

## Differential Chitinase Activity and Production within *Francisella* Species, Subspecies, and Subpopulations<sup>∇‡</sup>

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**Genotyping of *Francisella tularensis* (A1a, A1b, A2, and type B) and *Francisella novicida* has identified multiple differences between species and among *F. tularensis* subspecies and subpopulations. Variations in virulence, geographic distribution, and ecology are also known to exist among this group of bacteria, despite the >95% nucleotide identity in their genomes. This study expands the description of phenotypic differences by evaluating the ability of *F. tularensis* and *F. novicida* to degrade chitin analogs and produce active chitinases. Endochitinase activities were observed to vary among *F. tularensis* and *F. novicida* strains. The activity observed for *F. tularensis* strains was predominantly associated with whole-cell lysates, while the chitinase activity of *F. novicida* localized to the culture supernatant. In addition, the overall level of chitinase activity differed among the subpopulations of *F. tularensis* and between the species. Bioinformatic analyses identified two new putative chitinase genes (*chiC* and *chiD*), as well as the previously described *chiA* and *chiB*. However, the presence of these four open reading frames as intact genes or pseudogenes was found to differ between *Francisella* species and *F. tularensis* subspecies and subpopulations. Recombinant production of the putative chitinases and enzymatic evaluations revealed ChiA, ChiB, ChiC, and ChiD possessed dissimilar chitinase activities. These biochemical studies coupled with bioinformatic analyses and the evaluation of *chiA* and *chiC* knockouts in *F. tularensis* A1 and A2 strains, respectively, provided a molecular basis to explain the differential chitinase activities observed among the species and subpopulations of *Francisella*.**

The second most abundant polysaccharide found in nature is chitin, a biopolymer composed of repeating units of  $\beta$ -1,4 linked *N*-acetyl-D-glucosamine (35). Chitin is synthesized by many eukaryotes and is an essential component of arthropod cuticles, insect peritrophic membranes, and fungal cell walls. The abundance of this biopolymer allows it to serve as a major environmental reservoir of carbon and nitrogen, and chitin is especially concentrated in marine environments. Several bacteria produce chitinases that depolymerize chitin into metabolically accessible subunits. However, chitinases are not unique to prokaryotes and can be found in vertebrates, arthropods, plants, protozoa, and fungi (22, 41). All chitinases possess one or more glycosyl hydrolase (GH) superfamily domains of the 18, 19, 20, or 48 superfamilies (6, 11). Nonenzymatic domains commonly found in chitinases such as *N*-acetylglucosamine-binding protein A, carbohydrate binding, and fibronectin type 3 domains facilitate depolymerization of or adherence to chitin (2, 21, 53). Individual chitinases are also differentiated based on whether they act as endo- or exochitinases (6). Thus, the form of hydrolyzing activity, organization of functional domains, primary amino acid sequence, and protein folds are all used to distinguish and subgroup individual chitinases (5, 6).

Chitinases are especially abundant in the gammaproteobacteria. This is likely due to the association of this bacterial class with marine environments (4). The gammaproteobacteria subdivision includes both saprophytic and pathogenic species (7, 9, 13, 28, 32, 47). However, the involvement of chitinases in bacterial infections or vector transmission has received limited evaluation. A study by Kirn et al. (21) found that a chitin-binding protein of *Vibrio cholerae* allowed for enhanced binding to glycan moieties on the surface of human epithelial cells. In *Legionella pneumophila*, a chitinase was shown to enhance bacterial survival in the lungs of mice, but the specific mechanism remains unknown (7). Adding to the potential importance of chitinases in human disease is the observation that chitinases enable vector based transmission of the protist *Plasmodium falciparum* (the etiological agent of malaria) by degrading the peritrophic membrane that surrounds the mosquito blood meal (51).

Tularemia is a potentially severe zoonotic infection that occurs in the northern hemisphere and is caused by the gammaproteobacterium *Francisella tularensis* (43). Infection with this bacterium occurs via contact with infected animal tissues or ingestion of contaminated water, inhalation of infectious aerosols, or an arthropod bite (ticks, flies, and mosquitoes) (38). Three species of *Francisella* are recognized—*F. tularensis*, *Francisella novicida*, and *Francisella philomiragia*—that share >91% nucleotide identity between all species and >95% between *F. tularensis* and *F. novicida* (3, 42). *F. tularensis* contains three subspecies, *tularensis*, *holarctica*, and *mediasiatica*. Of these subspecies, *tularensis* (also known as type A) and *holarctica* (also known as type B) are clinically relevant human pathogens. Epidemiological, molecular, and virulence data

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demonstrate that *F. tularensis* type A and type B strains have divergent ecologies, biochemical properties, and virulence characteristics (3, 19, 34, 38, 46). *F. tularensis* type A strains cause disease exclusively in North America, are maintained zoonotically in lagomorph/tick cycles, and cause the most severe form of tularemia (38). Molecular data have identified four subpopulations within *F. tularensis* type A: A1a, A1b, A2a, and A2b (10, 24). Epidemiological analyses and virulence studies further support the separation of *F. tularensis* type A (24, 34). Specifically, *F. tularensis* A1a and A1b infections occur primarily in the Eastern United States and are associated with higher mortality rates and severity of infection (highest in A1b infections) compared to A2 infections. A2 infections occur primarily in the Western United States, and differences in geographic distribution or virulence among the two subpopulations (A2a and A2b) have not been found (24, 34). Conversely, *F. tularensis* type B strains occur throughout the Northern Hemisphere, have close associations to water and rodents, are transmitted by mosquitoes, and cause a milder usually self-limiting form of tularemia (38). *F. tularensis* type B subpopulations exist based on molecular typing (8, 16, 27, 37, 52) and geographic distribution (23, 39, 40, 52); however, virulence differences among subpopulations have not been shown (39). *F. novicida* and *F. philomiragia*, in contrast to *F. tularensis*, are most commonly associated with marine environments but are not associated with arthropod vectors, have larger coding capacities, and are rarely implicated in human disease (3).

We previously identified genomic regions of difference between the *F. tularensis* A1 and A2 subpopulations (33). One of these regions of genomic variance, denoted RD12, included a putative chitinase gene *chiA* that was present in *F. tularensis* A1, but not A2 genomes. In a separate study, ChiA was described as highly upregulated (>20-fold) *in vivo* during a murine infection with *F. tularensis* A1a (50). In addition, two *F. novicida* chitinases (ChiA and ChiB) were shown to be secreted by a type II secretion system, to aid in biofilm formation on chitin surfaces, and to facilitate nutrient acquisition (13, 31). In this present study, chitinase production and activity were evaluated across the *F. tularensis* subspecies and subpopulations (A1a, A1b, A2, and type B) and compared to that of *F. novicida*. This included two previously undefined chitinase gene products (ChiC and ChiD), as well as the previously studied ChiA and ChiB (13, 31). Despite the similarity among *Francisella* genomes, variability was observed between chitinase genes and gene products of the *Francisella* species and *F. tularensis* subspecies and subpopulations. These differences correlated to the chitinase activity of individual species and subpopulations, and individual chitinases presented unambiguous variability in activity.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Francisella* strains (Table 1) were grown from frozen stocks on cysteine heart agar supplemented with 9% chocolate sheep red blood cells (CHAB) at 35°C for 48 h, followed by subculture onto CHAB for 24 h at 35°C. *Francisella* liquid cultures were grown using a 24 h CHAB subculture to inoculate modified Mueller-Hinton (MMH) broth (1, 33). Liquid cultures of *Francisella* were incubated at 35°C overnight with shaking at 160 rpm. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth, with shaking at 160 rpm or on LB agar. When needed, appropriate antibiotics were added to the media.

