

# Characterization of Osmotically Induced Filaments of *Salmonella enterica*

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*Salmonella enterica* forms aseptate filaments with multiple nucleoids when cultured in hyperosmotic conditions. These osmotically induced filaments are viable and form single colonies on agar plates even though they contain multiple genomes and have the potential to divide into multiple daughter cells. Introducing filaments that are formed during osmotic stress into culture conditions without additional humectants results in the formation of septa and their division into individual cells, which could present challenges to retrospective analyses of infectious dose and risk assessments. We sought to characterize the underlying mechanisms of osmotic-induced filament formation. The concentration of proteins and chromosomal DNA in filaments and control cells was similar when standardized by biomass. Furthermore, penicillin-binding proteins in the membrane of salmonellae were active *in vitro*. The activity of penicillin-binding protein 2 was greater in filaments than in control cells, suggesting that it may have a role in osmotic-induced filament formation. Filaments contained more ATP than did control cells in standardized cell suspensions, though the levels of two F<sub>0</sub>F<sub>1</sub>-ATP synthase subunits were reduced. Furthermore, filaments could septate and divide within 8 h in 0.2× Luria-Bertani broth at 23°C, while nonfilamentous control cells did not replicate. Based upon the ability of filaments to septate and divide in this diluted broth, a method was developed to enumerate by plate count the number of individual, viable cells within a population of filaments. This method could aid in retrospective analyses of infectious dose of filamented salmonellae.

Numerous conditions cause rod-shaped bacteria to form filamentous cells by interrupting the normal process of division and growth. Repressing the function of any filamentation temperature-sensitive (Fts) protein prevents the division complex from developing but does not block growth and results in filaments with multiple chromosomes (10, 22). FtsZ is a well-studied member of this family of proteins; at nonpermissive temperatures, *ftsZ* mutants of *Escherichia coli* are incapable of cellular division (4, 9, 10, 33, 56). Likewise, hydrostatic pressure, the SOS response via SulA, and antimicrobials, such as berberine and cinnamaldehyde, induce filamentation of *E. coli* by preventing the polymerization of FtsZ (5, 12, 13, 24, 30, 39, 55). β-Lactam antibiotics that bind penicillin-binding protein 3 (PBP3, also known as FtsI) inhibit its enzymatic activity and septum formation but not cellular growth (46, 62).

Bacterial filaments are also generated when DNA replication is inhibited. For example, nalidixic acid binds bacterial DNA gyrase, thereby stalling replication (52). These filaments have a single copy of the bacterial chromosome (27, 52). Additionally, *dnaK* mutants of *E. coli* form filaments with lower levels of DNA, protein, and RNA synthesis than the parental strain (23, 44).

*Salmonella enterica*, *Listeria monocytogenes*, and *E. coli* can form filaments in hyperosmotic environments. These osmotically induced filaments are morphologically similar to *fts* mutants of *E. coli* in that they appear aseptate and have numerous chromosomes (1–3, 19, 31, 34, 36, 46, 58). Hyperosmotic conditions can be found in foods and the processing environment. Introducing the filaments into culture media without added humectants encourages the formation of septa and division of the filaments into daughter cells, each with a single chromosome (34). Filaments could have significant implications for public health, including their increased duration in hosts relative to nonfilamented cells (51), and present challenges to accurately monitor *S. enterica* if filaments form on food items.

Characteristics of osmotically induced filaments of *S. enterica* were examined in detail in order to determine the mechanisms

that regulate filamentous growth during osmotic stress. DNA and protein levels were similar in filaments and control cells when normalized by biomass. *In vitro*, the activity of PBP2 was greater in membranes of filaments than in control cells. Also, filaments had significantly more ATP than did control cells despite reduced levels of the β and γ subunits of the F<sub>0</sub>F<sub>1</sub>-ATP synthase. Potential mechanisms for the osmotic-induced filamentation of *S. enterica* are discussed.

