The First Purification of Functional Proteins from the Unculturable, Genome-Reduced, Bottlenecked α -Proteobacterium ‘*Candidatus Liberibacter solanacearum*’

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ABSTRACT

‘*Candidatus Liberibacter solanacearum*’ is an unculturable α -proteobacterium that is the causal agent of zebra chip disease of potato—a major problem in potato-growing areas, because it affects growth and yield. Developing effective treatments for ‘*Ca. L. solanacearum*’ has been hampered by the difficulty in functionally characterizing the proteins of this organism, largely because they are not easily expressed and purified in standard expression systems. ‘*Ca. L. solanacearum*’ has a reduced genome and its proteins are predicted to be prone to instability and aggregation. Among intracellular-dwelling bacteria, chaperone proteins are conserved and overexpressed to buffer against problems in protein folding. We mimicked this approach for expressing and purifying ‘*Ca. L. solanacearum*’ proteins in *Escherichia coli* by coexpressing them with chaperones. Neither of the representative ‘*Ca. L. solanacearum*’ enzymes, dihydrodipicolinate synthase (key in lysine biosynthesis) and pyruvate kinase (involved in glycolysis), were overexpressed in standard *E. coli*

expression plasmids or strains. However, soluble dihydrodipicolinate synthase was successfully coexpressed with GroEL/GroES, while soluble pyruvate kinase was successfully coexpressed with either GroEL/GroES, dnaK/dnaJ/grpE, or a trigger factor. Both enzymes, believed to be key proteins for the organism, were purified by a combination of affinity chromatography and size-exclusion chromatography. Additionally, both ‘*Ca. L. solanacearum*’ enzymes are active and have the canonical tetrameric oligomeric structure in solution, consistent with other bacterial orthologs. This is the first study to successfully isolate and functionally characterize proteins from ‘*Ca. L. solanacearum*’. Thus, we provide a general strategy for characterizing its proteins, enabling new research and drug discovery programs to study and manage the pathogenicity of the organism.

Keywords: biochemistry and cell biology, techniques

‘*Candidatus Liberibacter solanacearum*’ is an unculturable α -proteobacterium and the causal agent of zebra chip disease of potato. The disease is a major problem in potato-growing areas in North and Central America and New Zealand, because it affects crop growth and yield, resulting in millions of dollars of loss to the potato industries (Munyaneza 2010, 2012; Munyaneza et al. 2007; Secor and Rivera-Varas 2004). Zebra chip disease is characterized by browning and necrotic flecking of vascular and internal tissue that presents as dark stripes and streaks within the chip when fried, rendering them commercially unacceptable (Buchman et al. 2012; Crosslin et al. 2010; Miles et al. 2010; Munyaneza 2012; Munyaneza et al. 2007; Secor et al. 2009). The solanaceous-infecting clades of this bacterium are vectored by *Bactericera cockerelli* (Šulc) (Hemiptera: Trioziidae), also known as the tomato

potato psyllid (Hansen et al. 2008; Liefting et al. 2008a,b), which is the target of current disease management strategies. Thus, new management strategies that target the bacterial pathogen itself are required but this has been hampered by an inability to establish the bacterium in axenic culture and study the expression and purification of proteins that might be attractive targets for novel control strategies (Gilkes et al. 2018).

‘*Ca. L. solanacearum*’ has a reduced genome of 1.26 Mbp (Lin et al. 2011). Genomes of less than 1 Mbp have been found in host-dependent microorganisms, reflecting the fact that the richness of metabolites provided by the host allows for the loss of genes that were previously deemed essential (McCutcheon and Moran 2012). Genes maintained include those for cellular processes and for host interaction, which can be for either invasion or mutualistic benefit (McCutcheon and Moran 2012) such as the synthesis of essential amino acids, vitamins, and carotenoids (McCutcheon and Moran 2012; Shigenobu et al. 2000; Sloan and Moran 2012). Moreover, small genomes generally lack genes for DNA repair and have a bias toward A + T residues (Kelkar and Ochman 2013). This, in turn, translates to a mutational bias toward hydrophobic residues at the protein level, increasing the likelihood of protein aggregation (Kelkar and Ochman 2013).

Although all genomes acquire mutations (deletions, insertions, or substitutions), they are usually selected against and removed from the population if there is a negative effect on fitness (McCutcheon and Moran 2012). Asexual reproduction of intracellular bacteria, along with vertical transmission through the host, affects the endosymbiont population as it experiences frequent population “bottlenecks” (McCutcheon and Moran 2012; Wernegreen 2002). *Liberibacter* spp. experience a reduction in cell numbers that are transmitted to the

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insect offspring, resulting in a bottleneck in sustaining symbionts through successive generations. However, horizontal transmission (via the plant) offsets some of the consequences of the restrictions on vertical transmission (Hosokawa et al. 2007; Mira and Moran 2002). Workneh et al. (2016) released ‘*Ca. L. solanacearum*’-positive psyllids in covered field plots; however, the proportion of psyllids that tested positive for ‘*Ca. L. solanacearum*’ declined over time, which suggested that new generations of psyllids were devoid of the pathogen. It was proposed that this decline in ‘*Ca. L. solanacearum*’-positive psyllids could be the result of inefficient transovarial transmission of ‘*Ca. L. solanacearum*’ (Mira and Moran 2002; Workneh et al. 2016). Psyllids lay multiple eggs at one time and, thus, it is possible that some may harbor the symbiont while others may not (Workneh et al. 2016). Successive bottlenecks allow deleterious mutations to become fixed through genetic drift (McCutcheon and Moran 2012; Wernegreen 2002, 2015). Such mutations tend to affect the thermodynamic stability of encoded proteins (van Ham et al. 2003), destabilize protein secondary structure, and make the proteins prone to misfolding and aggregation (Mira and Moran 2002; Wernegreen and Moran 1999).