TABLE 1. *F. tularensis* and *F. novicida* strains used in this study

<i>Francisella</i> strain <sup>a</sup>	Species	Subspecies	Subpopulation <sup>b</sup>	Source	Geographic origin
OK01-2528	<i>tularensis</i>	A	A1a	Human	Oklahoma
MO02-4195	<i>tularensis</i>	A	A1a	Human	Missouri
SCHU S4	<i>tularensis</i>	A	A1a	Human	Ohio
MA00-2987	<i>tularensis</i>	A	A1b	Human	Massachusetts
MD00-2970	<i>tularensis</i>	A	A1b	Human	Maryland
WY96-3418	<i>tularensis</i>	A	A2a	Human	Wyoming
NM99-1823	<i>tularensis</i>	A	A2b	Human	New Mexico
MI00-1730	<i>tularensis</i>	B	UK	Human	Michigan
KY99-3387	<i>tularensis</i>	B	B.II	Human	Kentucky
LVS	<i>tularensis</i>	B	B.Br.LVS	Rat	Russia
GA99-3550 (U112)	<i>novicida</i>			Water	Utah
GA99-3548	<i>novicida</i>			Human	Louisiana

<sup>a</sup> The CDC accession number is indicated.

<sup>b</sup> That is, the classification based on Kugeler et al. (24) or Vogler et al. (52). UK, unknown.

**PCR and cloning of chitinase genes.** PCR amplification of chitinase genes was performed using 30 to 75 ng of genomic DNA, 2.5 U of PrimeSTAR HS DNA polymerase (Takara Bio, Inc., Otsu, Shiga, Japan), 1× PrimeSTAR buffer (Mg<sup>2+</sup> plus), 200 μM concentrations of each deoxynucleoside triphosphate, and 1 μM concentration of each primer in a final reaction volume of 50 μl. Primers (see Table S1 in the supplemental material) were designed by using VectorNTI advance 11.0 software (Invitrogen, Carlsbad, CA) and included specific restriction enzyme sites for use in downstream cloning. PCR conditions were 98°C for 2 min, followed by 30 cycles of 98°C for 10 s, 45°C to 65°C for 5 s, and 72°C for 2.5 min. Amplicons were cloned by using a Zero Blunt TOPO PCR cloning kit (Invitrogen) according to the manufacturer's instructions. Engineered restriction enzyme sites were used to isolate the cloned chitinase gene from the TOPO vector and for ligation into the pET23b-(+) expression vector (Novagen, San Diego, CA). The expression plasmids are listed in Table S2 in the supplemental material. All clones were sequence verified by the Proteomics and Metabolomics Facility at Colorado State University using the primers listed in Tables S1 and S3 in the supplemental material.

**Construction of *chiA* and *chiC* knockouts and complemented strains.** Constructs for in-frame deletions of *chiA* (FTMG\_00598) in *F. tularensis* strain MA00-2987 (A1b) and *chiC* (FTW\_0313) in *F. tularensis* strain WY96-3418 (A2) were made using the *sacB*-based allelic exchange vector pMP590 (29). Specifically, constructs pMP590- $\Delta$ *chiA* and pMP590- $\Delta$ *chiC* (see Table S2 in the supplemental material) were created by PCR amplification of 477- to 941-bp DNA fragments that encompassed both flanking regions and 65 to 120 bp of the 5' and 3' regions of *chiA* and *chiC*, followed by ligation of the corresponding amplicons into the multiple cloning site of pMP590. The primers used are listed in Table S4 in the supplemental material. Electroporation was used to transform competent *F. tularensis* cells with the pMP590-*chiA* or pMP590-*chiC* constructs (29). Plasmid DNA (0.1 to 1.0 μg) was added to 100 μl of competent cells, and electroporation was performed by using a Bio-Rad Gene Pulsar (Bio-Rad, Hercules, CA) with the following parameters: 2.0 kV, 25 μF, and 200 Ω. Electroporated cells were incubated in 1 ml of MMH broth for 3 h at 35°C with shaking (160 rpm). Transformants were selected by plating on MMH agar containing 5 μg of kanamycin/ml, followed by incubation at 35°C for 2 to 4 days. Individual clones were transferred to MMH plates containing 8% sucrose to select for homologous recombination events. Genomic DNA was isolated from the knockout strains, and the genomic fragment representing the regions of homologous recombination was amplified by PCR and sequence verified. The knockouts were termed  $\Delta$ *chiA* and  $\Delta$ *chiC*.

Deletion mutants were complemented by cloning the full-length target genes, *chiA* (FTMG\_00598) and *chiC* (FTW\_0313), into the shuttle vector pMP529 to create pMP529-*chiA* and pMP529-*chiC*, respectively (see Tables S2 and S4 in the supplemental material). Electroporation of the deletion mutants with the complementing plasmids was performed as described for wild-type (WT) *F. tularensis*. To select for transformants, electroporated cells were plated onto MMH plates containing 200 μg of hygromycin/ml and incubated for 2 to 4 days at 35°C. Complementation was confirmed by PCR amplification, sequencing, and Western blotting.

**Recombinant chitinase production and purification.** To produce recombinant chitinases, expression vectors possessing individual chitinase genes (see Table S1 in the supplemental material) were transformed into *E. coli* BL21(DE3)/pLysS cells (Invitrogen). Recombinant clones were grown in 2 liters of LB broth with

100 µg of ampicillin/ml and 34 µg of chloramphenicol/ml at 37°C for 3 h while shaking at 160 rpm prior to the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cultures were grown for an additional 4 h, and the cells were harvested by centrifugation at 3,500 × g. Cells were suspended in 10 ml of breaking buffer (phosphate-buffered saline [PBS; pH 7.4], 1.2 µg of DNase I/ml, 1.2 µg of RNase A/ml, 1 µg of lysozyme/ml, and one Complete EDTA-free protease inhibitor cocktail tablet [Roche Applied Sciences, Mannheim, Germany] per 50 ml of buffer), placed on ice, and lysed by probe sonication using a Vibra Cell VCX750 sonicator (Sonics and Materials, Inc., Newton, CT). Sonication was performed at an amplitude setting of 30 with seven 60-s pulses and a 60-s pause between the pulse cycles. Unbroken cells and debris were removed by centrifugation at 12,000 × g, and the clarified lysate was applied to a 0.8-by-0.4-mm Poly-Prep (Bio-Rad) column prepacked with 1.5 ml of His-Resin (EMD Chemicals, Gibbstown, NJ) pre-equilibrated in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). Unbound proteins were eluted with 15 column volumes (CV) of binding buffer followed sequentially with 10 CV of wash buffer A (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), 23 CV of wash buffer B (40 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), and 20 CV of 10 mM Tris-HCl (pH 8.0). The bound recombinant protein was eluted with 5 ml of elution buffer (0.5 M imidazole, 10 mM Tris-HCl [pH 8.0]). All purification steps were performed at 4°C. The eluent of purified protein was dialyzed at 4°C against 10 mM ammonium bicarbonate using a 3,500-Da molecular mass cutoff dialysis membrane and concentrated by using a 10,000-Da molecular mass cutoff Amicon Ultra centrifugal filter unit (Millipore, Billerica, MA). Protein concentrations were determined by using the bicinchoninic assay (44). Samples were divided into aliquots and frozen at -80°C until further use.

**Assays for chitinase activity.** To assess secreted chitinase activities of *Francisella* strains, the cells were grown in MMH broth (10 ml) overnight. Immediately prior to harvesting of the culture supernatant (CS), an aliquot (10 µl) of each culture was serially diluted and plated on CHAB agar in duplicate. The agar plates were incubated for 48 h at 35°C, and the colonies were enumerated. The CS was separated from the bacterial cells by centrifugation at 5,000 × g for 15 min, concentrated using a 10-kDa molecular mass cutoff Amicon Ultra centrifugal filter unit, and brought to a volume of 500 µl with PBS (pH 7.4). An aliquot of the processed CS corresponding to approximately  $6.0 \times 10^8$  CFU of the original 10-ml culture was assayed against three chitin analogs (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl *N,N'*-diacetyl-β-D-chitobioside, and *p*-nitrophenyl β-D-*N,N',N''*-triacetylchitotriose) provided in the colorimetric chitinase assay (Sigma) according to the manufacturer's instructions, except the activity was assessed after incubation of the enzyme reaction for 16 h at 35°C. The release of *p*-nitrophenol was measured at an absorbance of 405 nm using a Multiskan EX spectrophotometer with Ascent v2.6 software (Thermo-Fisher, Waltham, MA).

