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FEMS Microbiology Letters 229 (2003) 283–289

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Localisation of *Helicobacter pylori* catalase in both the periplasm and cytoplasm, and its dependence on the twin-arginine target protein, KapA, for activity

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Received 1 October 2003; received in revised form 4 November 2003; accepted 9 November 2003

First published online 28 November 2003

Abstract

Helicobacter pylori induces a severe inflammatory response in the gastric mucosa. It is able to withstand the inflammatory response by producing proteins such as KatA and KapA. The C-terminus of KatA possesses a unique tetra-lysine motif not found in other catalases or other known protein sequences. Mutants deficient in this motif were constructed by site-directed mutagenesis. Cytoplasmic and periplasmic catalase activities were measured for the parental strain, a truncated KatA mutant (deficient in the unique C-terminal tetra-lysine motif) and a previously constructed KapA-deficient mutant (confirming previous observations regarding the possible periplasmic localisation of KatA). No differences were observed in the cytoplasmic catalase activities, however, the KapA-deficient mutant had approximately 5.5 times less catalase activity in the periplasmic extract when compared to the periplasmic preparations of either parental strain or KatA truncated mutant. N-terminal sequencing of KatA revealed no cleaved N-terminal signal peptide, indicating Sec-independent transport. These findings support previous reports that there is some form of interaction between KatA and KapA of *H. pylori*, an interaction which still needs to be characterised.

Keywords: Oxidative stress; Catalase-associated protein; Site-directed mutagenesis; Periplasmic localization

1. Introduction

Helicobacter pylori is responsible for the development of peptic ulcer disease in humans and is a predisposing factor for the development of gastric carcinomas [1]. The infection induces both an acute and chronic inflammatory response in the gastric mucosa. Despite the aggressive inflammatory response that is elicited by the host, the infection continues to persist. It has long been believed that the ability to avoid toxic oxygen species, generated as a result of the inflammatory response, is crucial to the survival of *H. pylori* in vivo [2,3]. There is now evidence to support this theory through the use of animal models of infection and isogenic mutants in a number of antioxidant defence mechanisms including superoxide dismutase [4], catalase-associated protein (KapA) and catalase (KatA) [5]. Through co-culture with professional phagocytes in vitro, the importance of KatA in the survival of *H. pylori* has been demonstrated [6].

Catalase (EC 1.11.1.6) is a ubiquitous enzyme that is responsible for the enzymatic dismutation of hydrogen peroxide into water and molecular oxygen. Catalase protects organisms from the potentially damaging effects of hydrogen peroxide. The reaction between hydrogen peroxide and iron cations yields a more reactive oxygen species, the hydroxyl radical. Hydroxyl radicals rapidly react with organic molecules resulting in widespread damage [7,8].

KatA of *H. pylori* was purified and characterised by Hazell et al. [3] and was found to be expressed in the cytoplasm. It was also suggested that KatA is located in the periplasm, an observation that until now has not been validated. The

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0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00850-4

suggestion that KatA may be specifically expressed on the cell surface remains to be substantiated [9].

Upon sequencing *katA* of *H. pylori* [10–13] it was noted that the C-terminus of the protein possesses a unique tetra-lysine motif, not found in other catalases or other proteins where the amino acid sequence is known. The lipoproteins of *Escherichia coli* have C-terminal lysine residues that mediate covalent adherence to the peptidoglycan layer [14–16]; however, it is unlikely that KatA is covalently bound to the peptidoglycan of *H. pylori* as Costa et al. [17] and Krishnamurthy et al. [18], when investigating cell wall differences between the spiral and coccoid forms of *H. pylori*, were unable to detect any peptidoglycan-bound lipoproteins. This finding appears to be unique to *H. pylori* and it would be highly unlikely that other proteins are covalently linked to the peptidoglycan.

The predicted secondary structure of KatA reveals an unusual amphiphilic C-terminus and elements that might be involved in the export of proteins. It was thought that these novel features of the C-terminus of KatA from *H. pylori* might be involved in the localisation of the protein.

