Characterization of a haemolysin from *Mycobacterium tuberculosis* with homology to a virulence factor of *Serpulina hyodysenteriae*

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Scrutiny of sequence data from the *Mycobacterium leprae* genome sequencing project identified the presence of a gene encoding a 268-amino-acid polypeptide which is highly similar to a pore-forming haemolysin/cytotoxin virulence determinant, TlyA, from the swine pathogen *Serpulina hyodysenteriae*. Using degenerate oligonucleotide primers based on the TlyA sequences, the *Mycobacterium tuberculosis* homologue was amplified and this product was used to obtain the clone and sequence a 2.5 kb fragment containing the whole *M. tuberculosis* tlyA gene. tlyA encodes a 267-amino-acid protein with a predicted molecular mass of 28 kDa. TlyA homologues were identified by PCR in *M. leprae*, *Mycobacterium avium* and *Mycobacterium bovis* BCG, but appeared absent in *Mycobacterium smegmatis*, *Mycobacterium vaccae*, *Mycobacterium kansasii*, *Mycobacterium chelonae* and *Mycobacterium phlei*. The *M. tuberculosis* gene appeared to be the first gene in an operon containing at least two other genes. Introduction of the *M. tuberculosis* tlyA gene into *M. smegmatis* using a mycobacterial shuttle expression plasmid converted non-haemolytic cells into those exhibiting significant haemolytic activity. Similarly, inducible haemolytic activity was observed in sonicated bacteria when tlyA was expressed as a His₆-tagged fusion protein in *Escherichia coli*. tlyA mRNA was detected in both *M. tuberculosis* and *M. bovis* BCG using RT-PCR, confirming that this gene is expressed in organisms cultured *in vitro*.

Keywords: *Mycobacterium leprae*, tlyA, recN, BCG, contact-dependent haemolysin

INTRODUCTION

The mycobacteria include major human pathogens, notably *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively. Tuberculosis is the predominant infectious cause of morbidity and mortality worldwide, infecting 8 million and killing 3 million people annually (Snider *et al.*, 1994). This situation is exacerbated by the HIV pandemic and the increased prevalence of multiply resistant *M. tuberculosis* (Smith & Moss, 1994), while vaccine prophylaxis using *Mycobacterium bovis* BCG is unsatisfactory in many parts of the world

(Rodrigues & Smith, 1990). In addition, the *Mycobacterium avium* complex has emerged as a leading cause of infection in immunocompromised patients in developed countries (Porter, 1996). Despite a major research effort to characterize the pathophysiology of *Mycobacterium* species, few dedicated virulence determinants have been identified to date.

M. tuberculosis, *M. leprae* and *M. avium* are facultative intracellular organisms, having the capacity to survive in macrophages, and although the molecular basis of this property is not known, there have been reports suggesting that the bacteria are able to inhibit phagolysosome fusion (Armstrong & Hart, 1971), exist within tight vacuoles (Moreira *et al.*, 1997) and even escape into the cytoplasm (McDonough *et al.*, 1993). Haemolytic activity, associated with cytoplasmic development, has

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been documented in a wide variety of viral, bacterial and protozoan pathogens, including *Shigella*, *Rickettsia*, *Trypanosoma cruzi* and *Listeria monocytogenes* (reviewed by Andrews & Portnoy, 1994). Mutants of *L. monocytogenes* lacking the haemolysin listeriolysin O do not escape from the endosomal compartment and fail to become established in the cytoplasm. Furthermore, cloning the listeriolysin O gene into *Bacillus subtilis* produced a haemolytic organism which disrupted the phagolysosome membrane, permitting cytoplasmic growth. Thus a single gene product was sufficient to convert a common soil organism into an *in vitro* intracellular parasite (Bielecki *et al.*, 1990).