To evaluate cell-associated chitinase activity, *Francisella* strains were grown on CHAB agar for 24 h at 35°C. The cells were scraped from the agar and suspended in PBS to a turbidity of 0.6 to 0.7 measured by using a Microscan turbidity meter (Siemens Healthcare Diagnostics, Deerfield, IL). To ensure consistency and to normalize the enzymatic activity between each culture, the number of CFU in the cell suspension was determined as described for the CS. An aliquot (300 µl) of the cell suspension was subjected to seven freeze-thaw cycles using a dry ice-ethanol bath and a 37°C heat block. This process allowed for the lysis of *F. tularensis* without generating hazardous aerosols. Unbroken cells and the whole-cell lysates (WCL) were separated by centrifugation at 17,000 × g for 15 min. An aliquot of the WCL corresponding to approximately  $1.6 \times 10^8$  CFU of the original cell suspension was applied to the colorimetric chitinase assay (Sigma), and the chitinase activity was measured as described for the CS. Chitinase activity was reported as the average of six assays (two technical replicates from three independent experiments). The background activity from the substrate incubated in the absence of CS or WCL was subtracted from all the activity values reported.

The enzymatic activity of individual recombinant chitinases (ChiA, ChiB, ChiC, or ChiD) produced in *E. coli* was determined by using a variation of the colorimetric chitinase assay. An aliquot (10 µl) of the recombinant chitinase at 1 µg/µl in 0.1 M sodium phosphate buffer (pH 5.9) was added to 90 µl of the appropriate chitin analog at a concentration of 267 µM in 0.1 M sodium phosphate (pH 5.9). Separate assays were conducted at ambient temperature and 37°C. The release of the *p*-nitrophenol was measured at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480, and 540 s during the enzymatic reaction. Three technical replicates were used for each time point and activity was reported as the average of these replicates. The enzymatic activity of the individual recombinant chitinases was confirmed using a glycol chitin substrate as previously described (7).

**Antibody production, SDS-PAGE, and Western blotting.** Antisera were generated in rabbits to histidine tagged products of *F. tularensis* A1a ChiA (FTT\_0715), *F. tularensis* A1a ChiB (FTT\_1768c), and *F. tularensis* A2 ChiC (FTW\_0313). All antisera were produced by SDIX (Windham, ME).

Recombinant proteins (0.1 µg) and WCL (15 µg) of *F. tularensis* or *F. novicida* strains were separated by SDS-PAGE using NuPAGE 4 to 12% Bis-Tris SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes by electroblotting (45). The nitrocellulose membranes were incubated in PBS (pH 7.4) containing 5% nonfat milk for 1 h and washed in PBS. Incubation of the primary antibody (anti-chitinase serum diluted 1:500 in PBS with 0.1% Tween 20 plus 5% nonfat dry milk or anti-histidine antibody diluted 1:5,000 [Qiagen, Valencia, CA]) with the nitrocellulose membranes was performed at ambient temperature with gentle shaking for 3 h, followed by washing with PBS (pH 7.4). The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000; Calbiochem, Darmstadt, Germany), was applied for 1 h at room temperature with gentle shaking. A final series of PBS washes were performed, and antibody reactive proteins were detected using a solution of BCIP/NBT made with SigmaFAST tablets (Sigma). Western blot and SDS-PAGE images were digitized by using a ScanJet 4850 photo scanner (Hewlett-Packard, Palo Alto, CA).

**Bioinformatic analyses of *Francisella* chitinases.** DNA and protein similarity searches were performed using BLASTN and BLASTP, respectively, against the nonredundant and whole-genome shotgun reads databases of the National Center for Biotechnology Information. To predict similarity to other known products, the largest product from each chitinase class (ChiA, ChiB, ChiC, and ChiD) of *F. tularensis* or *F. novicida* was used for BLAST similarity searches. When multiple gene products of the same chitinase class were of the same size, the *F. novicida* gene product was used in the BLAST search. *Francisella* genomes that were available in GenBank and used in the present study were: *F. tularensis* subsp. *tularensis* A1b strain MA00-2987 (accession number ABRI00000000), *F. tularensis* subsp. *tularensis* A1a strain SCHU S4 (accession number AJ749949), *F. tularensis* subsp. *tularensis* A2 strain WY96-3418 (accession number CP000608), *F. tularensis* subsp. *holarctica* OSU18 (accession number CP000437), *F. tularensis* subsp. *holarctica* LVS (accession number AM233362), and *F. novicida* strain GA99-3550/U112 (accession number CP000439). DNA and protein sequence alignments were performed using LALIGN ([www.ch.embnet.org/software/LALIGN\\_form.html](http://www.ch.embnet.org/software/LALIGN_form.html)) and CLUSTAL W ([www.ebi.ac.uk/cluster/#](http://www.ebi.ac.uk/cluster/#)). Conserved domains within the individual chitinase sequences of *F. tularensis* were identified using the NCBI Conserved Domain search software (concise display) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (30). The presence of GH18, GH19, GH20, and GH48 domains were identified in *Francisella* genomes using the UniProt database ([www.uniprot.org](http://www.uniprot.org)). Signal peptide predictions were accomplished using the SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP>).

## RESULTS

***In vitro* chitinase activity of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida*.** To determine the chitinase activity of *Francisella* grown *in vitro*, endochitinase and exochitinase (chitobiosidase and *N*-acetylglucosaminidase) activities were evaluated by using a panel of 12 *Francisella* strains (Table 1) representing *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* (Fig. 1). Comparisons were normalized to the cell numbers used to generate the WCL and CS. Endochitinase activity differed between the *Francisella* species and subpopulations (Fig. 1A and B). The A2 strains showed the highest level of endochitinase activity in the WCL (average  $A_{405}$  of 0.27), but displayed minimal activity in the CS (Fig. 1A and B). Endochitinase activity was detectable in the WCL of *F. tularensis* A1a/A1b strains; however, this activity was minimal. The WCL of *F. tularensis* type B strains displayed approximately half of the endochitinase activity of the *F. tularensis* A2 strains (average  $A_{405}$  of 0.13). In contrast, the endochitinase activity of *F. novicida* strains was observed in the WCL and CS, with the CS possessing the greatest activity (average  $A_{405}$  of 0.57) and the WCL activity (average  $A_{405}$  of 0.11) similar to that of *F. tularensis* type B strains. Little to no chitobiosidase or *N*-acetylglucosaminidase activities were detected in the WCL of *F.*



