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Functional properties of Kch, a prokaryotic homologue of eukaryotic potassium channels

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Abstract

To test the hypothesis that the *Kch* gene of *Escherichia coli* encodes a potassium channel, we have transformed *E. coli* with an expression vector containing the Kch sequence and observed the effect of over-expression of Kch on *E. coli*. We found that: (i) over-expression of Kch is toxic to *E. coli*, but the toxicity could be prevented by supplementing the growth medium with K^+, Rb^+, and NH_4^+, but not Na^+, consistent with the properties of a potassium selective pore; (ii) Cs^+, a blocker of potassium channels, rescues the growth of Kch over-expressing cells; and (iii) when the putative pore-forming region of Kch, containing the signature sequence, was replaced with the corresponding region of the eukaryotic *Shaker* potassium channel, and the resultant construct expressed in *E. coli*, the cells became critically dependent on K^+ supply for survival. These data are consistent with the proposed function of Kch, i.e., K^+ conduction.

Keywords: Kch; Potassium channel; Protein expression; Chimaeric channels

The *Kch* gene of *Escherichia coli*, first reported by Milkman [1], encodes a protein, named as Kch, that possesses features reminiscent of voltage-gated potassium (Kv) channels: it contains the potassium channel signature sequence within the pore-forming P-loop (present between S5 and S6) and six putative transmembrane segments (S1–S6). However, apart from the P-region, the overall amino acid sequence identity between Kch and modern K channels is very low. Moreover, Kch lacks the positively charged S4 segment present in other members of this family, including those found in other bacteria [2–7]. These differences underscore the importance of establishing evidence that Kch conducts K^+ ions. Once this is established, one could begin studies on its physiological role and regulation. So far, Kch protein has been purified from *E. coli* using over-expression systems [8,9] and its predicted transmembrane topology has been confirmed [10]; however, the two major questions, viz. the function and regulation, still remain unanswered.

In the present study, we sought to address the first question: does Kch conduct K^+ ions? Our initial attempts to express Kch in *Xenopus* oocytes and examine its ability to conduct K^+ by electrophysiological means proved to be unsuccessful. We therefore examined its function in *E. coli* by studying the consequence of over-expression of Kch on the phenotype of the cell. We found that over-expression of Kch is lethal to cell survival and growth, and that supplementation of K^+ protects the cell against these effects. When we replaced the putative pore region of Kch with the corresponding sequence of the well-characterised eukaryotic *Shaker* potassium channel, the resultant chimaeric protein showed characteristics similar to those of Kch. These data provide evidence to the proposition that Kch conducts K^+ ions.

Materials and methods

Bacterial strain. Escherichia coli DH5α (Gibco-BRL) cells were used for cloning. *E. coli* BL21(DE3) harbouring pLysS (Novagen) was used for phenotype analysis.

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Plasmid constructs. Sequence encoding Kch, minus the start codon (GTG), was amplified from the *E. coli* DH5α genomic DNA by the polymerase chain reaction and subcloned in-frame with the ATG start codon of pT7.7 to generate the expression vector-construct, referred to as pT7.7-Kch. To facilitate other manipulations, the *Xba*I–*Hin*dIII fragment encompassing the ribosome binding site and the *Kch* sequence was subcloned into pBluescript KS downstream of the T7 promoter. The resulting construct, pKS-Kch, was used for over-expression of Kch protein in *E. coli*. The Kch insert in the final construct was fully sequenced; the sequence was identical to that reported previously [1] (GenBank Accession No. L10244; TC-Number: 1.A.1.13.1). To express Kch in *Xenopus* oocytes, the Kozak sequence (ccaccatgg) was added at the start codon by PCR and the resultant sequence was subcloned into the pKS-globin vector [11].