KapA is known to be involved in the oxidative stress response induced by hydrogen peroxide in *H. pylori* [5,19]. It has been reported in two separate studies that KapA appears to have no peroxidase activity [11,19], yet KapA-deficient mutants are more susceptible to the effects of hydrogen peroxide than the parental strain of *H. pylori* [19]. In addition to displaying increased sensitivity to hydrogen peroxide in vitro, KapA is also required for long-term survival in the SS1 murine model of infection [5]. In a protein–protein interaction study by Rain et al. [20], KapA was identified as the only protein that interacts with KatA. It has previously been speculated that KapA might be an accessory protein that could be involved in the translocation of KatA. Blastp [21] results (October 2003) reveal that the protein with the highest similarity to KapA is the orthologue of the human α-aminoadipate semialdehyde synthase (R02D3.1 protein) in *Caenorhabditis elegans*. With a score of only 35.4 and an *E*-value of 1.3, this similarity is very poor.

The aims of the current investigation were to confirm whether *H. pylori* KatA is in part localised in the periplasm and determine whether KapA and the C-terminal tetra-lysine motif are involved in the translocation of KatA. The current investigation also describes a novel method for introducing unmarked mutations into genes involved in the oxidative stress response using phenotypic selection and validates the use of a periplasmic protein extraction kit for use in *H. pylori*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. pylori strain 26695 parental strain was routinely maintained on *Campylobacter* selective agar (CSA) consisting of 7% (v/v) sterile defibrinated horse blood (Oxoid, Basingstoke, UK) as described previously [19]. Isogenic mutants of *H. pylori* 26695 *katA*::*aphA3* and 26695 *kapA*::*aphA3* (constructed previously [19]) were routinely cultured on CSA supplemented with 20 μg ml^{-1} kanamycin (CSAK). Cultures were incubated for 24–48 h in a Stericult incubator (Forma Scientific, USA) at 37°C in a reduced oxygen environment (microaerobic) that consisted of 10% CO_2 and 95% relative humidity.

E. coli JM109 (F′ *traD*36 *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/e14$^-$ (McrA$^-$) Δ(*lacC-proAB*) *thi gyrA*96 (Nal^r) *endA*1 *hsdR*17 (r_k^-, m_k^+) *relA*1 *supE*44 *recA*1) was used to propagate pKatA for site-directed mutagenesis. pKatA was constructed previously [19]. Cells harbouring pKatA were grown on LB agar or broth supplemented with 50 μg ml^{-1} ampicillin.

2.2. Site-directed mutagenesis

Site-directed mutagenesis of the C-terminal tetra-lysine motif of KatA was carried out using the Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Oligonucleotide primers (KatA Thr^{501}* Fwd GGAAAAGACATGCACCACACATAAAAGAAAAA-GTAACTC and KatA Thr^{501}* Rev GAGTTACTTTTT-CTTTTATGTGTGGTGCATGTCTTTTCC) were designed according to the specifications of the Quikchange™ kit. Constructs containing desired mutations (pKatA-Thr^{501}*) were created according to the manufacturer's instructions. The plasmid carrying the mutated gene was confirmed by nucleotide sequencing thrice in both directions using primers HpKatA 1001F GACGCTTG-TTCTCTTATGG and HpKapA 103R TTGCAGCAC-TCCCTTTGC.