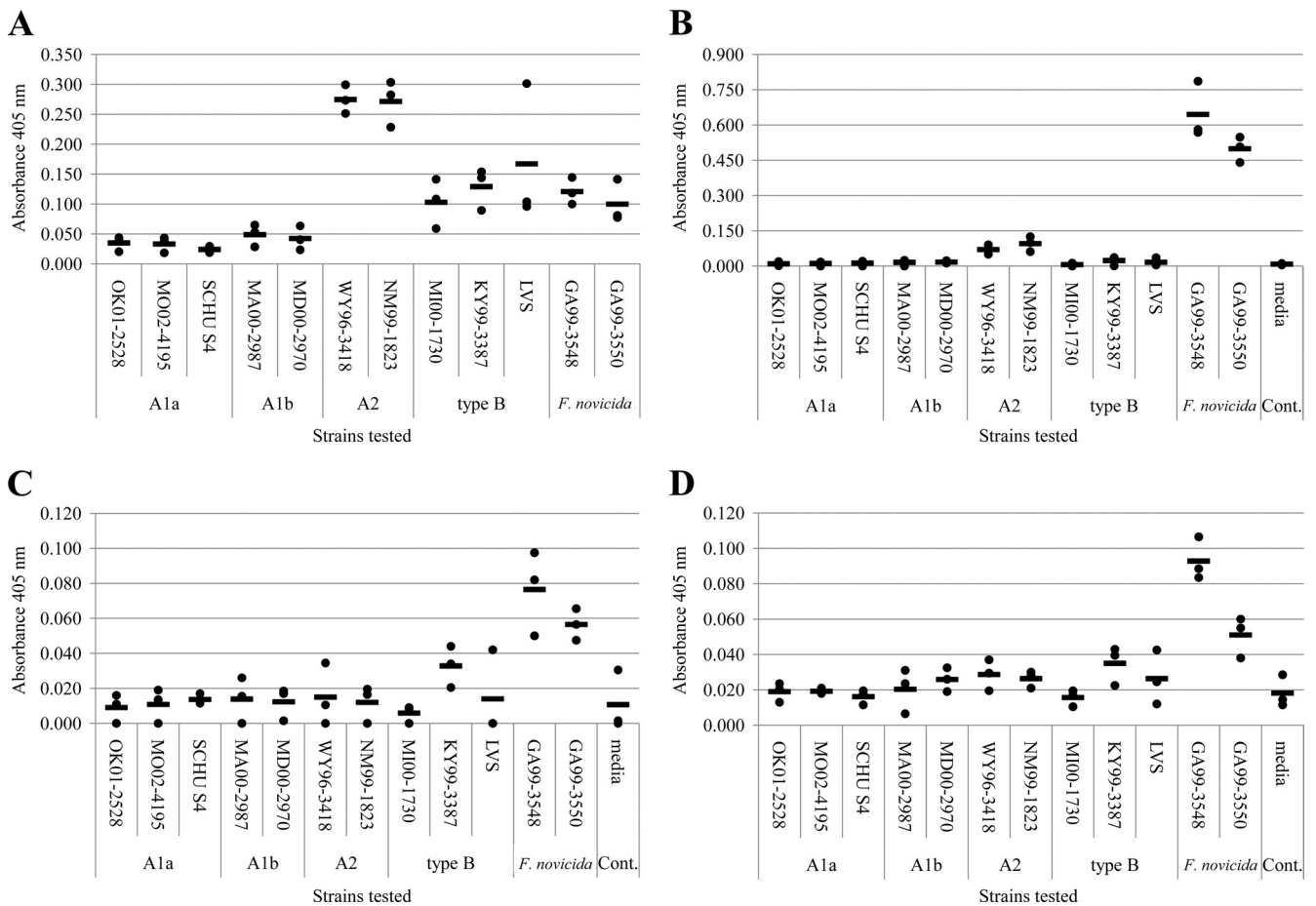


FIG. 1. *In vitro* chitinase activities of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* strains. A panel of 12 characterized strains (three A1a, two A1b, two A2, three type B, and two *F. novicida*) were evaluated for endochitinase, chitobiosidase, and *N*-acetylglucosaminidase activity. (A) endochitinase activities of WCL; (B) endochitinase activities of CS; (C) chitobiosidase activities of CS; (D) *N*-acetylglucosaminidase activities of CS. The average absorbance at 405 from three biological replicates (two technical replicates) are reported as a dot (●), with the average of all replicates indicated by a bar (—). Little to no chitobiosidase or *N*-acetylglucosaminidase activities was detected in WCL.

*tularensis* or *F. novicida* strains (data not shown). However, both types of exochitinase activities were detectable in the CS of *F. novicida* (Fig. 1C and D). These data provide strong evidence of differential production of chitinases among the *Francisella* species and subpopulations and suggest underlying differences in their chitinase genetic profiles.

***F. tularensis* and *F. novicida* encode four putative chitinase genes (*chiA*, *chiB*, *chiC*, and *chiD*).** To address whether genetic differences exist between *Francisella* species and subpopulations with respect to chitinase genes, a comprehensive bioinformatics analysis was performed. Genes encoding for *chiA* and *chiB* were previously described in *F. novicida* and *F. tularensis* (13, 26). Domain searches of the *chiA* and *chiB* gene products found they possessed a GH18 domain (PfamID PF00704), a characteristic domain of bacterial chitinases. Thus, a search for chitinase proteins was performed by BLAST analyses using the previously described *Francisella* ChiA and ChiB and their GH18 domains. This identified a total of four chitinase genes—*chiA*, *chiB*, *chiC*, and *chiD*—in *F. tularensis* and *F. novicida* (Fig. 2). The *chiA* gene product shared the greatest similarity with a probable chitinase of *Polysphondylium pallidum* ( $E$  value  $7 \times 10^{-51}$ ). The *Francisella* ChiB most

closely resembled a chitinase from *Saccharophagus degradans* ( $E$  value  $3 \times 10^{-11}$ ), and the ChiC displayed the greatest similarity to a hypothetical chitinase of *Aureococcus anophagefferens* ( $E$  value  $4 \times 10^{-56}$ ). *F. tularensis* and *F. novicida* ChiD showed limited similarity to other known chitinases but most closely resembled a chitinase from *Lactococcus lactis* ( $E$  value  $9 \times 10^{-8}$ ). The GH18 of the *Francisella* ChiD, however, is similar ( $E$  value  $2.65 \times 10^{-79}$ ) to a specific type of GH18 domain (cd02871) previously identified in *Bacillus circulans*.

The presence of these four chitinase genes differed among *F. tularensis* subpopulations (A1a, A1b, A2, and type B), and *F. novicida* (Fig. 2). *F. tularensis* A1a and A1b possessed genes for *chiA*, *chiB*, *chiC*, and *chiD* with an altered *chiB* and *chiC*. Specifically, the *chiC* of *F. tularensis* A1a possessed a point mutation causing a premature stop codon and two predicted open reading frames (FTT\_1592c and FTT\_1593c) encoding products of 387 and 207 amino acids, respectively. The *F. tularensis* A1a FTT\_1592c open reading frame encodes for the C-terminal portion of ChiC that includes a complete GH18 domain. This was found to be identical in the *F. tularensis* A1b genome (MA00-2987); however, it is currently annotated as one reading frame (FTMG\_01551). The ChiB of the *F. tular-*