Phenotype analysis. Competent *E. coli* BL21 DE3 (pLysS) cells were transformed with pKS-Kch and equal amounts (typically in a 100 μl volume) of transformed cells were plated on LB-agar-Amp-Cm plates (1% bactotryptone (Difco), 0.5% yeast extract (Difco), 50 mM NaCl, 1% agar (Difco), 50 μg/ml ampicillin, and 20 μg/ml chloramphenicol) with or without 0.1 mM isopropyl-β-thio-galactoside (IPTG). Components were included or omitted as required. The plates were incubated at 37 °C for 16–20 h. Survival was measured by counting the number of transformed cells that gave rise to colonies. Growth was monitored by measuring the diameter of well-separated colonies using a scale fitted to the eye-piece of the microscope. Alternatively, aliquots of serially diluted cultures of transformed cells, grown to A_{600} of 0.6, were plated on selection agar plates, as described by Blount et al. [12].

Biosynthetic labelling of Kch with [^{35}S]methionine. Pulse labelling of Kch with [^{35}S]methionine was performed essentially as described previously [13]. Briefly, BL21(DE3) cells harbouring pLysS and the pKS-Kch expression vector construct were grown in M9 medium supplemented with all amino acids, except cysteine and methionine to A_{600} of ~0.3. Following induction with IPTG (30 min), cultures were treated with rifampicin (0.2 mg/ml) for 30 min. The cells were then pulsed with [^{35}S]methionine (10 μCi/ml) for 5 min, collected by centrifugation, solubilised in SDS-sample buffer, and subjected to SDS–polyacrylamide (8%) gel electrophoresis and fluorography.

Construction of chimaeras. Exchange of P-loop sequences between Kch and the *Shaker* B1 potassium channel lacking the inactivation-ball domain (residues 6–46) [14] was carried out by recombinant PCR using *Pfu* DNA polymerase (Stratagene) as follows; the methods are as described previously [11]. The first chimaera contained the *Shaker* backbone, but the putative pore region of Kch. The sequence comprising residues 411–453 of *Shaker* channel was substituted with the corresponding region (158–195) of Kch. DNA sequence corresponding to residues 411–453 was removed from the pKS-*Shaker* B1 construct by deletion PCR [11], whilst introducing the *Sph*I and *Nhe*I sites. DNA corresponding to residues 158–195 of Kch was amplified from pKS-Kch using primers that allowed the addition of *Sph*I and *Nhe*I at the 5′ and 3′ ends of the product. After restriction, the two products were joined to generate the chimaera referred to as pSh-Kch. These manipulations altered residues ACV of *Shaker* (positions 454–456) to VGF. These positions are little conserved, so we assumed that these changes would not drastically affect the function. The second chimaera, referred to as pKch-Sh, contained the Kch backbone (residues 1–417, lacking residues 155–195), but the *Shaker* pore (residues 412–452). The procedure used was similar to that described above and the involved addition of *Spe*I and *Sac*I sites at the 5′ and 3′ ends of the pore sequence.

Electrophysiology. cRNA encoding Kch or the chimaera of *Shaker* with the Kch pore was prepared using the Megascript transcription kit (Ambion). Oocytes isolated from *Xenopus laevis* were micro-injected with 50 nl cRNA (15–25 ng); after 2–3 days of incubation at 18 °C, membrane currents recorded using two-electrode voltage-clamp (Geneclamp 500, Axon Instruments) [15]. Oocytes were held in a 50 μl chamber and continually superfused (2 ml/min) with Frog Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.2). Membrane potential was held at −80 mV and 500 ms test depolarizations were applied in 10 mV increments at an interval of 10 s. Data were filtered at 2 kHz and sampled at 4 kHz using CED software and a CED 1401 interface.