2.3. Transformation of H. pylori and phenotypic selection of KatA truncated mutants

The phenotypic selection of mutants containing the unmarked mutation relies upon the need for the truncated gene to be transformed into *H. pylori* 26695 *katA::aphA3* (catalase-negative (H_2O_2 sensitivity) and kanamycin-resistant phenotypes). An allelic exchange between the truncated gene and the disrupted gene resulted in catalase-positive (H_2O_2 tolerance) and kanamycin-sensitive phenotypes. These phenotypic differences are used to select transformants that have obtained the truncated *katA*. Briefly, cultures of *H. pylori* 26695 *katA::aphA3* were grown on CSA for 24 h and transformed with pKatA-Thr^{501}* as described previously [19]. Once transformed, cells were allowed to grow for a further 24 h before being transferred to fresh CSA and were allowed to grow for an additional 48 h. Cells were harvested in phosphate-buffered saline (PBS), washed three times and finally resuspended in 100 μl of PBS. After the final wash, cells were

exposed to 98 mM hydrogen peroxide for 20 min, sufficient time to kill all catalase-negative cells [19]. The hydrogen peroxide/cell suspension was mixed with an equal volume of 1% (w/v) bovine liver catalase (Sigma) to detoxify any remaining hydrogen peroxide. The cells were plated on CSA and allowed to grow for 48 h. Confirmation of *H. pylori* KatA truncated mutants (26695 KatA-Thr^{501}*) was determined by sensitivity to kanamycin, tolerance to hydrogen peroxide and by nucleotide sequencing thrice in both directions using HpKatA 1001F and HpKapA 103R.

2.4. Selective extraction of periplasmic proteins

Extraction of the periplasmic proteins of *H. pylori* was carried out using the PeriPreps™ periplasting kit (Epicentre Technologies, Madison, WI, USA), which relies upon the integrity of the peptidoglycan being compromised by lysozyme, followed by osmotic shock. Briefly, *H. pylori* was grown on CSA or CSAK for 48 h, harvested with PBS and centrifuged at 13 000 × *g* for 2 min at 4°C. The cells were resuspended in 50 μl of a lysozyme/sucrose buffer (200 mM Tris–HCl (pH 7.5), 20% sucrose, 1 mM EDTA, 30 U μl^{-1} Ready-Lyse™ lysozyme) and incubated for 5 min at room temperature. An equal volume of pre-chilled sterile Milli-Q™ water was combined with the cell suspension, mixed by inversion, incubated on ice for 5 min and followed by centrifugation at 13 000 × *g* for 2 min at room temperature. The supernatant (periplasmic fraction) was transferred to a clean tube and stored at −80°C until required. Periplasmic fractions were extracted on five separate occasions. The catalase activities of the periplasmic preparations were determined a minimum of two times per sample.

2.5. Malate dehydrogenase activity assays

Malate dehydrogenase (MDH) has been reported to be an exclusively cytosolic protein of *H. pylori* [22]; consequently, it was chosen as a marker for cytosolic contamination. Activity of the enzyme was determined using the method of Smith [23] measuring the synthesis of NADH, by an increase in absorbance at 339 nm. Failure of the absorbance to increase indicated the absence of MDH activity. Positive and negative controls were run as appropriate. All measurements were recorded using a Cary 50 Bio UV-Vis spectrophotometer. The temperature was kept at a constant 37°C using a Peltier device and the reaction was continually stirred using a Teflon-coated magnetic stirrer.

2.6. Catalase activity assays

Specific activities of catalase were determined spectrophotometrically using the method of Beers and Sizer [24]. Briefly, the time taken to degrade 3.45 μmol of hydrogen peroxide (mg protein)$^{-1}$ was determined a total of two times for each sample. Catalase activities were determined using a Cary 50 Bio UV-Vis spectrophotometer. The temperature was kept at a constant 25°C using a Peltier device and the reactions were continually stirred using a Teflon-coated magnetic stirrer.

2.7. Purification of catalase

Catalase was purified from cytoplasm and the cell envelope of *H. pylori* using the method of Hazell et al. [3] for N-terminal sequencing to determine whether KatA possesses an N-terminally cleaved signal peptide. Briefly this involved size exclusion chromatography using a K26/100 gel filtration column of Sephacryl S-300 HR followed by cation exchange chromatography. Cell envelopes were prepared as described previously [25]. The purity of catalase was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining with Coomassie brilliant blue R-250, [26].