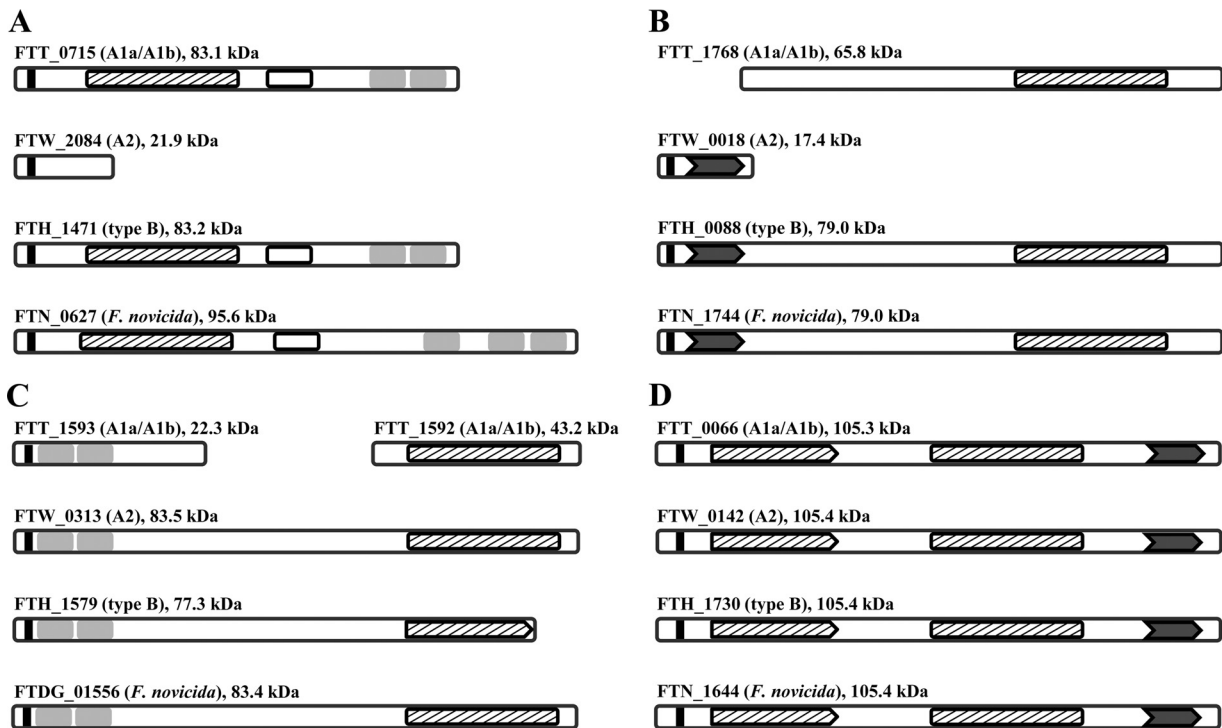


FIG. 2. Domain features of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* chitinases. The relative positions of conserved domains were identified in the chitinase gene products of *F. tularensis* and *F. novicida*. Solid black boxes indicate the predicted signal peptide cleavage site, striped boxes indicate the location and completeness of the GH18 domain (truncated GH18 domains appear as a pentagon), open boxes indicate the position of fibronectin type 3 domains, gray boxes indicate carbohydrate binding domains, and dark gray chevrons represent incomplete *N*-acetylglucosamine-binding protein A domains. (A) *chiA* gene products; (B) *chiB* gene products; (C) *chiC* gene products; (D) *chiD* gene products. A similar *chiC* gene product was identified in the genome of *F. novicida* GA99-3548 and GA99-3549 but not *F. novicida* GA99-3550.

*ensis* A1a/A1b subpopulations contained a 124-amino-acid *N*-terminal truncation that removed a partial *N*-acetylglucosamine-binding protein A domain and the conserved signal peptide for translocation. *F. tularensis* A2 appeared to encode a functional ChiC and ChiD. *F. tularensis* type B was predicted to have all four chitinase genes, but the *chiC* product possessed a 58-amino-acid C-terminal truncation that appeared to impact the essential GH18 domain. *F. novicida* strain U112 was predicted to only lack *chiC*. Two additional *F. novicida* genomes became available during the course of the present study: the genome for strain GA99-3548 (accession number ABAH00000000) and strain GA99-3549 (accession number AAYF00000000). Both of these *F. novicida* strains possessed a *chiC*. In addition to the overall absence or presence of the four chitinases, the domain structure between ChiA, ChiB, ChiC, and ChiD differed (Fig. 2). Detailed bioinformatic analyses are described in the supplemental material.

These *in silico* analyses predicted that each *Francisella* species, subspecies, and subpopulation possessed two or more functional chitinases. When applied to the differential endochitinase activities observed with the WCL of *Francisella* species, subspecies and subpopulations (Fig. 1), this bioinformatics data led to several hypotheses. (i) Enzyme kinetics of the individual chitinases or different levels of chitinase production in the various *Francisella* strains significantly influence the overall endochitinase activity; (ii) the strong chitinase activity of the A2 subpopulation is attributable to ChiC or ChiC in

combination with ChiD; and (iii) the moderate cell-associated endochitinase activity of *F. tularensis* type B and *F. novicida* is a result of activity from ChiA, ChiB, or ChiD or a combination of these proteins.

**Differential endochitinase activities of ChiA, ChiB, ChiC, and ChiD.** To establish the level of endochitinase enzyme activity associated with individual gene products, *F. tularensis* and *F. novicida* chitinases predicted to be functional (those with a complete or partially altered GH18 domain) were produced as recombinant proteins in *E. coli* and assayed for endochitinase, chitobiosidase, and *N*-acetylglucosaminidase function. The ChiA, ChiB, and ChiC proteins possessing a complete GH18 domain were positive for endochitinase activity (Fig. 3). The *F. tularensis* type B ChiC was found to be negative for all chitinase activity, a result that correlates with the 58-amino-acid C-terminal deletion that truncates the GH18 domain of this protein. The greatest endochitinase activity over a 9-min assay was observed for the ChiB product of *F. tularensis* A1a/A1b and type B and *F. novicida*. The full-length ChiC of *F. tularensis* A2 was slightly less active than the intact ChiB proteins. Interestingly, the recombinant *F. tularensis* A1a/A1b C-terminal ChiC fragment (FTT\_1592c) was active but at decreased levels compared to the full-length A2 ChiC. The ChiA of *F. tularensis* A1a/A1b and type B and *F. novicida* yielded modest endochitinase activity ( $A_{405}$  values of 0.40, 0.38, and 0.40, respectively), and none of the ChiD proteins were found to possess chitinase activity. Only ChiA and

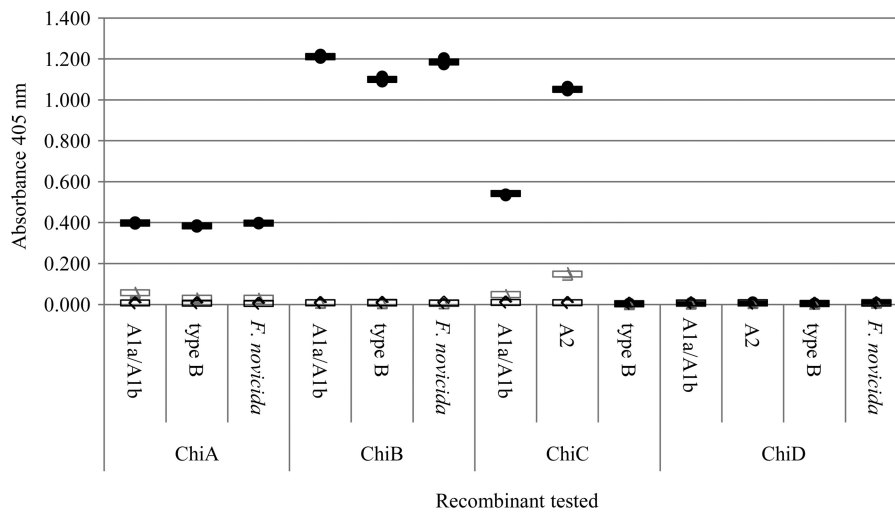


FIG. 3. Chitinase activity and substrate specificities of *F. tularensis* (A1a/A1b, A2, and type B) and *F. novicida* recombinant chitinases. *F. tularensis* and *F. novicida* chitinases with complete or partial GH18 domains were produced as recombinants in a heterologous system and assayed for 9 min to determine their degree and specificity to analogs capable of distinguishing endochitinase (●), chitobiosidase (△), and *N*-acetylglucosaminidase (◇) activities. The absorbance at 405 nm was determined for three technical replicates of each enzyme at 37°C. The average of the technical replicates is reported as a bar of the corresponding color and shading.

ChiC chitinases displayed minimal measurable activity to the chitobiosidase analog, and *N*-acetylglucosaminidase activity was not observed for any chitinase enzyme (Fig. 3).

The enzyme kinetics of individual chitinases were evaluated to further define and differentiate these proteins (Fig. 4). ChiD was excluded from these analyses since no chitinase activity was associated with this protein. The kinetics of the ChiA proteins of *F. tularensis* A1a/A1b and type B and *F. novicida* were similar to one another (Fig. 4A), but these chitinases yielded a much slower rate of catalysis than ChiB or ChiC (Fig. 4D). The ChiB recombinant proteins of *F. tularensis* A1a/A1b and type B and *F. novicida* all presented similar kinetics, and these recombinant products provided the greatest activity over the 9-min endochitinase assay (Fig. 4B and D). Although the maximum release of *p*-nitrophenol from the endochitinase substrate by ChiC of *F. tularensis* A2 was slightly less than that observed with the most active ChiB protein, the ChiC protein had a dramatically higher rate of catalysis, with activity plateauing after 90 s. The *F. tularensis* A1a/A1b C-terminal ChiC fragment (FTT\_1592c) containing the intact GH18 domain again presented significantly less activity than the full-length ChiC of *F. tularensis* A2, but its activity also plateaued at 90 s (Fig. 4C and D).

