Molecular and Cellular Probes 28 (2014) 65–72

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Protein markers for identification of *Yersinia pestis* and their variation related to culture

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ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form
27 November 2013
Accepted 2 December 2013
Available online 11 December 2013

Keywords:
Yersinia pestis
Culture environment
Protein expression
HPLC
Tandem mass spectrometry

ABSTRACT

The detection of high consequence pathogens, such as *Yersinia pestis*, is well established in biodefense laboratories for bioterror situations. Laboratory protocols are well established using specified culture media and a growth temperature of 37 °C for expression of specific antigens. Direct detection of *Y. pestis* protein markers, without prior culture, depends on their expression. Unfortunately protein expression can be impacted by the culture medium which cannot be predicted ahead of time. Furthermore, higher biomass yields are obtained at the optimal growth temperature (i.e. 28 °C–30 °C) and therefore are more likely to be used for bulk production. Analysis of *Y. pestis* grown on several types of media at 30 °C showed that several protein markers were found to be differentially detected in different media. Analysis of the identified proteins against a comprehensive database provided an additional level of organism identification. Peptides corresponding to variable regions of some proteins could separate large groups of strains and aid in organism identification. This work illustrates the need to understand variability of protein expression for detection targets. The potential for relating expression changes of known proteins to specific media factors, even in nutrient rich and chemically complex culture medium, may provide the opportunity to draw forensic information from protein profiles.

1. Introduction

Confident identification of the potential bioterrorism agent *Yersinia pestis* is critical for a rapid response to an incident. Guidelines for culture of suspected samples to yield colonies for microscopy and identification are provided to member labs of the laboratory response network (LRN), to demonstrate morphology and antigen expression characteristics [1]. In particular the recommendations call for culture of any suspected isolate at both 28 °C and 37 °C where the former is in a more optimal growth temperature range (between 28 °C and 30 °C) and the later is needed for production of the diagnostic F1 capsule antigen. The F1 capsule has also been the focus of clinical diagnostic assays as well [2]. The recommendations for culture conditions build on previous data describing the impact of temperature on growth rate and protein synthesis [4,3]. Complex growth media such as brain heart infusion (BHI), tryptic soy broth (TSB), nutrient broth (NB), sheep blood agar (SBA), and Maconkey agar are recommended for culture of clinical samples [5]. BHI is most useful for rapid growth of the organism while cefsulodin-irgasan novobiocin (CIN) agar has been investigated as a semi-selective agar for *Yersinia* isolation [6]. While temperature has been well described for its impact on growth rate and protein synthesis, the impact of different types of complex growth medium and their formulations has not. These factors have an important influence on the expression of diagnostic proteins and culture-based identification.

Beyond the diagnostic role of the plasmid-borne capsular F1 antigen (CaF1), it is important for virulence [7]. Additional virulence related proteins have been well-studied for their expression with both temperature and calcium concentration. Many of these proteins are plasmid-borne including the “low calcium response”(lcr) proteins found on pCD1 that are necessary for virulence and are inhibited in their expression with 2.5 mM Ca and culture at 26 °C [8]. The induction of these proteins was demonstrated at 37 °C by Perry and Fetherston [9]. These proteins include the V antigen and *Yersinia* outer membrane proteins (yops) including lcr proteins D-H, R, V, Q and yops B–F, H, and J-M [9]. The optimal growth temperature for expression of the V antigen (V) has been described at 37 °C [10]. Sub millimolar Ca concentrations and growth at 37 °C are thought to mimic the conditions within the

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http://dx.doi.org/10.1016/j.mcp.2013.12.001

mammalian host where these protein factors are needed for virulence [11,12].

In addition to toxins, Fe acquisition and storage has been shown to be critical for virulence [13]. Expression of hemin storage proteins and the siderophore yersiniabactin for acquisition of hemin bound or free Fe have been identified as specific factors necessary for virulence [9,13]. Loss of the 102 kB pigmentation (pgm) locus coding for these factors has been shown to severely attenuate the organism virulence [14]. Fe acquisition is an important aspect of bacterial survival in a variety of environments and so Fe-responsive transcriptional regulators (termed ferric uptake regulators [Fur]) have been found that control different mechanisms of Fe acquisition in a variety of pathogens [15]. Pieper et al. [16] performed a more extensive study on the impact of Fe and temperature on protein expression in *Y. pestis*. They demonstrated that Fur and RhyB controlled the expression of independent sets of proteins involved in Fe acquisition depending on whether the organism was grown at 26 °C or 37 °C. No data was presented on protein expression at 28–30 °C.

The pMT1 and pPCP1 plasmids are specific to *Y. pestis* among the *Yersinia* species and contain the CaF1 in addition to *Yersinia* murine toxin (ymt) and plasminogen activator protease (Pla) virulence factors. The impact of metals, alone or in conjunction, with changing temperature on the expression of these proteins has not been well defined. Even for those proteins whose expression has been well studied under defined conditions, the temperatures used have been intended to simulate the host environment [11]. Furthermore, the Fe or Ca concentrations studied have been intended to mimic host conditions or have a definitive impact on protein expression. While these studies have been vital to our understanding of the interaction between metals, temperature and protein expression, they do not reflect growth conditions that are optimal for organism production in rich growth media and therefore are not directly relevant for microbial identification and forensics.