## MATERIALS AND METHODS

**Strains, media, and growth conditions.** All strains of *Salmonella* used in this study were *S. enterica* subsp. *enterica*. LT2 is a laboratory strain of *S. enterica* serovar Typhimurium (43). *S. enterica* serovar Typhimurium strain M-09-0001A-1 (M-09) was isolated from an outbreak of salmonellosis involving peanut butter and obtained from the Minnesota Department of Health. *S. enterica* serovar Enteritidis strain E40 was obtained from the University of Pennsylvania and originally isolated from the ovary of a hen. *S. enterica* serovar Agona strain S-12 is a clinical isolate obtained from the Wisconsin State Laboratory of Hygiene. *S. enterica* serovar Tennessee strain 4539H was originally isolated by the United States Food and Drug Administration from an outbreak of salmonellosis involving dry milk. Δ*dps* Δ*recA* *E. coli* is a derivative of strain ATCC 43895 (*stxI stxII* serotype O157:H7) in which both *dps* and *recA* were deleted by homologous recombination (25). Strains were stored at –70°C in nutrient broth (BD Biosciences, Sparks, MD) with 15% (vol/vol) glycerol. Colonies of each strain were maintained on tryptic soy agar (TSA; BD Biosciences) for up to a month at 4°C.

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Tryptic soy broth (TSB; BD Biosciences) was inoculated with individual *S. enterica* colonies and incubated for 20 to 24 h at 37°C with shaking to generate stationary-phase cultures. The optical density at 600 nm ( $OD_{600}$ ) of cultures in a 1-cm polystyrene cuvette (VWR, Radnor, PA) was measured with a Biomate3 instrument (Thermo Fisher Scientific, Waltham, MA). The  $OD_{600}$  values of stationary-phase cultures were greater than 1.7. Exponential-phase cultures were generated by diluting a stationary-phase culture 1:1,000 in fresh TSB and incubating the culture with shaking for 3 to 4 h at 37°C until an  $OD_{600}$  of 0.4 to 0.6 was achieved.

TSA (BD Biosciences) and TSB were supplemented with 2 to 10% (wt/vol) NaCl (Thermo Fisher Scientific) to induce osmotic stress. These are denoted as TSA-*X*NaCl and TSB-*X*NaCl, where *X* represents the percentage of NaCl supplemented to the media. An AquaLab dew point water activity meter 4TE instrument (Decagon Devices, Inc., Pullman, WA) was used to measure water activity ( $a_w$ ) values at 25°C in three independent experiments. On average, TSA supplemented with 0, 2, 4, 6, 7, 8, or 10% NaCl had  $a_w$  values of 0.99, 0.98, 0.97, 0.96, 0.95, 0.94, or 0.92, respectively. TSB supplemented with 0, 2, 4, 6, 8, or 10% NaCl had  $a_w$  values of 0.99, 0.98, 0.97, 0.96, 0.95, or 0.93, respectively.

To generate filaments on agar, 0.1 ml of a stationary- or exponential-phase culture of *S. enterica* was cultured on TSA or TSA supplemented with NaCl and incubated for 4 days at 30°C. These conditions were found to produce a maximum number of filaments on TSA and 7% NaCl (TSA-7NaCl) (51). Plates were wrapped with parafilm to prevent evaporation. Cells were harvested from the plates in 1× phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 4.3 mM  $Na_2HPO_4$ , 1.47 mM  $KH_2PO_4$ , pH 7.4) and placed on ice to prevent growth or division. To generate filaments in broth, NaCl-supplemented TSB was inoculated with a stationary- or exponential-phase culture of *S. enterica* at 1:50 in individual wells of a 24-well plate (BD Biosciences) and cultured statically for 4 days at 37°C (51).

