Physiol Genomics 26: 1–7, 2006.
First published April 4, 2006; doi:10.1152/physiolgenomics.00026.2006.

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Cloning and functional characterization of a superfamily of microbial inwardly rectifying potassium channels

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Submitted 15 February 2006; accepted in final form 3 April 2006

Sun, Si, Jo Han Gan, Jennifer J. Paynter, and Stephen J. Tucker. Cloning and functional characterization of a superfamily of microbial inwardly rectifying potassium channels. *Physiol Genomics* 26: 1–7, 2006. First published April 4, 2006; doi:10.1152/physiolgenomics.00026.2006.—Our understanding of the mammalian inwardly rectifying family of K^+ channels (Kir family) has recently been advanced by X-ray crystal structures of two homologous prokaryotic orthologs (KirBac1.1 and KirBac3.1). However, the functional properties of these KirBac channels are still poorly understood. To address this problem, we cloned and characterized genes encoding KirBac orthologs from a wide variety of different prokaryotes and a simple unicellular eukaryote. The functional properties of these KirBacs were then examined by growth complementation in a K^+ uptake-deficient strain of *Escherichia coli* (TK2420). Whereas some KirBac genes exhibited robust growth complementation, others either did not complement or showed temperature-dependent complementation including KirBac1.1 and KirBac3.1. In some cases, KirBac expression was also toxic to the growth of *E. coli*. The KirBac family exhibited a range of sensitivity to the K^+ channel blockers Ba^{2+} and Cs^+ as well as differences in their ability to grow on very low-K^+ media, thus demonstrating major differences in their permeation properties. These results reveal the existence of a functionally diverse superfamily of microbial KirBac genes and present an excellent resource for the structural and functional analysis of this class of K^+ channels. Furthermore, the complementation assay used in this study provides a simple and robust method for the functional characterization of a range of prokaryotic K^+ channels that are difficult to study by traditional methods.

prokaryotic K^+ channel; cyclic nucleotide-gated channel

INWARDLY RECTIFYING K^+ (Kir) channels are found in a wide range of cell types and tissues, where they are important in controlling the resting membrane potential and a variety of K^+ transport processes (2, 17). The human genome contains 15 different Kir channel genes divided into 7 subfamilies, and their fundamental importance is highlighted by the fact that mutations in several Kir channel genes result in diseases such as Andersen's syndrome, Bartter's syndrome, congenital hyperinsulinemia, and forms of neonatal diabetes (1, 8, 16).

Many of the recent advances in our understanding of K^+ channel structure and function have been achieved through the study of prokaryotic homologs (11, 13, 14). But although many of these prokaryotic K^+ channels share extensive homology with mammalian channels in the "transmembrane pore" region, their homology outside of this region is less well defined, e.g., the KcsA K^+ channel from *Streptomyces lividans*, which has proven so useful in determining the mechanism of K^+ permeation and channel gating, has no obvious mammalian counterpart. In contrast, recent X-ray crystal structures of the prokaryotic KirBac channels KirBac1.1 (12) and KirBac3.1 (18) have revealed that these channels share extensive structural homology with the superfamily of mammalian Kir channels and can be regarded as true "prokaryotic homologs" (2). However, despite this wealth of structural information, little functional data exist for prokaryotic KirBac channels; the only functional studies have been macroscopic ion flux studies using purified KirBac1.1 protein in reconstituted liposomes (4, 5). Although these studies have been useful, they are labor intensive and are not particularly suited to high-throughput analysis. Attempts at more detailed electrophysiological analysis by reconstitution of purified protein into artificial lipid bilayers have also proven largely unsuccessful (S. J. Tucker, unpublished data). Alternative approaches to the study of this important family of ion channels are therefore required.

We undertook a systematic search of the wealth of microbial genomic information now available to search for novel KirBac genes that may serve as useful models for the structural and functional study of this superfamily of K^+ channels. In this study, we describe the cloning of a family of novel KirBac channels and their functional characterization by expression in a K^+ uptake-deficient strain of *Escherichia coli*. This family of novel KirBac channels appears to have a range of functional properties and provides an excellent resource for the analysis of Kir channel structure and function.

METHODS

Molecular biology. The Integrated Microbial Genomes (IMG) system (**http://img.jgi.doe.gov**) was used to identify KirBac genes by TBLASTN analysis with KirBac1.1 as the query sequence. Genomic DNA samples were the kind gifts of the following: *Magnetospirillum magnetotacticum*, Dirk Schueler, Max-Planck Institute for Marine Microbiology, Bremen, Germany; *Phytopthora ramorum*, Katy Hayden, University of California, Berkeley, CA; *Synechocystis* PCC6803, Nigel Robinson, University of Newcastle, Newcastle, UK; *Ralstonia solanacearum*, Simon Weller, Central Science Laboratory, York, UK; and *Solibacter usitatus* sp. Ellin6076, Cheryl Kuske, Los Alamos National Laboratory, Los Alamos, NM. *Chromobacterium violaceum* genomic DNA was obtained from the American Type Culture Col-

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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lection (no. 12472). The gene encoding KirBac2.1 (*Anabaena* PCC7120) was kindly provided by Chris Miller (Brandeis Univerisity, Waltham, MA). The cloning of KirBac1.1 has been described previously (12). On the basis of the genomic database sequences, specific oligonucleotides were designed to incorporate the initiator methionine into a *Nco*1 restriction site (5′-CCATGG-3′, where underlined sequence shows the start codon), whereas reverse primers incorporated a *Hin*dIII restriction site after the stop codon. Genes were amplified by PCR using high-fidelity *Tli* DNA polymerase and cloned into the pQE60-lac expression vector using a standard DH5α strain of *E. coli* for plasmid propagation. Sequences were then verified by automated sequencing of both strands. The pQE60-lac vector was constructed by inserting the β-galactosidase repressor gene encoded on the *Nde*1/*Xba*1 fragment of pQE80L into the *Nde*1/*Xba*1 sites of pQE60, thus generating a host-independent *cis*-repressed expression plasmid.