A series of studies suggest that intracellular bacteria have increased chaperone expression in order to compensate for deleterious mutations that affect protein folding and stability (Fares et al. 2002, 2004; McCutcheon and Moran 2012; Moran 1996; van Ham et al. 2003; Wernegreen 2015). Genetic drift should lead to a decline in genomic fitness (Muller’s ratchet) and eventual extinction (McCutcheon and Moran 2012; Wernegreen 2002, 2015). However, the persistence of endosymbiotic relationships implies a mechanism to buffer against the effect of deleterious genetic variation, preventing the organisms from experiencing a fitness decline (Fares et al. 2004). Importantly, chaperones are abundantly expressed and evolutionarily conserved among intracellular bacteria (McCutcheon and Moran 2012; van Ham et al. 2003). Shotgun proteomics demonstrate that GroEL and DnaK are the most abundant proteins in ‘*Ca. Hodgkinia cicadicola*’ and ‘*Ca. Sulcia muelleri*’ (McCutcheon and Moran 2012). van Ham et al. (2003) computationally demonstrated thermodynamic instability of *Buchnera* proteins and determined that the proteins would have reduced and slower protein folding efficiency, misfolding and aggregation, or instability in native conformation. In contrast, the DnaK chaperonin of *Buchnera* displayed thermodynamic stability similar to free living bacteria (van Ham et al. 2003). In addition, Fares et al. (2002) demonstrated that GroEL overproduction in *Escherichia coli* compensates for harmful mutations that accumulated in a simulated process of vertical transmission. Furthermore, Huang et al. (2008) showed that GroEL enhanced the function of degenerate anthranilate synthase of *Buchnera*.

Insights into *Liberibacter* pathogenesis and proteins have mainly been derived from genomic analysis (Duan et al. 2009; Fagen et al. 2014; Huot et al. 2018; Leonard et al. 2012; Levy et al. 2017; Lin et al. 2011; Wulff et al. 2014) and host transcriptomic and metabolomic responses to *Liberibacter* infection (Fan et al. 2011; Ibanez et al. 2014; Mafra et al. 2013; Slisz et al. 2012). However, an understanding of ‘*Ca. L. solanacearum*’ protein structure and function is limited, with several studies on recombinant expression of ‘*Ca. Liberibacter*’ proteins highlighting the difficulties of purifying proteins that are active. Ravindran et al. (2018) purified the serralysin-like protein (haplotype LsoA KJZ82330.1 and haplotype LsoB WP_013461860 LsoA) from ‘*Ca. L. solanacearum*’ haplotypes LsoA and LsoB (HenneA and ZC1) under denaturing conditions. However, the purified enzyme had no measurable protease activity, leading to the conclusion that either the putative gene does not encode a functional enzyme or the protein incorrectly folds during the purification and refolding process (Ravindran et al. 2018). Liu et al. (2017) purified six proteins from ‘*Ca. L. asiaticus*’ psy62 under native and denaturing conditions; however, the activity of these proteins was not studied. Furthermore, Lau et al. (2011) purified the partial outer membrane protein

of ‘*Ca. L. asiaticus*’ under denaturing conditions; however, the protein was not characterized. In general, the strategy has been to denature and then refold ‘*Ca. Liberibacter*’ proteins—a harsh process that can lead to misfolding or aggregation (Fink 1998).

The aim of this research was to develop a general method for expressing and purifying ‘*Ca. L. solanacearum*’ proteins in their native state, without the need for subsequent denaturation and refolding. We hypothesized that coexpressing chaperones would allow the isolation of correctly folded and functional proteins from organisms with reduced genomes, such as in ‘*Ca. L. solanacearum*’. To test this, we investigated chaperone coexpression with two ‘*Ca. L. solanacearum*’ proteins for characterization: dihydrodipicolinate synthase (DHDPS) (the rate limiting step in *L*-lysine biosynthesis and potential drug target) and pyruvate kinase (PK) (a core metabolic enzyme involved in glycolysis).

MATERIALS AND METHODS

Expression vector construction. The ‘*Ca. L. solanacearum*’ NZ1 (haplotype A, JMTK00000000) DHDPS and PK genes (CLso_dapA [KJZ81861] and CLso_pykF [KJZ82234.1], respectively) were cloned into the pOPINF vector (Addgene plasmid number 26042, a gift from Ray Owens) (Berrow et al. 2007) to generate expression vectors with a general structure of N-His₆-HRV3C protease cleavage site-protein of interest. Briefly, coding sequences for the proteins were codon optimized for *E. coli* expression and synthesized by Integrated DNA Technologies (Skokie, IL, U.S.A.). Synthesized DNA fragments were cloned into pOPINF (linearized with *KpnI* and *HindIII*) using an In-Fusion HD Cloning kit (Clontech, Mountain View, CA, U.S.A.), according to the manufacturer’s instructions. Plasmids were purified using a NucleoSpin Plasmid Miniprep kit (Macherey-Nagel, Düren, Germany) and DNA sequence was verified by Sanger sequencing (Macrogen, Seoul, Korea). Coding and translated protein sequences for all constructs are provided in Supplementary Dataset S1.

