Channel-mediated potassium uptake in *Helicobacter pylori* is essential for gastric colonization

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To date, the biological role of prokaryotic K⁺ channels remains unknown. *Helicobacter pylori* contains a gene encoding a putative K⁺ channel (HpKchA) of the two-transmembrane RCK (regulation of K⁺ conductance) domain family, but lacks known bacterial K⁺ uptake systems. A *H. pylori* Δ hpKchA mutant presented a strong growth defect at low K⁺ concentration, which was compensated by KCl addition. The role of the separate RCK domain was investigated in *H. pylori* by mutagenesis of its internal start codon, which led to a K⁺-dependent intermediate growth phenotype, consistent with RCK activating channel function. Tagging HpKchA C-terminally, we detected a 1:1 stoichiometry of the full-length HpKchA and the separate RCK domain. We constructed single amino-acid exchanges within the unusual selectivity filter of HpKchA (ATGFGA) in *H. pylori* and observed complete loss (G74A), a slight defect (G76A or F75G) or wild-type (A77D) channel function. HpKchA was essential for colonization of the murine stomach. These data show, for the first time, a biological function for a prokaryotic K⁺ channel, as a K⁺ uptake system, essential for the persistence of *H. pylori* in the gastric environment.

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Introduction

Potassium is the most abundant ion in the cytoplasm of all living cells. Cytoplasmic K⁺ is essential for many bacterial processes like regulation of cell turgor pressure, growth,

cytoplasmic pH homeostasis and protein synthesis. In *Escherichia coli*, K⁺ uptake occurs through several systems, the constitutive and low-affinity transporters, Trk and Kup, and the high-affinity, inducible transport system, KdpFABC complex (Epstein *et al*, 1993). At low millimolar K⁺ concentrations, the constitutively expressed low-affinity transporters are sufficient for K⁺ acquisition under fluctuating osmotic pressure. Upon depletion of K⁺, the KdpFABC complex (P-type ATPase) is induced and takes over the function of Trk and Kup. Some other bacteria contain a fourth type of K⁺ uptake system, the Na⁺-dependent Ktr system (Nakamura *et al*, 1998).

Helicobacter pylori is involved in several gastric diseases, including chronic gastritis, gastric and duodenal ulcers and cancer (Ernst and Gold, 2000). Upon oral uptake, the pathogen has to pass the extreme acidic gastric lumen in order to reach the mucosa, its sole niche for proliferation, where the pH is close to neutrality (Schreiber *et al*, 2000). The concentration of K⁺ in the gastric juice is estimated to be around 12 mM (Greger and Windhorst, 1996). At the mucosa, the abundance of K⁺ is likely to be lower, as it is assumed to resemble that of blood (3.5–5 mM) (Devlin, 1997).

The systems involved in K⁺ uptake of *H. pylori* are not known. Its genome lacks genes encoding homologues of Kdp, Trk, Kup or Ktr. Instead, it contains a gene coding for a putative K⁺ channel (Tomb *et al*, 1997). This open reading frame (hp0490, called here *hpKchA*) codes for a protein with homology to the family of two-transmembrane (2TM) K⁺ channels, which are found in most eukaryotes and prokaryotes. This family includes KcsA from *Streptomyces lividans*, the bacterial Kirba1.1 from *Burkholderia pseudomallei* and the inwardly rectifying MthK from the archaeon *Methanobacterium thermoautotrophicum*, for which crystal structures are available (Doyle *et al*, 1998; Jiang *et al*, 2002; Kuo *et al*, 2003). Functional K⁺ channels are homo-tetramers with the single subunits arranged in a four-fold symmetry around a water-filled core. In the 2TM K⁺ channels, the 2TM helices of each subunit are separated by a P-loop carrying the selectivity filter signature sequence, which contributes to the high specificity of K⁺ (Doyle *et al*, 1998). The canonical amino-acid sequence of the selectivity filter, TXGYGD, presents little variation among K⁺ channels. Frequently, the tyrosine is substituted by a phenylalanine, as in HpKchA. However, HpKchA exhibits further differences, as it presents the unusual selectivity filter sequence, ATGFGA. Strikingly, the negatively charged aspartate protruding at the external site of the channel is substituted by a neutral alanine. A non-conservative modification at this position is rare among K⁺ channels, as the aspartate is supposed to stabilize the filter structure (Chapman *et al*, 2001) and/or to be involved in the accumulation of the K⁺ ion close to the filter, increasing the rate of ion entry into the pore (Haug *et al*, 2004).

Like MthK, its close homologue, HpKchA contains a regulatory RCK (regulation of K⁺ conductance) domain of

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poorly defined function (Jiang *et al*, 2002). The MthK gene has its own internal start codon, which leads to the separate expression of the RCK domain in *E. coli*. From its crystal structure and from biochemical studies, a model for functional MthK was proposed, in which four full-length MthK subunits constitute the pore complex to which a tetramer of separate RCK domains is attached from the cytoplasmic side (Jiang *et al*, 2002).

So far, the biological role of K⁺ channels in prokaryotes is unknown (Kuo *et al*, 2005). On deleting the six-transmembrane K⁺ channel *kch* gene from *E. coli*, no 'loss-of-function' phenotype was obtained. Further work suggests that Kch is an inwardly rectifying K⁺ channel, functioning as a voltage valve for the cells under yet unknown conditions (Kuo *et al*, 2003). Two K⁺ channels from the archaeon *Methanococcus janaschii* may play a role in K⁺ uptake, as they restored growth at low K⁺ concentrations of an *E. coli* mutant deficient in K⁺ transport (Hellmer and Zeilinger, 2003). However, as *M. janaschii* possesses an additional Trk system (Bult *et al*, 1996), it remains unclear whether its K⁺ channels play a role in K⁺ uptake or fulfill a different function in this organism.