Growth media. The high-K^+ K115 media contained (in mM) 46 K_2HPO_4, 23 KH_2PO_4, 8 $(NH_4)_2SO_4$, 0.4 $MgSO_4$, 0.006 FeSO4, 1 sodium citrate, and 40 glucose, with 1 mg/l thiamine hydrochloride. K0 was identical to K115 with the exception that K^+ was replaced by equimolar quantities of Na^+ (10). For solid media, 1.5% bacteriological agar was added. For barium inhibition studies, TK0 media contained 10 g/l tryptone and 130 mM NaCl. The high-K^+ LBK130 media contained 10 g/l tryptone, 5 g/l yeast extract, and 130 mM KCl. K^+, Ba^{2+}, and Cs^+ concentrations were varied by supplementation from sterile stock solutions of the appropriate chloride salt to the concentrations indicated.

Growth assays. The TK2420 strain was kindly provided by Prof. Wolf Epstein (University of Chicago, Chicago, IL). Cultures were grown overnight in K115 media, washed in K0 media, and then diluted in K0 media supplemented with KCl to the concentrations indicated. The density of the culture was adjusted by measuring the absorbance at 600 nm [600-nm optical density (OD_{600})] and adjusted to obtain equal OD_{600} values for each sample of ~0.1. Expression at *time 0* was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Samples were grown at 37°C unless otherwise indicated, and growth was monitored by measurement of OD_{600} at the appropriate time points after induction. Nonsupplemented K0 media contained <0.05 mM K^+ as determined by flame photometry. For barium inhibition studies, TK0 media were used instead of K0 media, and growth was quantified by calculating the area under each growth curve. In each case, the background growth (pQE60-lac control) was subtracted from the calculated value and expressed as a percentage (means ± SE) of growth in the absence of barium. The same approach was taken to quantify the growth in different K^+ concentrations and expressed as a percentage of growth in 8 mM K^+. Drop tests were performed to investigate the growth of cells over a longer time period using solid K0 media containing 0.5 mM IPTG. Overnight cultures were spun down, washed, and resuspended in an equal volume of K0; 4 μl of undiluted and 1:100 and 1:10,000 dilutions were spotted onto the plates and allowed to dry before being grown overnight at the indicated temperatures.

RESULTS

Identification and cloning of the KirBac superfamily. A previous data mining search identified three KirBac channel genes: KirBac1.1 (*Burkholderia pseudomallei*), KirBac2.1 (*Anabaena* sp. PCC7120), and KirBac3.1 (*M. magnetotacticum*) (3). To identify novel KirBac genes, we searched the IMG system using the KirBac1.1 amino acid as a query sequence. Six sequences exhibiting >90% identity to the *B. pseudomallei* KirBac1.1 gene were found in the *Burkholderia* genus, including *B. ambifaria*, *B. vietnamiensis*, *B. thailandensis*, *B. cenocepacia*, *B. xenovorans*, and *B. cepacia* 383. These sequences were not pursued further due to their near identity to KirBac1.1. However, they clearly illustrate conservation of the KirBac1.1 gene in this particular genus.

Novel KirBac gene sequences were also identified in the genomes of *C. violaceum, P. ramorum, Synechocystis* PCC6803, *R. solanacearum*, and *S. usitatus* sp. Ellin6076. These were assigned the names KirBac4.1 to KirBac8.1, respectively, to extend the current nomenclature (3). The species in which these KirBac channels were found range from a model cyanobacterium (*Synechocystis*) to the simple eukaryotic oomycete (*P. ramorum*, which is responsible for sudden oak death syndrome). Such phylogenetic diversity makes it difficult to deduce any particular functional role for these channels from the types of species in which they are found.

An alignment of the KirBac1.1 to KirBac8.1 sequences is shown in the online supplementary information[1] and demonstrates that these different orthologs exhibit significant sequence homology throughout the "structural core" of the channel, but, like their mammalian counterparts, they differ extensively at their distal termini. Pairwise alignments revealed that KirBac2.1 to KirBac8.1 exhibited 65%, 56%, 69%, 47%, 63%, 82%, and 47% homology, respectively, with KirBac1.1. Thus, although similar, these gene products are clearly not identical, and these sequences therefore demonstrate the existence of a diverse superfamily of microbial Kir channels. To isolate these genes, the KirBac1.1 to KirBac8.1 sequences were first PCR amplified from either genomic DNA or plasmid DNA (see METHODS) and cloned into the pQE60-lac expression vector. This novel expression vector is a tightly regulated *cis*-repressed variant of the commercially available, host-independent pQE60 vector (see METHODS). In all cases, the cloned sequences were found to be identical to those in the database. The translational ability of each KirBac gene was also checked by in vitro transcription/translation using *E. coli* S30 extracts. In each case, full-length proteins were generated, thus demonstrating their suitability for expression in an *E. coli*-based system (not shown).