Recombinant expression of ‘*Ca. L. solanacearum*’ proteins. *pOPIN expression.* pOPINF_CLso_dapA and pOPINF_CLso_pykF plasmids were transformed into *E. coli* Tuner pLacI (Novagen, Darmstadt, Germany). Colonies were picked and cultured in Luria-Bertani (ampicillin at 100 µg/ml, chloramphenicol at 20 µg/ml, and 1% glucose) for 16 h at 37°C. Overnight cultures were used to inoculate 1 liter of Luria-Bertani (ampicillin at 100 µg/ml, chloramphenicol at 20 µg/ml, and 1% glucose) in 2-liter flasks, which was then grown at 37°C with shaking (180 rpm) to an optical density at 600 nm (OD₆₀₀) = 0.5, when expression of recombinant protein was induced with 300 µM isopropyl-β-D-thiogalactoside (IPTG). The cultures were incubated at 26°C for 14 h with shaking (180 rpm) and the bacteria were harvested by centrifugation for 20 min at 8,565 × *g*. The resulting pellets were either used immediately or stored at –20°C.

Recombinant protein expression in E. coli and coexpression of chaperones. CLso_dapA (KJZ81861) and CLso_pykF (KJZ82234.1) genes were codon optimized for expression in *E. coli* and synthesized by GenScript (Piscataway, NJ, U.S.A.), then cloned into the pET24-a plasmid at the *NdeI* and *XhoI* restriction sites, in frame with a C-terminal His₆ tag. The pET24_CLso_dapA and pET24_CLso_pykF plasmids were transformed into *E. coli* BL21(DE3) strains that harbored plasmids encoding chaperones for coexpression (Takara Bio Inc, Shiga, Japan) (Table 1). The three chaperone plasmids enable efficient expression of multiple molecular chaperones to work cooperatively for protein folding. The expressed chaperones are derived from *E. coli* but used to aid in folding of recombinant-expressed proteins from various organisms. The chaperone genes are downstream of the *araB* promoter. Thus, expression of target proteins and chaperones can be induced individually if the target gene is placed under the control of another promoter (e.g., *lac*).

Colonies of pET24_CLso_dapA and pET24_CLso_pykF in *E. coli* BL21(DE3) with chaperone plasmids BL21(DE3)pKJE7,

BL21(DE3)pGRO7, and BL21(DE3)pTF16 were cultured in Luria-Bertani (kanamycin at 50 µg/ml and chloramphenicol at 25 µg/ml) for 16 h at 37°C. These overnight cultures were used to inoculate 1 liter of Luria-Bertani (kanamycin at 50 µg/ml and chloramphenicol at 25 µg/ml) in 2-liter flasks, grown at 37°C with shaking (220 rpm) to OD₆₀₀ = 0.2, at which point chaperone expression was induced with L-arabinose at 0.5 mg/ml. The cultures were grown further to an OD₆₀₀ of 0.5, then expression of the protein of interest was induced with 1 mM IPTG. The cultures were incubated at 16°C for 18 h with shaking (220 rpm). Cells were harvested by centrifugation at 8,565 × g for 10 min and resuspended in phosphate-buffered saline (PBS) buffer with 1 mM dithiothreitol (DTT), 5% glycerol, and 40 mM imidazole (pH 7.4).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to analyze the expression and solubility of recombinant proteins. Cell pellets were resuspended in PBS (pH 7.4) buffer and the cells were lysed by sonication, resulting in a soluble and insoluble fraction present in solution as a whole-cell lysate. Lithium dodecyl sulfate (LDS) sample buffer (10 µl; Thermo-Fisher Scientific, Ottawa, ON Canada) was added to 5 µl of whole-cell lysate and was loaded onto the SDS-PAGE gel. The soluble fraction was prepared from the whole-cell lysate by centrifugation at 36,223 × g for 20 min, producing a cell pellet (insoluble) and the resulting supernatant (soluble fraction). LDS sample buffer (10 µl) was added to 5 µl of the resulting supernatant and loaded onto the SDS-PAGE gel.

Purification of recombinant protein by immobilized metal affinity chromatography. All purification steps were conducted at 4°C. Cells were lysed by sonication to produce soluble and insoluble fractions that were separated by centrifugation at 36,223 × g for 20 min. The resulting supernatant was loaded onto a 5-ml HisTrap FF nickel-affinity column controlled by an AKTA FPLC chromatography system (GE Healthcare Life Sciences, Uppsala, Sweden) equilibrated with PBS buffer with 1 mM DTT, 5% glycerol, and 40 mM imidazole (pH 7.4). Bound proteins were eluted with PBS buffer containing 1 mM DTT, 5% glycerol, and 500 mM imidazole (pH 7.4). Eluted samples were analyzed by electrophoresis on a 12% SDS-PAGE gel with a Novex molecular weight marker (Thermo-Fisher Scientific) and stained with Coomassie brilliant blue R250 to visualize the protein bands.