As *H. pylori* presents no other commonly found K⁺ transport system, we chose HpKchA as a model protein to study the function of a prokaryotic K⁺ channel in its *in vivo* context. In this study, we show that HpKchA is essential for K⁺ uptake in *H. pylori* at low external K⁺ concentrations and for colonization of the gastric environment, corroborating its role as a unique uptake system for K⁺ in the gastric pathogen. In addition, we demonstrate that the expression of a separate RCK domain in addition to the full-length HpKchA is necessary for wild-type function and we discuss modifications of its unusual selectivity filter analyzed by site-directed mutagenesis.

Results

Features of the HpKchA primary sequence

HpKchA, which presents a calculated molecular mass of 43 kDa, exhibits significant homology with a number of K⁺ channel proteins or protein domains (Figure 1). The closest homologues of HpKchA are found in *Helicobacter hepaticus* (Suerbaum *et al*, 2003) and *Wolinella succinogenes* (Baar *et al*, 2003), which are members of the Helicobacteraceae. Its next best hits are with the two 2TM-K⁺ channels MjK1 and MjK2 from the archaeon *M. jannaschii* (Bult *et al*, 1996) (25 and 26% identity, respectively; Figure 1, lines 3 and 4, respectively). These proteins closely resemble the inwardly rectifying K⁺ channel MthK from *M. thermoautotrophicum* (Jiang *et al*, 2002) (Figure 1, line 1) and consist of a 2TM-K⁺ channel domain and a C-terminal regulatory RCK domain.

Growth defect of the non-polar *H. pylori* Δ hpKchA mutant

To characterize the biological function of HpKchA, we constructed an isogenic deletion mutant of *hp0490* in *H. pylori* strain X47-2AL by insertion of a non-polar kanamycin cassette. In brucella broth supplemented with 10% FCS, the *hpKchA*-deficient mutant had a strong growth defect (Figure 2A, open circles). The K⁺ concentration of this medium was determined by flame spectroscopy and amounts to 7.3 ± 2.0 mM K⁺ (five independent measurements). Growth of the mutant was restored stepwise by addition of KCl. At K⁺ concentrations of around 22 mM, the growth defect of the Δ hpKchA strain was fully compensated to that of the wild-type level (Figure 2A and B).

Growth of wild-type *H. pylori* in the complex medium was completely inhibited in the presence of 10 mM CsCl, a well-known inhibitor of both eukaryotic inwardly rectifying K⁺

		--Outer helix (M1)--	--P-loop--	--Inner--
		--Helix--	Filter	Helix (M2)
2TMHpKchA	1-	MFEKLLKFKKIKKDDIEQEVNLNSEIYEQFKVFRPLILILQLLVLLGLTGLYFALENY-----	SLMQAFFQTTTMTATGFCALNESQFGPISIFLTSILM	
2TMMthK	1-	-----MVLVIEIIRKHLPRVLKVPATRIILLVLAVIYGTAGPHFIEGE-----	SWTVSLYWTFFVTIATVGMCDYSP--STPLGMYFTVTLI	
2TMMjK1	1-	-----METYEKIELGIIVILLILIESVILMTVEGW-----	DFFTAFYTAVVITISTVGVCDYTP--QTFLGKLSVIIYI	
2TMMjK2	1-	-----METSKKLVIVAVLSITLITLYAYLISIIIEGV-----	DYFTALYFSVITITTTTGVCDFTF--KTFGLGRTLTVVYL	
6TMEcKch	141-	-----IFAFISFTTLLFTSYGALYSEGFN--PRIESLMTAFYFSIETMSTVGMCDIVF--VSESARLFTISVI		
		Inner helix (M2)--		
2TMHpKchA	96-	FFGAGIIAFSVAILVSVVNKGTLTRILKEKGIYKIYARLKDHYVICYHNEYTIELSKQFRSAQIPFVVVDNDPSFEEEAIKHKYP--YYIIGDPHTNLAML		
2TMMthK	81-	VLGIITFAVAVERLLEFLIN----REOMKLMGLIDVAKSRHVVICWSESTLECLRELRG--SEVFLVAEDENVRKKVLRSG--ANFVHGDPTRVSDLE		
2TMMjK1	68-	FAGVGAVAYTMGNIAFFIEGHRKYFRLRKWMDRICKLNNHYIICGYRLGKVIAEFEKKCNIPFVIDS--DEKLLBEALEKDP--NLICIVGDAATSDILK		
2TMMjK2	68-	CVGVGIVMYLFLSLIAEFIVEGKFEFVRLKKMKNKIKTLKDHYIICGYRLGKVGVEKFIENIPFIAIDI--NEDVLKEEYKYPDKFLYIVGDAKKEEVLK		
6TMEcKch	207-	ISGITVFATSMTSIFGSLIRG---GFNKLKVGNNHMRKDHFIYVCGHSILAINITILQLNQRGQNVTVISNLPEDDIKQLEQLRGDNADVI PGDSNDSSSVLK		
		-αC-----βD--αD-----βE--αE-----βF--αF-----αG-----		
2TMHpKchA	195-	KTHLSSARGVVALSKILPVNVALMVSVRLFELKELKRKPYIITASHSDEGLEKLLKLGADMVVSPTKLMQVRSAAMAVRPD--MENILERFINKK-DTLL		
2TMMthK	172-	KANVRGARAVIVDLESSETIHCILGIRKIDES--VRIIAEAERYENIEQLRMAGADQVISPFVISGRILMSRIDG---YEAMFVQDVLAE-ESTR		
2TMMjK1	168-	KAKIEKAGKLISVSSDAENVFITLSAKKLNNP----IYIVAKAEKPSLTKLIKAGADRAVCPIYVGMETIARIANP--DIVEFIHSLVAT-EEDM		
2TMMjK2	169-	KAKIDKAGLIATLPSDADNVFLTLTARELNPN----ILITAKADEKEARKLKIAAGANRVSPYILIGGLRMAEVSVRPGILDPLSTFIKIAKDEYBEDI		
6TMEcKch	306-	KAGIDRCRAILALSDNDADNAFVVLAKDMSSD----VKTVLAVSDSKNENLKKMHPDIIILSPQLFGSEILARVLNGEINNMDMLVSMLLNSGHGIFSD		
		--βG--αH--αI--βH--βI--βJ--αJ--		
2TMHpKchA	292-	DLEEVIPTKTSWLVRLKLEAHFREIAKAFVIGITQKDGKYPMPDGETIITASSEKSLMVGTSEGVATCKQLISNHQRPKEVDYISL*-----378		
2TMMthK	263-	RMVEVPIPEGSKLEGVSLVDADIHDVTGVIIIGVGRGD-ELIIDPPRDYSFRAGDIILGIGKPEEIERLKNYISA*-----336		
2TMMjK1	259-	EVRRYIVKNKELDN-KLLKDSGIREKTGATILAVKKG-DFTITSPDPPTVINIGDIIYAFGTKEQLEKLRKYVEGVE*-----333		
2TMMjK2	265-	ELRKFIYEKDSLAYKSLKDNIRGKTGATILGIRREK-ETCINPYPEFILKPGDVYAFGTENLKYLENLVKKKKKL*-----343		
6TMEcKch	402-	NDEQETKADSKESAQK*-----417		