Functional characterization by complementation analysis. Traditional methods for analyzing prokaryotic ion channels rely on recombinant expression in *E. coli*, affinity purification, and reconstitution into either lipid bilayers for electrophysiological analysis or into liposomes for macroscopic ion flux studies (10, 11, 13, 14). A recent study (7) also reported successful expression of KcsA in mammalian cells using a codon-optimized synthetic gene. However, although these approaches have many advantages, they are labor intensive and not suited for the rapid analysis of ion channel function. Previous studies (9, 10, 19) have taken advantage of the ability of recombinantly expressed K^+ channels to complement the K^+ uptake deficiency of the TK2420 strain of *E. coli*. In wild-type *E. coli,* K^+ uptake is mediated by three principal pathways: Kdp, Trk, and Kup (6). The TK2420 strain has mutations in the genes responsible for these three pathways, and, consequently, the strain exhibits almost no growth in media containing low concentrations of K^+ (<10 mM). Instead, normal growth requires media supplemented with K^+ (typically 100 mM; see METHODS). However, this growth defect in low external K^+ concentrations can be complemented by

[1] Supplemental data for this article is available at the *Physiological Genomics* web site.

recombinant expression of a functional K^+ channel, which provides an alternative pathway for the entry of K^+. We therefore decided to test whether these different KirBac genes produced functional K^+ channels by complementation analysis in the TK2420 strain. Briefly, cultures of TK2420 transformed with different KirBac pQE60-lac plasmids (or empty vector) were grown overnight in high K^+ concentrations, washed in K0 media, and then diluted to an identical optical density (OD_{600} = 0.1) in K0 media containing 7 mM K^+. Expression was induced at *time 0* by the addition of 0.5 mM IPTG and growth at 37°C monitored by measuring OD_{600} at different time points.

Figure 1 shows a representative growth curve for each of the different KirBac channels. KirBac2.1, KirBac6.1, KirBac7.1, and KirBac8.1 all showed significant growth after induction. No growth for any KirBac clone was seen in the absence of IPTG (not shown) or for the empty control vector pQE60-lac. Interestingly, KirBac1.1 exhibited a weak but transient complementation, with OD_{600} failing to enter a rapid growth phase and declining after ~4 h. Similarly, the growth produced by KirBac7.1 and KirBac8.1 also appeared to be transient, and, although the cultures reached a higher OD_{600} than KirBac1.1, they plateaued and declined after 6–8 h.

Temperature-dependent complementation. An alternative method for measuring growth complementation in the TK2420 strain is the "drop test." In this method, washed overnight cultures are prepared in the same manner as above (see METHODS) and serial dilutions are then spotted onto solid K0 media (containing IPTG and 7 mM K^+) and allowed to grow. Serial dilutions of KirBac1.1 to KirBac8.1 cultures were therefore spotted and grown at either 30 or 37°C. Figure 2*A* shows that KirBac1.1 exhibited no growth at 37°C but showed robust complementation at 30°C. Although KirBac7.1 and KirBac8.1 did exhibit some growth at 37°C, there was stronger complementation at 30°C. In contrast, KirBac2.1 and KirBac6.1 appeared largely unaffected by temperature. KirBac3.1, KirBac4.1, and KirBac5.1 still exhibited no complementation at 30°C. The improved complementation at 30°C for KirBac1.1, KirBac7.1, and KirBac8.1 was also reflected in their ability to grow well in liquid culture at 30°C, as shown in Fig. 2, *B–D*.

Toxicity of KirBac expression in E. coli. The marked temperature dependence shown by KirBac1.1 and the "transient" complementation at 37°C seen in liquid culture would explain why no growth is observed on the solid media grown at 37°C. It also suggests that expression of KirBac1.1 may be toxic to the cells at 37°C. The similar effects seen with KirBac7.1 and KirBac8.1 also suggest a related mechanism. To test this, we examined the viability of TK2420 cells expressing KirBac8.1 grown at either 30 or 37°C. After induction, aliquots of cells were removed at various time points and grown on nonselective, high-K^+ LBK130 plates, and the numbers of colonies were counted. Figure 2*E* shows that the viability of cells expressing KirBac8.1 at 37°C rapidly decreased with time after expression was induced and that this reduction in viability correlated with the "plateauing" effect seen in the growth curves in culture. No such reduction in viability was seen when the cells were grown at 30°C. Therefore, the inability of the cells to enter a rapid growth phase and to reach a high optical density at 37°C is because overexpression of KirBac8.1 is toxic to the cells.