Size-exclusion chromatography. Size-exclusion chromatography (SEC) was carried out using a HiLoad 16/200 Superdex 200-pg column (GE Healthcare Life Sciences). This column was controlled by an ÄKTA-FPLC (GE Healthcare Life Sciences). The protein sample from nickel-affinity chromatography was concentrated from 10 to 2 ml for DHDPS and from 6 to 2 ml for PK using a Vivaspin 6 spin concentrator (Sartorius, Göttingen, Germany). The sample was injected onto the column at a rate of 1 ml/min. Once the sample was loaded, 120 ml of PBS buffer (pH 7.4) was passed through the column at 0.5 ml/min and 1-ml fractions were collected. Fractions of the elution peaks were analyzed by SDS-PAGE.

Protein characterization. *Differential scanning fluorimetry.* Differential scanning fluorimetry (DSF) was carried out on a QuantStudio 3 Real-Time PCR system (Thermo-Fisher Scientific) using OptiFlex software, 96 clear-well PCR plates, and clear seals. The fluorescence of SYPRO-Orange was measured from 3°C to 100°C in steps of 0.5°C. The temperature was increased for 10 s and

held at that temperature for 10 s, taking measurements at each temperature.

Circular dichroism. Circular dichroism (CD) spectra of ‘*Ca. L. solanacearum*’ DHDPS and PK were obtained using the Jasco J815 CD spectrometer. Wavelength scans were performed between 260 and 200 nm with 4.0 s averaging time in PBS (pH 7.4) in a 1-mm quartz cuvette. Data were analyzed using DichroWeb (Whitmore and Wallace 2004, 2008). Data were fitted using the CONTINLL algorithm (Sreerama and Woody 2000) and the CDSSTR algorithm (Compton and Johnson 1986).

Analytical ultracentrifugation. Sedimentation velocity experiments were performed at 20°C in a Beckman Coulter Model XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, U.S.A.) using double-sector quartz cells and an An50-Ti rotor. Reference buffer (400 µl) and sample (380 µl at varied concentrations) were loaded into the reference and sample sectors of the cells, respectively. The rotor was accelerated to 45,000 rpm and data were collected continuously at 280 nm. Initial scans were carried out at 3,000 rpm to determine the optimal wavelength and radial positions for the high-speed experiment. Solvent density and viscosity were fitted using SEDNTERP (Laue et al. 1992). Data were fitted using the SEDFIT16.1c software to a continuous size-distribution model (Brown and Schuck 2008; Schuck 2000).

Activity assays. (S)-ASA, pyruvate, (S)-lysine, NADPH, NADH, and fructose 1,6 biphosphate were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Lactate dehydrogenase was obtained from Pharmacia (Peapack, NJ, U.S.A.). Protein concentration was measured by the method of Bradford (1976). Enzymes were manipulated at 4°C or on ice, unless otherwise stated. Reaction

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of ‘*Candidatus Liberibacter solanacearum*’ A, dihydrodipicolinate synthase and B, pyruvate kinase expressed as His₆-tagged proteins from the pOPINF plasmid in *Escherichia coli* Tuner pLacI. Both expressed proteins are insoluble (indicated by arrows in lane 3 of both A and B). In both panels, lane 1 = Novex ladder, lane 2 = cells prior to induction with isopropyl-β-D-thiogalactoside, lane 3 = insoluble fraction of cell lysate following centrifugation, and lane 4 = soluble fraction of cell lysate following centrifugation.

TABLE 1. Plasmids used for coexpression of chaperones with ‘*Candidatus Liberibacter solanacearum*’ proteins

Plasmid	Chaperone	Expression host	Chaperone mass (kDa)	Promoter	Inducer
pGRO7	GroES	<i>Escherichia coli</i> BL21(DE3)	10	<i>araB</i>	L-arabinose
	GroEL	...	60	...	...
pKJE7	dnaK	<i>E. coli</i> BL21(DE3)	70	<i>araB</i>	L-arabinose
	dnaJ	...	40	...	...
	grpE	...	22	...	...
pTF16	Trigger factor	<i>E. coli</i> BL21(DE3)	56	<i>araB</i>	L-arabinose

measurements were carried out on an Agilent Cary 100 bio dual beam spectrophotometer (Agilent, Santa Clara, CA, U.S.A.).

Activity assay for DHDPS. The substrate (S)-aspartate β -semi-aldehyde was synthesized using the method of Roberts et al. (2003). DHDPS activity was measured using a dihydronicotinate reductase (DHDPR)-coupled assay, as previously described (Dobson et al. 2004a,b; Yugari and Gilvarg 1965). Stock solutions of (S)-ASA, pyruvate, (S)-lysine, NADPH, and NADH were prepared fresh for each experiment. An aliquot of purified CLso_DHDPS (0.4 mg/ml) was added to a solution containing 100 mM HEPES (pH 8), 2.5 mM pyruvate, 2.5 mM (S)-ASA, and 0.203 mM NADPH. Approximately 20 μ g of DHDPR was used in each assay to ensure that it was present in excess. Assays were performed in 10-mm pathlength, 1.5-ml reduced-volume acrylic cuvettes, in triplicate. The reaction was initiated with DHDPS, after the cuvette had preincubated at 30°C for 10 min. Activity was determined from the rate of absorbance decrease at 340 nm at 30°C.

