Recombinant Technology

Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*

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Abstract

*Brucella melitensis* is a highly infectious animal pathogen able to cause a recurring debilitating disease in humans and is therefore high on the list of biological warfare agents. Immunoglobulin genes from mice immunized with gamma-irradiated *B. melitensis* strain 16M were used to construct a library that was screened by phage display against similarly prepared bacteria. The selected phage particles afforded a strong enzyme-linked immunosorbent assay (ELISA) signal against gamma-irradiated *B. melitensis* cells. However, extensive efforts to express the respective single chain antibody variable region fragment (scFv) in soluble form failed due to: (i) poor solubility and (ii) in vivo degradation of the c-myc tag used for the detection of the recombinant antibodies. Both problems could be addressed by: (i) fusing a human kappa light chain constant domain (Ck) chain to the scFv to generate single chain antibody fragment (scAb) antibody fragments and (ii) by co-expression of the periplasmic chaperone Skp. While soluble, functional antibodies could be produced in this manner, phage-displaying scFvs or scAbs were still found to be superior ELISA reagents for immunoassays, due to the large signal amplification afforded by anti-phage antibodies. The isolated phage antibodies were shown to be highly

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; Ck, human kappa light chain constant domain; Cm, chloramphenicol resistance gene; ELISA, enzyme-linked immunosorbent assay; FPLC, fine performance liquid chromatography; HRP, horseradish peroxidase; IMAC, immobilized metal affinity chromatography; g3p, M13 gene III protein; g8p, M13 gene VIII protein; IPTG, isopropyl β-D-thiogalactopyranoside; LPS, lipopolysaccharide; phAb, phage displayed antibody fragment; MALDI/TOF, matrix-assisted laser desorption ionization/time of flight; M-MLV, Moloney-murine leukemia virus; Mab, monoclonal antibody; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; scAb, single chain antibody fragment; scFv, single chain antibody variable region fragment; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; TB, terrific broth; Tween-20, polyoxyethylene sorbitan monolaurate.

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specific to *B. melitensis* and did not recognize *Yersinia pseudotuberculosis* in contrast to the existing diagnostic monoclonal YST 9.2.1.

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### 1. Introduction

The *Brucellae* are Gram-negative facultative intracellular pathogens that are responsible for zoonoses worldwide (Corbel, 1997; Boschiroli et al., 2001; Moreno and Moriyon, 2002). Infection causes abortion in wild and domestic animals resulting in enormous financial losses especially in developing parts of the world. Contact with infected animals can result in human infection, which while rarely fatal, can cause long-term recurring illness marked by debilitating malaise and fever. *Brucellae* are easily dispersed in aerosols, have a low dose to infectivity ratio, and can survive extended periods of time separated from their host. As a result of these features, the *Brucellae*, particularly *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*, were among the first organisms to be considered as offensive biological warfare weapons (Franz et al., 1997). Indeed, these three of six very closely related species remain on the Centers for Disease Control and Prevention select agent list.

Rapid approaches for detecting *Brucellae* bypass the requirement for lengthy and fastidious culture conditions (Doern, 2000; Sumerkan et al., 2001) and include real-time PCR (Sreevatsan et al., 2000; Redkar et al., 2001), fluorescence whole cell nucleic acid hybridization (Fernandez-Lago et al., 2000), or immunoassays utilizing antibodies able to specifically recognize *Brucellae* cells. Current immunoassay methods employ antibodies to lipopolysaccharide (LPS), the immunodominant surface antigen (Berger et al., 1969). However, the immunologically distinguishing feature in the LPS of the *Brucellae*, the O:9 epitope, is also shared by certain *Yersiniae* (Caroff et al., 1984). As a result, many existing anti-*Brucellae* sera exhibit cross-reactivity with the *Yersiniae* (Bundle et al., 1984). It is important to be able to distinguish between these two organisms as double infections can be common (Kittelberger et al., 1995) and since certain *Yersiniae* are also considered potential biological warfare agents.

Recombinant antibody fragments are gaining ground as supplements to monoclonal and polyclonal animal sera in the biological agent diagnostic and detection arena (Emanuel et al., 2000). Genetic manipulation can be employed to tailor affinity and specificity (Chen et al., 1999; Short et al., 2001), stability (Reiter et al., 1994), avidity (Iliades et al., 1997), or to generate chimeras to reporter molecules (Kerschbaumer et al., 1997; Pearce et al., 1997; Casey et al., 2000). Antibody fragments can be readily expressed in *Escherichia coli* allowing low cost production and purification, an important advantage for many applications including proteomics (Hayhurst and Georgiou, 2001).