Figure 1 Alignment of HpKchA with RCK-domain-containing K⁺ channel proteins. 2TM and 6TM, two-transmembrane and six-transmembrane K⁺ channel, respectively. Line 1: HpKchA (O25233); line 2: MthK, inwardly rectifying K⁺ channel of *M. thermoautotrophicum* (O27564); lines 3 and 4: MjK1 (Q57604) and MjK2 (Q58752), two K⁺ channels of *M. jannaschii*; line 5: partial EcKch (Doye *et al*, 1998) and MthK (RCK domain) (Jiang *et al*, 2002). α, α-helix; β, β-strand. The internal start codon of the RCK domain (M127) in HpKchA and the selectivity core glycine residues are highlighted in white on a black background. Residues identical to the corresponding amino acids in HpKchA are highlighted by a gray background.

Figure 2 Growth of *H. pylori* $\Delta hpKchA$ strain is impaired at low K^+ concentrations. *H. pylori* wild-type (X47-2AL) and its $\Delta hpKchA$ derivative were grown in brucella broth supplemented with 10% FCS. (A) $\Delta hpKchA$ strain at different K^+ concentrations; $\circ$, 7.3 mM K^+ (basal K^+ concentration of the medium); ∇ , 10.3 mM KCl; ∇ , 12.3 mM KCl; $\blacklozenge$, 17.3 mM KCl; $\diamond$, 22.3 mM KCl; $\bullet$, 37.3 mM KCl. (B) growth of *H. pylori* wild-type in the presence or absence of the K^+ channel blocker CsCl; $\circ$, no addition (7.3 mM K^+); $\square$, 10 mM CsCl (and a basal level of 7.3 mM K^+); $\blacksquare$, 10 mM CsCl and 37.3 mM KCl.

channels (Gay and Standfield, 1977) and bacterial KcsA (LeMasurier *et al*, 2001) (Figure 2B, open squares). This growth inhibition was only partially restored by addition of 30 mM KCl (Figure 2B, closed squares) suggesting that CsCl had also additional effects on cell growth, as growth at high K^+ concentrations was independent of HpKchA (Figure 2A).

The K^+ content of *H. pylori* cells was about 95 nmol/ml and the cytoplasmic water space about 0.5 μ l/ml cell culture of OD_{600 nm} = 1 ($\sim 5 \times 10^8$ CFU/ml). This gives a calculated cytoplasmic K^+ concentration of 192 ± 23 mM (seven independent measurements), which is similar to that of other bacteria (Walderhaug *et al*, 1987). It indicates that *H. pylori* accumulated K^+ up to at least 30-fold from the liquid growth medium.

HpKchA is not required for pH homeostasis under acidic conditions

K^+ transport processes are crucial for pH homeostasis of the cytoplasm at different values of medium pH in most bacteria (Booth, 1985). Therefore, the capacity to maintain pH homeostasis was compared between *H. pylori* $\Delta hpKchA$ and the wild-type. Urease activity was used to monitor cytoplasmic pH, as acidification of the cytoplasm has been shown to result in loss of activity of this enzyme and strongly correlates with survival of the bacterium (Rektorschek *et al*, 2000). Survival

during 60 min of wild-type *H. pylori* at pH 2 was similar in brucella broth with a level of around 7 mM K^+ and in citrate-phosphate buffer without KCl (Stingl *et al*, 2001), indicating that survival at acidity was independent of the presence of potassium. Moreover, urease activity of the $\Delta hpKchA$ mutant and wild-type strain in citrate-phosphate buffer in the presence of urea at pH 2 was comparable, with 12.5 ± 3.6 μ mol urea/(min $\times$ mg) protein for $\Delta hpKchA$ as compared to 16.4 ± 3.9 μ mol urea/(min $\times$ mg) protein for the wild-type (five independent measurements). Hence, HpKchA was dispensable for pH homeostasis of *H. pylori* at acidic pH in the presence of urea.

Purified His-tagged HpKchA formed oligomers

HpKchA was C-terminally fused to six histidine residues and the gene was overexpressed in *E. coli* C43 (Miroux and Walker, 1996). A total of 90% of the protein was solubilized from membranes with a 5:1 detergent mixture of *N*-dodecyl-maltoside and SDS (Figure 3A and B, lanes 2–4). After binding to Ni^{2+} -agarose, the eluted fraction (Figure 3A and B, lane 6) consisted of the 40 kDa HpKchA protein, substoichiometric amounts of the 28 kDa RCK domain from HpKchA (both identified by N-terminal sequencing) and minor contaminants (partially HpKchA degradation products). As with MthK from *M. thermoautotrophicum* (Jiang *et al*, 2002) and MjK2 from *M. jannaschii* (Hellmer and Zeilinger, 2003), the conserved amino-acid residue M127 in HpKchA (Figures 1 and 3C) appears to serve as a start codon in *E. coli*, giving rise to the separate translation of the HpKchA-RCK domain. In a second purification step, the 40 kDa protein was electroeluted from the gel and re-chromatographed by SDS-PAGE (Figure 3D). At high SDS concentrations and in the presence of β -mercaptoethanol, we observed that a minor fraction of the electroeluted monomer formed a dimer at 80 kDa (Figure 3D, lane 1). In the absence of reducing agent and at low concentrations of SDS, the same electroeluted monomer formed two distinct bands at 40 and 35 kDa and several oligomerization states up to one tetramer (Figure 3D, lane 2). In further experiments, we verified that this migration difference was caused by the presence or absence of reducing agent and not by the concentration of SDS. We concluded that monomeric HpKchA exhibits two conformations under non-reducing conditions and that these monomers spontaneously formed dimers, trimers and tetramers, the latter being the functional unit of HpKchA.