Activity assay for PK. The enzyme assay was modified from that described by Bücher and Pfeleiderer (1955) and recently adapted in Donovan et al. (2016). An aliquot of purified CLso_PK (0.4 mg/ml) was added to a solution containing 10 mM HEPES (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 2 mM phosphoenolpyruvate, 2 mM ADP, 2 mM fructose-1,6-bisphosphate, 0.12 mM NADH, and 2.2 units of lactate dehydrogenase. Activity was determined from the rate of absorbance decrease at 340 nm at 25°C.

RESULTS

Protein expression in standard *E. coli* system yields insoluble protein. '*Ca. L. solanacearum*' DHDPS and PK were

Fig. 2. Chaperone coexpression produces soluble '*Candidatus Liberibacter solanacearum*' **A**, dihydronicotinate synthase (DHDPS) and **B**, pyruvate kinase (PK). Chaperone proteins are indicated by dashed arrows while expressed DHDPS and PK are denoted by solid arrows. In lanes 2 and 3 of both panels, dashed arrows at 70 and 40 kDa denote the expression of chaperone proteins dnaK and dnaJ, respectively. In lanes 4 and 5 of both panels, the dashed arrow at 60 kDa indicates the expression of chaperone protein GroEL. In lanes 6 and 7 of both panels, the dashed arrow at 56 kDa indicates the expression of trigger factor chaperone protein. **A**, Overexpression of '*Ca. L. solanacearum*' DHDPS (32.6 kDa) with coexpressed chaperone GroEL (lane 5) in *Escherichia coli* BL21(DE3) pGRO7. Coexpressed chaperones with the *E. coli* BL21(DE3) pKJE7 and with *E. coli* BL21(DE3) pTf16 did not produce soluble '*Ca. L. solanacearum*' DHDPS. **B**, Overexpression of '*Ca. L. solanacearum*' PK (52.6 kDa) with coexpressed chaperones dnaK/dnaJ/grpE (lane 2) in *E. coli* BL21(DE3) pKJE7, with GroES and GroEL (lane 5) in *E. coli* BL21(DE3) pGRO7, and with trigger factor in *E. coli* BL21(DE3) pTf16 (lane 7). **A** and **B**, Lane 1 = Novex ladder, lane 2 = whole-cell lysate with both the soluble and insoluble fraction of *E. coli* BL21 pKJE7 cells with chaperone induction only and expressed protein of interest, lane 3 = soluble fraction of *E. coli* BL21(DE3) (pKJE7) after expression of chaperones and protein of interest, lane 4 = whole-cell lysate with both the soluble and insoluble fraction of *E. coli* BL21(DE3) pGRO7 cells with chaperone induction and expressed protein of interest, lane 5 = soluble fraction of *E. coli* BL21(DE3) pGRO7 after expression of chaperones and protein of interest, lane 6 = whole-cell lysate with both the soluble and insoluble fraction of *E. coli* BL21(DE3) pTf16 cells with chaperone induction and expressed protein of interest, and lane 7 = soluble fraction of *E. coli* BL21(DE3) pTf16 after expression of chaperones and protein of interest.

initially overexpressed in *E. coli* Tuner pLacI cells from the pOPINF plasmid but the proteins were insoluble (Fig. 1). SDS-PAGE analysis showed that '*Ca. L. solanacearum*' DHDPS was clearly overexpressed as an approximately 35-kDa insoluble protein (Fig. 1A, lane 3), consistent with its predicted molecular mass of 32.6 kDa. PK was overexpressed as a 53-kDa insoluble protein (Fig. 1B, lane 3), also consistent with its predicted molecular mass of 52.8 kDa. Extensive screening of expression temperature and IPTG concentrations failed to produce soluble proteins (data not shown).

Coexpression of chaperones produces soluble protein.

We hypothesized that both proteins are insoluble because they are misfolded. To test this, we coexpressed each protein with different combinations of chaperones, to mimic the high expression levels of chaperones observed in '*Ca. L. solanacearum*' in vivo. Consistent with this hypothesis, '*Ca. L. solanacearum*' DHDPS was expressed as a soluble 32.6-kDa protein when coexpressed with GroEL and GroES from the pGRO7 plasmid (Fig. 2A, lane 5). Similarly, '*Ca. L.*