Phage display is routinely used for the isolation of antibody fragments to a wide variety of antigens including haptenes, carbohydrates, peptides, proteins, and surface antigens of intact mammalian cells (Griﬃths et al., 1993; Hoogenboom et al., 1998, 1999). Panning of phage display libraries on intact pathogens has also been employed to generate diagnostic and therapeutic antibodies to a wide range of disease causing agents including bacteria. Single chain antibody variable region fragment (scFv) speciﬁc for Gram-negative, complement-resistant *Moraxella catarrhalis* were isolated by competitive selection of semi-synthetic library with complement-sensitive strains (Boel et al., 1998). Similarly, scFv speciﬁc to a surface component of Gram-positive, pathogenic *Streptococcus suis* were generated by subtractive panning (de Greeff et al., 2000). Finally, scFv directed toward surface associated antigens of *Chlamydia trachomatis*, including some host cell components, were derived from a naïve scFv library (Lindquist et al., 2002).

Here, we used phage display to isolate scFv to gamma-irradiated *B. melitensis* strain 16M. After panning a biased library on immobilized antigen,
two phage clones were shown to be specific for the antigen and not cross-reactive with *Yersinia*. Efforts to express scFv antibodies revealed several complications with common epitope tags used for detection of scFv. These problems were successfully addressed by converting the scFv into the single chain antibody fragment (scAb) format (McGregor et al., 1994; Hayhurst, 2000) and co-expression of the chaperone Skp (Bothmann and Pluckthun, 1998; Hayhurst and Harris, 1999) to alleviate protein aggregation in the bacterial periplasm. This strategy will be useful for the preparative production of other recombinant antibodies including those specific for biological warfare agents especially since scAb have recently been shown to have improved pharmacokinetic properties over scFv (Maynard et al., 2002).

2. Materials and methods

2.1. Antigens

Inactivated *B. melitensis* strain 16M was provided by Edgewood Chemical and Biological Center (ECBC, Aberdeen Proving Ground, MD). Prior to inactivation, cells were grown in soy trypticase broth, washed, and treated by one of three methods: cobalt gamma-irradiation (3.0 × 10⁶ rad total dose). Concentration of cells prior to inactivation was 4.5 × 10⁹ cfu/ml; inactivation was confirmed by culture of 1.0 ml of suspension on solid media. The following *Yersinia* species were provided by ECBC to test for scFv cross-reactivity: *Yersinia enterocolitica* (ATCC 23715), *Yersinia pseudotuberculosis* (ATCC 6905, 29833, and NADC 5561, 5560), *Yersinia rhodei* (ATCC 43380), *Yersinia ruckeri* (ATCC 29473).

2.2. Antibodies

Mouse monoclonal antibody YST 9.2.1 specific for LPS was provided by ECBC. Anti-mouse-HRP was from BioRad (Hercules, CA) and anti-M13 HRP was from Amersham Pharmacia (Piscataway, NJ). Anti-FLAG M1, anti-His⁶ clone-1, anti-myc clone 9E10 and anti-His⁶ clone-1 HRP conjugate were all from Sigma (St. Louis, MO).

2.3. Immunizations

Five-week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed at the Texas Center for Infectious Diseases (TCID, San Antonio, TX) on a 12h light/dark cycle and given food and water ad libitum. All animal handling procedures were performed according to TCID guidelines. For the first immunization, bacterial suspensions in saline were mixed with an equal volume of Freund’s complete adjuvant (Sigma) to form a stable emulsion. Mice were anesthetized via inhalation of Metofane and injected intramuscularly at 0.1 ml per site with each animal receiving a total of 1 × 10⁹ bacteria per immunization. The mice were boosted with bacterial cells emulsified in Freund’s incomplete adjuvant 1, 4 and 8 weeks later. Control mice were inoculated with saline in place of bacterial suspension following the same schedule. Six days after the final boost, mice were anesthetized, bled via cardiac puncture, and sacrificed for tissue harvest. Spleens were removed and placed into RNALater™ storage buffer on ice (Ambion, Austin, TX) prior to processing and RNA extraction.