Results

Expression of exogeneous Kch is toxic to E. coli

To investigate the physiological function of Kch, we sought to over-express the Kch gene in *E. coli*. The rationale was that over-expression would alter the phenotype of the host cell thereby revealing the function of Kch. However, when competent BL21 (DE3) *E. coli* cells transformed with pKS-Kch were plated on LB-ampicillin-agar plates no growth was observed, although cells transformed with the vector lacking the *Kch* gene gave rise to colonies (data not shown). Assuming that the transformed cells might have expressed the *Kch* gene due to leaky expression and that the expressed protein is toxic to the cell, we transformed pKS-Kch into competent BL21(DE3) cells harbouring pLysS; the latter plasmid expresses T7 lysozyme, which prevents any leaky expression [16]. As anticipated, the transformed cells not only survived but grew into colonies (Fig. 1, plate A) similar in size to cells, transformed with the control plasmid (pKS) lacking the *Kch* gene (Fig. 1, plate D). To further confirm that over-expression of Kch is toxic to *E. coli*, we plated an aliquot of the transformed cells on agar plates containing the inducer, IPTG. As can be seen from Fig. 1 (plate B), no growth was seen on these plates, compared to normal growth on control (vector alone) plates (plate E). Identical results were obtained when we used the more defined M9 medium (data not shown).

The toxic effect of over-expressed Kch can be prevented by supplementing the growth medium with potassium ions

Since the predicted function of Kch is that of a potassium channel [2–7], we suspected that the inability of transformed cells to survive on this medium could be due to loss of intracellular K^+ or excessive accumulation of K^+ from the medium via the over-expressed Kch. To distinguish between the two possibilities, we plated the transformed cells on agar medium supplemented with 50 mM K^+ and IPTG. Fig. 1 (plate C) shows that supplementation of K^+ promoted the survival of cells transformed with pKS-Kch; this suggests that the poor survival of Kch over-expressing cells may be due to the loss of intracellular K^+, down the electrochemical K^+ gradient. If the cause of the cell death were to be due to accumulation of K^+, one would expect increasing the K^+ concentration of the medium would promote influx of K^+ into the cell, thereby enhancing, rather than

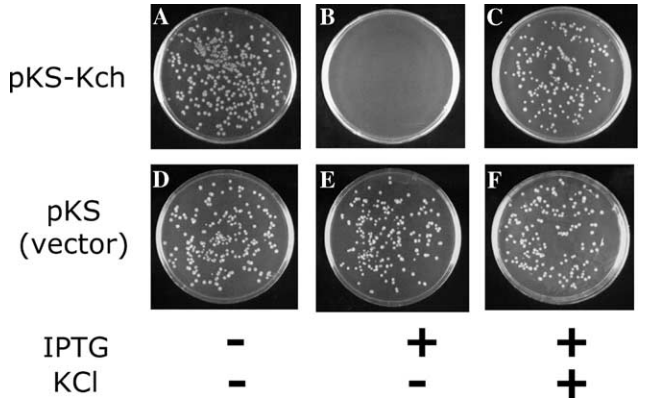

Fig. 1. Over-expression of Kch is toxic to *E. coli*, but the toxicity could be prevented by potassium ions. Competent BL21 DE3 (pLysS) cells transformed with the pKS-Kch (A–C) or pKS vector lacking the Kch gene (D–F) were grown on agar plates (LB-agar-Amp-Cm) containing (+) or not containing (−) the indicated additives. IPTG and KCl concentrations were 0.1 and 50 mM, respectively.

preventing, the cell death. Thus, the K^+ content (7.5 mM as estimated by flame photometry) of the LB-agar medium, not supplemented with K^+, appears to be too low to support the growth of Kch over-expressing cells.