Detection of the full-length channel and a separate RCK domain in *H. pylori*

In order to test the expression of a separate RCK domain in *H. pylori*, we exchanged the methionine codon at the N-terminus of the predicted RCK domain (M127; Figure 1) in *hpKchA* by leucine. An M127L version of *hpKchA* was introduced into strain X47-2AL- $\Delta hp490^{KmR}$. Subsequently, we tagged the wild-type *hpKchA* and the M127L-*hpKchA* mutant gene C-terminally with a tandem affinity purification (TAP) tag that we recently adapted for utilization in *H. pylori* (manuscript in preparation). As the TAP tag carries the antibody-binding domain of Protein A, its fusion to any *H. pylori* protein leads to specific detection of the target protein and its expression level by immunoblot analysis. We detected two separate protein bands in stoichiometrically similar amounts in the *hpKchA*-TAP strain, corresponding to the full-length

Figure 3 Purification of recombinant HpKchA. (A) Coomassie blue-stained 10% SDS-polyacrylamide gel. (B) Immunoblot using anti-penta-His antibodies. In panels A and B: lane 1, MW standard; lane 2, membranes before solubilization; lane 3, solubilized material; lane 4, membranes after solubilization; lane 5, flowthrough of the Ni²⁺-NTA resin; lane 6, eluted protein using 500 mM imidazol. Protein samples were dissolved in buffer with 2.7% SDS and 0.7% β -mercaptoethanol, heated at 50°C for 30 min and loaded on the gel. The relative amounts with respect to total cell protein in lanes 2–5 are comparable. Molecular weights are given in kDa. The 40 kDa HpKchA monomer (1xHpKchA) and its separate 28 kDa RCK domain are indicated with arrows; they were identified by N-terminal Edman sequencing. (C) Cartoon of the *hpKchA* gene illustrating its two start codons, one for the full-length protein and one for a separate RCK domain; the structural model modified after Jiang *et al* (2002) is depicted below in which a tetramer of the full-length channel interacts at the cytoplasmic site with an additional tetramer of the separate RCK domain. (D) Electroeluted monomeric HpKchA was mixed with loading buffer containing 0.7% SDS without reducing agent. After 1 h incubation at room temperature, the sample was divided into two equal aliquots. One aliquot received an additional 2% SDS and 0.7% β -mercaptoethanol and was heated for 30 min at 50°C. Both aliquots were run on a 10% SDS-polyacrylamide gel and stained with silver. Lane 1, HpKchA at 2.7% SDS plus 0.7% β -mercaptoethanol; Lane 2, HpKchA at 0.7% SDS. The oligomerization states are indicated on the right; the molecular weight is given on the left.

HpKchA and the separate RCK domain (Figure 4, lane 1). In the M127L-*hpKchA*-TAP strain, only the full-length HpKchA was detected (Figure 4, lane 2), indicating that the RCK domain was indeed expressed separately from the second methionine in position 127 *in vivo*. This shows that in *H. pylori*, HpKchA is expressed as a full-length protein plus a separate RCK domain in a 1:1 stoichiometry, which is in agreement with the proposed model of a tetrameric full-length pore complex associated with a tetrameric RCK domain complex (Jiang *et al*, 2002; Albright *et al*, 2006) (Figure 3C).

In order to evaluate the impact of the expression of this separate RCK domain in the channel function of *H. pylori*, we studied growth of the M127L-*hpKchA* mutant in brucella broth. To accentuate the growth defect of HpKchA mutants, we supplemented the broth in all following experiments with β -cyclodextrin instead of FCS. This medium contained a lower and more reproducible concentration of 4.5 ± 1 mM K⁺ (five different measurements). Growth of the wild-type *H. pylori* was independent of the concentration of additional KCl, whereas Δ *hpKchA* was strongly impaired in growth without supplementation of KCl (6% of OD_{600 nm} at 30 h compared to the wild-type; Figure 5B, white bars, first two sets of columns), confirming and strengthening the data in FCS-supplemented medium (Figure 2). As expected, the *hpKchA* reinsertion mutant showed a similar growth behavior

compared to the wild-type. Interestingly, the mutant carrying the single exchange of methionine 127 to leucine, lacking the expression of the separate RCK domain, exhibited a phenotype intermediate between that of the wild-type and the Δ *hpKchA* mutant (25% of OD_{600 nm} at 30 h compared to the wild-type; Figure 5B, white bars, first and third sets of columns). This suggests that the separate RCK domain is essential for full function of HpKchA, and that the mutant channel conducts potassium less efficiently.