KirBac channel block by Ba^{2+}. Barium is a commonly used blocker of Kir channels (17). We therefore investigated the effect of Ba^{2+} on the growth complementation produced by KirBac expression. However, the K0 media used in this assay contains high concentrations of phosphate and sulphate ions, which preclude the use of Ba^{2+}. We therefore used TK0 media containing only NaCl and tryptone (see METHODS). Flame photometry revealed this to have a K^+ concentration of ~0.1 mM, and, in the presence of 7 mM K^+, the growth complementation exhibited by KirBac1.1 to KirBac8.1 was identical to that seen

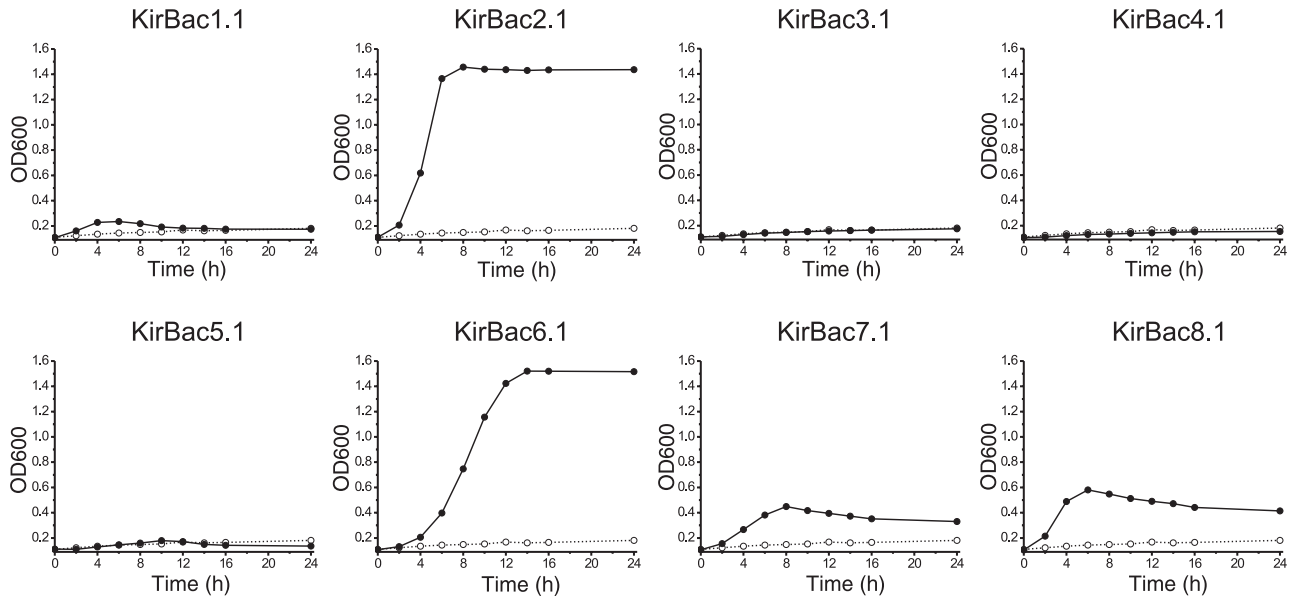

Fig. 1. Functional complementation of the TK2420 growth defect by expression of inwardly rectifying KirBac channels. Expression was induced at *time 0* by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; see METHODS), and growth in culture (K0 media containing 7 mM KCl) was monitored by measuring 600-nm optical densiometry (OD_{600}) over 24 h at 37°C. In each graph, KirBac growth is indicated by solid lines and filled circles and growth of the control strain containing the empty pQE60-lac vector shown by open circles and dotted lines.