Specificity of Kch

We next investigated the specificity of Kch by testing the ability of other monovalent cations, including Na^+, Rb^+, and NH_4^+, to support the growth of Kch-transformed cells. For this, we included these test ions at 90 mM in agar plates and plated the transformed cells onto two sets of plates, one containing (induced) and the other lacking (uninduced) IPTG. No colonies were seen on Na^+ and IPTG containing plates (Fig. 2A). In contrast, there were no obvious differences in the number of colonies between the induced and uninduced plates when K^+, Rb^+, or NH_4^+ was included in the plates. This suggests that both Rb^+ and NH_4^+ were able to substitute for K^+ in supporting the growth of Kch over-expressing *E. coli*. The growth (measured as diameter of colonies) of induced cells relative to the uninduced cells for K^+, Rb^+, and NH_4^+ was 0.76, 0.83, and 0.59, respectively. The data are consistent with the known ion selectivity of a K^+-selective pore [4,17].

Fig. 2B shows that the ability of K^+ to support the survival and growth of Kch transformed cells is dose dependent. The viability of transformed cells improved with the increase in K^+ concentration of the medium, reaching optimum value at about 20 mM (added concentration, total concentration being ~28 mM).

Effect of potassium channel blockers on Kch

Pharmacology of Kch was examined by testing the ability of known potassium channel blockers, tetraethylammonium (TEA^+), 4-aminopyridine (4-AP), Cs^+, and Ba^{2+}, to support the growth of Kch expressing cells. For this, a filter disc assay was used. Transformed cells

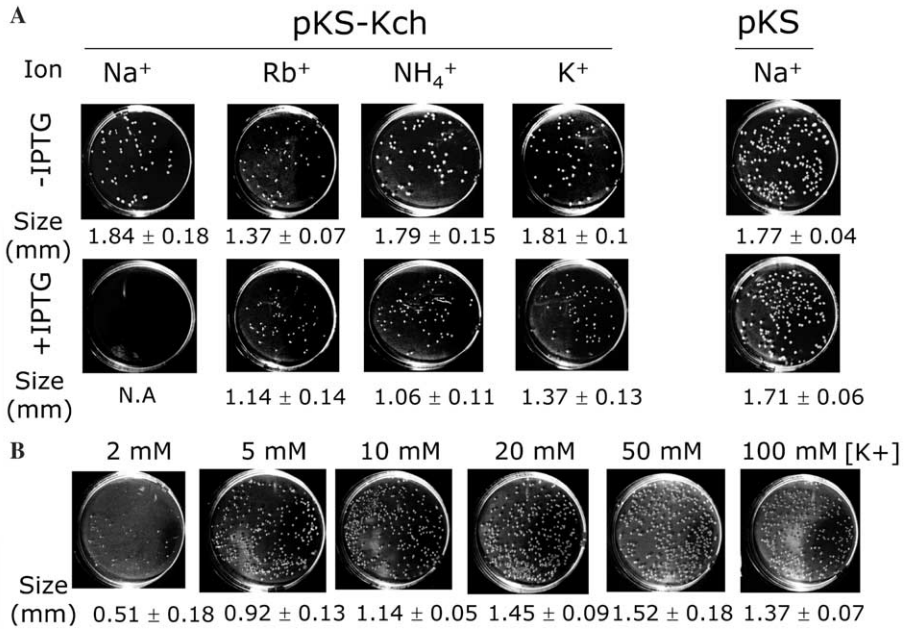

Fig. 2. Kch facilitates selective transport of K^+. (A) Survival and growth of Kch over-expressing *E. coli* are supported by K^+, Rb^+, and NH_4^+, but not Na^+. BL21 (DE3) (pLysS) cells were transformed with pKS-Kch and grown on agar plates (LB-agar-Amp-Cm) containing the chloride salts of the indicated test cations (90 mM) plus 10 mM NaCl, with or without IPTG. Data for the control plasmid, pKS, lacking the *Kch* gene, are shown for sodium ions. (B) The growth of Kch over-expressing *E. coli* increased with the increase in the concentration of K^+ in the medium. Transformed cells were plated as in (A) on plates containing IPTG and the indicated concentrations of KCl. NaCl was included so that the final concentration of the two salts was 100 mM. Mean ± SEM of the diameter (mm) of 10 colonies of the transformed cells is indicated for each plate. Data shown are a representative of three independent experiments.