2.4. scFv library construction

Spleens were teased apart in ice-cold PBS, transferred to guanidinium denaturation solution, and disrupted using a Dounce homogenizer. Total RNA was extracted using the ToTALLY RNA™ kit (Ambion) with twice the recommended volume of denaturation buffer to reduce the viscosity of the extract. Total RNA (3.7 µg/50 µl reaction) was subjected to RT-PCR using the RetroScript kit (Ambion) with oligo-dT₁₈ and M-MLV reverse transcriptase either at 42 °C (V₁ samples) or 54 °C (Vₗ samples). PCR and scFv assembly was performed using standard conditions (Krebber et al., 1997) to generate a library of 5 × 10⁵ clones which was subsequently rescued with helper phage M13KO7.

2.5. Phage display and panning

 Gamma-irradiated *B. melitensis* 16M (6 × 10⁷ cfu/ml), in a total volume of 4 ml PBS, was coated overnight in MaxiSorp Immunotubes (Nunc, Roches-
ter, NY) at 4 °C. The tube was blocked with 1% BSA/
PBS for 2 h at 37 °C. Phage particles ($10^9$) were
added in BSA/PBS, incubated for 1 h at room temper-
ature on an end-over-end turntable, followed by over-
night at room temperature without agitation. The
immunotube was washed 15 times with PBS/0.1%
Tween-20, followed by 15 times with PBS alone.
Bound phage were eluted for 10 min in 1 ml 100
mM triethylamine, neutralized with 0.5ml 1M Tris–
HCl pH 7.5, and used to infect exponentially growing
TG1 cells. Infected cells were plated overnight on 2
TY/30 Ag/ml chloramphenicol + 1% glucose. The next
day, cells were scraped from the plates, grown to
exponential phase, and rescued with M13KO7 helper
phage. Infected cells were grown overnight in 2
TY/30 Ag/ml chloramphenicol + 1 mM IPTG at 30
°C. Phage were purified by polyethylene glycol/NaCl precipitation
and resuspended in a total volume of 2 ml PBS.
One milliliter was used for the next round of panning,
for a total of four rounds.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Wells of MaxiSorp microtitre plates (Nunc) were
coated with 100 μl the same dilution of gamma-
irradiated B. melitensis suspension as used for pan-
ning. 100 μl of OD$_{600}$ = 1.0 suspensions of the other
bacterial antigens were used to coat. Standard
ELISA blocking and washing procedures were used
throughout for both phage and antibody fragments
(Chen et al., 2001) with antibodies used at the
manufacturer’s recommended dilutions. Buffer con-
taining 1 mM calcium chloride was used throughout
for FLAG-M1 antibody experiments to satisfy
the requirement for calcium ions. Data points are
the average of duplicate dilutions with each experi-
ment being done at least twice. Antibody fragment
and phAb ELISAs were also carried out with BSA-
coated wells as control for each dilution and these
blanks subtracted to counter background-binding
effects.

2.7. Antibody fragment expression vector construction

Primers AHX77 (5′-TATATAGGCTCCGG-
GGCGGAATTCGCGCGCAGTCGACCATCAT-
CATCACCAC-3′) and AHX78 (5′-
GCGCAAGCTTCGAGATCATGCGCGCGCG-
CACCATGAGCTCCTTC-3′) were used for PCR
amplification of the His$^6$-c-myc tag region from
pHEN2 (Griffin library protocol, Medical Research
Council, UK) which in turn was used to replace the
His$^6$ tag in pAK400 (Kreber et al., 1997) via Sfi/
HindIII to create pMoPac1. Subsequently, the chlor-
amphenicol resistance gene (Cm) of pMoPac1 was
replaced with an ampicillin resistance gene. Kunkel
template of pMoPac1 was generated by co-transfec-
tion of vector and M13KO7 replicative form into
CJ236Fem, a derivative of E. coli CJ236 cured of
the chloramphenicol resistant F episome by repeated
growth in the absence of chloramphenicol. Kunkel
mutagenesis using both AHX71 (5′-CCAGTGATT-
TTTTCTCATTTAAATCTTCTAGTCTCT-
GAAAATC-3′) and AHX72 (5′-GGGCACC-
AAATAACTGCCTATTTAAATTTACGCCCCG-
CCTGCCC-3′) introduced unique SwaI restriction
sites either side of the pMoPac1 Cm gene enabling
easy unidirectional resistance gene exchange em-
ploying the vectors own Cm promoter. In this manner,
the β-lactamase gene was amplified from pUC19
using AHX73 (5′-ATATAATTTAAATGAGTATTT-
ACACATTCCG-3′) and AHX74 (5′-GCGCGAT-
CATTAAAATTTACATGCTTACATG-3′), introduced via
SwaI sites and clones selected upon
media containing 200 μg/ml ampicillin designated
pMoPac10.