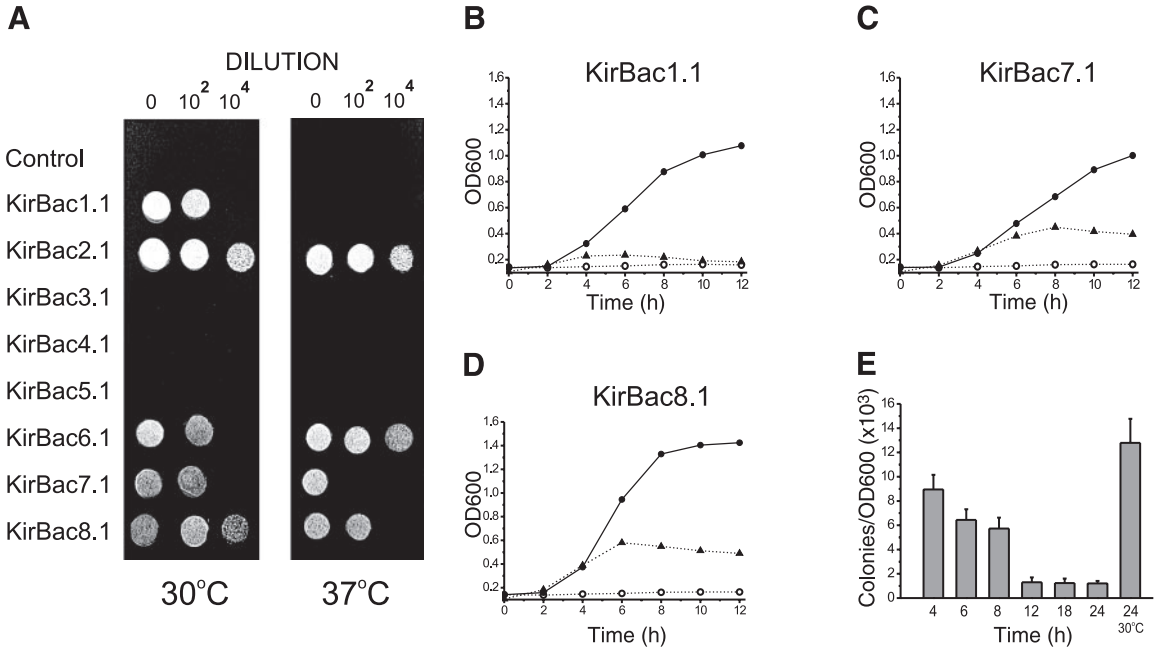

Fig. 2. KirBac toxicity to *Escherichia coli* growth is temperature dependent. *A*: drop tests of indicated dilutions of overnight cultures on solid K0 media containing IPTG and 7 mM K^+. Plates were grown at either 30 or 37°C for 18 h. *B–D*: KirBac1.1, KirBac7.1, and KirBac8.1 complementation when grown in culture at 30°C (filled circles) or 37°C (filled triangles). Open circles indicate the control strain with the empty pQE60-lac vector. *E*: cell viability of KirBac8.1 cultures grown at 37 and 30°C measured over 24 h. Viability was measured as the number of viable colonies and adjusted relative to $OD_{600} = 1$. Values are means ± SE; $n = 3$.

above with K0 media (not shown). Figure 3 shows that Ba^{2+} had a clear concentration-dependent inhibitory effect on the growth complementation shown by KirBac1.1, KirBac2.1, KirBac6.1, KirBac7.1, and KirBac8.1 at 30°C.

The inhibitory effect of Ba^{2+} on KirBac1.1 appeared to be very weak, with 1 mM Ba^{2+} having almost no effect. The effect of Ba^{2+} on KirBac8.1 appeared even weaker, with only ~50% inhibition caused by 10 mM Ba^{2+}. In contrast, the inhibitory effects of Ba^{2+} on KirBac2.1, KirBac6.1, and KirBac7.1 were much stronger. In particular, 0.1 mM Ba^{2+} caused >80% inhibition of KirBac6.1, and 1 mM Ba^{2+} inhibited KirBac2.1 and KirBac7.1 by >50%. Under non-K^+-selective conditions, 10 mM Ba^{2+} had no effect on the growth of TK2420 (not shown), implying that the effects of Ba^{2+} are specific. Furthermore, the inhibitory effect of Ba^{2+} was dependent on the external K^+ concentration, as would be expected from a blocker that acts as a competitive permeant ion; 5 mM Ba^{2+} caused 78 ± 12% block of KirBac8.1 growth when the extracellular K^+ concentration was reduced to 2 mM compared with ~10% block at 7 mM K^+, as seen in Fig. 3. Cs^+ also had a similar inhibitory effect; in the presence of 7 mM external K^+, the growth of KirBac1.1 and KirBac8.1 was largely unaffected by the addition of 5 mM CsCl, whereas growth of KirBac6.1 was inhibited 85 ± 9% by 1 mM CsCl. Like Ba^{2+}, these effects were also dependent on the external K^+ concentration, consistent with the role of Cs^+ as a permeant ion (not shown).

Effect of extracellular K^+. In all of the cases above, the extracellular K^+ concentration was held constant at 7 mM. We therefore tested the complementation of KirBac1.1 to Kir-

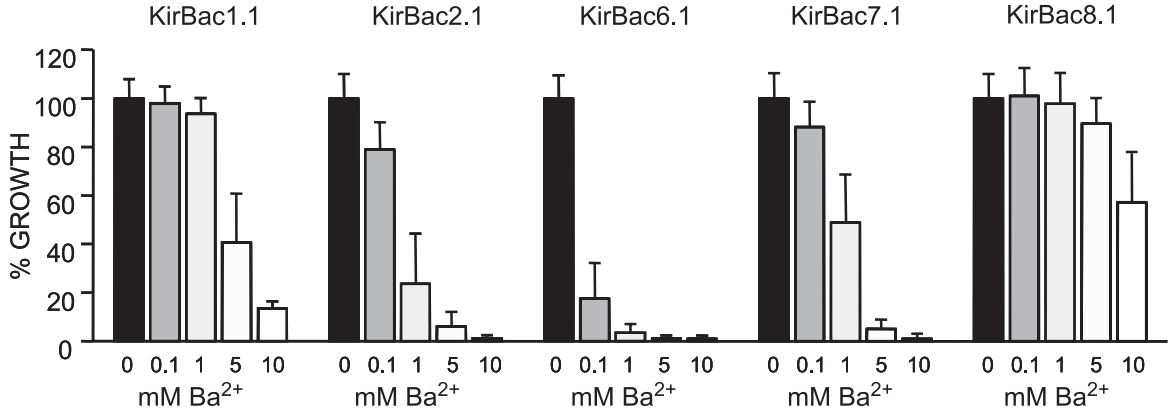

Fig. 3. Inhibition of growth by Ba^{2+}. The relative growth at 30°C over 12 h of different KirBac-expressing strains was measured in the presence of the indicated concentrations of Ba^{2+}. Values are expressed as percentages of growth in the absence of Ba^{2+} ($n = 3$; see METHODS).

Bac8.1 in a range of different extracellular K^+ concentrations. As expected, the amount of growth increased with the external K^+ concentration, presumably because this increased the driving force for K^+ entry. However, as seen in Fig. 4*A*, this effect saturated at markedly different concentrations for the various KirBacs. This suggests that sufficient K^+ entry is achieved to obtain a maximal growth and any further increases in K^+ concentration have no effect. Furthermore, the "minimum" K^+ concentration required for complementation varied significantly between KirBacs. For example, growth of KirBac1.1 and KirBac8.1 was observed at concentrations as low as 0.5 mM K^+. In contrast, at least 6 mM K^+ was required before complementation was observed with KirBac6.1.