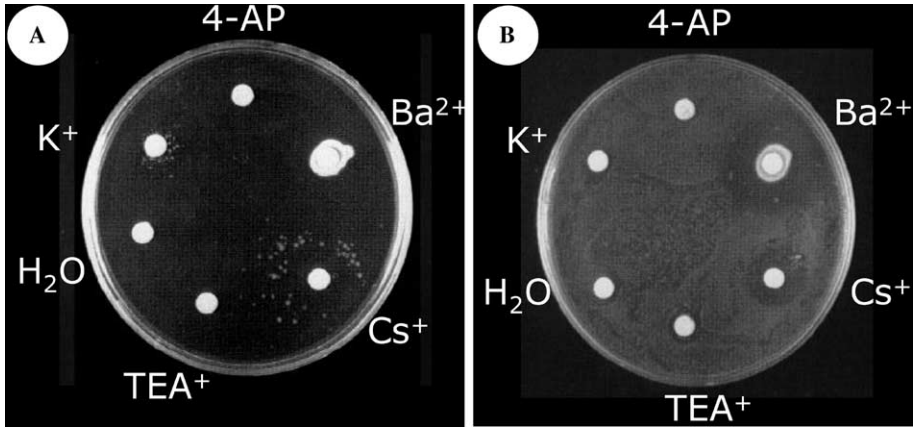

Fig. 3. Effect of known potassium channel blockers on Kch. BL21 DE3 (pLysS) cells transformed with pKS-Kch were plated onto IPTG+ (A) or IPTG− (B) plates. Sterile filter discs saturated with 1 M test substances (indicated), made up in LB or ethanol (4-AP), were placed on the media. Representative data ($n = 3$) are shown.

were first plated on agar plates with (Fig. 3 panel A) or without (Fig. 3 panel B) the inducer. Filter discs saturated with 1 M test substances were then placed on the plates and incubated at 37 °C. If the test substance inhibits Kch, growth surrounding the test substance would be expected. The results show that apart from Cs^+, none of the other substances supported growth. Ba^+ was found to be toxic to the control cells, so its effect could not be determined. TEA^+ and 4-AP were unable to support the growth. KCl was used as a positive control; as expected, growth was seen surrounding the disc containing KCl. The lack of effect by TEA^+ and 4-AP suggests that these substances were either not effective in blocking the Kch activity or were only partially effective.

Detection of plasmid-mediated Kch expression

To distinguish between the Kch protein expressed from the plasmid construct from Kch expressed from the native *Kch* gene of the bacterial chromosome, we have carried out pulse labelling experiments with [^{35}S]methionine in the presence and absence of rifampicin. Since rifampicin prevents the synthesis of proteins from the bacterial chromosome, but not from the T7 promoter of the plasmid construct [13,16], it was possible to uniquely label the Kch protein expressed from the plasmid construct. Fig. 4 shows a number of labelled bands corresponding to bacterial proteins (lane U) and bacterial proteins plus proteins encoded by the plasmid (lane I). In the presence of rifampicin, however, only one band with an approx Mr of 43 kDa was seen (lane R). The size of this band corresponds to the calculated mass of Kch (45.9 kDa). We were unable to grow cells in liquid cultures for prolonged periods to be able to assess the level of expression using Coomassie blue staining. Using slightly different constructs and conditions, however, others [8,9] have demonstrated that Kch can be expressed in large quantities in *E. coli*.

Kch is not functional in oocytes

In an attempt to study the electrophysiological properties of Kch, we added the Kozak sequence to Kch and expressed the resultant construct in *Xenopus* oocytes. Currents were then recorded in both low (2 mM) and high (100 mM) K^+ media at various voltages.