Y. pestis specific proteins exist for detection but their expression has not been studied in rich media at growth temperatures optimal for that organism. Beyond species-level identification, differentiation among the *Y. pestis* isolates has been proposed through identification of outer membrane proteins using a proteomic analysis [17]. However, strain identification and differentiation depends on both the presence and sequence variability of the detected proteins. The presence of essential proteins may be affected by growth medium, so evaluating the impact of common media is essential. In addition to identification, the profile of identified proteins may provide a means to determine attributes of the original culture medium as a forensic signature.

To that end, we have investigated two versions of two different culture media recipes formulated with components from different sources for cultivation of a *Y. pestis* strain. The growth characteristics and secreted protein profiles were examined in relation to medium recipe. The ability to discern between *Y. pestis* sequence homologues in the database was also investigated.

2. Methods and materials

2.1. Chemicals and reagents

The HPLC grade water, urea, acetonitrile, acetone, ammonium bicarbonate, trichloroacetic acid and formic acid were purchased from Sigma chemical Co (St. Louis MO). Sequencing grade trypsin was purchased from Promega (Madison WI).

2.2. Bacterial strain and growth conditions

Y. pestis KIM D27 (pgm-) was maintained in glycerol at −80 °C prior to streaking onto brain heart infusion agar (BHI) plates and incubated at 30° for 18 h. Broth cultures were started by inoculating a single colony into 10 mL of appropriate media in a 50 mL glass flask. This starter culture was then sub-cultured 1:100 into 75 mL of the appropriate media in a 250 mL glass flask.

Two medium recipes were used for organism culture, Brain Heart Infusion (BHI) and Tryptic Soy Broth (TSB). BHI medium was formulated with brain heart extract (17.5 g/L), peptone (10 g/L), dextrose (2 g/L), NaCl (5 g/L), and Na_2PO_4 (2.5 g/L). TSB medium was formulated with tryptone casein digest (15 g/L), soytone protein digest (3 g/L), NaCl (5 g/L), and K_2HPO_4 (2.5 g/L). The BHI and TSB media were formulated using components purchased separately to create "in-house" recipes of each. BHI 1 and BHI 2 were both formulated using the Heart & Brain Extract of Pork from the Solabia Group (Pantin, Edex France). BHI1 contained a Bacto Brand Peptone (BD Biosciences, San Jose, CA USA) while BHI 2 was formulated with proteose peptone #3 (Global Bioingredients, Tampa FL, USA). The TSB 1 recipe was formulated with a tryptone, "pancreatic digest, peptone from casein" and a soytone, "peptone from soymeal papain-digested for microbiology" both from Merck KGaA, (Darmstadt, Germany). TSB 2 was formulated with the tryptone, "Bacto Tryptone" (BD Biosciences, San Jose, CA USA) and soytone, "SE 50 M enzymatic digest from soy flour" peptone (DMV International bv. Veghel, Netherlands).

Cultures of each media were grown in triplicate at 30 °C and duplicate at 26 °C or 37 °C using a rotator incubator at 210 rpm. Late log phase cultures were harvested at 11 h at OD_{600} 0.8–1.0, stationary phase were harvested at 32 h at OD_{600} 1.4–1.8 and again at 50 h. Cultures were pelleted by centrifugation at 6000 *rcf* for 10 min and washed in sterile water. The cell supernatant was collected for protein precipitation. Final cell suspensions were enumerated on the appropriate solid media. One mL of the supernatant for each medium was precipitated in a 1.5 mL tube by trichloroacetate (20% final volume) and placing the sample at −20 °C overnight. The precipitate was pelleted by centrifugation at 16,000 *rcf* for 5 min and 4 °C. The pellet was washed in 3 × 200 μL of ice-cold acetone then resuspended in 100 μL of phosphate buffered saline and 0.01% SDS.

2.3. Inductively coupled plasma mass spectrometry

Sterile media samples were prepared for inductively coupled plasma mass spectrometry (ICP-MS). A calibration curve for Be, Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sn, Sb, Ba, Tl, and Pb was used for elemental composition determination. One half mL of each media sample was digested with 4.5 mL of two percent concentrated nitric acid prior to sample introduction into the ICP-MS Model 7500 (Agilent Technologies, Santa Clara CA). Each media sample was analyzed in triplicate with mean and standard deviation reported for biologically relevant elements in Fig. 3.

2.4. Protein separation and digestion

Samples were prepared for separation by 1-D polyacrylamide gel electrophoresis (PAGE) by adding 10 μL of precipitate to 1 μL of NuPAGE reducing agent and 2.5 μL of NuPAGE loading buffer as recommended by the manufacturer (Invitrogen, Torrance, CA). 10 μL of Precision Plus Protein™ Standards (BioRad, Hercules, CA) was loaded into an outside lane for size estimate. The proteins were separated on a NuPAGE Bis-Tris 4–12% pre-cast polyacrylamide gel. The gels were run at 200 V for 35 min and stained with SimplyBlue SafeStain (Invitrogen) for 1 h and destained using deionized water overnight.

Gel bands were excised using an ethanol cleaned razor blade and placed into a 1.5 mL microcentrifuge tube. The gel slices were