Nonfunctional KirBac channels. The above results demonstrate that both temperature and extracellular K^+ concentration can have a profound effect on the ability of different KirBacs to complement the growth defect of TK2420. We therefore reexamined the effect of temperature and K^+ concentration on the "nonfunctional" KirBacs shown in Fig. 1, namely, KirBac3.1, KirBac4.1, and KirBac5.1. Figure 4*B* shows that when cells were grown at 30°C and in the presence of 15 mM K^+, KirBac3.1 clearly exhibited complementation. However, at this relatively high K^+ concentration, some growth also began to occur in the control. In contrast, no difference in growth rates was seen between KirBac4.1 or KirBac5.1 and controls even when they were grown in either 15 or 20 mM extracellular K^+ at 30 or 25°C (not shown).

A screen for novel K^+ channels? This complementation assay provided vital functional data about the KirBac superfamily of K^+ channels. We therefore tested whether this assay could be used to examine the functional properties of other novel K^+ channels that have also proven difficult to study by conventional means. Ohndorf and MacKinnon (15) recently reported the cloning of a cyclic nucleotide-gated K^+ channel (RP-CNG) from *Rhodopseudomonas palustris*, which shares extensive homology with the eukaryotic family of cation-selective CNG channels. Although the RP-CNG channel expressed well in *E. coli* and could be purified, no functional channels were observed when this purified protein was incorporated into lipid bilayers; functional channels were only observed when a chimera was made between RP-CNG and KcsA (15). We therefore cloned the RP-CNG channel into the pQE60-lac vector and tested its ability to complement the growth defect of TK2420. Figure 5*A* shows that growth

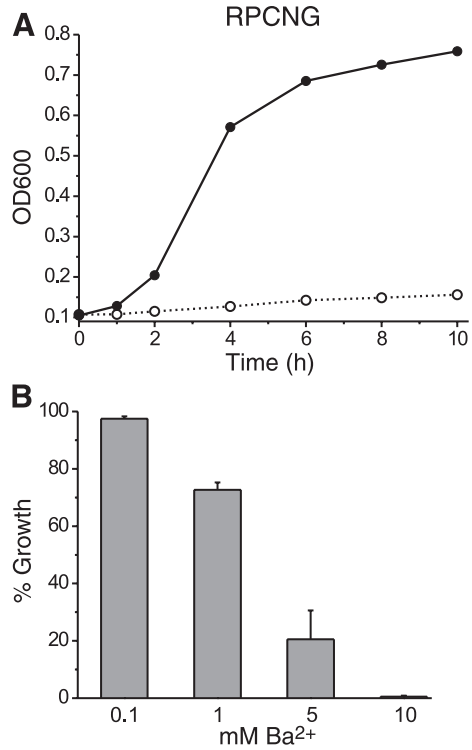

Fig. 5. A useful screen for novel prokaryotic K^+ channels. *A*: complementation can be observed by the cyclic nucleotide-gated K^+ channel RP-CNG (filled circles) when cells were grown at 30°C with 3 mM external K^+ compared with the control (open circles) *B*: complementation by RP-CNG was inhibited in a dose-dependent manner by Ba^{2+} ($n = 3$; see METHODS).

complementation was observed by RP-CNG in 3 mM extracellular K^+ in cells grown at 30°C. Furthermore, Fig. 5*B* shows that this complementation could be blocked in a dose-dependent manner by extracellular Ba^{2+}.

DISCUSSION

In this study, we identified a diverse family of microbial KirBac channels and functionally characterized them by complementation analysis in a K^+ uptake-deficient strain of *E. coli*. The study is important for two main reasons. First, although these prokaryotic orthologs have proven particularly informative about the structure of the related mammalian Kir

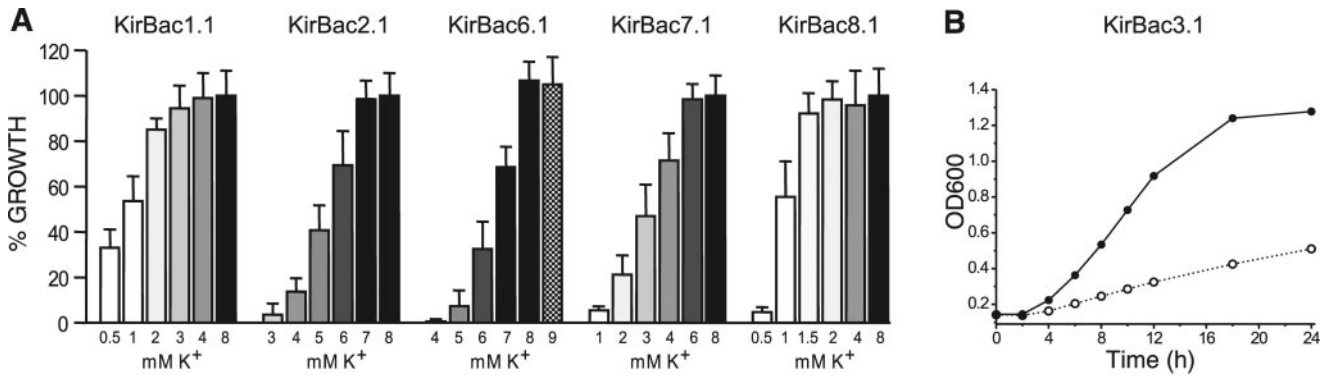

Fig. 4. Effect of external K^+ on KirBac complementation. *A*: growth in different concentrations of external K^+ shown as percentages of growth observed in 8 mM K^+ ($n = 3$; see METHODS). *B*: complementation by KirBac3.1 (filled circles) can be observed when cells were grown at 30°C and external K^+ was raised to 15 mM. At this K^+ concentration, growth was also observed in the control strain (open circles).