Selectivity filter mutants

The K⁺ filter sequence of HpKchA (ATGFGA; A72–A77; Figure 1) deviates from the canonical motif TXGYGD (T75–D80 in KcsA) (Figure 6, upper panel). Therefore, we studied the effect of mutations within this filter on the activity of the channel, monitored by the growth behavior of these *H. pylori* mutants at different K⁺ concentrations. Four different *hpKchA* versions, each carrying single codon exchange in the selectivity filter, were constructed and replaced the native gene on the chromosome of the *H. pylori* strain X47-2AL. The aspartate residue (of the canonical filter sequence) was shown to be important for accumulation of K⁺ before entering the pore (Haug *et al*, 2004) as well as for stabilization of the pocket structure of the filter (Chapman *et al*, 2001). An alanine is found at this position in HpKchA. When this

Figure 4 Immunoblot of TAP-tagged HpKchA in *H. pylori*. A 5 μ g portion of *H. pylori* crude protein extracts was dissolved in loading buffer supplemented with 1% SDS and 50 mM DTT, separated on a 12.5% polyacrylamide gel and blotted to PVDF membrane. TAP-tagged protein was detected using the PAP antibody. Lane 1, HpKchA-TAP; lane 2, M127L-HpKchA-TAP.

alanine was exchanged to aspartate to fit the canonical sequence, we observed the same growth behavior on different K^+ concentrations as for the wild-type (Figure 6B and C, first set of columns), indicating that at minimal K^+ concentrations of around 5 mM, selectivity was not changed by altering the filter at this position. We then focused on the highly conserved canonical central motif, GYG, which, in wild-type HpKchA, is altered in a conservative manner to GFG. Exchanging the phenylalanine (F75) to a glycine (GFG $\rightarrow$ GGG) led only to a slight K^+ -dependent growth phenotype, indicating that K^+ was still conducted by this mutant channel in a nearly as efficient way as by wild-type HpKchA (Figure 6B and C, second set of columns). Exchanging the first glycine (G74) to alanine (GFG $\rightarrow$ AFG) led to a complete loss of channel activity, as the growth behavior resembled that of the deletion mutant (Figures 5, 6B and C, third set of columns). In contrast, when the second glycine (G76) was substituted by alanine (GFG $\rightarrow$ GFA), growth of the mutant was only affected at the lowest concentration of K^+ tested but reached wild-type level at 10 mM K^+ (Figure 6B and C, fourth set of columns). The latter two results confirm the relative importance of these two glycine residues in coordination of K^+ within the selectivity filter, as proposed from the crystal structure of KscA. As the first glycine is situated in the center of the filter pore, it is consistent with the model that modification to alanine leads to complete loss of activity. However, the second glycine was proposed to be located near the external site of the filter; thus, its minor change to alanine might be better tolerated.

Colonization of the gastric murine mucosa is dependent on HpKchA

We have demonstrated above that the K^+ channel of *H. pylori* is essential for growth at low K^+ concentrations *in vitro*. In order to test whether *H. pylori* encounters low K^+ concentrations in its natural environment and whether HpKchA is essential for persisting in the host, we compared

Figure 5 Growth phenotype of *H. pylori* lacking the separate RCK domain. Growth of *H. pylori* X47-2AL, its $\Delta hpKchA$ deletion mutant, the M127L- $hpKchA$ mutant lacking the separate RCK domain and the $hpKchA$ reinsertion mutant ($hpKchA-RE$) in brucella broth with β -cyclodextrin and different concentrations of K^+ (added as KCl). (A) Representative growth curves. (B) Average OD_{600 nm} after 30 h of growth (data from four independent growth curves for each strain); error bars correspond to standard deviation. Symbols are as follows: white, around 5 mM K^+ (basal level of K^+ in the medium); light gray, 10 mM K^+ ; dark gray, 20 mM K^+ ; black, 35 mM K^+ .

the capability of *H. pylori* wild-type and its isogenic $hpKchA$ deletion mutant to colonize the gastric mucosa. For this purpose, we used the murine model for orogastric infection (Lee *et al*, 1997), counting the bacterial load at the gastric mucosa after 4 weeks of infection.

First, we used the X47-2AL- $\Delta hpKchA$ mutant resistant to kanamycin. In two independent experiments, the wild-type *H. pylori* X47-2AL colonized the murine gastric mucosa to a mean bacterial load of 10^5 – 10^6 CFU/g of stomach (Figure 7A and B). In contrast, none of the $\Delta hpKchA$ ($X47^{KmR}$) mutant cells was detected in the stomach of mice after 4 weeks of orogastric infection. In order to strengthen this result and use the same antibiotic resistance cassette for the complementation mutant, we constructed deletion mutants carrying a gentamycin resistance cassette in two different *H. pylori*

Figure 6 Modification of the selectivity filter in HpKchA. (A) Structural model of the selectivity filter of KcsA from *S. lividans* (modified by N Tholema according to the cover of *Nature* 414, 2001) and the corresponding amino-acid residues in HpKchA. Both hexameric filter sequences are summarized below. (B) Representative growth curves. (C) Average OD_{600 nm} after 30 h of growth (data from 3 to 5 independent growth curves for each strain) of the selectivity filter mutants of *H. pylori* X47-2AL in brucella broth with β -cyclodextrin and different concentrations of K⁺ (added as KCl). Symbols are as follows: white, around 5 mM K⁺ (basal level of K⁺ in the medium); light gray, 10 mM K⁺; dark gray, 20 mM K⁺; black, 35 mM K⁺; error bars correspond to standard deviation.

strain backgrounds, the X47-2AL and the SS1 strain. These deletion mutants were then complemented by reinserting the native *hpKchA* into the same genetic locus using the kanamycin resistance cassette. In the X47-2AL genetic context, the gentamycin-resistant Δ *hpKchA* (X Δ ^{GmR}) was not capable of colonizing the mouse mucosa (Figure 7C). For the SS1 genetic background, we detected only minor amounts of Δ *hpKchA*(S Δ ^{GmR}) in three out of seven mice (2–3 log less than median of wild-type; Figure 7D), leading to a geometrical median below the detection limit. For the reinsertion mutants of both strains, SS1 (S-RE^{KmR}) and the X47-2AL (X-RE^{KmR}), colonization of the gastric mouse mucosa (Figure 7C and D) was comparable to that of the wild-type, corroborating the result that the absence of HpKchA itself caused the dramatic decrease (for SS1) or even loss (X47-2AL) of capacity for colonization of the murine gastric environment. In conclusion, our results showed that HpKchA is essential for efficient colonization of the gastric environment, probably because of the presence of a concentration of potassium lower or equal to around 5 mM (as tested *in vitro* as minimal K⁺ concentration) at the site of bacterial proliferation.