2.8. N-terminal sequencing of catalase

N-terminal protein sequencing was carried out on the purified catalase from the cell envelope and from the cytoplasm. Purified catalase was fractionated by SDS–PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane using a CAPS-based buffer described by Matsudaria [27]. Blotted membranes were stained with amido black and the N-terminal sequence (first 10 amino acids) of the protein was obtained using a Hewlett-Packard G100A protein sequencer using a standard PVDF programme. Amino acid sequencing was performed by the Australian Proteome Analysis Facility (APAF).

2.9. Protein concentration determination

The concentrations of protein samples were determined using the bicinchoninic acid microtitre plate protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the protein standard.

2.10. Statistical analysis

To analyse the statistical significance of the catalase activities observed between the different groups, the results were subjected to Kruskal–Wallis analysis and Tukey's test. All statistical analyses were performed using SPSS statistical analysis software (SPSS, Chicago, IL, USA, ver. 10.0.5).

2.11. Secondary structure prediction

Secondary structures of peptide sequences were determined using the PSIpred server (http://bioinf.cs.ucl.ac.uk/psipred/) [28,29].

3. Results

3.1. Construction of KatA truncated mutants

To determine whether the unique C-terminal tetra-lysine motif of KatA was involved in the translocation or localisation of the protein, a construct carrying the specific mutation, encoding an early stop codon (pKatA-Thr^{501}*), was created. The planned substitution of T^{1504} for A^{1504} in the first position of the codon that corresponded to the first of the four terminal lysine residues was achieved successfully introducing a premature stop codon; however, C^{1507} had also been incorporated as an unplanned substitution, replacing A^{1507} into the first position of the codon that corresponds to the second lysine of the C-terminal tetra-lysine motif. The reason for this unplanned substitution was never investigated as the change had no practical impact as the new stop codon was immediately upstream of this unwanted mutation (Fig. 1).

Genomic DNA extracted from four successfully transformed isolates of *H. pylori* was subjected to nucleotide sequencing. Each isolate had identical sequences in this region. Both mutations (planned and unwanted) were successfully introduced into the genome of *H. pylori*. The two base variations were the only differences identified between the 3′ end of *katA* from parental strain *H. pylori* 26695 and the transformants.

3.2. Validation of periplasmic protein extraction protocol

The periplasting™ kit has previously been used to isolate periplasmic proteins from *E. coli*. To date, the use of this kit has not been reported in relation to the isolation of periplasmic proteins from *H. pylori*. To validate the periplasting™ kit, periplasmic proteins need to be extracted without the risk of cytoplasmic proteins contaminating the preparation. MDH of *H. pylori* is exclusively found in the cytoplasm, making this enzyme an ideal marker of cytoplasmic contamination [22]. When periplasmic preparations of *H. pylori* were tested for MDH, there was no apparent increase in absorbance measured at 339 nm, indicating that the periplasmic preparations contained no detectable MDH. However, MDH activity was observed

```
Hp.Kat
      GAC ATG CAT GGA AAA GAC ATG CAC CAC ACA AAA AAG AAA AAG TAA
        D   M   H   G   K   D   M   H   H   T   K   K   K   K   *

KatA Thr501 F Primer
                  GGA AAA GAC ATG CAC CAC ACA [T]AA AAG AAA AAG TAA CTC
                    G   K   D   M   H   H   T     *   K   K   K   *   L

pKatA Thr501
      GAC ATG CAT GGA AAA GAC ATG CAC CAC ACA [T]AA [C]AG AAA AAG TAA CTC
        D   M   H   G   K   D   M   H   H   T     *   K   K   K   *   L

26695 KatA-Thr501
      GAC ATG CAT GGA AAA GAC ATG CAC CAC ACA [T]AA [C]AG AAA AAG TAA CTC
        D   M   H   G   K   D   M   H   H   T     *   K   K   K   *   L
```

Fig. 1. The C-terminal sequence of KatA from *H. pylori* 26695, the primer used to create the truncated protein and the sequence of plasmid and 26695 KatA-Thr^{501}. The boxed T and C residues indicate the planned and unwanted mutations, respectively.