There have been a number of reports of haemolytic activity in *M. tuberculosis*. King *et al.* (1993) detected a contact-dependent haemolysin activity in strain H37Rv which was much reduced in the avirulent derivative H37Ra, and not present in BCG. Two genes encoding phospholipase C enzymes, which have haemolytic properties, have been cloned (Johansen *et al.*, 1996; Leao *et al.*, 1995). Recently Deshpande *et al.* (1997) purified a 66 kDa haemolysin from *M. tuberculosis* which showed limited homology to the haemolysin A precursor of *Vibrio cholerae*. It has also been shown that *Mycobacterium haemophilum*, which causes disease, most frequently in immunocompromised people, has a contact-dependent haemolysin (Fischer *et al.*, 1996). These haemolysins are all contact-dependent, while there has been a report of a heat-stable extracellular haemolytic activity in fast-growing mycobacteria (Udou, 1994).

Using data from the *M. leprae* genome sequencing project (Genome Therapeutics Corporation, Waltham, USA), we identified the presence of a 268-amino-acid polypeptide which shows striking similarity (40% identity) to a haemolysin/cytotoxin, TlyA, from the spirochaete *Serpulina hyodysenteriae*. The *S. hyodysenteriae* protein is a 26.9 kDa protein and is a major factor in the pathogenesis of swine dysentery (Hyatt *et al.*, 1994; ter Huurne *et al.*, 1994). In this paper, we describe the sequencing and initial characterization of the *M. tuberculosis* tlyA gene and its product.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Most mycobacterial strains were cultured in Middlebrook growth medium, except *Mycobacterium smegmatis* mc²155, which was grown in Lemco or modified Dubos medium (Difco), at 37 °C with the addition of 25 µg kanamycin ml⁻¹ or 100 µg hygromycin ml⁻¹ for strains containing plasmids. *M. leprae* DNA was provided by Dr M. J. Colston, National Institute for Medical Research, UK. *Escherichia coli* strains were routinely grown in LB broth or on LB agar. The antibiotics used for selection purposes were ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) and hygromycin (50 µg ml⁻¹).

DNA manipulations and PCR. Plasmid and genomic DNA extractions, restriction enzyme digests, DNA ligations, and transformations into *E. coli* were performed by standard

procedures (Sambrook *et al.*, 1989) using enzymes supplied by Promega. Mycobacterial DNA was isolated by the method of Santos *et al.* (1992). Hybridization probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) during PCR, and hybridization and detection were performed according to the manufacturer's instructions. The *M. tuberculosis* tlyA gene fragment was amplified by PCR with degenerate oligonucleotide primers using a method modified from that described by Wren *et al.* (1992). The oligonucleotides (CGCTGCAGCNWSSACNGGNTTYAC and CC-AAGCTTCNRCYTCRAAYTGNGGYTT) were based on *M. leprae* and *S. hyodysenteriae* sequence data and were designed to incorporate *Pst*I and *Hind*III restriction endonuclease sites (underlined) into the amplified product. Mycobacterial chromosomal DNA (100 ng) was added to the PCR reaction mixture and submitted to 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 40 °C and 2 min extension at 72 °C in a Hybaid Omnigene thermal cycler. The amplified product of predicted size was digested with *Hind*III and *Pst*I, ligated into similarly digested pUC18 to form pCIG7, and transformed into *E. coli* XL2-Blue MRF' cells (Stratagene). All DNA sequences were determined using an ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer). The *Sma*I fragment containing the tlyA gene was sequenced on both strands using primer walking.

Cosmid library screening. A cosmid library of *M. tuberculosis* H37Rv DNA in pYUB328 (provided by W. R. Jacobs, Albert Einstein College of Medicine, NY, USA) was used for screening. The cosmid library phage lysate was transduced into *E. coli* NM554 by the method of Eiglmeier *et al.* (1993). Colonies were picked onto gridded nylon membranes (Amersham), replicated, and membranes for screening were treated to lyse the colonies and immobilize the DNA according to Sambrook *et al.* (1989). A probe was generated from pCIG7 using the degenerate primers described above, and used for hybridization. Positive colonies were identified, picked from duplicate filters, grown, and cosmid DNA was isolated using the Wizard Minipreps DNA Purification System (Promega). Cosmid DNA was analysed by Southern blotting to confirm positive hybridization to the digoxigenin-labelled probe.

