiagen) or antipeptide antibodies specific for YbdG followed by anti-rabbit-HRP secondary antibody (Perbio).

Blue Native PAGE. A total of 60 μg protein from an MJF612 cell membrane suspension, after overexpression of YbdG, was solubilized in 1.5% dodecylmaltoside (DDM; Anatrace) for 30 min on ice (30 μL volume). After centrifugation for 10 min at 14,000 × g, 15 μL of the supernatant was mixed with native sample buffer (Invitrogen) and 0.15% Coomassie G250 and immediately applied to a preformed native Novex 4–16% Bis-Tris gel (Invitrogen). Purified YbdG protein samples were adjusted to 0.5% DDM, mixed with sample buffer and 0.15% Coomassie G250, and applied to the gel. Conditions of the run have been described previously (5). This procedure was followed by Western blot detection (using Penta-His antibody) or blue staining with Coomassie R250. Molecular masses, obtained by comparison with soluble protein standards, were corrected by a factor of 1.8 to account for bound lipids, detergent, and dye (6).

Generation of YbdG Mutants. Point mutations in pTrcYbdGH4 were introduced by site-directed mutagenesis following the QuikChange protocol (Stratagene). Primers (Table S3) contained restriction sites for selection before sequencing. All mutants were sequenced at least twice on both strands using the ABI 3730 capillary DNA sequencer at The Sequencing Service (University of Dundee, Dundee, UK).

Random mutants were isolated by transforming XL1-Red cells (Agilent Technologies) with pTrcYbdGH4 and after growth in complex media, plasmids were isolated following the manufacturer’s instructions. Mutants were selected by transforming a ybdG-deleted derivative of TK2309 (kp::Tn10, kup, trkA405, ΔybdG::apr; MJF622) with the plasmid pools derived from XL1-Red cells and selecting for growth on minimal medium K3, which contains only 5 mM K+, which is insufficient to allow the growth of strain TK2309 alone (7). Plasmids allowing growth were purified and their sequence was determined on both strands.

Gain-of-Function Assays. Growth in K+ assay. TK2309 ΔybdG::apr (MJF622) cells were grown alone or expressing wild-type or V229A mutant YbdG. Cells were precultured in K+ (40 mM K+) medium to OD650 ~ 0.4. After washing in K+-free medium, cells

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were diluted 1 in 10 into either K$_5$ or K$_40$ medium and grown in the presence of 0.1 mM IPTG at 37 °C. OD$_{650}$ was measured every 30 min.

**Transformation assay.** Transformation assay was performed as described previously (4), with the following details. MJF612 ($\Delta$ybdG) cells were transformed with YbdG wild-type or mutant V229A plasmid. After recovery of cells in either LB or LBK (LB + 30 mM K$^+$) at 37 °C for 1 h, cells were serially diluted to 10$^{-3}$ and 5-μL spots of each dilution were plated onto either LB and LB + 1 mM IPTG or LK and LK + 1 mM IPTG, respectively, and incubated overnight at 37 °C. As positive controls, known MscS gain-of-function mutations, L109S and T93R, were transformed into MJF465 cells in parallel and the same protocol was applied. After overnight growth, pictures were taken using a Bio-Rad ChemiDoc Hood.

**Quantitative Real-Time PCR (qRT-PCR) Analysis.** Primers for qRT-PCR were designed within the ORF of nfb and ybdG; rpoB was used as internal standard. Cells were grown to the required optical density (exponential phase, OD$_{650}$ = 0.5; early stationary, OD$_{650}$ = 3) and total RNA was isolated. Contaminating genomic DNA was removed (DNA-free kit; Ambion). cDNA was obtained by reverse transcription of 4 μg of total RNA using the First-Strand cDNA Synthesis Kit (GE Healthcare). Reactions for qRT-PCR were prepared as instructed by the manufacturer. Standard curves were prepared by serial dilution of cDNA from 10- to 100,000-fold and run in duplicate. Individual cDNA samples were diluted 100-fold and run in triplicate. Relative fluorescence and concentrations were determined using the Light-cycler 480 software and analyzed in Excel. All samples were normalized to the rpoB expression level of the individual sample. Low osmolarity culture of Frag1 grown to exponential phase was chosen as a reference point and therefore expression levels of all other samples are presented relative to this sample.