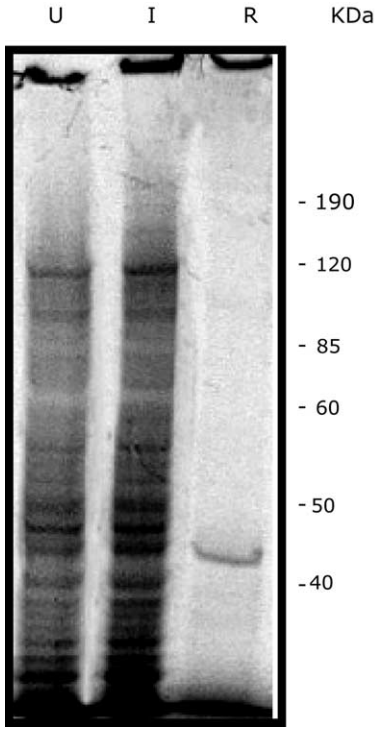

Fig. 4. Pulse-labelling of Kch with [^{35}S]methionine. BL21(DE3) cells harbouring pKS-Kch were treated with [^{35}S]methionine for 5 min to radiolabel the proteins. The labelled proteins were separated on a 8% SDS–polyacrylamide gel and detected by fluorography. Labelling was performed on cells before (lane U) and after (lane I) induction with IPTG. Lane R shows proteins isolated from induced cells but treated with rifampicin. Positions of marker proteins are indicated on the right.

However, no currents above the endogenous levels could be detected (data not shown). The lack of expression may be due to the absence, in oocytes, of auxiliary subunits or factors required to support functional expression of Kch or, alternatively, due to poor expression of Kch in oocytes.

To circumvent these potential problems, we substituted the pore region of the *Shaker* potassium channel with the corresponding region of Kch and expressed the resultant chimaeric construct (*Shaker* channel with the Kch pore) in oocytes. Since *Shaker* channels can be functionally expressed in oocytes, we reasoned that in the *Shaker* background the Kch pore loops could function without the need for any auxiliary subunits or factors. However, currents recorded from oocytes injected with the chimaeric cRNA were indistinguishable from those of the water-injected oocytes (Fig. 5A). The absence of currents is not due to the lack of expression because when cRNA encoding the chimaeric construct was co-injected with cRNA for *Shaker* channel, currents through *Shaker* channel were suppressed (Figs. 5B and C). Current amplitudes recorded from oocytes injected with a 5:1 *Shaker* to chimaera cRNA ratio (Fig. 5C) were about 50% less than those obtained from the equivalent amount of *Shaker* cRNA alone (Fig. 5B); at high ratios, there was a total loss of current from the *Shaker* subunits (Fig. 5D). This suggests that the chimaeric protein was expressed in oocytes and was able to associate with the *Shaker* subunits to confer a dominant negative effect on the *Shaker* channel function.