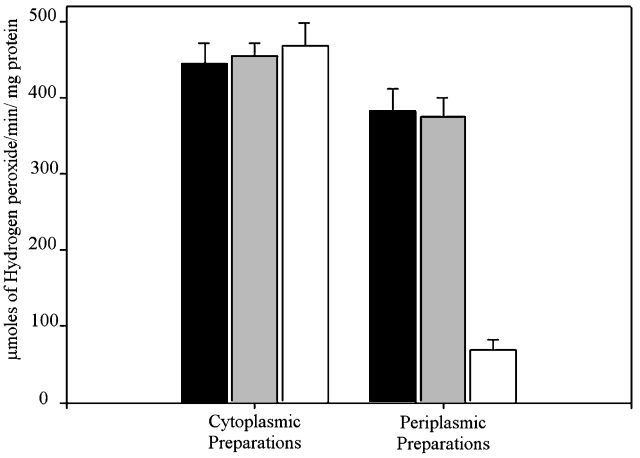

Fig. 2. Bar graph showing the specific catalase activities, calculated according to the method of Beers and Sizer [24], of cytosolic and periplasmic preparations of the parental 26695 (black bars), 26695 KatA-Thr^{501}* truncated mutant (grey bars) and the KapA-deficient mutant (white bars). The height represents the mean activity with the error bars representing the standard deviation.

in cell-free extracts, confirming the viability of the assay (specific activities not determined).

3.3. Catalase activities

There were no significant differences between the catalase activities of cytoplasmic preparations of the KapA-deficient mutant, the 26695 KatA-Thr^{501}* mutant or the parental strain (Fig. 2). There was no detectable catalase activity of the *H. pylori* KatA-deficient mutant. There was also no apparent difference in catalase activities of periplasmic preparations between the parental strain and the 26695 KatA-Thr^{501}* truncated mutant. However, there was a statistically significant difference ($P \leq 0.05$) between the periplasmic catalase activities of the KapA-deficient mutant (Fig. 2) and those of the parental strain and the 26695 KatA-Thr^{501}* truncated mutant. The KapA mutant had approximately 5.5 times less catalase activity in the periplasmic preparation.

3.4. N-terminal sequencing of catalase

KatA purified from the cytoplasm and cell envelope were N-terminally sequenced. The sequence data show that KatA had an N-terminus identical to the inferred amino acid sequence, that being: MVNKDVKQTT.

4. Discussion

Catalase is a key enzyme involved in the response to oxidative stress in many different organisms, accounting for approximately 1% of the total protein in *H. pylori* [3], and has also been identified as an excellent candidate vaccine antigen [30].

Reactive oxygen species are capable of killing organisms that lack effective mechanisms of detoxifying these compounds. This was the basis for the selection of isolates with unmarked mutations in catalase. The construction and selection of *H. pylori* mutants with a truncated catalase was based on their ability to survive for 20 min in the presence of 98 mM hydrogen peroxide. Catalase-deficient mutants of *H. pylori* (*katA::aphA3*), into which pKatA-Thr^{501}* was transformed, were unable to survive in such conditions [5,19]. Thus only those cells with an active catalase, the successful transformants of the truncated catalase, survive the exposure to hydrogen peroxide. Several transformants were sequenced, and all were found to have the desired truncation of catalase (Fig. 1). This method of introducing unmarked mutations and the use of phenotypic selection can be applied to any gene where the protein product is involved in the detoxification of a lethal compound provided the isogenic mutant is viable.

During the enzymatic characterisation of KatA, Hazell et al. [3] indicated that KatA of *H. pylori* appeared to be localised in part to the periplasm. This was determined by treating the bacteria with polymyxin B. This method had not been validated as a method of extracting periplasmic proteins from *H. pylori*. Using the periplasting™ kit it was possible to extract periplasmic proteins from *H. pylori* without cytosolic proteins contaminating the samples.