**Electrophysiology.** Strains MJF429 and MJF611 were transformed with YbdG plasmids or left untransformed. Protoplasts were prepared as described previously (8) and cells containing a YbdG plasmid were induced for expression by incubating in 1 mM IPTG for 60–100 min during the exposure to cephalexin. Excised membrane patches were analyzed at room temperature in symmetrical solutions containing 200 mM KCl, 90 mM MgCl$_2$, 10 mM CaCl$_2$, and 5 mM Hepes buffer (pH 7). Data were acquired at a holding potential of 20 mV (pipette positive) and a sampling rate of 50 kHz with 5 kHz filtration, using an AxoPatch 200B amplifier and PClamp9 software (Axon).

Fig. S1. Comparison of YbdG and MscS protein properties. (A) Crystal structure of the MscS protein (1) with one subunit highlighted in black and major domains labeled. Image was prepared using Protein Explorer (2). (B) Alignment of the MscS pore-lining residues with the putative pore-lining region of YbdG as determined by BLASTP (http://www.ncbi.nlm.nih.gov). The prominent Ala-Gly pattern of MscS and corresponding residues of YbdG are indicated in black and pore-seal Leucine residues are in blue. (C) Hydrophobic profiles (3) of both protein sequences were created using the DNASTAR Lasergene software (window = 19 residues). Positive values indicate strong hydrophobicity and ruler numbers represent residue position. The plots were aligned according to BLASTP alignments. The MscS domain organization, as determined by the crystal structure, is indicated with gray boxes; boxes indicating the MscS TM spans are extended to mark the putative aligning TM spans present in YbdG. Two additional TM helices of YbdG (TM\textsubscript{N1} and TM\textsubscript{N2}) are indicated with open boxes. The inserted region in the YbdG protein is indicated with a dashed box.

Fig. S2. Comparison of the MscS/MscK family homologs. (Inset) ClustalW alignment of full-length sequences was used to predict the equivalent region of each homolog to MscS TM1\text{start}−TM3\text{end} (V29−F127). The inset shows percentage of identical (I) and similar (S) residues from pairwise alignments of the equivalent regions to MscS TM1\text{start}−TM3\text{end} (Lipman–Pearson, MegAlign; DNASTAR Lasergene) and the length of the consensus sequence for which these values were calculated. Hydrophobicity plots were created in Lasergene using the Kyte–Doolittle scale (1) (window equals nine residues) and aligned by the last three predicted transmembrane spans for each homolog with the three transmembrane spans known for MscS (*---------*).

Fig. S4. Schematic illustration of the strategy used to create a ybdG mutant strain. The genomic region of the ybdG gene flanked by genes nfnB and pheP is shown, with the terminator sequence for pheP marked as a vertical gray box. The ybdG gene was replaced with the Apramycin resistance cassette such that the 114 nucleotides of the 5’ end of the ybdG gene were left in place, ensuring that a potential nfnB terminator sequence would not be disrupted. A stop codon was inserted following the first 114 nucleotides of ybdG to ensure translational termination of potential ybdG transcripts. The Apramycin recombination fragment was obtained by PCR and recombination was carried out as described (1).

**Fig. S5.** YbdG protein expression. (A) Blue native PAGE of the YbdG protein complex. Cell membranes were extracted from MJF612 cells overexpressing YbdG and solubilized with 1.5% dodecylmaltoside (DDM). The sample was run on a native Novex 4–16% Bis-Tris gel, transferred to polyvinylidene fluoride (PVDF), and detected with Penta-His antibody. On the basis of the predicted molecular mass of 46.6 kDa for a subunit of YbdG, and a correction factor of 1.8 (Materials and Methods), our data indicate an oligomeric complex of between 6 and 7 subunits. (B) Strains MJF611 and MJF612 are deleted for YbdG. Frag1, MJF465, MJF429 (all YbdG⁺), MJF612, and MJF611 (both YbdG⁻) were grown in LB medium to midexponential phase. MJF612 cells expressing YbdG from a plasmid (without induction) were used as a control (pTrcYbdG). Membrane fractions were isolated and 25 μg (15 μg for the control) of membrane protein was separated by SDS/PAGE. Western blots were probed with antibodies specific for YbdG followed by incubation with anti-rabbit-HRP antibodies. Blots were developed using the standard ECL method. (C) Cephalexin-treated cells (precursors to protoplasts) contain YbdG protein in their membranes. Membrane samples were collected from MJF612 cells grown alone (lanes 1) or with plasmids expressing wild-type MscS (lanes 2), wild-type YbdG (lanes 3), or mutant YbdG V229A (lanes 4), in the absence (cells) or presence (cxn) of 60 μg/mL cephalexin. Cell samples were induced with 1 mM IPTG for 2.5 h and cxn-treated samples were induced for 1 h before harvesting and membrane extraction. Total protein was measured (3) and 15 μg was loaded per lane for SDS/PAGE. Western blots were probed with Penta-His antibody and developed using standard ECL. YbdG protein is seen to express to similar levels irrespective of whether cells are treated with cephalexin and, notably, levels are similar to those of MscS (readily recorded by patch-clamp assays) in both conditions.
Fig. S6. Gain-of-function analysis of YbdG V229A mutant protein. (A) Strain MJF622 is deficient in K\textsuperscript+ uptake and is ΔybdG and requires ≥30 mM K\textsuperscript+ for rapid growth. MJF622 cells were grown either alone (TKΔ) or expressing YbdG wild-type (TKΔ WT) or V229A (TKΔ V229A) channels, in K\textsubscript{5} and K\textsubscript{40} media, in the presence of 0.1 mM IPTG. OD\textsubscript{650} was recorded every 30 min. As expected, all cells grow well in 40 mM K\textsuperscript+ (Inset). In 5 mM K\textsuperscript+, YbdG V229A allows growth of the K\textsuperscript+ transport-deficient cells but wild-type YbdG fails to support growth under these conditions. (B) A gain-of-function transformation assay tested growth of MJF612 (ΔybdG) cells expressing YbdG wild-type (WT) or mutant V229A plasmids, on LB or LK media in the presence and absence of 1 mM IPTG. Controls, MscS L109S and T93R transformed into MJF465 cells, were also tested. L109S is a strong gain-of-function mutation causing severe growth inhibition even when uninduced and T93R is a weak gain-of-function mutation reducing growth of cells when overexpressed (4). YbdG wild-type protein allowed healthy colony growth.