Discussion

Although a considerable amount of data are available on structural and mechanistic features of prokaryotic K⁺ channels, till now no clear biological function has been assigned to any of these systems (Kuo *et al*, 2005). In the present study, we identified for the first time an unequivocal biological role of a prokaryotic K⁺ channel, by demonstrating that HpKchA from *H. pylori* serves as bulk K⁺ uptake system for this gastric pathogen at low K⁺ concentrations. Moreover, HpKchA was essential for the efficient colonization of the mouse gastric mucosa (Figure 7), indicating that HpKchA serves as sole uptake system in this gastric pathogen.

The primary sequence shows that HpKchA belongs to the K⁺ channels of the 2TM RCK domain type family (Figure 1). Purified His-tagged monomeric HpKchA oligomerized spontaneously (Figure 3D), which is consistent with a K⁺ channel function, as the active form of K⁺ channels is a homotetramer (Doyle *et al*, 1998).

Unlike KcsA, HpKchA contains a regulatory RCK domain at its C-terminus. As for other related proteins, a potential internal second start codon (M127; Figure 1) for the RCK domain is found in HpKchA, and like MthK and MjK2, expression of HpKchA in *E. coli* leads to an additional 28 kDa RCK domain (Figure 3A and B; Jiang *et al*, 2002; Hellmer and Zeilinger, 2003; Parfenova *et al*, 2006). Till now, it was not known whether the separate RCK domain is expressed *in vivo*, and whether it complexes in a 1:1 stoichiometry with the complete channel subunit, forming a hetero-octamer as the K⁺-conducting channel entity (Jiang *et al*, 2002; Figure 3C). Using a tag fused to wild-type HpKchA and M127L-HpKchA, we showed that in *H. pylori* a separate RCK domain is indeed expressed in a 1:1 stoichiometry with the full-length HpKchA protein (Figure 4, lane 1). Moreover, loss of this separate RCK domain led to an intermediate growth phenotype at defined external K⁺ concentrations (Figure 5), indicating that the separate RCK domain is essential for full HpKchA activity. This could be explained by assuming that the open probability in the M127L-HpKchA

Figure 7 *H. pylori* Δ hpKchA fails to efficiently colonize the mouse gastric mucosa. The following *H. pylori* strains were tested for their capacity to colonize the murine stomach: two different wild-type *H. pylori* (X47-2AL and SS1), their isogenic Δ hpKchA deletion mutants carrying either a kanamycin or gentamycin resistance cassette ($X\Delta^{Kmr}$, $X\Delta^{Gmr}$ and $S\Delta^{Gmr}$) as well as their hpKchA reinsertion strains ($X-RE^{Kmr}$ and $S-RE^{Kmr}$). Each panel (A–D) corresponds to an independent experiment. Each data point represents the gastric colonization load of one mouse; horizontal bars, the geometric mean for each group of mice. The lower detection limit was 400 bacteria/g stomach (dashed line); points below this detection limit correspond to mice, in which no colonization of *H. pylori* was detected.

variant is lower, and that the role of the separate RCK domain is activation of channel activity.

A highly conserved hexapeptide sequence within the pore-forming P-region determines the cation selectivity of K^+ channels. HpKchA exhibits the unusual selectivity filter motif ATGFGA instead of the commonly found TXGYGD (Kuo *et al*, 2005). The sequence GYG is the critical determinant for K^+ selectivity and conductance in KcsA (Heginbotham *et al*, 1992, 1994) and is conservatively modified to GFG in HpKchA. When we exchanged the phenylalanine to glycine (F75G), we observed a nearly wild-type growth phenotype of this *H. pylori* mutant (Figure 6). For the *E. coli* Kch channel, exchange of the corresponding tyrosine residue to anything else than phenylalanine led to loss of function (Kuo *et al*, 2003). However, in a eukaryotic 4TM K^+ channel, the aromatic amino acid is replaced by leucine in one of the two P-loop motifs, which does not affect K^+ selectivity (Lesage *et al*, 1996). As only the negatively charged carbonyl oxygens of the main polypeptide chain are involved in direct coordination of K^+ in the filter pore, tolerance of amino-acid variations is supposed to be determined by the overall constitution of the filter and not only by the GYG core motif.

The two glycines in the filter core region are conserved in nearly all K^+ channels, with the first glycine being the most important residue for K^+ channel activity (Lu and Miller, 1995). The KcsA crystal structure shows that this residue (G77) is in a left-handed helical conformation, normally observed for D-amino acids, and that its essentiality at this position was explained by being the only natural amino acid that can exhibit this unusual conformation (Valiyaveetil *et al*, 2004). Accordingly, exchange of the corresponding glycine to alanine (G74A) in HpKchA led to complete loss of function of the K^+ channel (Figure 6). However, when the second glycine (G76) was changed to alanine, we observed a growth defect at 5 mM K^+ , which was nearly completely compensated at 10 mM K^+ (Figure 6). This only slight decrease in channel function is in agreement with glycine 76 being situated near to the external site of the filter pore, where an exchange to alanine might better be tolerated.

Changing the last alanine (A77) in the filter motif of HpKchA to the commonly used aspartate had no effect on channel function as observed under our conditions. Ionizable side chains in the vicinity of the filter pore are thought to affect the energetics of ion permeation (Ranatunga *et al*, 2001). At pH 7, aspartate is likely to be mainly deprotonated and therefore negatively charged. This might not be the case at slightly acidic pH, found in the natural environment of the pathogen. Thus, we speculate that under these conditions, the aspartate at the entry of the pore does not confer a major advantage for the conductance of K^+ through the filter and was evolutionarily lost (i.e. exchanged to an alanine).