channel family, very little is known about the functional properties of these prokaryotic KirBac channels. Therefore, the identification of a family of functionally diverse KirBac channels opens up a range of detailed structural and functional studies that are now possible. In particular, this is the first reported functional activity of the KirBac3.1 channel for which a high-resolution X-ray crystal structure is now available (18). Second, this study demonstrated that these KirBac and other novel prokaryotic K^+ channels that are difficult to analyze by conventional methods can be functionally characterized in a simple and robust *E. coli* assay that has the potential to be adapted for the high-throughput screening of prokaryotic K^+ channel function.

The IMG system database currently contains 674 genomes, of which >80% are finished sequences. Even if only the fully sequenced genomes are included, KirBac genes are found in <3% of these microbial genomes, and no genome was found to contain >1 KirBac gene. Thus, although K^+ channel genes are widespread throughout prokaryotic genomes, KirBac genes are relatively rare, making any kind of phylogenetic analysis difficult. Despite this, KirBac genes appear to be most common among cyanobacteria and proteobacteria, in particular, the *Burkholderiaceae*, which all appear to possess KirBac genes. This observation may be of significance because many of the *Burkholderiaceae* species are pathogenic, in particular, *B. pseudomallei*, which underlies the infectious tropical disease melioidiosis (20). Therefore, the existence of a relatively specific ion channel in the *Burkholderia* genus may prove a potential pharmacological target.

Although the complementation assay used in this study cannot provide the type of detailed functional data that ion channel electrophysiologists have become used to, the assay still provides an enormously useful method for the analysis of prokaryotic K^+ channel function and has many advantages, in particular, its ease and simplicity. A similar genetic screen based on a K^+ uptake-deficient strain of yeast has also successfully been used for the structure/function analysis of mammalian Kir channels (21). However, KirBac channels are inhibited by the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (5), which may explain why KirBac channels do not appear to function when expressed in eukaryotic expression systems, including *S. cerevisiae*. Therefore, in addition to its relative speed and simplicity, the fundamental advantage of this *E. coli* K^+ uptake complementation assay is that the *E. coli* membranes and lipid composition are more representative of a prokaryotic K^+ channel's native environment than any eukaryotic expression system. This may also account for the successful functional expression of the RP-CNG channel in this system.

The assay can also be easily adapted for the high-throughput screening of agents that modulate channel function, e.g., novel KirBac activators/inhibitors, as well as for the rapid screening of mutations that up/downregulate channel activity. Furthermore, with the huge increase in microbial genomic information now becoming available, the assay can also be used as a high-throughput screen for the functional analysis of putative K^+ channel (or transporter) clones.

The temperature-dependent complementation exhibited by some KirBacs could be due to several factors. Our studies with KirBac8.1 demonstrated that expression at 37°C (compared with 30°C) permitted initial growth to occur but then proved toxic to the continued growth of *E.coli*. Thus, continued overexpression at 37°C is toxic either due to a build up of incorrectly assembled/folded proteins or the expression of too many functionally active channels, which would ultimately depolarize the cell. In contrast, the inability of KirBac3.1 to complement growth except at high K^+ concentration and low temperatures (30°C) suggests that either only a few channels are inserted into the membrane at this temperature or that they are not very active; hence, the requirement for the high K^+ concentration. Previous experience purifying KirBac1.1 and KirBac3.1 for structural studies has shown that membrane fractions contain more tetrameric protein when grown at lower temperatures (12, 18). Either way, the ability to use this assay to monitor functional expression may prove useful for optimizing the growth conditions necessary for the purification of functionally active channels for functional and/or structural studies.

The inability of KirBac4.1 and KirBac5.1 to complement growth, even when grown at low temperature or very high K^+ concentration, suggests that these channels are either not inserted into the membrane or that they are nonfunctional under normal physiological conditions in *E. coli*. If they are simply nonfunctional, then these channels may prove a useful substrate for the identification of pharmacological tools that open these KirBac channels or the identification of mutations that enhance channel activity.

The inhibition of growth by blockers such as Ba^{2+} and Cs^+ permits quantitative assessment because the channel affinity for these blockers does not depend on the relative number of channels. Our results showed a clear difference in the Ba^{2+} sensitivity of KirBac1.1 and KirBac8.1 compared with KirBac6.1, a difference that also correlated with their "sensitivity" to extracellular K^+; both KirBac1.1 and KirBac8.1 were capable of complementation even at very low (1 mM) K^+ concentrations and were relatively insensitive to Ba^{2+}. In contrast, KirBac6.1 required much higher concentrations of K^+ (>6 mM) to exhibit significant growth yet appeared very sensitive to Ba^{2+} (0.1 mM Ba^{2+} caused >80% inhibition). These results reflect the important differences in the functional properties of these KirBac channels, which may now be exploited to examine their structural basis. The existence of X-ray crystal structures for KirBac1.1 and KirBac3.1 will also greatly facilitate the design and interpretation of such studies.