**Enumeration of *Salmonella*.** CFU were enumerated by serially diluting cultures 10-fold in 1× PBS and plating 0.1 ml on TSA. Plates were incubated for 20 h at 37°C prior to enumeration. To enumerate CFU in filamented populations, samples were cultured statically in 0.2× Luria-Bertani (0.2LB) broth (BD Biosciences) at a starting  $OD_{600}$  of ~0.2 at 23°C for up to 24 h to enumerate viable units.

**Isolation of genomic DNA.** Genomic DNA was isolated from control or filamented cells harvested from TSA or TSA-7NaCl, respectively. Briefly, cells suspended in 1× Tris-EDTA (TE) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 0.5% (wt/vol) SDS, 167 µg/ml of RNase A, and 50 µg/ml of proteinase K were incubated at 37°C for 1 h. NaCl was added to a final concentration of 1.25 M, and the supernatant was transferred to a new tube following centrifugation in an Eppendorf 5417C centrifuge (18,000 × *g*, 10 min, 25°C). DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction with chloroform. The DNA was precipitated with 0.6× volumes of isopropanol and washed with 70% (vol/vol) ethanol. DNA was resuspended in 1× TE and the quantity measured with a Nanodrop 1000 (Thermo Fisher Scientific). The number of chromosomes per CFU was calculated using the equation  $X \times (1 \text{ mol} \cdot \text{bp}/660 \text{ g}) \times [1 \text{ chromosome}/(4.6 \times 10^6 \text{ bp})] \times [(6.02 \times 10^{23} \text{ chromosomes})/1 \text{ mol}]$ , where *X* is the concentration of DNA/CFU. The size of the chromosome of *S. enterica* is approximately  $4.6 \times 10^6$  bp, and 1 mol of bp is 660 g.

**Digestion of genomic DNA with BAL 31.** Genomic DNA was digested with BAL 31 nuclease as previously described (25). Briefly, DNA was digested with 0.2 units of BAL 31 nuclease in 1× BAL 31 nuclease buffer (TaKaRa, Mountain View, CA) for 30 min at 30°C. Afterward, the nuclease was denatured at 70°C and DNA was separated on a 0.8% Tris-acetate-EDTA (TAE) (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5) agarose gel at 9 V/cm. Digested DNA was visualized with ethidium bromide and its migration measured with ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ). DNA isolated from  $\Delta dps \Delta recA$  *E. coli* O157:H7 that had been cultured for 2 h at 37°C in LB broth, pH 7.0 or 2.0,

was used as the negative or positive control, respectively, for the digestion of DNA with BAL 31.

**Isolation of WCLs.** Cells harvested from TSA or TSA-7NaCl were suspended in 1× PBS and pelleted via centrifugation using an Eppendorf 5417C centrifuge (18,000 × *g*, 10 min, 25°C). Pellets were resuspended in 1× lysis buffer (62.5 mM Tris-HCl, 2% [wt/vol] SDS, pH 6.8) and incubated for 10 min at 95°C. Samples were centrifuged again and the supernatants transferred to new tubes. The concentration of protein was quantified with bicinchoninic acid (Pierce, Rockford, IL) as per the manufacturer's instructions. A standard curve was generated with known concentrations of BSA (Pierce). Afterward, samples were diluted to a desired concentration in 1× lysis buffer and 2× sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 20% [vol/vol] glycerol, 0.1 M dithiothreitol [DTT], 0.05% [wt/vol] bromophenol blue) to a final concentration of 1×. Whole-cell lysates (WCLs) were stored at -20°C.