Fig. 3. Purification of '*Candidatus Liberibacter solanacearum*' **A**, dihydronicotinate synthase (DHDPS) and **C**, pyruvate kinase (PK) to homogeneity after coexpression of GroEL and GroES in *Escherichia coli* BL21(DE3) pGRO7. UNIND indicates cells with no prior induction, IND shows cells with chaperone expression only, WCL indicates the whole-cell lysate when both chaperones and the protein of interest were expressed, immobilized metal affinity chromatography (IMAC) indicates protein purified by nickel-affinity chromatography, and size-exclusion chromatography (SEC) represents protein purified by size exclusion chromatography. **B**, SEC determined soluble '*Ca. L. solanacearum*' DHDPS eluted at 75 ml; the peak highlighted in gray represents a fraction of the total pooled DHDPS after SEC and corresponds to lane 6 in **A**. **D**, SEC, which revealed that soluble PK largely eluted at 70 ml. Peak 1 indicates a fraction of PK that eluted as aggregate and peak 2 specifies the proportion of PK that eluted as nonaggregated protein. The peak highlighted in gray corresponds to lane 6 in **C**. **A**, Lane 1 = Novex molecular weight ladder, lane 2 = *E. coli* BL21(DE3) pGRO7 preinduction, lane 3 = *E. coli* BL21(DE3) pGRO7 with the induction of GroEL and GroES, lane 4 = *E. coli* BL21(DE3) pGRO7 with the induction of '*Ca. L. solanacearum*' DHDPS, lane 5 = first purification step using IMAC, and lane 6 = SEC purification. **C**, Lane 1 = Novex molecular weight ladder, lane 2 = *E. coli* BL21(DE3) pGRO7 preinduction, lane 3 = *E. coli* BL21(DE3) pGRO7 with the induction of GroEL and GroES, lane 4 = *E. coli* BL21(DE3) pGRO7 with the induction of '*Ca. L. solanacearum*' PK, lane 5 = first purification step using IMAC, and lane 6 = SEC purification.

solanacearum' PK was also expressed as a soluble 53-kDa protein when coexpressed with GroEL/GroES (pGRO7) (Fig. 2B, lane 5), DnaK/DnaJ/GrpE (pKJE7) (Fig. 2B, lane 2), or trigger factor (pTF16) (Fig. 2B, lane 7).

Protein purification. '*Ca. L. solanacearum*' DHDPS and PK were both purified to homogeneity by a combination of immobilized metal affinity chromatography (IMAC) and SEC (Fig. 3A and C). IMAC was used a purification step to avoid the copurification of contaminating *E. coli* chromosomally derived enzymes that were not His₆-tagged. '*Ca. L. solanacearum*' DHDPS eluted from the SEC column at approximately 80 ml (Fig. 3B), whereas PK eluted at both 50 and 70 ml (Fig. 3D). The fraction of PK that eluted at 50 ml (Fig. 3D, peak 1) was before the void volume of the SEC column, indicating that this portion of the protein was aggregated. Estimation of protein thermostability using DSF indicated that both proteins were relatively stable, with DHDPS showing a melting temperature of 65.6°C and PK having a melting temperature of 56.4°C (Supplementary Figure S1).

Demonstration of functional DHDPS and PK. The specific activity of the enzymes increased during purification (Table 2, DHDPS and Table 3, PK). The specific activity of DHDPS increased from 0.56 to 2.4 $\mu\text{mol}/\text{min}/\text{mg}$ after SEC, while the specific activity of PK increased from 2.6 to 5.6 $\mu\text{mol}/\text{min}/\text{mg}$, demonstrating that the proteins had been purified. The purification protocol produced 9.6 mg of purified '*Ca. L. solanacearum*' DHDPS and 2.9 mg of purified '*Ca. L. solanacearum*' PK per liter of culture. However, the total DHDPS activity decreased from 789 $\mu\text{mol}/\text{min}$ in the crude lysate to 23 $\mu\text{mol}/\text{min}$ after SEC (a yield of just 2.9%), whereas the total PK activity decreased from 2,480 $\mu\text{mol}/\text{min}$ in the crude lysate to 16 $\mu\text{mol}/\text{min}$ after SEC (a yield of just 0.64%). Although the yields were low, there was sufficient enzyme for initial functional and structural characterization.

The presence of *L*-lysine, a known allosteric inhibitor for other bacterial DHDPS enzymes (Griffin et al. 2012; Karsten 1997), significantly reduced the rate of reaction for DHDPS (Fig. 4A, dashed lines), suggesting that '*Ca. L. solanacearum*' *L*-lysine biosynthesis is also allosterically regulated by *L*-lysine, as in other bacteria and plants. Additionally, PK demonstrated a rate of reaction of 0.085 $\Delta\text{A340}/\text{min}$ with 1.4 μg of the enzyme, and the reaction doubled to 0.17 $\Delta\text{A340}/\text{min}$ with 2.9 μg , demonstrating that the rate of the reaction was dependent on the concentration of enzyme (Fig. 4B). To ensure that the assay was working effectively, we conducted a positive control with the *E. coli* DHDPS and PK isozymes. Interestingly, the rates of reaction for both DHDPS and PK from '*Ca. L. solanacearum*' were considerably slower compared with that of *E. coli* (Supplementary Fig. S5). The rate of reaction for *E. coli* DHDPS (4.4 $\Delta\text{A340}/\text{min}$ with 0.3 μg) is increased approximately 32-fold compared with the rate of '*Ca. L. solanacearum*' DHDPS (1.1 $\Delta\text{A340}/\text{min}$ with 2.5 μg). Similarly, the

rate of reaction for *E. coli* PK (10.5 $\Delta\text{A340}/\text{min}$ with 0.6 μg) is increased approximately 300-fold compared with the rate of '*Ca. L. solanacearum*' PK (0.17 $\Delta\text{A340}/\text{min}$ with 2.9 μg).