RT-PCR. Total RNA from *M. bovis* BCG and *M. tuberculosis* H37Rv was isolated from mid-exponential-phase cultures using the method of Mangan *et al.* (1997) with primers P_{K1} (5'-GATCCTCGGGTGGTGGTC; nt 1026–1043 in the published sequence) and P_{K2} (5'-CCTCAAACCTGGGGCTTCA-CC; nt 1212–1193), which were designed to give a 187 bp product. Total RNA was treated with 1 unit RNase-free DNase I (Life Technologies) in 1 × reaction buffer (20 mM Tris/HCl, pH 8.4, 2 mM MgCl₂, 50 mM KCl) for 15 min at room temperature. After digestion, the DNase I was inactivated by the addition of EDTA to 2 mM followed by incubation at 65 °C for 10 min. The RNA was heat-denatured at 85 °C for 5 min and quenched on ice prior to adding reverse transcriptase. The RNA was reverse-transcribed in a final volume of 20 µl containing 2.5 µM P_{K2}, 40 units RNasin (ribonuclease inhibitor), 1 mM each dATP, dCTP, dGTP and dTTP, 5 mM DTT and 200 units Superscript II reverse transcriptase (Life Technologies) in a reaction buffer consisting of: 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂. Ten microlitres of heat-denatured RNA was added to 10 µl of above mix and incubated at 42 °C for 30 min. One microlitre of the reverse transcription mix was used for hot start PCR and added to 9 µl water and 40 µl PCR master mix. The master mix for one reaction contained 1 µl primer P_{K1} (50 µM), 1 µl primer P_{K2} (50 µM), 5 µl 10 × Taq polymerase reaction buffer (100 mM Tris/HCl, pH 9.0, 500 mM KCl,

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source/reference*
Strains		
<i>E. coli</i>		
XL2-Blue MRF'	Cloning strain	Stratagene
BL21	Cloning strain	
JM109	Cloning strain	Stratagene
NM554	Cosmid host	Stratagene
<i>M. smegmatis</i> mc ² 155		Snapper <i>et al.</i> (1990)
<i>M. phlei</i> NCTC 8156		Graham McIntyre
<i>M. chelonae</i> ATCC 1977		Graham McIntyre
<i>M. vaccae</i> clinical isolate		Graham McIntyre
<i>M. kansasii</i> USA patient		Graham McIntyre
<i>M. avium</i> NCTC 8559		Graham McIntyre
<i>M. bovis</i> BCG Pasteur		Graham McIntyre
<i>M. tuberculosis</i> H37Rv		Graham McIntyre
Plasmids		
pUC18	Amp ^R cloning vector	Pharmacia
pCIG7	Amp ^R plus 303 bp <i>M. tuberculosis</i> <i>tlyA</i> gene fragment	This study
pCIG19	pUC18 plus 2.5 kb <i>M. tuberculosis</i> <i>Sma</i> I fragment carrying <i>tlyA</i> and flanking DNA	This study
c4I8	Cosmid clone containing <i>tlyA</i>	This study
pSMT3	Hyg ^R , mycobacterial shuttle vector	Paedar O'Gaora
pRSETC	Amp ^R , expression plasmid	Invitrogen
pRAS3.2	pRSETC plus 1199 bp <i>tlyA</i> ORF	This study
pDORF	pSMT3 plus 2.5 kb <i>Sma</i> I fragment carrying <i>tlyA</i> and flanking DNA	This study
pTA _g	Amp ^R Kan ^R , PCR cloning	Novagene

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15 mM MgCl₂, 1% Triton X-100), 8 µl dNTPs (each 1.25 mM), 25 µl RNase-free water and 0.2 µl *Taq* polymerase (Promega). PCR was performed for 40 cycles at 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min followed by 10 min at 72 °C. PCR products were analysed directly on agarose gels.

Sequence data analysis. Amino acid homology searches were performed using BLAST (Altschul *et al.*, 1990). DNA sequence analysis was performed using GeneJockey software (BioSoft) and amino acid sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994).