To validate the observations made with the small chitin analogues and the colorimetric assay, a second assay with glycol chitin (a soluble polymeric chitin) as the substrate was performed (7). The presence or absence of chitinase activity for each of the recombinant chitinases was the same as that observed using the colorimetric assay (data not shown). To account for the neutral pI of the ChiD products, the glycol chitin assay was also performed at a neutral pH, and no activity was observed. In addition to test whether the form of the recombinant protein influenced activity, each chitinase was also produced as a recombinant product without a predicted signal peptide or without a histidine tag and then assayed for chitinase activity. The relative endochitinase activities between

ChiA, ChiB, ChiC, and ChiD did not change with these other recombinant forms (data not shown).

**Production of individual chitinases by *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida*.** Western blot analyses of WCL from *in vitro* grown *F. tularensis* and *F. novicida* were performed with antiserum generated against the recombinant proteins possessing endochitinase activity (Fig. 5). Antiserum specificity was confirmed using the individual recombinant chitinases (data not shown). A protein band of the correct molecular mass (83 kDa) reactive to anti-ChiA antiserum was detected in WCL of *F. tularensis* A1a/A1b and type B, but, as predicted by bioinformatics, not to *F. tularensis* A2. A ChiA product of ~120 kDa was detected in *F. novicida* WCL. This was greater than the predicted mass of 95.5 kDa. The ChiB antiserum recognized products at a predicted mass of 79 kDa in the WCL of *F. tularensis* type B and *F. novicida* but did not identify a product in the *F. tularensis* A1a/A1b or A2. A protein band corresponding to ChiC was only detected in *F. tularensis* A2. Products corresponding to the *F. tularensis* A1a/A1b ChiC fragments or the inactive *F. tularensis* type B ChiC were not detected.

It is noted that the ChiA of *F. novicida* migrated at a molecular mass of ~120 kDa, although its predicted mass was ~96 kDa. This aberrant migration is hypothesized to be a result of the chemical properties of the protein's amino acid sequence or an uncharacterized posttranslational modification. The recombinant *F. novicida* ChiA with a poly-His tag had a predicted molecular mass of 97 kDa but also migrated at ~120 kDa as determined by SDS-PAGE (data not shown). Thus, we are confident that the protein observed in Fig. 5 is the actual native ChiA and not an artifact. The ChiA gene of *F. novicida* also possesses several tandem repeat sequences that might have resulted in errors in the assembly of the gene sequence or annotation. However, we sequenced the PCR product used to generate the recombinant *F. novicida* ChiA and did not find differences with the reported gene sequence. This further sug-

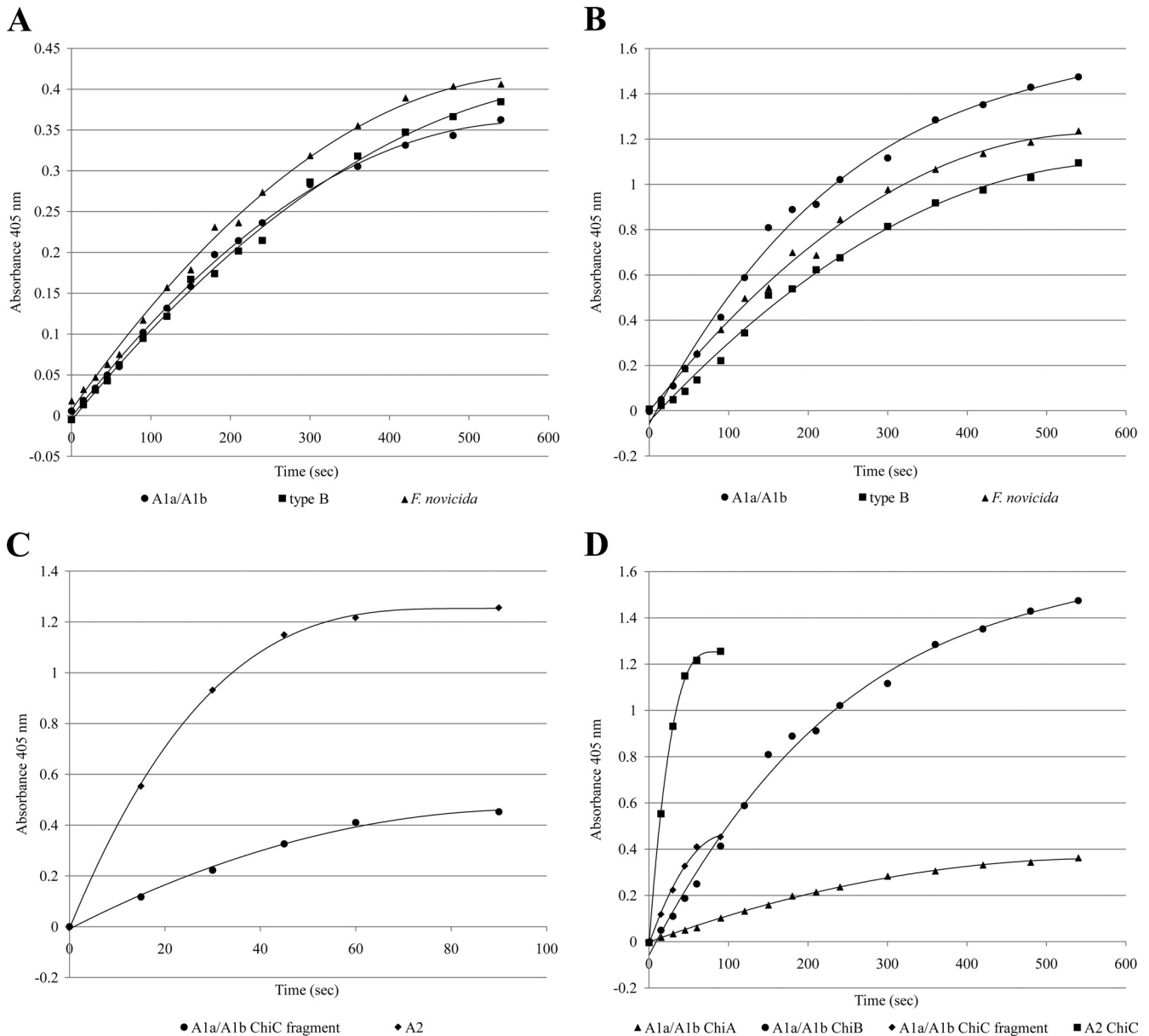


FIG. 4. Comparative endochitinase kinetics of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* recombinant chitinases. Recombinants that tested positive for endochitinase activity (Fig. 3) were assayed with an endochitinase analog to determine the relative activity of each functional enzyme at 37°C. The average absorbance at 405 nm of three technical replicates is reported for time points at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480, and 540 s, except for ChiC, where activity plateaued at 90 s. The kinetics of ChiA recombinant proteins (A), ChiB recombinant proteins (B), and ChiC recombinant proteins (C) are presented, as well as a relative comparison of all functional chitinases (D).

gests that the aberrant migration by SDS-PAGE is an intrinsic property of this protein.