Both enzymes are tetramers in solution. To characterize the quaternary structure in solution and to test for the presence of '*Ca. L. solanacearum*' PK and DHDPS aggregate, sedimentation velocity experiments were conducted in an analytical ultracentrifuge. The absorbance versus radial position profile for both proteins at initial concentrations of 0.2, 0.4, and 0.6 mg/ml show a distinct sedimentation boundary profile consistent with the presence of a single species (Supplementary Fig. S2).

The *c*(*M*) distributions for both '*Ca. L. solanacearum*' DHDPS and PK show a dominant, symmetrical peak, confirming that the proteins largely exist as a single species in solution, without the presence of any aggregated protein (Fig. 5A and B). For '*Ca. L. solanacearum*' DHDPS (Figs. 5A), the data fitted to an apparent molar mass of 144 kDa, consistent with the theoretical tetrameric mass of 130 kDa. For '*Ca. L. solanacearum*' PK (Fig. 5B), the data fitted to apparent molar mass of 195 kDa, consistent with the theoretical tetrameric mass of 213 kDa. These data are consistent with *E. coli* PK, which also shows a single tetrameric species of approximately 195 kDa (Zhu et al. 2010). For both proteins, a single peak dominated at all concentrations, suggesting that the proteins were not in a concentration-dependent self-association. Continuous size-distribution analyses of the data from '*Ca. L. solanacearum*' DHDPS determined that it had a standardized sedimentation coefficient of 6.8 S, whereas a continuous size-distribution analysis of the data from '*Ca. L. solanacearum*' PK determined that it had a standardized sedimentation coefficient of 8.5 S (Supplementary Fig. S3).

DISCUSSION

DHDPS regulates the first committed step in lysine biosynthesis (Atkinson et al. 2012, 2013; Dobson et al. 2004a, 2008; Dogovski et al. 2009; Soares da Costa et al. 2010). This enzyme is expressed in both plants and microorganisms but not in animals and, therefore, is a desirable target for the antimicrobial development (Boughton et al. 2008a,b; Turner et al. 2005). PK is the key glycolytic enzyme responsible for transferring phosphate from phosphoenolpyruvate to ADP, accompanied by the formation of ATP and pyruvate (Mazurek et al. 2002), and is readily subject to adaptation in bacterial evolution experiments (Peng et al. 2018). Both PK and DHDPS are proposed to play an important role in the survival of '*Ca. L. solanacearum*'.

Current purification strategies for '*Ca. L. solanacearum*' proteins expressed in *E. coli* have used methods that require denaturation and refolding of the recombinant protein (Liu et al. 2017; Ravindran et al. 2018). These methods can result in the protein assuming

TABLE 2. Purification table for '*Candidatus Liberibacter solanacearum*' dihydrodipicolinate synthase

Purification step ^a	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Total activity ($\mu\text{mol}/\text{min}$)	Yield (%)	Purification factor
Crude lysate	60	23.7	1,419	0.56	789	...	...
IMAC	10	2.2	22	0.51	11	1.5	0.92
SEC	7.5	1.3	9.6	2.4	23	2.9	4.3

^a IMAC = immobilized metal affinity chromatography and SEC = size-exclusion chromatography.

TABLE 3. Purification table for '*Candidatus Liberibacter solanacearum*' pyruvate kinase

Purification step ^a	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Total activity ($\mu\text{mol}/\text{min}$)	Yield (%)	Purification factor
Crude lysate	60	16	954	2.6	2,480	...	...
IMAC	6	1.5	9.0	1.8	16	0.65	0.69
SEC	6	0.48	2.9	5.6	16	0.64	2.2

^a IMAC = immobilized metal affinity chromatography and SEC = size-exclusion chromatography.

nonnative secondary structures, incurring loss of activity and protein misfolding or aggregation with the removal of the denaturant (Tsumoto et al. 2003). Consistent with this, expression of '*Ca. L. solanacearum*' DHDPS and PK with a hexahistidine tag

produced insoluble protein, even after extensive screening of expression conditions, further suggesting that this method might not be conducive to expression of soluble proteins from '*Ca. L. solanacearum*' proteins. In contrast, recombinant protein expression in *E. coli* and purification of DHDPS from several species, including *Vitis vinifera* (Atkinson et al. 2011), *Bacillus anthracis* (Domigan et al. 2009) and *Mycobacterium tuberculosis* (Evans et al. 2011), and PK from *Salmonella typhimurium* (Garcia-Olalla and Garrido-Pertierra 1987), *Leishmania mexicana* (Tulloch et al. 2008), and *Toxoplasma gondii* (Bakszt et al. 2010), produced active enzymes that were soluble and easily purified.

Our results demonstrate the difficulty in obtaining soluble, stable '*Ca. L. solanacearum*' proteins without the use of chaperones. This is probably caused by an inherent disposition of these proteins to aggregate or misfold. Both PK (glycolysis) and DHDPS (lysine biosynthesis) are core metabolic enzymes. The need to overexpress these '*Ca. L. solanacearum*' genes with chaperones to generate functional proteins, unlike their bacterial orthologs that do not require chaperones, suggests that these '*Ca. L. solanacearum*' genes may have been affected by genetic drift, caused by the bottleneck effect and resulting in a reduced genome environment in which '*Ca. L. solanacearum*' exists. It is likely that the '*Ca. L. solanacearum*' chaperone proteins are under strong purifying selection to buffer against the deleterious effects of accumulating mutations within the '*Ca. L. solanacearum*' genome (i.e., poor protein structure or folding).