Protein expression studies. A 1332 bp sequence containing the complete *tlyA* gene, 124 bp of upstream sequence and 401 bp of downstream sequence was amplified using primers C9P3RE3 (GACCCCGCCGACGCCGAGAAC) and C9P3-RE2 (CCGGTAATCCTGTGCGACAAC) and cloned into the pTA_g vector. A *Bam*HI-*Pst*I fragment containing the 1332 bp sequence was directionally cloned in-frame into the pRSETC vector, making pRAS3.2. These clones were used to express the *M. tuberculosis* *tlyA* according to manufacturer's instructions. Samples were grown until the OD₆₀₀ was 0.3 (Pharmacia LKB Ultraspec III spectrophotometer) and expression was induced with IPTG (1 mM). Samples (1 ml) were taken at hourly intervals between 0 and 4 h. Lysed samples (30 µg) were electrophoresed through a 12% SDS-polyacrylamide gel and stained with Coomassie blue.

Haemolysis assay. Contact-dependent haemolysin assays on intact cells were performed essentially as described by King *et al.* (1993). *M. smegmatis* transformants were grown up for 2 d

in liquid medium plus hygromycin and frozen down. Frozen stocks were then diluted into liquid medium containing hygromycin and grown for 24 h, to an OD₆₀₀ > 1.0. *E. coli* strains were inoculated into broth and grown at 37 °C for 24 h; 3 × 10¹⁰ bacteria were pipetted into 50 ml plastic tubes, centrifuged, washed and resuspended at 10¹⁰ bacteria ml⁻¹ in PBS, and 3 × 10⁹ sheep erythrocytes were added. A final centrifugation ensured close contact between bacteria and red blood cells. The assays were incubated at 37 °C for 24 h with 1 ml samples removed after 4 and 24 h intervals. For bacterial cell lysates, cultures were sonicated on ice, centrifuged at 13000 g and sterilized using a 0.45 µm filter. Samples (200 µl) were added to 800 µl of washed sheep erythrocytes and incubated at 37 °C for 16 h. Haemolytic activity was assayed by haemoglobin release and measured spectrophotometrically at A₅₄₅.

RESULTS

Identification of mycobacterial homologues of *S. hyodysenteriae* TlyA

Analysis of *M. leprae* cosmid L247 (U00021) revealed an ORF with a predicted amino acid sequence showing 34% identity to the *S. hyodysenteriae* TlyA. To investigate the prevalence of this gene in other mycobacterial species, degenerate oligonucleotide primers were designed to regions conserved between *M. leprae* and *S. hyodysenteriae* TlyA proteins. These primers suc-

Fig. 3. *tlyA* gene organization. The gene organization of the 2.5 kb *SmaI* fragment is shown. The *tlyA* gene is shown in black, and other ORFs are cross-hatched. Putative non-coding regions are shown in white. *npp*, end of gene encoding putative nitrophenylphosphatase; *orf*, ORF with homology only to genes of unknown function; *recN*, start of gene encoding putative RecN protein.

Table 2. Detection of *tlyA*-encoded haemolytic activity

Strain	<i>n</i>	Contact haemolytic activity $\pm$ SE*
<i>M. smegmatis</i> /pSMT3	10	0.498 $\pm$ 0.025
<i>M. smegmatis</i> /pDORF	5	0.739 $\pm$ 0.103†
<i>E. coli</i> JM109	3	0.226 $\pm$ 0.015
<i>E. coli</i> JM109/pRSETC	3	0.248 $\pm$ 0.018
<i>E. coli</i> JM109/pRAS3.2	3	0.913 $\pm$ 0.031‡

* Read after 24 h incubation.

† *P* value (unpaired two-tailed *t*-test) was <0.01 .

‡ *P* value (unpaired two-tailed *t*-test) was <0.0001 for pRAS3.2 compared to pRSETC.

codon, yielding a 267-amino-acid polypeptide with a predicted molecular mass of 28 kDa. There was one other GTG in the *tlyA* ORF at position 495, but we think it is unlikely to be relevant because the *M. leprae* TlyA protein shows high similarity only from the start codon equivalent to GTG₆₅₄, and this is the first possible start in that ORF. The sequence just upstream of the *M. tuberculosis* GTG₆₅₄ is AGGGTTGAGTG(N)₄, which contains some matches with *M. tuberculosis* 16S rRNA, and may therefore function as a ribosome-binding site.

