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Characterization and purification of proteins suitable for the production of antibodies against '*Ca*. Liberibacter asiaticus'

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ABSTRACT

The citrus disease huanglongbing (HLB), which is caused by '*Candidatus* Liberibacter asiaticus' (CaLas), is one of the most devastating pathogens of citrus, and with no effective method of control, poses a serious threat to citrus production throughout the world. In a previous study we described the production of single chain antibodies against several CaLas proteins that provide the basis for efficient and accurate detection of CaLas in citrus tissues. The isolation of a sufficient amount of purified antigen is a key step in the production of functional antibodies. The current report details purification procedures for six protein antigens used to select recombinant and produce polyclonal antibodies. These proteins include a flagellar biosynthesis protein (FlhA), a dinucleoside polyphosphate hydrolase (InvA), a portion of the major outer membrane protein (OmpA), a component of type IV pilus (CapB), the polysialic acid capsule expression protein (KpsA) and the outer membrane efflux protein (TolC). Results of purification under completely native or denatured conditions were not satisfactory. Therefore different hybrid purification conditions were optimized for each of the different proteins. The results of bioinformatic analysis also indicated that the six proteins contained a great diversity of potential antigenic epitopes, which varied in number, and that the antigenic clusters were not uniformly distributed throughout the proteins. The purified proteins are useful for the development of highly specific antibodies capable of differentiating specific strains of Liberibacter.

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1. Introduction

The non culturable α-proteobacteria '*Candidatus* Liberibacter asiaticus' (CaLas), is strongly associated with the huanglongbing disease (HLB) of citrus trees [2]. The disease is transmitted in citrus groves by the Asian citrus psyllid (*Diaphorina citri* Kuwayama) [3], and experimentally by grafting [17]. Once infected, citrus trees typically enter a progressive decline and die within a few years. Since the symptoms of leaf mottle and yellowing associated with the disease were first described in southern China and India [18,20], it is estimated that HLB has destroyed more than 100 million trees globally [23]. Although HLB was limited to the old world throughout the 20th century it has recently spread to both Brazil [6] and Florida [11], which are key citrus producing locations that sustain the highest production of sweet orange in the world [7].

The development of accurate and rapid PCR- and qPCR- and LAMP-based detection and identification methods for HLB have provided useful tools to detect and monitor the global expansion of CaLas [13–15,19], which has an explosive epidemiology once it enters a new grove or region [11]. Our research group has recently described the use of antibodies to detect CaLas in plant tissue using tissue print and dot blot assays [8,9,22]. The tissue print assay is especially valuable for detecting CaLas in practice because it can specifically track viable CaLas within the phloem of plant tissues [8], is complementary to DNA-based assays, and scales well to many samples. This is especially important for detection of CaLas because of the erratic distribution of the pathogen in diseased plant tissue [16,21].

The goal of the current research program was therefore to develop antibodies capable of recognizing different epitopes with potential for use in various avenues of research. Different purification strategies are known to optimize the purification of individual proteins [12]. The current study optimized the conditions of a Ni-NTA purification system to purify six antigens from extracts of *Escherichia coli* that expressed the CaLas antigens. The antigens, which were fragments of proteins found on the surface of CaLas or

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Table 1
Antigens selected to generate antibodies for detection of '*Ca.* Liberibacter asiaticus' in citrus tissue samples.

Antigen[a]	Protein ID	Gene product
FlhA	WP_015452646.1	Flagellar biosynthesis protein
InvA	WP_015825974.1	Dinucleoside polyphosphate hydrolase
OmpA	WP_015452389.1	Outer membrane protein
CpaB	WP_015452434.1	Component of type IV pilus
KpsA	WP_015824923.1	Polysialic acid capsule expression protein
TolC	WP_015452761.1	Nodulation outer membrane efflux protein

[a] Abbreviated protein name.

secreted by CaLas, were initially cloned in a protein expression vector before optimization of different purification variables including: the ratio of the recombinant proteins to the agarose purification resins, the length of the incubation period of lysates and resins, the duration of the washing and elution steps, and the pH the buffers, as well as the optimization of the concentration of imidazole in the buffers. Bioinformatic analysis was used to gain a greater understanding of the structure of the antigens in order to predict the utility of the antibodies for highly specific or broad spectrum detection of members of the *Candidatus* genus Liberibacter.

