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Functional significance of the highly conserved Glu^{570} in the putative pore-forming helix 3 of the *Bordetella pertussis* haemolysin toxin

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ABSTRACT

Adenylate cyclase-haemolysin toxin (CyaA) is a virulence factor secreted from the etiologic agent of whooping cough, *Bordetella pertussis*. Previously, the haemolysin or pore-forming domain (CyaA-PF) has been shown to cause cell lysis of sheep erythrocytes independently, and the predicted helix $3_{(570-593)}$ within the PF-hydrophobic stretch could be a pore-lining constituent. Here, a plausible involvement in haemolytic activity of polar or charged residues (Glu^{570}, Gln^{574}, Glu^{581}, Ser^{584} and Ser^{585}) lining the hydrophilic side of CyaA-PF helix 3 was investigated *via* single-alanine substitutions. All the 126-kDa mutant proteins over-expressed in *Escherichia coli* were verified for toxin acylation as the results are corresponding to the wild-type toxin. When haemolytic activity of *E. coli* lysates containing soluble mutant proteins was tested against sheep erythrocytes, the importance of Glu^{570}, which is highly conserved among the pore-forming RTX cytotoxin family, was revealed for pore formation, conceivably for a general pore-lining residue involved in ion conduction.

1. Introduction

Bordetella pertussis, a Gram-negative bacterium causing whooping cough in human, secretes a variety of toxins including the adenylate cyclase-haemolysin toxin (CyaA) which is important for initiating respiratory tract infection (Carbonetti et al., 2005). CyaA (~177 kDa) is a typical member of the pore-forming RTX cytotoxins (a subgroup of the **R**epeats-in-**T**o**X**in (RTX) protein family) that contains an adenylate cyclase (AC) domain attached to the *N*-terminus of the relatively conserved haemolysin or pore-forming (PF) domain (Fig. 1A). The CyaA-PF domain (~126 kDa) shares some common features with other RTX cytotoxins in that it contains an *N*-terminal hydrophobic region, a Gly–Asp-rich nonapeptide-repeat region and an unprocessed *C*-terminal signal peptide sequence (Welch, 1991; Linhartova et al., 2010). Moreover, these RTX toxins require post-translational acylation at an internal lysine, *e.g.* palmitoylation at Lys^{983} for CyaA by CyaC acyltransferase, to turn into an active form (Hackett et al., 1994) and be secreted subsequently by the type I secretion system (Welch, 2001; Linhartova et al., 2010). Following secretion, the CyaA toxin is stabilised by extracellular calcium ions which might act as a structural stabilising bridge in a β-roll motif of the Gly–Asp-rich repeats (Rose et al., 1995; Knapp et al., 2003; Chenal et al., 2009; Pojanapotha et al., 2011).