**Analysis of ChiA and ChiC knockouts of *F. tularensis*.** To demonstrate that the observed endochitinase activity of *F. tularensis* A2 was attributable solely to ChiC, a  $\Delta chiC$  mutant and the corresponding complement  $\Delta chiC$ /comp were generated in *F. tularensis* A2 strain WY96-3418 and evaluated for chitinase activity (Fig. 6B). The  $\Delta chiC$  strain displayed minimal to no endochitinase activity ( $A_{405}$  of 0.005). When this mutant was complemented with *chiC*, the endochitinase activity was restored ( $A_{405}$  of 1.088) to a level greater than that of the WT *F. tularensis* A2 strain ( $A_{405}$  of 0.239). Western blot analysis with

anti-ChiC antiserum verified the production of ChiC in WCL of the WT *F. tularensis* A2 and the  $\Delta chiC$ /comp strains and confirmed its absence in WCL of the  $\Delta chiC$  mutant (Fig. 6D). In addition, to determine whether the low endochitinase activity observed for *F. tularensis* A1a and A1b strains was provided by ChiA and not the fragmented ChiC, a  $\Delta chiA$  mutation in A1b strain MA00-2987 and the corresponding complement  $\Delta chiA$ /comp were generated (Fig. 6A). The endochitinase activity of the WT strain was low ( $A_{405}$  of 0.054) but was reduced further to an  $A_{405}$  of 0.004 in the  $\Delta chiA$  mutant. The  $\Delta chiA$  strain complemented with *chiA* yielded a level of chitinase activity moderately higher ( $A_{405}$  of 0.097) than the WT *F.*



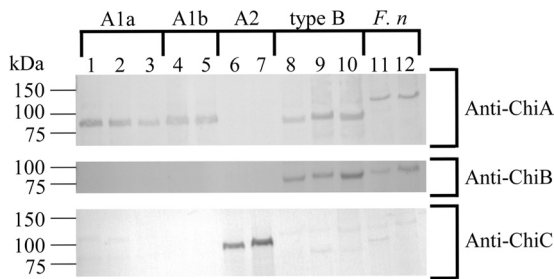


FIG. 5. Chitinases produced *in vitro* by *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida*. Western blots of WCL (15  $\mu$ g) of each *Francisella* strain with anti-ChiA antiserum, anti-ChiB antiserum, and anti-ChiC antiserum. Lanes 1 to 3, A1a strains OK01-2528, MO02-4195, and SCHU S4, respectively; lanes 4 and 5, A1b strains MA00-2987 and MD00-2970, respectively; lanes 6 and 7, A2 strains NM99-1823 and WY96-3418, respectively; lanes 8 to 10, type B strains KY99-3387, LVS, and MI00-1730, respectively; lanes 11 and 12, *F. novicida* strains GA99-3548 and GA99-3550, respectively.

*tularensis* A1b strain (Fig. 6A). Western blot analyses with anti-ChiA serum verified the production of ChiA in WCL of the WT *F. tularensis* A1b and the complemented  $\Delta$ *chiA* strains and demonstrated its absence in the  $\Delta$ *chiA* mutant (Fig. 6C). These results indicate that the ChiC fragment of *F. tularensis* A1a/A1b is not active under the conditions tested and that the low endochitinase activity observed is due to ChiA, a protein that yields modest endochitinase activity as a purified recombinant product.

## DISCUSSION

The genomes of *F. tularensis* and *F. novicida* present >95% identity (3), and the genomes of *F. tularensis* strains are considered monomorphic with a pairwise average nucleotide identity of >99.2% (25). Despite this, genetic differences do exist among the *Francisella* species, and *F. tularensis* subspecies and subpopulations, and are reflected in the documented pheno-

typic variations within members of this genus (43). The present study now expands this phenotypic variability to include endochitinase activity. A detailed comparison of *F. tularensis* A1a, A1b, A2, and type B strains and *F. novicida*, coupled with evaluation of two previously identified chitinase genes (*chiA* and *chiB*) and two newly identified putative chitinase genes (*chiC* and *chiD*), provided a molecular basis for the observed chitinase phenotypes. Table 2 provides a summary of the chitinase gene products for each of the *Francisella* species, subspecies, and subpopulations. Among the four predicted chitinases, ChiD was not a factor in defining the variable chitinase activity. The *chiD* gene was ubiquitous in all strains evaluated, but the purified recombinant ChiD protein was the only putative chitinase that failed to display activity. Examination of the other predicted chitinases (ChiA, ChiB, and ChiC) revealed that ChiC was the only active chitinase in *F. tularensis* A2, accounting for the relatively robust endochitinase activity of the *F. tularensis* A2 strains. Both *F. tularensis* A2a and A2b strains were tested, indicating that this phenotype is conserved among the A2 subpopulations. In contrast, *F. tularensis* A1 strains had two predicted functional chitinases (ChiA and ChiB) and a fragment of ChiC with a functional GH18 family domain but barely detectible enzymatic activity. This weak activity was a result of ChiA, since neither ChiB nor the ChiC fragment were detected in the WCL of *F. tularensis* A1 strains grown *in vitro*. Compared to the *F. tularensis* type A1 and A2 subpopulations, *F. tularensis* type B had a dissimilar chitinase profile comprised of a predicted functional ChiA and ChiB. The differences between the chitinase profiles of *F. tularensis* type A and type B were not surprising given the number of phenotypic variations that exist among these two subspecies (3, 19, 34, 38). The low endochitinase activity of purified *F. tularensis* ChiA proteins and the dominant activity of the ChiB protein support the conclusion that the robust endochitinase activity observed for *F. tularensis* type B is attributable to ChiB. A subset of the type B subpopulations identified by Vogler et

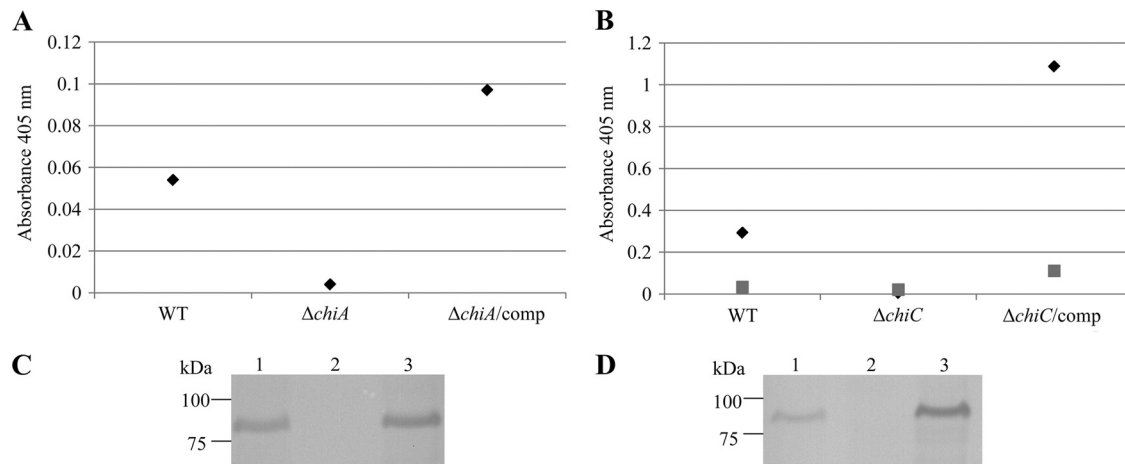


FIG. 6. Analyses of ChiA and ChiC chitinase knockouts in *F. tularensis* A1b strain MA00-2987 and *F. tularensis* A2 strain WY96-3418, respectively. (A) Endochitinase ( $\blacklozenge$ ) activity of WCL from WT strain MA00-2987,  $\Delta$ *chiA* MA00-2987, and  $\Delta$ *chiA/comp* MA00-2987; (B) endochitinase ( $\blacklozenge$ ) and chitobiosidase ( $\blacksquare$ ) activity of WCL from WT strain WY96-3418,  $\Delta$ *chiC* WY96-3418, and  $\Delta$ *chiC/comp* WY96-3418. (C) Western blot with anti-ChiA against WCL from WT MA00-2987 (lane 1),  $\Delta$ *chiA* MA00-2987 (lane 2), and  $\Delta$ *chiA/comp* MA00-2987 (lane 3) strains. (D) Western blot with anti-ChiC against WCL from WT WY96-3418 (lane 1),  $\Delta$ *chiC* WY96-3418 (lane 2), and  $\Delta$ *chiC/comp* WY96-3418 (lane 3) strains.