2. Materials and methods

2.1. Cloning and DNA sequence analysis

Six CaLas antigens (Table 1) were cloned in *E. coli* BL21 (DE3) using the pET102/D-TOPO vector system (Thermo Fisher, Pittsburgh, PA) [22], which incorporates a C-terminal 6 X His tag that allows purification with a nickel-conjugated agarose resin. The six antigens were cloned based on sequence data from the complete CaLas genome [10] and bioinformatic analysis, which predicted hydrophilic epitopes likely to be exposed on the surface of the protein and CaLas cell. Bacterial cultures were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 mg/L kanamycin at 37 °C. The sequence of the cloned protein encoding inserts was confirmed as follows. The cloned protein encoding fragments were isolated from the pET102/D-TOPO vector using PCR and the T7/TrxFus primer set: T7: 5′-TAGTTATTGCTCAGCGGTGG-3′ and TrxFus: 5′-TTCCTCGACGCTAACCTG-3' (IDT, Coralville, IA). PCR was conducted in 20 μl reaction mixtures containing 1.0 μl bacterial culture, 0.5 μl each of the forward and reverse primers (10 μM), 10.0 μl 2 × Taq Mix, and 8 μl ddH_2O in a CFX 100 thermo cycler (Bio-Rad, Richmond, CA) with the following program: initial cell lysis and DNA denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 40 s. The PCR products were visualized by electrophoresis in a 1% agarose gel and recovered using a gel extraction kit (Thermo Fisher, Pittsburgh, PA), before being cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced (Genewiz, Rockville, MD). The resulting sequence data were used to search the National Center for Biotechnology Information (NCBI) database to confirm the identity of the cloned products.

2.2. Expression of recombinant proteins in E. coli

The recombinant pET102/D-TOPO plasmids containing the antigenic sequences were transformed into competent *E. coli* BL21 cells. Fresh single colonies were picked and sub cultured at 37 °C for 12 h before being used to inoculate 25 mL LBAG (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, 0.01% ampicillin, 2% glucose) liquid cultures in 125 mL flasks, which were grown at 37 °C with shaking (220 rpm) to a density of $OD_{600} = 0.8$. The cultures were then transferred to 250 mL Nalgene bottles and centrifuged for 15 min at 8500 rpm, and the pellets re-suspended in 30 mL fresh LBAG containing 30 μl IPTG (1 mM) to induce expression of the recombinant proteins. After incubating the cultures at 37 °C for 14 h with shaking (220 rpm), the cultures were centrifuged for 20 min at 8500 rpm. The resulting pellets were either used immediately for protein purification or stored at −20° C for later use.

2.3. Purification of recombinant proteins

The protocol of the manufacturer of the Ni-NTA purification system were followed (Ni-NTA purification system (Thermo Fisher, Pittsburgh, PA)), with empirically determined modification for the hybrid conditions.

2.4. Native conditions

The bacterial pellets from the IPTG-induced cultures were suspended in native binding buffer, incubated with lysozyme on ice. The cell lysates were then subjected to sonication (Vir Sonic 300) in an ice bath using six 10-s bursts at high intensity and 10-s cooling intervals. The resulting lysates were centrifuged at 8500 rpm for 15 min and the supernatants mixed by inversion before being loaded onto a 10 mL column that contained 4 mL Ni-NTA resin that had been washed with 6 mL of ddH_2O, and equilibrated with native purification buffer (250 mM NaH2PO4, 2.5 M NaCl, pH 8.0) and in different experiments washed with native wash buffer (native purification buffer plus 10, 20 or 250 mM imidazole) and finally eluted with native elution buffer (native purification buffer plus 10, 20 and 250 mM imidazole). This protocol produced unsatisfactory results and different purifications under either denaturing or under a variety of hybrid conditions were carried out.

2.5. Denaturing conditions

Bacterial lysates prepared using guanidinium based lysis buffer (pH 7.8) were subjected to sonication and loaded onto a 10 mL column that contained 4 mL Ni-NTA resin that had been washed with 6 mL of ddH_2O and equilibrated with denaturing binding buffer (8 M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and allowed to settle completely by gravity. The column with the proteins bound under denaturing conditions were then washed with 4 mL denaturing wash buffers at pH 6.0 and 5.3 followed by elution with denaturing elution buffer.

2.6. Hybrid conditions

Bacterial lysates prepared using guanidinium based lysis buffer and sonication were prepared and bound to the columns as described above for purification under denaturing conditions. The columns with the proteins bound were then washed two times with denaturing wash buffer, then in different experiments two times with 6 mL native wash buffer plus imidazole at concentrations of 0–150 mM and the proteins were eluted using 8 mL native elution buffer plus imidazole (0–150 mM). The parameters of this basic protocol were systematically modified to optimize the purification of the different antigens by varying incubation times, pH of the elution buffer and concentration of imidazole in the elution buffers (Table 2).

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE with 15% polyacrylamide gels (Bio-Rad, Hercules, CA)