Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 411–420
Protein Expression & Purification
www.elsevier.com/locate/yprep

Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins

Donghee Cho [1], Sung-Jae Shin, Adel M. Talaat, Michael T. Collins *

School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706, USA

Received 20 December 2006, and in revised form 26 December 2006
Available online 9 January 2007

Abstract

Fourteen proteins of potential diagnostic value for bovine paratuberculosis were identified in the culture filtrate of *Mycobacterium paratuberculosis* JTC303 by immunoblot and mass spectrometry. The goals of the present study were to express these 14 ORFs in *Escherichia coli* and evaluate their antigenicity. All 14 proteins were expressed in *E. coli* BL21(DE3) after transformation with the pET-22b(+) vector. Yields of insoluble proteins were higher than those of the soluble proteins. Polyclonal rabbit antibodies directed against culture filtrate of JTC303 strain confirmed that five of the expressed and purified proteins are culture filtrate components: ModD, Antigen 85C, PepA, MAP1693c, and MAP2168c. Evaluation of ModD as an ELISA solid-phase antigen on a set of bovine sera from well-characterized paratuberculosis cases and infection-free controls revealed that there was strong serum antibody reactivity to rModD in many infected cattle. However, the overall rModD ELISA sensitivity and specificity for bovine paratuberculosis was not greater than those of ELISAs using crude antigens such as cellular extract or culture filtrate for plate coating, as judged by area under the curve (AUC) of Receiver-operating curve (ROC) analysis. However, an ELISA using natural ModD as the solid-phase antigen had a higher sensitivity and AUC than did rModD suggesting diminution of antigenicity in rModD. Taken together, our results showed that the natural forms of the identified proteins may be useful for diagnosis of bovine paratuberculosis.

Keywords: *Mycobacterium paratuberculosis*; ModD; Johne's disease; Diagnosis; FAP; ELISA; *Mycobacterium avium* subspecies *paratuberculosis*; MAP; Serodiagnosis; Bovine

Bovine paratuberculosis is a chronic inflammatory bowel disease caused by *Mycobacterium paratuberculosis* (also known as *Mycobacterium avium* subsp. *paratuberculosis*) [1]. Based on serologic testing, 3.4% of cows and 21.6% of US dairy herds are infected with *M. paratuberculosis* [2]. Clinical signs in cattle include persistent diarrhea, weight loss and decreased milk production [3,4]. Antibiotic therapy is ineffective and cost-prohibitive; the vaccine available in the United States has not performed sufficiently well to support its use in any but the most infected herds [5]. Thus, infection control depends upon herd management to limit the spread of the infection [6] and identification of infectious adult cattle by diagnostic testing for segregation or removal from the herd [7].

The most frequently used test for paratuberculosis is based on serum antibody detection using an ELISA platform. While inexpensive, fast and able to handle numerous samples, its low diagnostic sensitivity during much of the course of the infection is a drawback. The development of humoral immunity only at late stages of the infection and the variability of individual animals' immune responses are partly responsible for this low sensitivity [1,8,9]. Compromised specificity can also be a problem due to the extensive sharing of antigens or epitopes among *M. paratuberculosis* and other mycobacteria [10,11].

Two approaches have been tried to enhance paratuberculosis ELISA sensitivity and specificity. Enhanced specificity

* Corresponding author. Fax: +1 608 265 6463.
E-mail address: mcollin5@wisc.edu (M.T. Collins).
[1] Present address: (430-824) 480 Anyang 6-dong, Anyang-si, Kyunggi-do, Republic of Korea.

1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.12.022

can be achieved by removing cross-reactive antibodies from clinical samples by absorption of sera with *Mycobacterium phlei* antigens prior to performing the ELISA [11–13]. This technique, first reported by Yokomizo in 1986, is an integral part of most commercially available paratuberculosis ELISA kits in use today. These kits have a sensitivity and specificity for detection of fecal culture-positive cattle of roughly 28% and 99%, respectively [14]. In raising specificity, serum absorption may, however, lower assay sensitivity.

