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Evolution

An *Escherichia coli* homologue of eukaryotic potassium channel proteins

(potassium channels/sequence comparisons/common ancestry)

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ABSTRACT **A DNA sequence in *Escherichia coli* K-12 contains an evident gene, *kch*, which predicts a protein 417 residues long with extensive similarity to a group of eukaryotic potassium channel proteins in amino acid sequence, in the presence of six apparent transmembrane (S) regions, and in the potassium-specific P (or H5) "pore" region found between S5 and S6. Most of the *kch* gene, including all of these regions and the 5′ flanking region, have been sequenced in 38 wild reference (ECOR) strains as well; variation is conservative, indicating the protein's importance to the species, possibly as a defense against osmotic shock. Since the major family of eukaryotic potassium channel proteins is thought to have evolved from a common ancestor, the evolutionary position of this evident bacterial homologue is of interest, particularly since its function may have changed less than those of eukaryotic channels in the last billion years. While cases of probable importation of eukaryotic genes into bacteria are known, there is no evidence that *kch* has been imported. The relevant properties of the Kch protein and further ways to investigate its evolutionary position are discussed.**

The molecular ancestry of eukaryotic voltage-gated cation channels is obscure, beyond the likelihood that a potassium channel came first. This likelihood is based on the similarity between potassium channel proteins and other cation channel proteins in a set of six transmembrane domains with a cation-specific pore located between the fifth and sixth domains. While potassium channel proteins contain no more than one such set of domains, sodium and calcium channel proteins contain four sets in linear order, implying a history of two duplication events (1). Here I report evidence of a bacterial homologue which appears from sequence to be a potassium channel protein.* This protein, Kch, exists in *Escherichia coli* strain K-12 and in 38 broadly representative wild *E. coli* reference (ECOR) strains (2, 3); its variation is conservative, implying its importance to the cell.

The similarities to be described make it evident that Kch is homologous to eukaryotic potassium channel proteins and, by extension, to sodium and calcium channel proteins. The subject of ancestry calls for some stage-setting. First, we wish to know whether the most recent common ancestor of Kch and the eukaryotic proteins was a bacterial protein. In other words, do the structural properties of Kch imply that the eukaryotic potassium channels have evolved from a bacterial precursor? This would be important to know, since it is likely that bacterial channels, like bacteria themselves, have changed *relatively* little in the last 1 or 2 billion years, so that Kch might resemble the ancestral channel protein. Second, as we now understand that cellular life-forms are divided into three major approximately equidistant groups (4), Archaea (archaebacteria), Bacteria (bacteria), and Eucarya (eukaryotes), an origin in the Archaea is also possible. Third, the connection between similarity and homology is complicated by adaptation/selection (conservation and convergence are both caused by natural selection), by the recombination of nonhomologous protein domains (two proteins may be homologous in only one domain of many), and by horizontal transfer (there is good evidence that bacteria occasionally have imported eukaryotic proteins; ref. 5). Finally, the separation of the Archaea, Bacteria, and Eucarya may have occurred 2 billion years ago or earlier (6).

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METHODS

Computer Programs. Data-base searches, analysis, and protein characterization were carried out with the Genetics Computer Group software package (7). Kch was compared initially to translations of virtually all known nucleotide sequences by using the program TFASTA (8, 9). (TFASTA was used rather than FASTA because of the availability of a much larger data base.) The amino acid sequences of a few proteins of interest were subsequently compared directly, using the programs FASTA (8, 9) and (for multiple alignments) PILEUP (10). Putative transmembrane regions were suggested by Kyte–Doolittle hydropathy analysis (11) initially using a criterion hydrophilicity value of −1.3, determined as a running 7-residue average. The programs PEPTIDE STRUCTURE and (for graphics) PEPPLOT (12) and FIGURE were used.

Divergence Time. Divergence time from a common ancestor was calculated with the formula $t = -[\ln(1 - 2x)]/2k$, derived from $x = (1 - e^{-2kt})/2$, where x is the proportion of substitutions per codon, k is the divergence rate (twice the *retained* nucleotide substitution rate per codon per generation), and t is time in generations. This formula assumes an average of two neutral alternatives per codon, so that of the nine possible substitutions per codon, one can be retained on average. With an estimated nucleotide substitution rate 3×10^{-10} per nucleotide per generation (13), and therefore 1×10^{-10} per specific substitution (e.g., C → T) per generation, k is thus 2×10^{-10}. Numerous other clock formulas exist (14). With comparable assumptions, they should lead to similar results. Real time is based on the assumption of 200 generations per year.