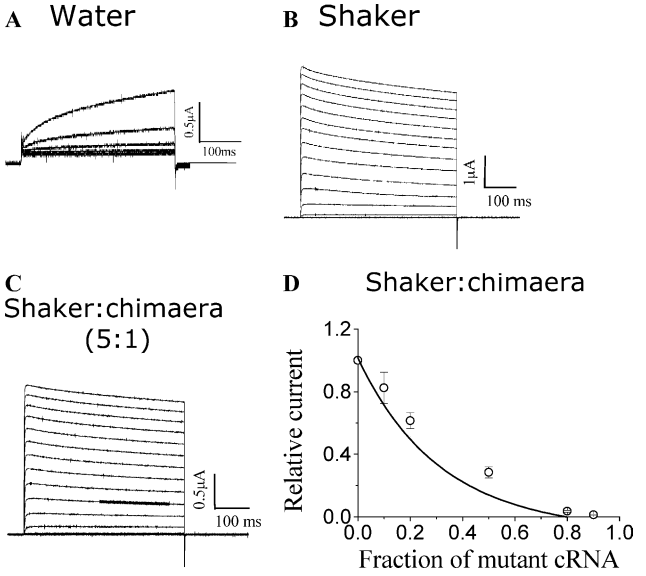

Fig. 5. The *Shaker* potassium channel containing the pore region of Kch is nonfunctional. Electrophysiological properties of *Shaker* channel containing the Kch pore loop (Sh-Kch chimaera). (A–C) *Xenopus* oocytes were injected with water (A), or cRNA encoding the *Shaker* channel (B) or a 5:1 mixture of cRNAs encoding the *Shaker* and Sh-Kch chimara (C). Current families shown were elicited by a series of 500 ms depolarising pulses, given at 10 mV intervals up to +80 mV, from a holding potential of −80 mV. (D) Currents were recorded from oocytes injected with the indicated ratio of chimaeric to wild-type *Shaker* cRNA. Points (◯) represent the mean of currents (measured at +40 mV; $n = 3$) expressed as a fraction of currents from the wild-type *Shaker* alone. The line represents the theoretical curve for a tetramer, using the equation, relative current $(I/I_{wt}) = (1 - p)^3$ [26], where I = current from mixtures, I_{wt} = current from *Shaker* channel alone, and p = fraction of mutant cRNA injected.

The pore region of the eukaryotic Shaker potassium channel can be functionally substituted into Kch

We next made a reverse construct, where we substituted the pore region of Kch with that of the *Shaker* channel and expressed in *E. coli*. The rationale was that if the function of Kch was to conduct K^+ ions, it should be possible to functionally substitute its pore loops with pore loops derived from any potassium channel. To test this, cultures of BL21 (DE3) (pLysS) cells harbouring the chimaeric construct (Kch with *Shaker* pore), Kch (positive control), and the parent vector (negative control) were first grown in liquid medium in the absence of IPTG. Aliquots of serially diluted cultures were then plated on agar plates, with and without IPTG, and with IPTG plus 50 mM K^+. As can be seen from Fig. 6, bacteria containing the vector alone grew well on all plates. However, cells harbouring the chimaeric construct, like those containing the Kch plasmid construct, showed no growth on plates containing the inducer (middle panel). Supplementation of K^+ to the inducer containing plate (bottom panel) fully restored growth

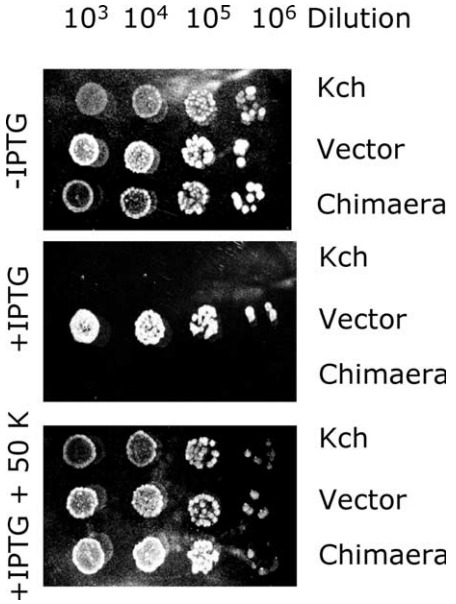

Fig. 6. Phenotype of *E. coli* transformed with Kch containing the Shaker pore region (Kch-Sh chimaera). BL21 (DE3) cells harbouring the indicated vector-gene (Kch or chimaera) constructs or the vector alone were grown to A_{600} of 0.6 and serially diluted (10^3 through 10^6) cultures were plated on agar plates (LB-agar-Amp-Cm) with or without IPTG or with IPTG plus 50 mM K^+. Growth after 16 h at 37 °C is shown.

(compare with growth on plate (top panel) lacking the inducer). Thus, cells expressing the chimaeric construct displayed a phenotype that is similar to cells expressing Kch, supporting the argument that the P-loop of Kch functions in K^+ conductance.