**Isolation of membrane proteins.** Membrane proteins were isolated as previously described from *S. enterica* cultured on TSA or TSA-7NaCl for 4 days at 30°C (15, 50). Briefly, cells harvested in 1× PBS were pelleted via centrifugation using a Sorvall SS34 rotor in a Sorvall RC 5B centrifuge (12,000 × *g*, 10 min, 4°C) and resuspended in ice-cold 50 mM sodium phosphate buffer, pH 7.0. Cells were sonicated in an ice bath for 20 s every minute for a total of 5 min. Cellular debris was cleared from the supernatant by centrifugation. Membrane proteins were pelleted via centrifugation (100,000 × *g*, 30 min, 4°C) of the supernatant in a TLA110 rotor in an Optima TLX centrifuge (Beckman Coulter, Brea, CA). Pellets were washed by first resuspending them with a 26-gauge needle in fresh 50 mM sodium phosphate buffer, pH 7.0, and then repeating the centrifugation. The membrane pellets were resuspended in 50 mM sodium phosphate buffer, pH 7.0, and the concentration of protein was measured with bicinchoninic acid. Samples were stored at -70°C.

**Binding of PBPs to BOCILLIN-FL.** A total of 1.33 µg/µl of membrane proteins was labeled with 25 µM BOCILLIN-FL (Molecular Probes, Eugene, OR) in 50 mM sodium phosphate buffer, pH 7.0, for 15 min at 37°C (18, 63). Afterward, proteins were denatured at 95°C in 1× sample buffer. Equal amounts of protein were separated via SDS-PAGE on a 10% Bis-Tris NuPAGE gel in 1× NuPAGE buffer (Invitrogen, Carlsbad, CA). Bound BOCILLIN-FL was imaged at a resolution of 100 µm with excitation at 473 nm and a long pass blue emission filter (>510 nm) on a Typhoon FLA 9000 scanner (GE Healthcare). The intensity of active PBPs labeled with BOCILLIN-FL was quantified using ImageQuant TL (GE Healthcare). The activity of each PBP was normalized to the total amount of protein detected with Coomassie stain. Values in Table 2 are represented as the normalized fluorescent units of each PBP bound to BOCILLIN-FL relative to the normalized fluorescent units of PBPs 5 and 6 in strain 4539H cultured on TSA. Afterward, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2-µm pores; Bio-Rad, Hercules, CA) and immunoblotted for total PBP3.

**Immunoblotting.** Equal amounts of proteins were loaded into individual wells of a 4 to 12% Bis-Tris NuPAGE gel and separated by SDS-PAGE in 1× NuPAGE buffer (Invitrogen). Proteins were transferred to PVDF membrane (0.2-µm pores) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% [vol/vol] methanol). Membranes were blocked overnight in Tris-buffered saline Tween 20 (TBST) (150 mM NaCl, 2.7 mM KCl, 25 mM Tris base, 0.05% [vol/vol] Tween 20, pH 7.4) supplemented with 5% (wt/vol) dried skim milk (BD Biosciences) at 4°C. Membranes were incubated with a primary antibody and subsequently incubated with a secondary antibody conjugated to alkaline phosphatase. Bands were visualized with a solution of 0.165 mg/ml of 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (Promega, Madison, WI) and 0.33 mg/ml of nitro blue tetrazolium chloride (Promega) in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.0], 150 mM NaCl, 1 mM  $MgCl_2$ ). Western blots were scanned to digitalize the image and quantified using ImageQuant 5.2 software.

Primary antibodies included rabbit anti-FtsZ polyclonal antibody (Acris Antibodies, Inc., San Diego, CA) at 1:100, mouse anti-DnaK mono-

clonal antibody (Enzo Life Science, Farmingdale, NY) at 1:2,000, and rabbit anti-PBP3 antisera (kindly provided by David Weiss) at 1:15,000 (58). Secondary antibodies included goat anti-rabbit antibody (Promega) at 1:1,000 and goat anti-mouse antibody (Promega) at 1:6,000. Primary and secondary antibodies were diluted in TBST.