RESULTS AND DISCUSSION

The *kch* locus was cloned as part of an effort to extend a region of comparative sequencing (2) that includes the well known tryptophan (*trp*) operon and the nearby *tonB* locus. A 17-kb region including *tonB* was subcloned to yield a 4-kb region, most of which had not been sequenced. Some 3 kb were sequenced initially by the Maxam–Gilbert method, and PCR primers were generated for the amplification and dideoxy sequencing of K12 and other genomes from the ECOR

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L12044).

strains. A link to the next large sequenced region was subsequently completed via PCR.

The deduced Kch protein contains 417 amino acid residues. It has six hydrophobic membrane-spanning (S) regions like most potassium channel proteins. [Inward rectifying (IRK, ROMK) channels have only two (15, 16).] Between the fifth and sixth S domains is a pore sequence, P, that is strikingly similar to those of many eukaryotic potassium channels and different from those of channel proteins that are selective for other cations.

The evidence of homology came initially from three sources. (*i*) TFASTA searches revealed about 20% identity, in at least 200 residues, between Kch and each of eight known eukaryotic potassium channel proteins (Table 1). (*ii*) Hydropathy analysis demonstrated six hydrophobic stretches (Fig. 1) that match well with the inferred transmembrane S1–S6 regions of other voltage-gated potassium channel proteins; the comparison was anchored in the P region in its characteristic position between S5 and S6. These hydrophobic stretches are taken to form the major part of larger (at least 22 residues) transmembrane domains. A seventh hydrophobic region is apparent further along. (*iii*) A TFASTA search for matches to a 13-residue stretch in the P region (Kch residues 182–194) showed 60–75% identity with corresponding regions in various potassium channel proteins. These, together with the KCH/ECOKCH self-match and one uncharacterized open reading frame found in two *Bacillus* species (27), constitute the top 81 TFASTA matches. There was then an abrupt drop in the scores of matches, and no further mention of potassium-related proteins, channels or not, in the 69 subsequent cases making up the top 150-score sample. Evidence indicates that this P region is part of the putative channel's pore (1) and that the sequence defines its ion selectivity (28). Furthermore, 6 consecutive residues (numbered 185–190) are of particular interest. Fifty-five of the top TFASTA matches are identical to Kch in these residues, 24 have a leucine instead of a valine at position 186, and the *Bacillus* open reading frame codes for a serine instead of threonine at position 185. No other sequences in the data base have more than four identities to Kch in this set of 6.

A number of comparative and descriptive studies (17–19, 22, 26, 29–31) have included alignments of certain eukaryotic potassium channels, emphasizing the six transmembrane regions and the potassium-specific region. The general similarity is stronger in these regions than in the intervening and peripheral parts, in which there is considerable length variation. The corresponding stretch of the Kch sequence (Fig. 2) is not more similar to the eukaryotic channel proteins in its transmembrane regions than in its other central parts, with the striking exception of the P region. While the TFASTA program used does not permit a cumulative shift in alignment

Table 1. Comparison of Kch with various eukaryotic potassium channel proteins

Organism	Gene, accession no. (ref.)			No. of residues aligned	% I	% CR
Rat	k13	M81784	(17)	212	21.2	42.5
Rat	RK5	M59980	(18)	212	20.3	44.3
Mouse	mShal	M64226	(19)	219	20.5	42.9
Mouse	mSlo	L16912	(20)	359	21.2	41.2
Drosophila	*slo*	M96840	(21, 22)	318	22.0	42.8
Drosophila	*eag*	M61157	(23)	236	20.8	47.9
Aplysia	ak01	M95914	(24)	101	24.8	45.5
Arabidopsis	kat1	M86990	(25)	200	19.5	42.5
Arabidopsis	akt1	X62907	(26)	55	30.9	52.7
Mouse	irk1	X73052	(16)	214	22.9	44.9

% I, percentage of identities; % CR, percentage of conservative replacements.