A cistron encoding the periplasmic chaperone Skp
was placed downstream of the scFv expression cas-
ette. Primers AHX155 (5′-ATATAAGCTTTAAGG-
AGATATATATGAAAAAGTGGTTATTAG-
CTGCA-3′) and AHX156 (5′-GTGGGGATCCG-
TGACGAGTGACGGTAAACCAGGCGA-
CATAAAAATGTGCACATGTCGCGC-
CATTTTTTACCTTCACAGGCTTTATTATT-
TAACCTGTTCGACGTCGTC-3′) were used to
amplify the skp gene from pHELP1 (Hayhurst and
Harris, 1999) and the product inserted into MoPac10
via HindIII and BamHII to create pMoPac12.

A human kappa constant domain (Ck) (McGregor
et al., 1994) was inserted downstream of the scFv to
enable scAb (scFv-Ck) expression. A pel-specific
primer and AHX88 (5′-CCGCGCTCGACTGA-
CTCCTCGCGGGTG-3′) were used to amplify a scAb
from pMS100 (Hayhurst, 2000) and the Ck domain
isolated inserted into MoPac10 and MoPac12 via NotI
and SalI to create pMoPac15 and 16 respectively. The
constructs are schematically shown in Fig. 1 with
detail of the linker from pMoPac16 from which all
constructs can be deconvoluted.

The super-short g3p gene in plasmid pAK100
(Krebber et al., 1997) was amplified using AHX104
(5′-TATAGGCAGGCAGGATGCTCATG-GGTGCTCTGGTTC-3′) and AHX105 (5′-
GGCCAAGCTTTCGATATCAAGACTCCTTTAT-
TACG-3′) and mobilized into pMoPac10, 12, 15,
and 16 via AscI and HindIII to form phage display
equivalents pMoPac23, 24, 25 and 26, respectively.

C10 and H1 scFv were mobilized between vectors
using SfiI. All constructs employing PCR amplified
products were sequenced through that region after
being first isolated.

2.8. Antibody fragment expression, analysis and
purification

Cell growth, fractionation and analyses were per-
formed essentially as described previously (Chen et
al., 2001) except that the cells were grown in glucose-
free TB and induced with 1 mM IPTG. Antibody
fragments were isolated from mid-scale 400 ml cul-
tures with 0.5 ml of nickel charged 6FF resin (Amers-
ham Pharmacia) and eluted in 1 ml of which 0.5 ml
was then applied to a Superdex 200 HR10/30 column
(Amersham Pharmacia) and gel filtration performed
using an Äkta FPLC system (Amersham Pharmacia).
Fractions (0.5 ml) were collected and those represent-
ing monomeric scAb pooled and quantified using
micro-BCA (Pierce, Rockford, IL). Proteins used
to calibrate the column were from the molecular
weight gel filtration standard kits from Amersham
Pharmacia.

2.9. MALDI/TOF mass spectrometry of purified scAb

Mass spectrometry was performed on a Voyager
MALDI/TOF mass spectrometer (PerSeptive Biosys-
tems, Framingham, MA) in the Protein Microanalysis
Facility of the University of Texas at Austin. Protein
samples were diluted into freshly prepared saturated
sinapinic acid dissolved in 50% acetonitrile, 0.3%
TFA. Sample aliquots of 0.5 or 1.0 ml were spotted
onto stainless steel sample plates and spectra were
collected by averaging 10–20 laser shots. Samples
were irradiated with a 377-nm nitrogen laser (Laser
Science, Franklin, MA) attenuated and focused on the
sample target using the built-in software. Ions were
accelerated with a deflection voltage of 30 kV. Ions
were differentiated according to their
\( m/z \) using a
time-of-flight mass analyzer.