Legend continued on following page.
growth on LB or LK, whereas YbdG V229A impaired colony growth on both LB and LK when overexpressed with IPTG. Colonies were smaller than in uninduced samples or cells containing wild-type protein, indicating a slow growth phenotype, and colonies appeared translucent (as seen for MscS T93R). These data demonstrate the gain-of-function nature of YbdG V229A channels and suggest they gate more frequently than wild-type YbdG channels.

Table S1. Pairwise alignment of the YbdG and MscS proteins

<table>
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<tr>
<th>Alignment</th>
<th>E value</th>
<th>Identity</th>
<th>Similarity</th>
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</thead>
<tbody>
<tr>
<td>YbdG</td>
<td>164</td>
<td>7E-70</td>
<td>22/93</td>
</tr>
<tr>
<td>YbdG*</td>
<td>161</td>
<td>2E-07</td>
<td>44/184</td>
</tr>
<tr>
<td>MscS</td>
<td>147</td>
<td>179</td>
<td></td>
</tr>
<tr>
<td>MscS*</td>
<td>147</td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

YbdG and YbdG* were pairwise aligned to MscS (BLAST b2seq; ref. 1) and the regions giving significant similarity are shown. The YbdG* protein sequence was obtained by in silico deletion of the 50 nucleotides identified as an insertion in the YbdG protein. Expect (E), identity, and similarity values over the aligning regions are listed.


Table S2. Bacterial strains used in this study

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<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>JM109</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17 (rK-, mK+), supE44, relA1, Δ(lac-proAB), [F, traD36, proAB, lacIq ZM15]</td>
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</tr>
<tr>
<td>JM110</td>
<td>Rpsl (strr), thr, leu, thi, hsdR17 (rK-, mK+), lacIq, galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ(lac-proAB), [F, traD36 proAB, lacIq ZM15]</td>
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</tr>
<tr>
<td>Frag1</td>
<td>F-, rha, thi, gal, lacZ</td>
<td>(2)</td>
</tr>
<tr>
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<td>(3)</td>
</tr>
<tr>
<td>MJF429</td>
<td>ΔmscS, ΔmscK::kan</td>
<td>(4)</td>
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<td>MJF465</td>
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<td>MJF611</td>
<td>ΔmscS::kan, ΔybdG::apr</td>
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<tr>
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<td>This study</td>
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<tr>
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<td>MJF622</td>
<td>ΔybdG::apr</td>
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<tr>
<td>DY330</td>
<td>Δi258, Δi258, Δi258</td>
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### Table S3. Primers used in this study

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<td>YbdGRT-F</td>
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<td>A388C§</td>
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*Introduced mutations are shown in boldface type.
†Underlined sequences are specific for ybdG; the BglII site is marked in italics.
‡Only the forward primer sequences are given here; a second reverse complement primer was also used.
§This construct carries an I386M mutation additional to the indicated mutation.