It has been predicted that the presence of a single K^+ uptake channel might not be sufficient for K^+ acquisition by *H. pylori*, and that yet unidentified transporters have to be present in this pathogen (Van Vliet *et al*, 2001). Deletion of all K^+ transport systems in *E. coli* generates an *E. coli* mutant, requiring ≥ 15 mM K^+ for growth (Stumpe and Bakker, 1997), similar to that necessary to support growth of the *H. pylori* Δ hpKchA. For both organisms, it is unclear how potassium at high concentrations reaches the cytoplasm but it is likely that it enters less specifically through other ion transporters. At K^+ concentrations lower than 20 mM, HpKchA is the sole K^+ uptake system of this gastric bacterium. Assuming a membrane potential of around -180 mV at pH 7, a bacterial cell is capable of accumulating K^+ about 1000-fold via such a K^+ -specific diffusion pathway. In most bacteria, the cytoplasmic K^+ concentration amounts to 200 mM. Thus, a K^+ channel would be sufficient for the cells to capture K^+ down to a K^+ concentration in the medium of 0.2 mM. In *H. pylori*, we measured a comparable internal K^+ concentration (192 ± 23 mM) and its membrane potential at pH 7 was previously determined to be at least -150 mV (Stingl *et al*, 2002), suggesting that *H. pylori* probably can grow down to K^+ concentrations of at least 0.4 mM. The colonization site of *H. pylori* is the human gastric mucosa, where concentrations of K^+ are expected to be 3.5–5 mM, which is far above the calculated minimal K^+ concentration. Thus, under such conditions, HpKchA activity

Figure 8 Function of prokaryotic potassium channels in the context of the lifestyle of three representative species of ϵ -proteobacteria. This model proposes the existence of a relation between the K⁺ content of single or multiple environmental niches and the need for a K⁺ channel, a K⁺ transport system or both. *H. pylori*, inhabiting a single, K⁺-rich niche, uses a channel for bulk potassium import; *C. jejuni*, inhabiting the intestine of different species and encountering high bacterial loads, faces low K⁺ concentrations. It uses efficient transport systems for K⁺ acquisition and no K⁺ channel; *W. succinogenes*, colonizing the rumen of cattle and presenting the potential for free-living as proposed by Baar *et al* (2003), has both, a K⁺ transporter for bulk K⁺ import and a K⁺ channel in order to adapt metabolic pathways upon environmental changes.

must be sufficient to satisfy the cells' need for K⁺. Consistently, HpKchA was essential for efficient colonization of the mouse gastric mucosa (Figure 7). This observation can be explained by assuming that at this site, the K⁺ concentration is indeed as low as 3.5–5 mM, conditions under which the $\Delta hpkchA$ strain is not able to sufficiently grow.

A role for a bacterial K⁺ channel as uptake system for bulk K⁺ might be an adaptation of the human pathogen to its K⁺-rich, competitor-free gastric environment, which is its sole proliferation niche. There are probably few other bacteria having only a K⁺ channel, like *Ureaplasma urealyticum*, which inhabits the human urogenital tract, a site at which potassium is excreted. In contrast, it is hypothesized that most bacterial K⁺ channels affect the membrane potential and respond to changes in the environment (Kuo *et al*, 2005). The distribution of K⁺ transporters and K⁺ channels in closely related ϵ -proteobacteria in relation to their lifestyle led us to propose the following model (Figure 8). Bacteria such as *H. pylori* dwelling in one single, K⁺-rich niche have only a K⁺ channel for bulk K⁺ import. *Campylobacter jejuni* (Parkhill *et al*, 2000), being restricted to the mammalian or avian intestine, in which it faces high bacterial load and low external K⁺ concentrations, possesses only efficient K⁺ transporters for K⁺ acquisition. Furthermore, *H. hepaticus* (Suerbaum *et al*, 2003) and *W. succinogenes* (Baar *et al*, 2003) both possess a homologue of HpKchA in addition to other K⁺ transporters. *H. hepaticus* lives in the murine intestine and is found in liver tissue, whereas the commensal, *W. succinogenes*, occurs in the bovine rumen and has the potential for free living (Baar *et al*, 2003). In these two latter organisms, K⁺ transporters are proposed to accumulate K⁺, whereas their K⁺ channels can be utilized to respond to environmental changes. Consistently, the recently sequenced free-living ϵ -proteobacterium *Thiomicrospira denitrificans* also possesses a K⁺ transporter in addition to its K⁺ channel (Copeland, 2005, EMBL gene databank).

In conclusion, HpKchA is an excellent model protein, as it allows to link structural characteristics of a prokaryotic K⁺ channel to its biological function. Electrophysiological studies on HpKchA are under way to characterize original properties of this K⁺ channel, which might be a reflexion of adaptation to the gastric environment and might contribute

to the development of specific inhibitors appropriate for anti-*Helicobacter* therapy.

Materials and methods

Bacterial strains and culture conditions

H. pylori strains DSM4867 (Deutsche Stammsammlung für Mikroorganismen, Braunschweig), X47-2AL (Ermak *et al*, 1998) and SS1 (Lee *et al*, 1997) were grown either on blood agar plates (Oxoid) supplemented with 10% defibrillated horse blood (bioMérieux) or in liquid culture using brucella broth with 10% fetal bovine serum (GibcoBRL) or with 0.2% β -cyclodextrin (Sigma). An antibiotic/anti-fungal cocktail was used at the following final concentrations per ml: 12.5 μ g vancomycin, 3.1 μ g polymyxin B, 6.25 μ g trimethoprim and 2.5 μ g amphotericin B. When appropriate, 20 μ g/ml kanamycin, 4–8 μ g/ml chloramphenicol or 50 μ g/ml apramycin was used. Selection and precultures were performed on blood agar plates and liquid medium supplemented with 15 or 30 mM KCl, respectively. Plates were incubated at 37°C under a microaerobic atmosphere in jars using CampyGen (Oxoid). Liquid cultures were shaken at 140 r.p.m.

E. coli strains DH5 α (Woodcock *et al*, 1989), C43 (Miroux and Walker, 1996) and MC1061 (Casadaban and Cohen, 1980) were grown at 37°C in Luria broth (Miller, 1992) or KML medium (10 g/l KCl, 10 g/l bactotryptone and 5 g/l yeast extract) with 100 μ g/ml ampicillin or carbenicillin, 100 μ g/ml spectinomycin or 20 μ g/ml kanamycin, when appropriate.