'*Ca. L. solanacearum*' DHDPS was expressed as a soluble protein when coexpressed with *E. coli* GroEL and GroES, whereas '*Ca. L. solanacearum*' PK was soluble when coexpressed with GroEL/GroES chaperones (pGRO7 plasmid), DnaK/DnaJ/GrpE (pKJE7 plasmid), and trigger factor (pTF16 plasmid). Interestingly, transcriptomic analysis of infected psyllids determined that GroEL, DnaK, and GrpE were highly expressed genes in '*Ca. L. solanacearum*' (Nachappa et al. 2012). Thus, the pGRO7 plasmid with GroEL expression and the pKJE7 plasmid encoding GrpE appear to provide conditions that are sufficiently similar to those found in '*Ca. L. solanacearum*' in vivo to allow in vitro production of soluble, functional protein.

Previous characterization of '*Ca. L. solanacearum*' proteins under denaturing conditions produced either nonfunctional protein

Fig. 4. Activity assays for '*Candidatus Liberibacter solanacearum*' **A**, dihydrodipicolinate synthase (DHDPS) and **B**, pyruvate kinase (PK). Rates of reaction are displayed for each plot. Both enzymes were active and DHDPS showed expected inhibition of DHDPS activity in the presence of lysine. Both DHDPS and PK displayed an increase in rate of reaction with increasing enzyme concentration.

Fig. 5. **A**, Sedimentation velocity analysis of '*Candidatus Liberibacter solanacearum*' dihydrodipicolinate synthase (DHDPS) (0.6, 0.4, and 0.2 mg/ml) demonstrates a homogeneous sample with a mass of 144 kDa. **B**, Sedimentation velocity analysis of '*Ca. L. solanacearum*' pyruvate kinase (PK) (0.6, 0.4, and 0.2 mg/ml) demonstrates a homogenous sample with a mass of 195 kDa. **A**, Molecular weight distribution of DHDPS plotted as a function of molar mass (kDa). The fit was obtained using a resolution of 100 species between 0 and 350 kDa with $P = 0.95$, $\bar{v} = 0.73$. The Root Mean Square Deviation (RMSD) and runs test average for the fit were 0.0047 and 1.91, respectively. **B**, Molecular weight distribution of PK plotted as a function of molar mass (kDa). The fit was obtained using a resolution of 100 species between 0 and 500 kDa with $P = 0.95$, $\bar{v} = 0.73$. The r.m.s.d and runs test average for the fit were 0.0062 and 1.2, respectively.

or insoluble protein (Liu et al. 2017; Ravindran et al. 2018). In contrast, we demonstrated that coexpression of 'Ca. L. solanacearum' DHDPS and PK with chaperones resulted in active, tetrameric enzymes. Utilization of chaperone coexpression circumvents the problems of protein refolding, because the formation of undesirable protein intermediate states is avoided. The activity of both 'Ca. L. solanacearum' DHDPS and PK corroborates that these proteins are in their native conformation. Furthermore, analytical ultracentrifugation determined that both enzymes were present as tetramers in solution. Development of a method to obtain correctly folded, functional 'Ca. L. solanacearum' proteins provides a platform for further investigation into proteins that could play a key role in pathogenicity and understanding the requirements for survival of this genome-reduced organism.

The purification methods yielded sufficient quantities of protein (9.6 mg of 'Ca. L. solanacearum' DHDPS and 2.9 mg of 'Ca. L. solanacearum' PK) from 1 liter of culture. However, the specific activity of both DHDPS and PK were significantly lower than their *E. coli* counterparts. The specific activity of 'Ca. L. solanacearum' DHDPS was 2.4 $\mu\text{mol}/\text{min}/\text{mg}$, with a yield of 2.9%; these values are lower than the recorded specific activity of 108 $\mu\text{mol}/\text{min}/\text{mg}$ and yield of 39% for *E. coli* after purification (Dobson 2003). Similarly, the 'Ca. L. solanacearum' PK-specific activity of 5.6 $\mu\text{mol}/\text{min}/\text{mg}$, with a yield of 0.64%, was lower than the recorded specific activity for purified *E. coli* PK of 270 $\mu\text{mol}/\text{min}/\text{mg}$ and 41% yield (Zhu 2008). This could reflect a loss of function during purification, if the proteins are less stable than *E. coli* counterparts, or that the crude lysate contains allosteric effectors not present in the subsequent purification steps.

The economic impact of 'Ca. L. solanacearum' on the potato industry in Central and North America and New Zealand is significant. New strategies to control this recently emerging plant pathogen require the characterization of correctly folded and functional proteins. Here, we have demonstrated the first purification of functional 'Ca. L. solanacearum' proteins expressed in *E. coli* and propose that chaperone coexpression may represent a general approach for producing 'Ca. L. solanacearum' proteins in their native conformation that can then be used for further functional or structural analysis. Such studies will further our understanding of the mode of pathogenicity of the organism and assist in the design of antimicrobials against this economically significant pathogen.

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