**Identification of membrane proteins.** Proteins from bacterial membranes were separated by SDS-PAGE on a 4 to 12% Bis-Tris NuPAGE gel in 1× NuPAGE buffer, stained with 0.1% (wt/vol) Coomassie brilliant blue G-250 (Bio-Rad), and destained with 10% (vol/vol) acetic acid. In two independent experiments, proteins were sequenced from *S. Tennessee* strain 4539H cultured either on TSA or TSA-7NaCl (26, 28). Briefly, detectable proteins were excised from the gel, destained with 50% (vol/vol) methanol in 100 mM (NH<sub>4</sub>)HCO<sub>3</sub>, and dried with acetonitrile. Proteins were reduced with 25 mM DTT in 25 mM (NH<sub>4</sub>)HCO<sub>3</sub> for 20 min at 55°C and alkylated for 20 min at 23°C in the dark with 55 mM iodoacetamide in 25 mM (NH<sub>4</sub>)HCO<sub>3</sub>. Samples were washed, dehydrated, and suspended in 25 mM (NH<sub>4</sub>)HCO<sub>3</sub> containing 200 ng of trypsin (Promega) and 0.01% (vol/vol) ProteasMax (Promega). Proteins were digested for 3 h at 42°C, after which the supernatant was transferred to a new tube. ProteasMax was cleaved and trypsin inactivated with 0.3% (vol/vol) trifluoroacetic acid. The proteins were identified via matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry at the University of Wisconsin-Madison Mass Spectrometry facility.

**Enumeration of filaments via microscopy.** An Olympus BHS microscope (Center Valley, PA) was used at ×1,000 magnification for all microscopy. Images were captured with an Olympus DP70 digital camera. To enumerate filaments (defined as cells longer than 10 μm), *S. enterica* cells were fixed in 5% (vol/vol) formalin, fixed to a glass slide (Gorilla Scientific, Silver Spring, MD), stained with 0.3% (wt/vol) crystal violet (BD Biosciences), and viewed with a bright-field objective. On average, more than 40 fields were viewed and 300 to 1,000 cells were enumerated. In the experiments shown in Fig. 2, 1 μg/ml of DAPI (4',6'-diamidino-2-phenylindole) was used to stain filaments on a black polycarbonate filter with pore size of 0.45 μm (Thermo Fisher Scientific). DAPI was visualized with the Olympus UG-1 excitation filter (330 to 385 nm) and L420 emitter (>420 nm). The method of staining did not affect the enumeration of filaments. Bacterial membranes were detected by staining cells with 15 μg/ml of FM4-64 (Molecular Probes) on glass slides coated with poly-L-lysine (Sigma). FM4-64 was visualized with the XF91 filter set (Omega Filters, Brattleboro, VT).

**Quantifying amounts of ATP.** Levels of ATP in populations of *S. enterica* grown for 4 days at 30°C on TSA or TSA-7NaCl were measured using the BacTiter-Glo microbial cell viability assay (Promega). Briefly, harvested cells were suspended in 1× PBS to an OD<sub>600</sub> of ~0.2. Equal volumes of cells and BacTiter-Glo reagent were mixed in individual wells of a black, flat-bottom 96-well plate (Thermo Fisher Scientific) and incubated for 5 min at 23°C. Luminescence was measured with a BioTek Synergy HT multidetection microplate reader (Winooski, VT). Known amounts of ATP (Roche Applied Science, Indianapolis, IN) were used to generate a standard curve.

**CTC redox assay.** The BacLight RedoxSensor 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) vitality kit (Molecular Probes, Eugene, OR) was used to visualize bacterial respiration as described previously (49). Briefly, *S. enterica* harvested from TSA or TSA-7NaCl was suspended in 1× PBS to an OD<sub>600</sub> of ~0.1 and then diluted 1:100 in 1× PBS. Samples were incubated with 5 mM BacLight RedoxSensor CTC for 30 min at 37°C. Afterward, cells were fixed in 5% (vol/vol) formalin and nucleic acids were counterstained with 1 μM SYTO-24. Cells were wet mounted onto a glass slide and viewed at ×1,000 magnification on an Olympus BHS microscope. SYTO-24 (Molecular Probes) and the red, fluorescent formazan product of reduced CTC were visualized with the XF91 filter set.