Construction and purification of the His-tagged HpKchA protein

The homologue of the open reading frame, *hp0490*, from *H. pylori* 26695 was amplified from chromosomal DNA of strain *H. pylori* DSM4867, which was purified using the DNEasy Tissue Kit (Qiagen). The oligonucleotides Hp490L and Hp490R (Supplementary Table S1) were used to amplify the 1.1 kb *hpKchA* gene and to fuse six histidine codons to its 3'-end. Using these primers, the original TTG start codon of *hp0490* was converted into ATG and an additional glycine was inserted as the second codon in order to create a NcoI restriction site. The PCR was performed using the high fidelity kit (Roche) as described by the manufacturer and the obtained sequence was checked. The fragment was ligated with plasmid pET16b opened with NcoI and BamHI. *E. coli* C43 was transformed with the ligation product, giving strain C43 with plasmid *phpKchA*. The purification of the His-tagged HpKchA was performed over Ni²⁺-NTA. For details see Supplementary data.

Construction of *H. pylori* strains carrying *hpKchA* mutations or C-terminal TAP fusions

Deletions, point mutations, fusions or reinsertions were introduced in *H. pylori* strains X47-2AL and/or SS1 by allelic exchange using

plasmids in which *H. pylori* DNA regions are flanking antibiotic resistance cassettes, either the non-polar kanamycin cassette (Skouloubris *et al*, 1998) or the gentamycin resistance cassette (Bury-Moné *et al*, 2003). For the C-terminal TAP fusion (TAP tag composed of Protein A and a calmodulin-binding domain both separated by a TEV cleavage site; Rigaut *et al*, 1999), we used a plasmid, pILL851-C, in which the tag was fused to the non-polar kanamycin cassette (manuscript in preparation). Construction of the plasmids used is detailed in Supplementary data, with the corresponding PCR primers described in Supplementary Table S1. *H. pylori* mutants were obtained by natural transformation as previously described (Bury-Moné *et al*, 2001). Genomic DNA of the recombinant *H. pylori* mutants was isolated using the QIAamp DNA Mini Kit (Qiagen). All insertions into the chromosome were verified by PCR and sequenced with an ABI 310 automated DNA sequencer (Perkin-Elmer). Site-directed mutagenesis of the *hpKchA* was performed using the divergent PCR strategy described by Bury-Moné *et al* (2001) using *phpKchA* as template and the primers detailed in Supplementary Table S2.

Immunoblot analysis and N-terminal sequencing

For the analysis of TAP-tagged protein, *H. pylori* cells were sonicated twice for 30 s, debris was removed by centrifugation at 20000 g for 5 min and 5 µl of protein extract was separated by 10 or 12.5% SDS-PAGE. Proteins were blotted onto nitrocellulose (Biometra, Göttingen, Germany) or polyvinylidene difluoride membrane (PVDF, Millipore). TAP-tagged protein was detected using a peroxidase-coupled anti-peroxidase antibody (Sigma) and the ECL reagent (Amersham) according to the manufacturer's instructions. His-tagged proteins were detected using an anti-penta-His-antibody (Qiagen, Hilden, Germany) at a concentration of 0.2 µg/ml and a second goat anti-mouse antibody (Pierce Biotechnology Inc., IL, USA) coupled to alkaline phosphatase.

For N-terminal sequencing, protein was blotted onto a PVDF membrane, from which the protein was N-terminally sequenced by Edman degradation (Applied Biosystems, 473 A apparatus).

Electroelution of monomeric HpKchA

Ni²⁺-NTA purified proteins were dissolved in the low SDS sample buffer without reducing agent, immediately separated by SDS-PAGE on a 10% gel and visualized by negative staining with 3 M KCl (Prussak *et al*, 1989). Gel pieces containing the 40 kDa HpKchA monomer were excised and transferred to an electroeluter (Bio-Rad Laboratories Inc., CA, USA). Protein was eluted into 500 µl of Laemmli buffer (0.1% SDS, 192 mM glycine and 25 mM Tris) by applying 10 mA per tube for 3 h at room temperature.

Determination of K⁺ concentrations

The volume of the cell pellet was determined as described previously (Stingl *et al*, 2002). For the determination of the

cytoplasmic K⁺ concentration, the cell pellet was added to 1 ml of 5% TCA, frozen at -20°C overnight and boiled for 10 min. After addition of 3 ml of 3.7 mM CsCl, the K⁺ concentration was determined using an ELEX 6361 flame photometer (Eppendorf, Germany) and KCl as a standard. The K⁺ concentration of liquid medium was determined accordingly.

Determination of urease activity

H. pylori cells were harvested in logarithmic phase (at OD_{600 nm} ~ 0.3) by centrifugation at 5000 g for 10 min. After resuspension in 1/10 volume of 0.9% NaCl, the cells were 10-fold diluted in 100 mM citric acid (pH 2) supplemented with 100 mM NaCl and 20 mM urea. Urease activity was determined by measuring the depletion of urea in the medium according to Rahmatullah and Boyde (1980).

Mouse model of colonization

H. pylori was grown on blood agar plates for 24 h and suspended in peptone broth at a concentration of 10⁹ bacteria/ml. For the mutants, three independently selected clones were mixed before inoculation of the mice. The precise infection dose was determined by CFU enumeration. Female NMRI mice (4 weeks old, Iffa-Credo) were inoculated orogastrically with aliquots of 100 µl bacteria (10⁸ bacteria) as described previously (Ferrero *et al*, 1998). Four mice were inoculated with peptone broth as a negative control. After 4 weeks, the mice were killed and the gastric tissue was homogenized in peptone broth. Bacterial counts were obtained by plating serial dilutions on blood agar plates containing per ml 200 µg bacitracine and 10 µg nalidixic acid in addition to the antibiotic/anti-fungal cocktail described before.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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