3. Results and discussion

3.1. Isolation of Brucella-specific antibody fragments

Mice were immunized with gamma-irradiated B.
melitensis 16M to provide a source of biased immu-


Fig. 1. (a) Schematic diagram of the expression cassettes employed
to rationalize and solve the poor expression characteristics of the
antibody fragments C10 and H1. The parental vector is pAK400
(Krebber et al., 1997) where the antibody fragments are targeted to
the E. coli periplasm using a pelB leader sequence. Abbreviations:
F, FLAG tag; H, His\(^6\) tag; M, myc tag; scFv single chain antibody
variable region fragment; Ck, human kappa light chain constant
domain; Skp, seventeen kilo Dalton protein, the periplasmic
chaperone. (b) Detail of the pMoPac16 vector region C-terminal
to scFv inserts with pertinent restriction sites highlighted. S/D
represents a Shine Dalgarno sequence. The inclusion of NotI allows
NcoI/NotI scFv mobilization from older pHEN style libraries as well
as newer unidirectional SfiI based assemblies (Krebber et al., 1997).
The arrow after the His\(^6\) tag indicates the likely C terminus of
\( \Delta \)scAb which lacks the c-myc tag.
response to gamma-irradiated *B. melitensis* yet no reactivity to *E. coli*, with serum from control saline-injected mice showing no reactivity against either bacterium (data not shown).

A scFv library was constructed from immunoglobulin genes amplified by RT-PCR from the spleens of two mice. Subsequently, the scFv genes were fused to M13 g3p and the resulting phage display library was panned on immobilized gamma-irradiated *B. melitensis*. Following binding of the phage, the cells were incubated overnight to reduce potential for selection of LPS-binders exhibiting high off-rates (Deng et al., 1994). Monoclonal phage ELISA of 94 clones from the round 4 population identified eight clones giving ELISA signals approximately five times above background. These were sequenced to reveal two unique clones, designated C10 and H1 (sequences submitted to Genbank).

### 3.2. Expression of Brucella-specific antibody fragments

The C10 and H1 scFv genes were subcloned from the phage display vector pAK100 to the periplasmic expression vector pAK400 (Krebber et al., 1997). pAK400 was designed for antibody fragment expression in *E. coli* and consists of a pUC replicon with a tightly regulated lac promoter (Krebber et al., 1996) that, upon induction, results in relatively high scFv expression levels thanks to the presence of a strong ribosomal-binding site. However, no signal was obtained in ELISA assays using gamma-irradiated *B. melitensis* 16M as capture antigen and osmotic shockates from *E. coli* BL21 cells containing pAK400 C10 or H1 scFv as the analytes. The capture assay used anti-FLAG-M1 or anti-His<sup>6</sup> as primary antibodies and anti-mouse-HRP as secondary antibody-conjugate.

![Fig. 2. Gamma-irradiated B. melitensis antibody fragment capture ELISA of antibody fragments in osmotic shockates of normalized small-scale expressions for C10 or H1 expressed as scFv from pMoPac10, scFv with Skp from pMoPac12, scAb from pMoPac15 and scAb with Skp from pMoPac16. Shockates were twofold serially diluted in PBS/BSA. Antibody fragment detection was using anti-FLAG M1 serum, anti-myc 9E10 serum or anti-His<sup>6</sup> serum followed by anti-mouse HRP conjugate. Development was 2 h for C10 and overnight for H1.](image-url)
We reasoned that the absence of ELISA signal could be due to a combination of two factors: (i) the occlusion of the N-terminal FLAG peptide epitope and (ii) the relatively low affinity of anti-His<sup>6</sup> sera for the C-terminally fused hexahistidine tag. To address these issues, a c-myc peptide epitope that is recognized by the 9E10 monoclonal antibody was placed at the C terminus of the scFv adjacent to the hexahistidine tag in the style of the commonly used display vector pHEN2 (Fig. 1). Still, no ELISA signal could be detected from osmotic shock fractions of cells expressing C10 or H1 scFv antibodies fused to the C-terminal c-myc tag. Co-expression of the periplasmic chaperone Skp which is known to improve the solubility of scFv antibodies (Bothmann and Pluckthun, 1998; Hayhurst and Harris, 1999) did not remedy the situation. Only the fusion of a human Ck domain between the scFv and His<sup>6</sup>-myc tags resulted in detectable ELISA signals for both C10 and H1 clones using secondary anti-His<sup>6</sup> sera or anti-myc sera but not anti-FLAG M1 sera (Fig. 2).