TABLE 2. Summary of *Francisella* chitinase bioinformatics and activities

<i>Francisella</i> group	Chitinase class	Predicted pseudogene <sup>a</sup>	Recombinant activity	<i>In vitro</i> production	
<i>F. tularensis</i>	A1a/A1b	ChiA	No	+	+
		ChiB	No*	+	-
		ChiC	Yes/Yes†	-/+	-
		ChiD	No	-	NT <sup>b</sup>
	A2	ChiA	Yes‡	NT	-
		ChiB	Yes§	NT	-
		ChiC	No	+	+
		ChiD	No	-	NT
	Type B	ChiA	No	+	+
		ChiB	No	+	+
		ChiC	No¶	-	-
		ChiD	No	-	NT
<i>F. novicida</i>	ChiA	No	+	+	
	ChiB	No	+	+	
	ChiC	No#	NT	-	
	ChiD	No	-	NT	

<sup>a</sup> The pseudogene designation is based on existing notations made to the annotated genome sequences. \*, *F. tularensis* A1a/A1b ChiB has an N-terminal truncation resulting in deletion of the signal peptide and the *N*-acetylglucosamine-binding protein A domain but does not impact the GH18 domain. †, *F. tularensis* A1a/A1b *chiC* contains a point mutation that causes a premature stop codon and results in two predicted open reading frames. One reading frame (FTT\_1592c) encodes an unaltered GH18 domain. ‡, *F. tularensis* A2 *chiA* is truncated due to a mutation resulting in a premature stop codon; the GH18 domain is missing. §, *F. tularensis* A2 *chiB* is truncated due to a mutation resulting in a premature stop codon; the GH18 domain is missing. ¶, *F. tularensis* type B ChiC contains a C-terminal truncation of that impacts the GH18 domain, but its gene is not annotated as a pseudogene. #, *F. novicida* strain GA99-3550 did not encode *chiC*. However, *F. novicida* GA99-3548 encoded a *chiC* whose product was predicted functional.

<sup>b</sup> NT, not tested.

al. (52) were tested in the present study, suggesting that that ChiB activity is conserved among at least 2 of the 10 type B subpopulations. Unlike *F. tularensis*, *F. novicida* displayed high chitinase activity in the CS with less activity detected in the WCL. However, the WCL activity of *F. novicida* was similar to that of *F. tularensis* type B, and there were similar patterns of reactivity to the chitinase-specific antibodies. These data, along with the enzymatic assays of the recombinant products, suggest that the WCL endochitinase activity *F. novicida* is attributable to ChiB, just as in *F. tularensis* type B.

It was interesting that ChiB of *F. tularensis* A1 only differed from that of *F. tularensis* type B and *F. novicida* by a short truncation at its N terminus but was not observed by Western blotting. Likewise, the ChiC of *F. novicida* and *F. tularensis* type B were predicted to be nearly identical or only slightly truncated compared to the ChiC of *F. tularensis* A2, but only the ChiC of *F. tularensis* A2 was detected by Western blotting. It is possible that beyond the genetic lesions resulting in truncated chitinase proteins or pseudogenes, the regulatory mechanisms also may differ between species and subspecies of *Francisella* and result in differential chitinase gene expression or protein stability. These potential regulatory mechanisms were not a focus of these studies or would not be uncovered by the experimentation presented. However, they could possibly explain the unexpected Western blot data and should be a focus of future experiments.

The differential chitinase phenotypes of the *Francisella* spe-

cies, subspecies, and subpopulations were not a simple reflection of the number of active chitinases produced but also resulted from variable activity among the predicted chitinases. Nevertheless, all of the putative intact *F. tularensis* and *F. novicida* chitinases contained GH18 family domains. The *chiC* fragments identified in *F. tularensis* A1a and A1b were annotated as pseudogenes, but the C-terminal encoding fragment contained an intact GH18 domain and was active as a recombinant product. On the contrary, *chiC* of *F. tularensis* type B was annotated as a functional gene; however, its GH18 domain was truncated and displayed no activity. The inactivity of ChiD is also likely a result of a nonfunctional GH18 domain. Functional GH18 domains contain a conserved catalytic motif of "DXDXE" where the glutamate residue acts as an acid critical for catalysis (14). Examination of this motif in the ChiD products (487-NFDLS-491) revealed the glutamic acid was replaced by a neutral serine residue. This, along with a nonconserved aspartic acid-to-asparagine substitution, likely inactivated this catalytic domain in ChiD.

Beyond GH18 domains, other regions of the individual *F. tularensis* chitinases are hypothesized to contribute to their variable activity. A fibronectin type 3 domain, was present only in the functional ChiA proteins and was positioned between the GH18 and carbohydrate binding domains, a common observation in other chitinases (48). This domain is thought to serve as a linker that adjusts the relative position of the chitinase catalytic and carbohydrate binding domains (48). Fibronectin type 3 domains of cellulases are also known to help disorganize polymers of cellulose (18, 53). Thus, it may play a similar role in chitin depolymerization. Tandem bacterial (type 3) carbohydrate binding domains were identified in ChiA and ChiC. These domains localize chitinases to their substrate and aid in chitin depolymerization (15, 49). The carbohydrate binding domains were not essential for the chitinolytic activity, as demonstrated with the C-terminal ChiC fragment of *F. tularensis* A1 strains, but a comparison of activity from this C-terminal fragment and the intact ChiC of *F. tularensis* A2 strains demonstrated that the presence of the nonenzymatic carbohydrate binding domains enhanced activity. A second binding domain (*N*-acetylglucosamine-binding protein A domain) was identified in ChiB and ChiD, though in a truncated form. This domain is present in the chitin-binding protein of *V. cholerae* and enhances bacterial attachment to chitin and human epithelial cells (21). The linkage of these bioinformatic analyses to the different activities observed for ChiA, ChiB, and ChiC further explains the dissimilar chitinase phenotypes of the various *Francisella* species and subpopulations. It also strongly suggests that each chitinase may act on different substrates or provide different biological functions.

Putative signal peptides were identified in all of the chitinases, except the ChiB and C-terminal fragment of ChiC in *F. tularensis* A1a/A1b. Despite this, *F. tularensis* chitinase activity was dominant in the WCL in contrast to *F. novicida*, where activity was focused in the CS. *F. novicida* chitinase secretion is dependent on a type II secretion system and in particular four "pilus" proteins (PilA [FTN\_0415], PilB [FTN\_1115], PilC [FTN\_1116], and PilQ [FTN\_1137]) (13). Homologues of these proteins are encoded by all *Francisella* strains analyzed in the present study, except *F. tularensis* LVS that lacks PilA (12). Thus, the absence of chitinase secretion in *F. tularensis* is

hypothesized to result from differences in the expression and production of secretion machinery between *F. novicida* and *F. tularensis*. Alternatively, with the larger number of pseudogenes in *F. tularensis* compared to *F. novicida*, it is possible that one or more unidentified products essential for secretion are absent from *F. tularensis* (3, 42).

Our studies demonstrated that chitinase activity differed between *Francisella* species, subspecies, and subpopulations. Nevertheless, at least one chitinase was found to be functional within all *Francisella* strains examined, thus implying a need for this enzyme. *F. tularensis* type A strains are considered more virulent than type B strains, and virulence differences occur among the *F. tularensis* type A subpopulations (A1a, A1b, and A2) (24, 34). The *chiA* gene is highly upregulated (>20 times) in mice infected with an *F. tularensis* A1 strain FSC033 (50), but a knockout of *chiA* in a *F. tularensis* A1 strain revealed no difference in virulence compared to WT strains (17). These data suggest that *F. tularensis* A1 strains produce ChiA during *in vivo* growth for a function not directly linked to pathogenesis. No studies have been conducted to decipher the potential roles of ChiB and ChiC in *F. tularensis* virulence. However, virulence differences that exist between *F. tularensis* A1, A2, and type B strains (34) provide a justification to study ChiB and ChiC mutants in animal models. Ecological niche modeling also predicts that *F. tularensis* A1 and A2 subpopulations occupy distinct habitats (36). Thus, the differences in chitinase phenotypes between these two subpopulations should be studied with respect to pathogen maintenance and survival. Likewise, the more complete repertoire and higher *in vitro* secreted chitinase activities of *F. novicida* may reflect this bacterium's ecological niche as an environmental organism that only rarely causes infections within humans (20). The variability we have described in the structure and function of specific chitinases now provides a foundation to investigate the role of these proteins in the growth and survival of pathogenic *F. tularensis* subpopulations (A1a, A1b, A2, and type B) and the environmental *F. novicida* strains.

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