A second approach to improving paratuberculosis ELISA accuracy is identification and purification of antigens most critical for serodiagnosis of *M. paratuberculosis* infections. Although several antigens inducing strong antibody responses have been identified [15,16], most have been found unsuitable for serodiagnosis because they are highly conserved within mycobacteria and hence cross-reactive with other mycobacterial pathogens. Strategies used to identify species-specific proteins of *M. paratuberculosis* have included phage library screening, DNA hybridization, crossed immunoelectrophoresis, and comparative genomics. Several *M. paratuberculosis*-specific antigens have been identified using these approaches. Among them are the immunodominant 35-kDa protein, 65 K heat shock protein, 34-kDa protein, and two alkyl hydroperoxide reductases [17–21]. In addition, Bannantine et al. found 21 *M. paratuberculosis* genes that are absent from all other mycobacteria [22,23].

In a previous study, 14 *M. paratuberculosis* culture filtrate (CF)[2] proteins were identified by immunoblot and mass spectrometry [24]. The bovine sera used to detect these proteins had been absorbed with *M. phlei* before immunoblot, thus the proteins are likely to be antigenic, expressed in vivo, and relatively specific for *M. paratuberculosis*. The goals of the present study were to clone, express, and purify these 14 *M. paratuberculosis* proteins of potential serodiagnostic value. The purified proteins were then screened by ELISA using bovine sera from paratuberculosis cases and infection-free controls. The sensitivity and specificity of the best of these 14 proteins were then evaluated in comparison with crude antigens such as *M. paratuberculosis* cellular extracts and culture filtrates.

Materials and methods

Bacterial strains

A clinical isolate of *M. paratuberculosis,* strain JTC303, was propagated in modified Watson-Reid (WR) broth supplemented with mycobactin J (Allied Monitor, Fayette, MO) [25]. *M. avium* ATCC 35712 was cultured in WR broth without mycobactin J. Bacterial cells and culture filtrates were harvested when growth reached the stationary phase. *M. phlei*, ATCC 11758, was cultivated in WR and cellular protein extracts were used to remove cross-reacting antibodies prior to ELISA (pre-absorption). Both *Escherichia coli* BL21(DE3), purchased from Novagen (Madison, WI) and *E. coli* DH5α were grown at 37 °C in LB broth (FisherBiotech, Fair Lawn, NJ). After plasmid transformation, *E. coli* were cultured in ampicillin-containing (100 μg/ml) LB broth.

Plasmids

The cloning vector plasmid pGEM®-T Easy was purchased from Promega (Madison, WI), and the expression vector, pET-22b(+) from Novagen (Madison, WI). Both plasmids contained an ampicillin resistance marker. The expression vector contained a T7 promoter and a C-terminal 6× His-tag coding sequence of the multiple cloning region.

PCR amplification and cloning

From previous work 14 proteins were selected for expression (Table 1) [24]. For gene amplification, genomic DNA was prepared from *M. paratuberculosis* JTC303 strain using a commercial kit (QIAGEN, Chatsworth, CA). Sequences for each ORF were amplified with primer pairs designed to cover the entire mature-secreted portion. Each primer pair was flanked by restriction enzyme sites for directional ligation into the pET-22b(+) vector (Table 2). After purification of PCR products using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI), the PCR products were ligated with pGEM®-T Easy vector. Recombinant clones were then transformed into competent *E. coli* DH5α. After sequencing the inserted ORFs, the fragments were cloned into the pET-22b(+) vector after digestion with *Nde*I and *Xho*I enzyme at their 5′ and 3′ ends, respectively. The ORFs carrying the *Xho*I restriction site were digested with *Not*I instead of *Xho*I. The ORFs carrying the *Nde*I restriction site at their 5′ end were preceded by the ATG initiation codon and followed by the ORF and then nucleotide sequences encoding six histidines. The ORF of Antigen 85C was digested with *Bam*HI and *Hin*dIII. Ligation products were then transformed into *E. coli* BL-21 (DE3) host cells (Novagen, Madison, WI) for expression.

Protein expression

Each transformed *E. coli* clone was grown in LB medium containing 100 μg/ml ampicillin at 37 °C with vigorous shaking. When the culture reached an $OD_{600\,nm}$ of 0.4–0.8, expression of cloned proteins was induced for 1–5 h by adding IPTG to a final concentration of 1 mM. Cells were harvested from 1000-ml batch cultures by centrifugation at 5000*g* for 5 min, and were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Soluble proteins were extracted by first disrupting the cell suspension with sonication. Cellular debris was removed by centrifuga-

[2] *Abbreviations used:* CF, culture filtrate; WR, Watson-Reid; Ni–NTA, Ni–nitrilotriacetic acid; rModD, recombinant ModD; HRP, Horseradish peroxidase; CE, cellular extract; ROC, Receiver-operating characteristic; AUC, areas under the curves.