Western blot analysis (Fig. 3) of scFv and scAb fragments from the osmotic shock and spheroplast fractions (the latter also contains aggregated membrane-bound proteins which centrifuge together with spheroplasts) revealed that:

(a) N-terminal FLAG: The FLAG epitope is detectable in both the scFv and the scAb fragments indicating that the N terminus of the proteins is not subject to degradation but is most likely occluded during the ELISA assay. Co-expression of Skp increases the production of soluble C10 modestly and that of H1 dramatically in both scFv and scAb formats. A doublet is visible for each format with a lower molecular weight species ΔscFv predominant and ΔscAb a relatively minor species.

(b) C-terminal proximal His<sup>6</sup> tag: The His<sup>6</sup> tag is not subject to degradation and is present on scFv and scAb. The His<sup>6</sup> tag is also present on ΔscFv and ΔscAb.

(c) C-terminal myc tag: The absence of a band corresponding to soluble scFv indicates that the c-myc epitope is subject to extensive degradation. Conversion of the scFv into the scAb format partially alleviated this problem and co-expression of Skp resulted in a further increase in the

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**Fig. 3.** Western blot of osmotic shockates (o) as used in ELISA in Fig. 2 and the remaining spheroplast fractions (s). + indicates Skp coexpression and — indicates no Skp supplement. Electrophoresis was on a 12% Laemmli gel and the Western blots were probed sequentially with three different antisera to establish a correlate between ELISA signal and immuno-reactive protein content. Following each probing was a 2 × 10-min room temperature stripping step (Kaufmann et al., 1987) with overnight reblocking with milk. Exposures represent approx. 5s. Approximate positions of molecular weight markers are shown in kDa and were deduced from the positions of Rainbow markers (Amersham Pharmacia) on the Immobilon-P membrane. Anti-β-lactamase serum was used to confirm the fractionation had succeeded on a separate blot (data not shown).
production of soluble material. c-myc-specific signal is not evident at locations expected for ΔscFv or ΔscAb indicating these species have lost the c-myc epitope.

The data in Fig. 3 indicate that the fusion of the Ck domain greatly enhanced the ability to detect the antibody protein as well as the overall expression level especially when combined with Skp co-expression. Indeed, following IMAC and FPLC purification scAb yields were far in excess of scFv yields (Fig. 4) allowing us to generate several hundred micrograms of scAb fragment per liter of pMoPac16 expression cultures. Yields of the corresponding scFv were consistently at least 10-fold lower.

3.3. Mass spectral analysis of IMAC-FPLC purified scAb

The monomeric scAb isolated by FPLC was found to migrate as a doublet in Coomassie stained SDS-PAGE gels (Fig. 5a). The abundance of the lower molecular weight species increases over time suggesting copurification of a contaminating protease. Both proteins possessed the FLAG tag and His\textsuperscript{6} tag yet only the higher molecular weight species possessed the c-myc tag as determined by Western blotting (data not shown). As the entire c-myc sequence is not required for recognition by anti-c-myc monoclonal 9E10 (Hilpert et al., 2001), it was pertinent to determine precisely where the myc tag was being “lost”. Therefore, the scAb was analysed by MALDI/TOF spectrometry yielding the masses of the doublet components (Fig. 5b). Since the N-terminal DYKD sequence is present as determined by anti-FLAG M1 reactivity, BioEdit (Hall, 1999) was used to deduce the masses of C-terminal truncations of scAb indicating that ΔscAb most likely ended immediately after the last histidine residue of the His\textsuperscript{6} as shown in Fig. 1b. The predicted mass of 39,689.58 Da is within experimental limits of the MALDI/TOF spectrometry determination of 39,667 ± 30 Da. We have since found several scFv isolated from a semi-synthetic library (the Griffin library) and expressed from pHEN2 can be subject to the same degradation pattern indicating that the cleavage is not a vector-specific artifact but rather a global problem with early tag design (data not shown).

Fig. 4. FPLC chromatography of IMAC eluates resulting from mid-scale expression culture of C10 scAb from pMoPac16 (solid line) and C10 scFv from pMoPac12 (dotted line). Molecular weight markers: (1) aldolase—158 kDa; (2) bovine serum albumin—67 kDa; (3) ovalbumin—43 kDa; (4) chymotrypsin—25 kDa. The expression and purification experiments were carried out three times and the same pattern observed in that scAb material by far exceeded scFv material.