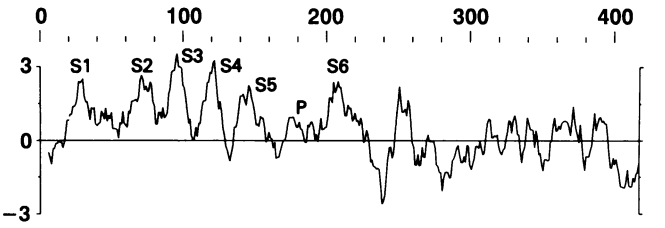

FIG. 1. Hydropathy plot of Kch. Mean hydropathy (ordinate) was plotted against amino acid residue number (abscissa) by using a moving window 11 amino acids wide. Hydrophobic regions are above the zero line, and hydrophilic below. Putative transmembrane and pore regions are labeled S1–S6 and P.

register longer than 32 residues, comparisons made with individual or consecutive Kch transmembrane regions did not reveal a significant number of new affinities, suggesting that this observation is not due to alignment limitations. Similarity in the peripheral regions is less frequent. Also, the S4 region in Kch does not show the regular spacing of basic residues and of leucine residues characteristic of a voltage-gated channel (1).

Eukaryotic potassium channel proteins are classified according to the electrophysiological and pharmacological properties of the channels they form, as well as their amino acid sequences and domain structure. They are grouped in several conserved families (31) that arose early in eukaryotic evolution or, in some cases, perhaps before the origin of eukaryotes. A summary diagram of the overall alignment similarity of 25 representative channel proteins is given in Fig. 3. It gives a clear indication of the relative sequence similarities of the proteins compared. In all cases, expectations based on prior information are met. The nine known members of the outward-rectifier family are grouped together with an *Aplysia* channel. The outward-rectifier family's four subdivisions have *Drosophila*-based (Shaker, Shab, Shaw, and Shal) and corresponding mammalian (Kv1, Kv2, Kv3, and Kv4) names (32). Members of each subfamily are ex-

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  1  MSHWATFKQT ATNLWVTLRH DILALAVFLN GLLIFKTIYG MSVNLLDIFH
        * |      *                  S1
 51  IKAFSELDLS LLANAPLFML GVFLVLNSIG LLFRAKLAWA ISIILLLIAL
                              S2                          S3
101  IYTLHFYPWL KFSIGFCIFT LVFLLILRKD FSHSSAAAGT IFAFISFTTL
                         S4                          S5
151  LFYSTYGALY LSEGFNPRIE SLMTAFYFSI ETMSTVGYGD IVPVSESARL
                                     P
201  FTISVIISGI TVFATSMTSI FGPLIRGGFN KLVKGNNHTM HRKDHFIVCG
           S6          *
251  HSILAINTIL QLNQRGQNVT VISNLPEDDI KQLEQRLGDN ADVIPGDSND
      *                                  *
                                         *
                                         #
301  SSVLKKAGID RCRAILALSD NDADNAFVVL SAKDMSSDVK TVLAVSDSKN
                                                        |
351  LNKIKMVHPD IILSPQLFGS EILARVLNGE EINNDMLVSM LLNSGHGIFS

401  DNDELETKAD SKESAQK
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FIG. 2. Amino acid sequence of Kch. Putative transmembrane regions are underlined. The potassium-selective P region (1) is doubly underlined, and residues 182–194 are in boldface. The limit of comparative sequencing of 39 *E. coli* strains is indicated (#). Amino acid replacements are shown by asterisks. (There are two different replacements at position 288.) Extreme limits of alignments with proteins listed in Table 1 are given by vertical bars, but most run between residues 19 and 266.