Periplasmic preparations of *H. pylori* had marked catalase activity. This is the first valid report of the catalase of *H. pylori* being a periplasmic protein, confirming the suggestion of Hazell et al. [3]. Having catalase localised (in part) in the periplasm is likely to confer an advantage to pathogens that elicit an aggressive inflammatory response, such as *H. pylori*. Dismutation of exogenous hydrogen peroxide before it damages essential proteins and nucleic acids either directly, or indirectly by generation of hydroxyl radicals via the Fenton reaction, is clearly beneficial.

This is not the first report of a periplasmically localised catalase. Catalase has been demonstrated as being periplasmic in *Brucella abortus*, *Vibrio fischeri* and *Pseudomonas syringae* [31–33]. N-terminal sequences of *H. pylori* KatA purified from the cell envelope and cytoplasm were found to be identical to the inferred amino acid sequence (Fig. 2) [10–13] indicating that like the catalase of *B. abortus* catalase, *H. pylori* KatA is exported to the periplasm in a *Sec*-independent manner. Sha et al. [31] noted some similarity between the export of catalase to the periplasm of *B. abortus* and the ATP-dependent import of catalase to the eukaryotic peroxisome. This import of eukaryotic catalase into the peroxisome is mediated by an apparent 'SKL' motif located near the C-terminus of the protein [34]. This motif is absent from the catalase of *B. abortus* and *H. pylori*. Sequence analysis indicates that there appears to be no consistent process in Gram-negative bacteria for the translocation of catalase into the periplasm. Interestingly, *Neisseria gonorrhoeae*, which also encounters significant oxidative stress as a result of the immune response, has a cytochrome *c* peroxidase lipoprotein (Ccp) located in the periplasm of the cell. Turner et al. [35] have demonstrated that Ccp offers some protection to catalase-deficient *N. gonorrhoeae* when exposed to hydrogen peroxide, suggesting a parallel physiological role for the Ccp and the periplasmically localised catalase of other Gram-negative bacteria.

Helices and a tetra-arginine motif at the C-terminus of the dengue virus capsid protein are believed to be involved in tethering the protein to cellular membranes [36]. The predicted secondary structures of the C-terminus of KatA and the capsid protein are quite similar (Fig. 3) and there are obvious similarities between the tetra-lysine and tetra-arginine motifs at the C-termini of KatA and the capsid protein, respectively. It is possible that by using a similar process, the C-terminal region of KatA might mediate membrane retention, anchoring KatA within the periplasm. The lack of difference between the periplasmic catalase activities of the parental strain and 26695 KatA-Thr^{501}* truncated mutant (Fig. 2) indicates that the C-