Discussion

In the present study, we have sought evidence for the proposition that the *Kch* gene of *E. coli* encodes a protein that might function as a potassium channel [1,17,18]. For this we have used two approaches. First, we expressed the *Kch* gene from the T7 promoter of a bacterial expression vector in *E. coli* and examined changes in the phenotype of the cell. Second, we expressed the Kch protein, and a chimaera containing the Kch P-loop region in the *Shaker* potassium channel backbone, in *Xenopus* oocytes and investigated if they conduct K^+ currents.

We found that over-expression of Kch was detrimental to the survival and growth of *E. coli* (Fig. 1). However, when the growth medium was supplemented with potassium channel selective ions including K^+, Rb^+, and NH_4^+, but not Na^+, the toxic effect of over-expressed Kch could be completely overcome (Fig. 2). The toxicity could also be prevented by Cs^+, a known blocker of certain types of potassium channels (Fig. 3). These findings are consistent with the suggestion that Kch might conduct K^+ ions. Moreover when the putative pore region of Kch was replaced with the well-characterized pore region of the eukaryotic *Shaker* potassium channel, the resultant chimaera conferred the same phenotype change on *E. coli* as Kch (Fig. 6). The ability of an authentic K^+ channel pore-forming sequence to substitute the function of the Kch pore provides further evidence to the hypothesis that Kch conducts K^+ ions.

Kch, however, could not be functionally expressed in *Xenopus* oocytes, preventing detailed studies on its biophysical and regulatory properties. This is not surprising given the fact that there are no reports on the expression of bacterial channels in *Xenopus* oocytes, with the exception of lctB of *Bacillus stearothermophilus* [19]. Attempts to express a chimaera of the *Shaker* potassium channel containing the Kch pore region were also unsuccessful (Fig. 5). This was not due to poor expression because the chimaeric protein suppressed the *Shaker* channel currents, perhaps by forming heterotetramers with the *Shaker* subunits (Fig. 5D). It is important to note that although the membrane topology of Kch is similar to that of the *Shaker* channel in having six transmembrane segments, it lacks the voltage sensing charged residues in S2–S4 and is unlikely to be gated by voltage. As such, the pore loop of Kch might lack residues required for coupling the voltage sensor to pore opening. Thus, the inability of the Kch pore loop to function in the *Shaker* background is more likely to be due to lack of coupling rather than the inability to conduct K^+ ions.

The lack of expression of Kch and the chimaera in *Xenopus* oocytes prevented us from studying its rectification properties. As a result, it is not clear whether Kch mediates K^+ uptake or efflux in *E. coli*. Since the resting potential of bacterial cell membranes is very negative (−100 to −150 mV) [20,21] assuming that Kch is an ohmic potassium channel, the electrochemical gradient for K^+ would be strongly in favour of K^+ entering into the cell. This means that any rise in extracellular K^+ concentration would be expected to increase the influx of K^+, hence of water, and cause the cell to burst. However, we observed the opposite effect: increasing the extracellular K^+ concentration actually protects the cell (Figs. 1 and 2). This suggests that Kch, like most other six transmembrane potassium channels, may be an outwardly rectifying potassium channel, favouring only K^+ efflux (assuming that the reversal potential for K^+ is more negative than the nesting potential). Similar outwardly rectifying potassium channels have been described in the yeast (Tok1) [22,23] and plant guard cells [24,25] whose membrane potentials are also very negative, yet capable of supporting K^+ efflux at as low as −200 mV.

In summary, we have shown that Kch conducts K^+ ions and that the pore loop of Kch can be functionally replaced with that of the eukaryotic *Shaker* potassium channel. The ability to express eukaryotic pore sequences in *E. coli* using Kch as a supporting structural framework and examine the pore function using a simple phenotype assay provides a new opportunity to investigate the pharmacology of potassium channel pores derived from channels of diverse origin and subtypes in a prokaryotic background.

Acknowledgments

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