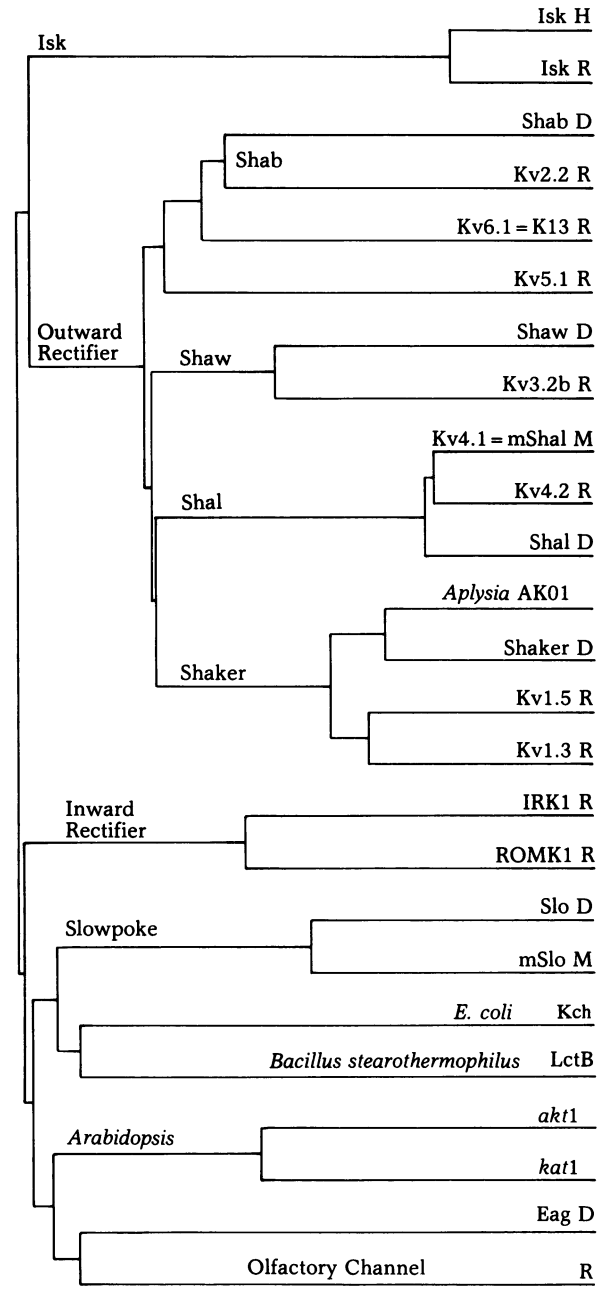

FIG. 3. Diagram of relative amino acid sequence similarity derived from the Genetics Computer Group multiple-alignment program, PILEUP, and plotted with the program FIGURE. This is not a precise evolutionary tree but is likely to reflect evolutionary relationships. D, *Drosophila*; H, human; M, mouse; R, rat.

pected to be more similar, even in different species, than proteins found in a given species but belonging to different channel families. The other eukaryotic channel proteins except for the *eag* gene product have at least one moderately close eukaryotic relative. The rat (cyclic nucleotide-gated) olfactory channel has very close relatives in mice, cattle, and humans (data not illustrated). The *Bacillus stearothermophilus* protein is very similar to one in *Bacillus caldotenax* (27). The finding that Kch is no more similar to any of the eukaryotic channels than the families of channels are to each other is perfectly consistent with the possibility that the most recent ancestor of Kch and the eukaryotic cation channel proteins existed in a bacterium. There is no evidence of a recent importation. Pore sequences are aligned in Table 2, perhaps suggesting that the Isk proteins are not complete channel proteins after all.

Table 2. Alignment of pore sequences (as defined in ref. 17) of 23 of the 25 channel proteins in Fig. 3 by means of the Genetics Computer Group program PILEUP

Protein	Amino acid sequence
Consensus	P*AFWW***TMTTVGYGD**P
D.m. Shaker†	-D-----AVV----------MT-
Rat Kv1.3	-D-----AVV----------MH-
Rat Kv1.5	-D-----AVV----------MR-
Aplysia AK01†	-D-----AVV----------MR-
D.m. Shaw†	-LGL---ALV----------MA-
Rat Kv3.2b	-IG----AVV-----L-----MY-
D.m. Shal†	-A----YTIV-----L-----MV-
Mouse Kv4.1 (mShal)	-A----YTIV-----L-----MV-
Rat Kv4.2 (= RK5)†	-A----YTIV-----L-----MV-
Rat Kv6.1 (= K13)†	-ACY---AVI----------MV-
D.m. Shab†	-E-----AGI----------IY-
Rat Kv2.2	-AS----ATI----------IY-
Rat Kv5.1	-QS----AII----------IY-
Arabidopsis AKT1	VTSMY-SIT-L--------LH-
Arabidopsis KAT1	VT-LY-SIT-L---T-----FHA
E. coli Kch	MT---YFSIE---S------IV-
D.m. Eag	VT-LYFTMTC---S---F-NVAA
D.m. Slowpoke	WTCVYFLIV---S------VYC
Mouse mSlo	WECVYLLMV---S------VYA
B.st. LCTB	EDSLYLSGM-LLS------VT-
Rat IRK1	TA---LFSIE-Q---I---FRCV
Rat ROMK	TS---LFSLE-QV-I---FRFV
Rat olfactory channel	IYCLY-STL-L---I-ETPPPV

Hyphens indicate residues that are identical to the consensus residues. Human and rat Isk show no meaningful alignment with the pore sequences. *D.m.*, *Drosophila melanogaster*; *B.st.*, *Bacillus stearothermophilus*.
*No consensus residue at this position.
†Also aligned in ref. 17.