In conclusion, this study identified a functionally diverse superfamily of microbial KirBac channels that now provide an excellent resource for the study of KirBac channel structure and function. Such studies will ultimately lead to a greater understanding of mammalian Kir channel function. In addition, we demonstrated that a simple and versatile complementation assay in *E. coli* can be used to study the functional properties of KirBac channels and other novel prokaryotic K^+ channels that have so far proven difficult to study by conventional methods.

GRANTS

This work was funded by the Royal Society and the British Heart Foundation. S. J. Tucker is a Royal Society University Research Fellow.

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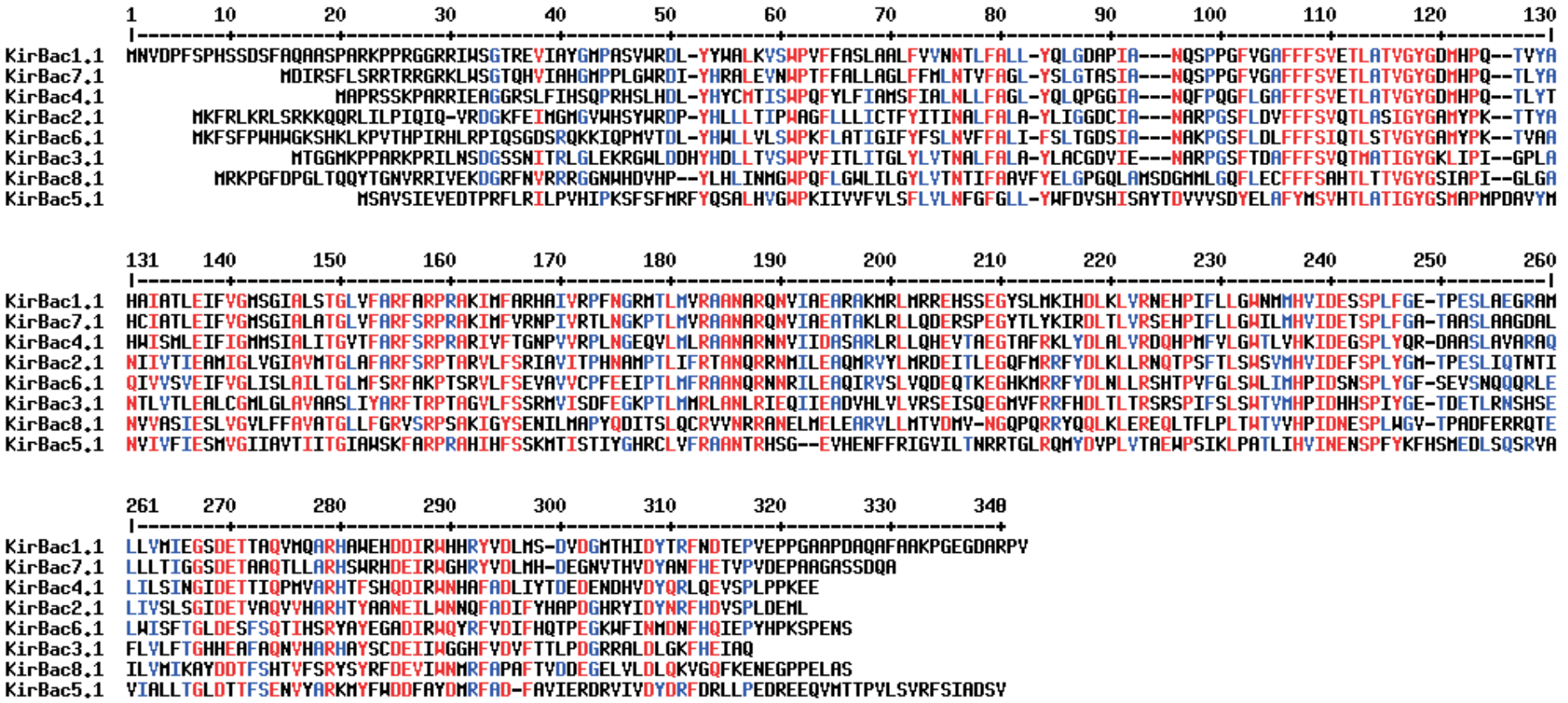

Alignment of KirBac sequences used in this study. Genes were isolated from the following species and assigned the following names to extend the current nomenclature: KirBac1.1 *Burkholderia pseudomallei* (100%), KirBac2.1 *Anabaena sp.* PCC7120 (65%), KirBac3.1 *Magnetospirillum magnetotacticum* (56%), KirBac4.1 *Chromobacterium violacaeum* (69%), KirBac5.1 *Phytophthora ramorum* (47%), KirBac6.1 *Synechocystis* sp. PCC6803 (63%), KirBac7.1 *Ralstonia solanacearum* (82%) and KirBac8.1 *Solibacter usitatus* Ellin6076 (47%). The percentage figures in brackets indicate the % similarity with KirBac1.1. Alignment was performed using Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html) with highly conserved residues being coloured either red or blue according to the degree of conservation (70% and 40% respectively).