Abbreviations: 3D, three-dimensional; AC, adenylate cyclase; CyaA, adenylate cyclase-haemolysin toxin; CyaA-PF, CyaA pore-forming; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; RTX, Repeats-in-ToXin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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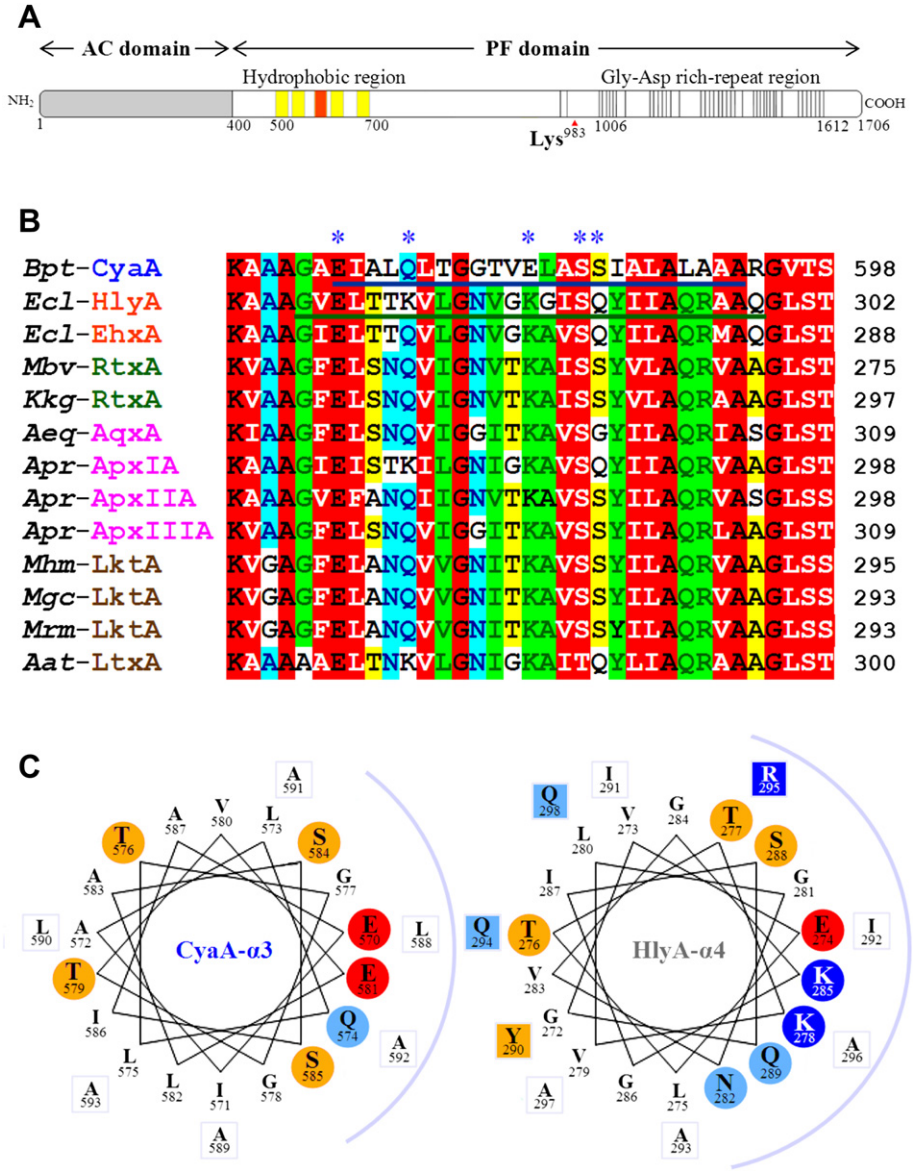

Fig. 1. A: Schematic diagram of CyaA showing adenylate cyclase (AC) and haemolysin or pore-forming (PF) domains. Five putative helices in the hydrophobic region (residues 500–700) are shown by yellow blocks, with red colour for α3. The calcium-binding region (residues 1006–1612) shows lines corresponding to each nonapeptide repeat (Gly–Gly–X–Gly–X–Asp–X–U–X). Lys^{983} indicates the palmitoylation site. B: Multiple sequence alignment of amino acid sequences covering the putative transmembrane $\alpha3_{(570–593)}$ of CyaA aligned with corresponding sequences of twelve pore-forming RTX cytotoxins. CyaA-$\alpha3_{(570–593)}$ and the putative membrane-spanning HlyA-$\alpha4_{(272–298)}$ are underlined. Degree of conservation among the sequences is highlighted by shading residues with red (black characters denote identical), blue, green and yellow for 100%, 80–90%, 65–75% and 50–60% homology, respectively. Asterisks indicate CyaA-PF residues selected for alanine substitutions. C: Helical wheel projections comparing CyaA-$\alpha3_{(570–593)}$ and HlyA-$\alpha4_{(272–298)}$. Amino acids were plotted every 100° consecutively around the helix axis (360° for 3.6 residues). Negatively and positively charged side-chains are shaded with red and blue, respectively. Orange and light-blue shadings represent oxygen- and nitrogen-containing uncharged side-chains, respectively. Relatively hydrophilic surface is indicated by arc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CyaA toxin affects mainly human macrophages by binding to the $\alpha_M\beta_2$-integrin receptor through its non-apeptide-repeat region (El-Azami-El-Idrissi et al., 2003). Recently, the involvements of lipid rafts as well as the sugar moiety on the β_2 integrin in CyaA toxin binding were also reported (Morova et al., 2008; Bumba et al., 2010). Upon binding, the AC domain was internalised into the target-cell cytoplasm to catalyse the uncontrolled production of cAMP, disturbing the transcription of many inflammatory- and cell signalling-associated genes in apoptotic pathways that lead to cell death (Hewlett et al., 1989; Carbonetti et al., 2005; Cheung, 2008). However, the CyaA toxin can also