**Statistics.** All experiments were performed independently at least three times, with the exception of the CTC assay, which was performed twice. Student's *t* tests were performed to identify significant differences (two-tailed, *P* < 0.05).

## RESULTS

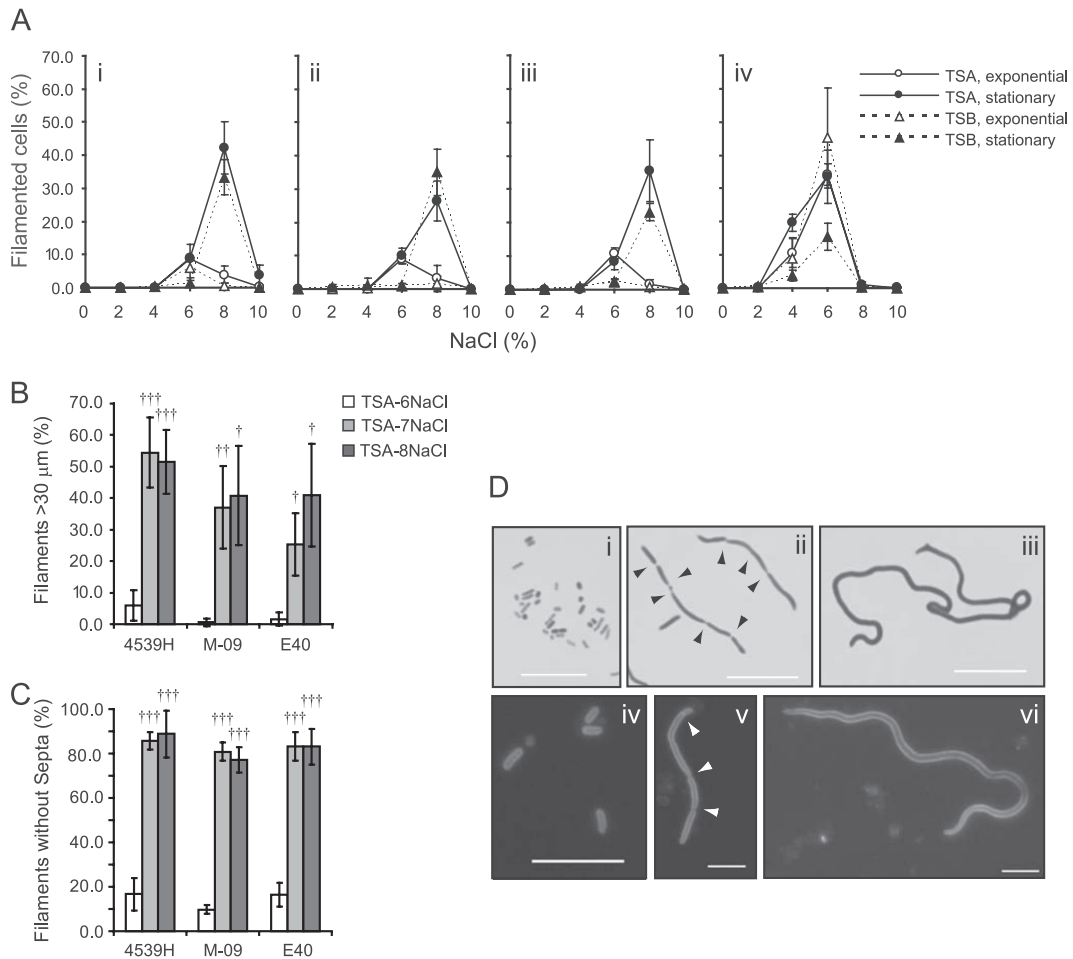
**Filamentation was dependent on osmotic stress.** To develop a system for the study of filamentous growth during osmotic stress, four strains of *S. enterica* (*S. Tennessee* strain 4539H, *S. Enteritidis* strain E40, and *S. Typhimurium* strains M-09 and LT2) were cultured on TSA or in TSB supplemented with various concentrations of NaCl. Strains 4539H, E40, and M-09 formed filaments, which were defined as cells longer than 10 μm, when stationary-phase inocula were cultured on TSA or TSB supplemented with 6 to 10% NaCl (Fig. 1Ai to iii and D). Statistics were performed to identify conditions in which the percentage of filaments was significantly different from the percentage that formed when cells were cultured on TSA or TSB not supplemented with NaCl (see Table S1 in the supplemental material). The maximum frequency of filaments relative to the total number of cells occurred when stationary-phase cells were cultured on TSA-8NaCl or in TSB-8NaCl. For example, 42% and 33% of cells of strain 4539H filamented on TSA-8NaCl and TSB-8NaCl, respectively (Fig. 1Ai). The frequency of filamentation for each strain was less than 5% on TSA-10NaCl or in TSB-10NaCl.