The ECOKCH DNA sequence containing the *kch* gene is 3951 nucleotides long and connects *tonB* with the region adjacent to *opp*, completing a 33-kb stretch running from *trp* to beyond *adh*. It is consistent with expectations from the Kohara physical map (33, 34), except for the absence of a *Kpn* I site at about 1316.5 kb. Putative −35 and −10 promoter regions, ribosomal attachment site, and terminator are evident. In the course of a comparative sequencing project (2), sequences from a diverse set of 38 ECOR strains have been obtained for most of the coding region and all of the 5′ flanking region. There are no substitutions in the putative −35, −10, and ribosome-binding sequences; in the coding region, substitutions at 39 sites are synonymous at 32 third positions and 1 first position. The remaining 6 substitutions all cause conservative replacements (Fig. 2). The average number of replacements per 100 bp is lower in *kch* (0.7) than in other genes in the region that have been sequenced comparatively. These include *trpC* (3.2), *trpB* (0.8), *trpA* (2.5), and *tonB* (2.2). This conservation indicates that the Kch protein is also functional and important in *E. coli*. Two replacements are found exclusively in a set of 10 related "Green" strains, listed in figure 1 of ref. 2. Each of the other four amino acid replacements is found in a different single strain.

Beginning with the proportion of sequence identity (35–37), the evidence for homology is strengthened by similarity at higher structural levels, and also by the fact that Kch is being compared with not one, but a set of proteins, whose function and structural relationship has been demonstrated. Nevertheless, bacteria have had a long time to evolve, and convergence is not out of the question. It is possible that a short, highly adaptive amino acid sequence could have evolved more than once. As to higher-level structure, there is a well-known case of the lysozymes from bacteriophage T4

and from hen egg white (38, 39), in which tertiary structures correspond impressively while the sequences do not correspond at all. Is this contrast the result of gradual replacement of residues without disturbing the three-dimensional structure, or is it due to convergence? Have Kch and the eukaryotic potassium channel proteins diverged less than the lysozymes have, or have they independently evolved the six-transmembrane-domain pattern with its included pore and then converged to a more constrained range of sequences? The latter seems quite improbable, especially without the full understanding of the pertinent structure–function relationships that makes, for example, the convergence of cephalopod and vertebrate eyes credible.

The difference between the most divergent *kch* genes sequenced in *E. coli* strains suggests an ancestral gene dating back about 1.2×10^8 generations, perhaps 600,000 years. Six hundred thousand years is a short time in the history of *E. coli* (40), but far longer than it would take for a favorable gene to spread throughout the species (41). The open reading frames of the two *Bacillus* species differ from each other by 76 of 402 nucleotides (and 17 of 134 amino acid residues). This works out to about 1.2×10^9 generations, or perhaps 6 million years. The presence of Kch-like proteins in a greater diversity of other bacteria would lend additional support to the conclusion that Kch is not a recent import. As genomic data bases grow, and also with the application of DNA hybridization, it will be of interest to make a more complete test by determining the distribution of homologous potassium channels in other bacteria and in the Archaea, as well as in the lowest eukaryotes, which lack mitochondria. In the same vein, occasional mitochondrial sequences show sequence similarity to Kch in TFASTA comparisons. However, cases examined to date indicate no functional parallels, and no mitochondrial sequences appeared in the top 150 matches to the 13-residue core of the Kch P region.

Further along the *E. coli* chromosome, there is an open reading frame of unknown function and affinity upstream from *kch*. Beyond it is the bacteriophage $\phi 80$ attachment site and then *tonB*, which is transcribed in the opposite direction. Downstream, following clearly evident −35, −10, and ribosome-binding sequences, is a gene that shows 28% identity over 363 residues with an uncharacterized open reading frame near the lipoamide dehydrogenase gene in *Pseudomonas putida* and 25% identity over 274 residues with an open reading frame adjacent to the *wapA* gene in *Streptococcus mutans*. [A novobiocin-related gene recently reported by Rakonjac *et al.* (42) appears to coincide physically with this *E. coli* gene.] These observations tend to rule out a potassium transport operon in this region. A number of *E. coli* genes related to potassium transport have been reported and mapped long ago (43, 44). The nearest one, *trkE*, was localized well beyond the start of the *trp* operon (43) in convincing detail by transduction experiments (45).

Finally, there is the question of the function of Kch. What do bacteria need potassium channels for? The work of Kung, Adler, and coworkers (46, 47, 49), as well as that of Ghazi's laboratory (48), suggests that stretch-activated channels help bacteria avoid bursting due to hypoosmotic shock. This question can be addressed by expressing *kch* in suitable systems or by knocking it out in *E. coli*.

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