ORFs were found on both sides of the *tlyA* gene. There is the end of an ORF 181 bp upstream of the putative start GTG₆₅₄ of *tlyA*; this ORF encodes a protein showing similarity to *p*-nitrophenylphosphatase of *Schizosaccharomyces pombe* (Yang *et al.*, 1991). Another ORF starts at the 3'-end of *tlyA*, with a GTG overlapping the *tlyA* stop codon; the encoded protein is 32.9 kDa, and homology searches pick up good matches with hypothetical proteins from other genomes, but none of known function. Just downstream of this gene is the start of a gene encoding a protein which shows homology to *E. coli* RecN; the gap between stop and start codons is just 14 bp, again suggesting that they are part of the same transcriptional unit. Thus *tlyA* appears to be the first gene in an operon containing at least three genes (see Fig. 3). The TlyA protein does not have a classical N-terminal signal sequence.

Fig. 4. RT-PCR analysis of *tlyA* expression. DNase-treated total RNA was extracted from in mid-exponential-phase growth from *M. bovis* BCG and *M. tuberculosis* H37Rv, and *tlyA*-specific RT-PCR was carried out. Lanes: 1, *M. tuberculosis* H37Rv; 2, *M. bovis* BCG; 3, *M. tuberculosis* H37Rv PCR without RT; 4, *M. bovis* BCG PCR without RT; 5, *M. tuberculosis* H37Rv RT-PCR following RNase A treatment; 6, *M. bovis* BCG RT-PCR following RNase A treatment; 7, RT-PCR negative control lacking nucleic acid; 8, *M. bovis* BCG PCR using genomic DNA (100 ng).

Expression of TlyA and haemolysis assays

We wished to determine whether *M. tuberculosis* TlyA possesses haemolytic activity, as has been shown with the *S. hyodysenteriae* protein. We therefore cloned the 2.5 kb *SmaI* fragment containing *tlyA* into the mycobacterial expression plasmid pSMT3, downstream of the strong BCG *hsp60* promoter, which is highly active even without heat shock (Stover *et al.*, 1991), producing pDORF, which was introduced into *M. smegmatis* mc²155. Other investigators have observed that recombinant protein expression may vary among mycobacterial transformants carrying the same plasmid (D. Young, personal communication), so five separate colonies were picked and contact-dependent haemolysin activity was assayed. We did indeed find variable expression, but the results shown in Table 2 demonstrate that colonies carrying pDORF were significantly more haemolytic than those carrying the vector alone ($P < 0.01$).

To obtain further evidence of the activity of *M. tuberculosis* TlyA, a 1332 bp PCR fragment containing the complete *tlyA* gene was cloned into pRSETC and expressed in *E. coli* JM109 as a fusion protein with a short N-terminal His₆ tag. We also included 125 bp of sequence upstream of what we now believe to be the start codon, because at the time we were unsure where translation began. This plasmid, pRAS3.2, was sequenced to confirm that the complete *tlyA* gene had been cloned, and was in the correct reading frame for expression. SDS-PAGE analysis on sample preparations of *E. coli*/pRAS3.2 revealed a product of the predicted size (not shown). Cell extracts of *E. coli*/pRAS3.2 exhibited significant haemolytic activity when induced by IPTG compared to control extracts (Table 2).

Analysis of *tlyA* transcription

To determine if *tlyA* is actively transcribed, specific mRNA was measured from mid-exponential-phase cultures of *M. tuberculosis* and *M. bovis* BCG using RT-PCR. A band of the correct size (187 bp) was detected

with both species, indicating the presence of *tly* mRNA (Fig. 4, lanes 1 and 2). Controls showed that the bands were produced by mycobacterial RNA and not genomic DNA (lanes 3–6).

DISCUSSION

We have identified an *M. tuberculosis* 268-amino-acid polypeptide which shows high homology to the TlyA haemolysin/cytotoxin from the spirochaete *S. hyodysenteriae*. We have demonstrated that the *M. tuberculosis* TlyA exhibits contact-dependent haemolytic activity when introduced into a non-haemolytic *M. smegmatis* strain, and when cloned into *E. coli* as a fusion protein. We have also shown that the *tlyA* gene is expressed in both *M. tuberculosis* and *M. bovis* BCG.