Fig. 5. (a) SDS-PAGE of IMAC/FPLC purified monomeric C10 scAb protein revealing the two species scAb and ΔscAb following Coomassie blue staining. (b) MALDI/TOF analysis of the scAb sample to elucidate the site at which the c-myc epitope is lost.
3.4. Detection of B. melitensis with the recombinant antibodies

Purified scAb protein was used in ELISA detection of B. melitensis alongside phage displayed scFv from pAK100. scAb protein capture detection employed anti-His6-HRP conjugate and phAb capture detection employed anti-M13 HRP. We consistently noticed improved signals for phage displayed antibodies compared to their soluble counterparts even though the latter were present in the assay at a significant molar excess (Fig. 6). The use of various anti-His sera and polyclonal anti-Ck sera failed to improve upon the sensitivity of scAb mediated B. melitensis detection (data not shown). Unlike purified antigens, the target to which C10 and H1 are specific may be relatively rare and/or occluded and so the one-to-one stoichiometry of secondary antibody HRP conjugate to detection tag on the scAb may be limiting sensitivity. However, when scFv are displayed as g3p fusions, the secondary antibody–HRP conjugate specific for the major coat protein of M13, g8p, is able to amplify the scFv/antigen binding event by virtue of each phage possessing several thousand copies of the g8p protein. The close packing of g8p (Makowski, 1993; Kneissel et al., 1999) and bivalency of the IgG based anti-M13 HRP conjugate will most likely prevent each and every copy of g8p being bound by conjugate. Yet even if 1 in 10 g8p were bound it would result in a 100-fold signal amplification.

Having established that phage-borne scFv were the superior detection reagent and having elevated periplasmic expression levels of scFv by conversion to
scAb, we sought to combine the two approaches as periplasmic solubility of antibody fragments can be proportional to the efficiency of their display (Coia et al., 1997; Bothmann and Pluckthun, 1998, 2000). However, we consistently observed that phage displaying scFv were almost an order of magnitude more effective in detecting \textit{B. melitensis} than phage displaying scAb (Fig. 7). Skp co-expression improved the capacity for detection even further. It appears that the larger size of the scAb (42 kDa as opposed to 27 kDa for an scFv) and its propensity to dimerise (Fig. 4 and McGregor et al., 1994) may be decreasing the frequency with which scAb-g3p fusions can assemble onto virions.

The specificity of the phAb-C10 antibody towards \textit{B. melitensis} was evaluated. Reactivity was observed only with gamma-irradiated \textit{B. melitensis} sample and not with any of the \textit{Yersiniae} tested (Fig. 8a). This is in contrast to an existing diagnostic monoclonal antibody YST9.2.1 which reacts not only with \textit{B. melitensis} but \textit{Y. pseudotuberculosis} (NADC 5561) by virtue of the shared O:9 LPS epitope for which YST 9.2.1 is specific (Fig. 8b). Thus, phAb C10 is clearly able to distinguish species sharing the same major LPS antigen. This is most useful in the diagnostic setting as bacteria sharing common LPS epitopes are often co-isolated and difficult to distinguish.

4. Conclusions

Recombinant antibody methodology was used to generate antibody fragments specific for the potential bioweapon \textit{B. melitensis}. Specific washing steps were included in the phage panning procedure to eliminate phage that might be cross-reactive with strains expressing the same dominant LPS epitope. The isolated \textit{Brucella}-specific scFvs were poorly expressed in \textit{E. coli}, and they displayed a propensity to lose the commonly used C-terminal c-myc peptide tag due to proteolytic degradation. Gene fusion with a human immunoglobulin kappa constant domain to produce scAb constructs as well as chaperone co-expression using Skp were employed to elevate significantly the production of purified antibody fragment.

Despite these improvements, we consistently observed that phAb were two orders of magnitude more sensitive in capture ELISA formats compared to the homologous scAb constructs. Indeed, similar phAb signal amplification has recently been shown to be of use in detecting low levels of \textit{Bacillus} spores (Zhou et al., 2002) and can doubtless be further improved by polyvalent display (Rondot et al., 2001). Another technical advantage of the phAb format is that production of phage particles is significantly more convenient than recombinant scFv or even scAb expression and isolation from \textit{E. coli}.
The isolated antibody fragment referred to as C10 was able to discriminate between B. melitensis and other bacteria that share the same major surface LPS antigen. C10 did not appear to be LPS specific and we are currently elucidating the nature of the antigen. This same strategy of avoiding LPS-specific antibodies while using phAbs to detect relatively rare but unique epitopes should allow rapid definition of bacteria within mixed populations and be of application to environmental sensing.

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