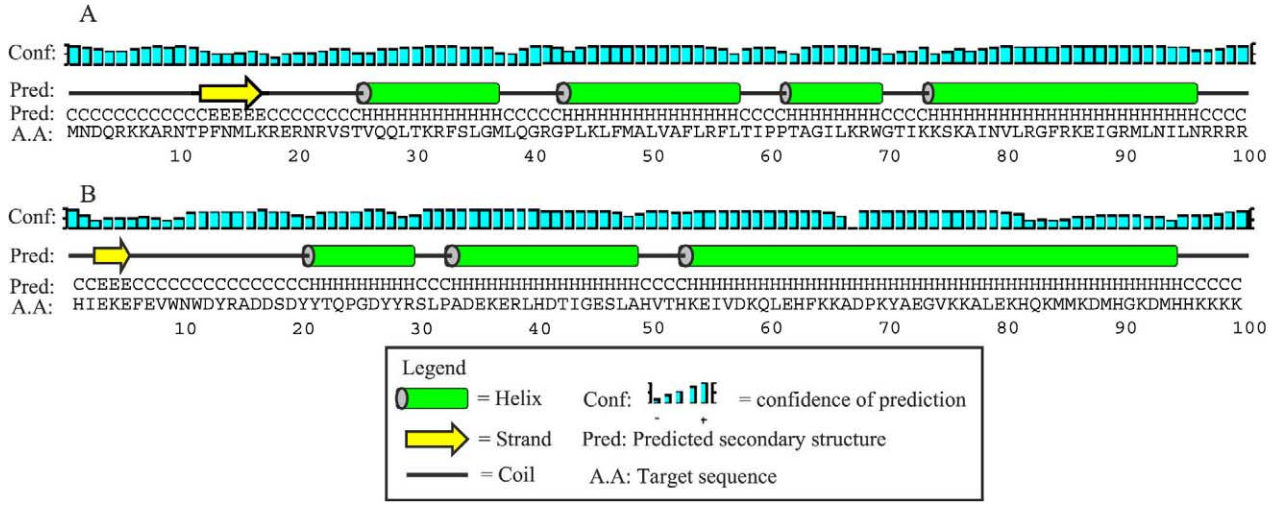

Fig. 3. Predicted secondary structure of (A) the dengue virus capsid protein and (B) KatA using PSIpred (http://bioinf.cs.ucl.ac.uk/psipred/) [28,29].

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  1 MKRRDFIKTT TLGATGAVLG AQILQAEESK GSVAKYKIEA QYSIDFDSAE HTSLFIPMPS
 61 VVASNVHLQG NHASYKSMLN FGVPYLQVDF LKSTQKKQVH LSYEIASYQL NERLFETSDF
121 VAMGRYERDD ASVANIANQL KGTTPKESVR NFYAFIKHEM PKRQKALEGK ENLPKRESLP
181 WFATISKESM FVSLCHACGI KSAEVQGLKL GQNSVVKNAP RVEVYLKDSF LAFDFQNNHK
241 EVFIPLNRHK DMQLDSALLA TFGDAFALVD GRDLGNYESK LFEKRVSYTI V
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Fig. 4. Inferred amino acid sequence of KapA. The region having homology to the TAT motif SRRXFLK is underlined, and amino acids having an exact match are in bold.

terminal tetra-lysine motif has little or no impact on the periplasmic localisation of KatA in *H. pylori*; however, this motif might have other functions yet to be determined.

The TAT system of protein translocation depends upon the presence of an (S/T)RRXFLK motif located at the N-terminus of the protein to be translocated [37]. Neither KatA from *H. pylori* nor catalases from other Gram-negative bacteria have this motif; however, at the N-terminus of KapA exists a TAT motif suggesting periplasmic translocation by the TAT system (Fig. 4). Using site-directed mutagenesis, Stanley et al. [38] were able to show that the serine and leucine residues of the motif do not play a major role in the transport of TAT-targeted proteins. An interaction between KatA and KapA, identified by Rain et al. [20], may allow KapA to carry the fully assembled KatA into the periplasm. This hypothesis would explain why the catalase activities of the periplasm extracted from the KapA isogenic mutants are 5.5 times lower than the catalase activities of the periplasm extracted from the parental strain, yet the cell-free extract had comparable activities. It would also explain why KapA isogenic mutants appear more sensitive to hydrogen peroxide in vitro [19]. KapA isogenic mutants appear to be less fit than KatA isogenic mutants and parental strain SS1 in inflamed murine gastric mucosa [5], suggesting other functions for KapA. This is an area that warrants further investigation.

This report supports the hypothesis that there appears to be an interaction between KapA and KatA, the exact nature of which remains unknown. Experimental data presented in this report suggest that the role of KapA might be chaperone-like or possibly a transport accessory protein involved in the cellular localisation of KatA; however, it remains unknown whether this is specific for KatA.

Acknowledgements

This work was supported in part by the Medical Research Council, UK (G0100109) and by the National Health and Medical Research Council of Australia.

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