S. hyodysenteriae is the aetiological agent of swine dysentery, an infection characterized by a mucohaemorrhagic diarrhoea. Three genes encoding haemolysins have been found (*tlyA*, *B* and *C*). Several lines of evidence suggest that in *S. hyodysenteriae* haemolysin activity is a major virulence determinant. These are that (1) purified TlyA is haemolytic and cytotoxic for a number of cell types (ter Huurne *et al.*, 1994), (2) the gene is absent in naturally occurring non-pathogenic strains, such as *Serpulina innocens* (Muir *et al.*, 1992), and (3) defined *tlyA* mutants from two independent strains were less virulent in both mice (ter Huurne *et al.*, 1994) and pigs (Hyatt *et al.*, 1994). There is evidence that the haemolytic activity of TlyA is due to pore formation, rather than through proteolytic or enzymic activity (Hyatt & Joens, 1997).

The distribution of the gene among bacterial species is interesting, in that while it is found in some pathogens (*S. hyodysenteriae*, *H. pylori*), homologues are also present in non-pathogens (*B. subtilis*, *Synechocystis* sp.), and other bacteria have no homologue (*Haemophilus influenzae*, *Methanococcus jannaschii* and *E. coli*). While we cannot predict the role of this protein in these different species, we have shown that whatever its function, the *M. tuberculosis* gene is expressed, and the protein possesses haemolytic activity. Furthermore, our preliminary evidence suggests that this gene is restricted to pathogenic species within the mycobacteria. It will be necessary to compare a virulent strain of *M. tuberculosis* with a *tlyA* mutant to prove or disprove a role in virulence.

Analysis of the sequenced region raises a number of questions. Firstly, it appears that *tlyA* is the first gene in an operon in both *M. tuberculosis* and *M. leprae*. The roles of the other genes are not clear; one only shows homology to ORFs with unknown function in other bacteria, while the third shows homology to *E. coli* *recN*. As yet, we have no rationale for the role played by these genes. Secondly, if TlyA functions as a haemolysin *in vivo*, one would expect it to be extracytoplasmic. The protein does not contain a classical N-terminal signal sequence, so we do not know how the protein passes through the cytoplasmic membrane. However, the same

is true for the *S. hyodysenteriae* protein, which is secreted from the treponeme, and is exported to the periplasm of *E. coli*, suggesting that it is exported by a different route.

There is as yet no direct evidence that haemolysins are important virulence factors in mycobacterial infections, but several proteins with haemolytic activity have been reported (see Introduction). Such proteins might alter the membrane encompassing the bacteria in macrophage phagosomes, allowing the bacteria to interfere with the host cells' killing mechanisms. Even if the bacteria remain within a phagosomal membrane, as appears to be the norm (see review by Clemens, 1996), a haemolysin may alter the properties of the membrane to allow molecules to pass through more readily. The presence of bacterial proteins in the cell cytoplasm might explain the evidence that the major histocompatibility complex class I pathway of antigen presentation is important in the immune response to *M. tuberculosis*. For example, CD8 cytotoxic T cell responses to live BCG have been reported (Turner & Dockrell, 1996), and mice lacking a functional β_2 microglobulin gene are more susceptible to *M. tuberculosis* than *M. bovis* BCG (Flynn *et al.*, 1992). These results suggest that class I presentation is important in both *M. tuberculosis* and *M. bovis* BCG, but more so in the former. This may correlate with the detection of greater haemolysin activity in *M. tuberculosis* (King *et al.*, 1993). Our results show that *tlyA* is present and expressed in *M. bovis* BCG. Thus either TlyA is not the major haemolysin detected by King *et al.* (1993), or its expression differs in the two organisms. Our RT-PCR results were not quantitative, so we do not know the relative levels of expression; also, our experiments used organisms cultured *in vitro*, and expression may differ *in vivo*.

The *tlyA* mutant of *S. hyodysenteriae* offered partial protection against challenge with the wild-type strain (Hyatt *et al.*, 1994; ter Huurne *et al.*, 1994), and if we can demonstrate attenuation of an equivalent mutant of *M. tuberculosis*, inactivation of the gene may be desirable in a rational vaccine strain. Alternatively, if haemolytic activity is important for production of a CD8 cytotoxic response, it may be better to retain this function, and concentrate on attenuation of other pathways.

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