Exponential- and stationary-phase inocula of strains 4539H, E40, and M-09 filamented equally well when cultured on TSA-6NaCl or on TSB-6NaCl (Fig. 1Ai to iii). Exponential-phase inocula of strains 4539H, E40, and M-09 did filament when cultured on TSA or in TSB supplemented with 8 or 10% NaCl, albeit less frequently than stationary-phase cells. The percentage of filaments that grew from exponential-phase inocula on TSA or in TSB supplemented with 8 or 10% NaCl was not statistically different from the percentage of filaments generated on TSA or TSB, respectively. The differing ability of exponential- and stationary-phase cells to filament during osmotic stress was not dependent on the concentration of the inoculum. Stationary-phase cultures of strain 4539H diluted prior to inoculation filamented as frequently on TSA-8NaCl and TSB-8NaCl as did undiluted inocula (data not shown).

LT2 encodes a rare start codon for *rpoS*, which represses the expression of its protein, RpoS (32). LT2 formed filaments as frequently as strains 4539H, E40, and M-09 (Fig. 1Aiv). LT2 cultured on TSA-4NaCl and in TSB-4NaCl filamented, while the other strains that were tested did not. Additionally, LT2 maximally filamented when grown on TSA-6NaCl and in TSB-6NaCl. Unlike E40, M-09, and 4539H, inocula prepared from exponential-phase cells of LT2 filamented more often than inocula prepared from stationary-phase cells in TSB-6NaCl (Fig. 1Aiv).

**The frequency of filaments without septa correlated with osmotic stress.** Filaments that formed on TSA-6NaCl or TSA-8NaCl had different morphologies. First, filaments were longer on TSA-8NaCl than TSA-6NaCl (Fig. 1B and Dii and iii). Whereas 6% ± 5% of filaments of strain 4539H grown on TSA-6NaCl were longer than 30 μm, 52% ± 10% of filaments that grew on TSA-8NaCl were longer than 30 μm. Second, the percentage of filaments without septa correlated with the concentration of NaCl (Fig. 1C and Dii and iii). A total of 80% of filaments generated on TSA-8NaCl did not have visible septa, while only 15% of filaments on TSA-6NaCl were without septa. This observation was confirmed with an independent staining method, in which FM4-64 was used to stain the bacterial membrane; a greater proportion of filaments generated on TSA-6NaCl had septa than filaments generated on TSA-8NaCl (Fig. 1Div to vi). These findings indicated that the





**FIG 1** *S. enterica* filamented during osmotic stress. (A) Strains 4539H (i), M-09 (ii), E40 (iii), and LT2 (iv) were inoculated from exponential-phase (open symbols) or stationary-phase (closed symbols) cultures onto TSA (circles) or into TSB (triangles) supplemented with 0 to 10% (wt/vol) NaCl. The percentages of filaments (cells greater than 10  $\mu\text{m}$ ) generated were calculated after culturing cells for 4 days at 30 (TSA) or 37°C (TSB). In each strain, the maximal percentage of filamented cells was significantly different from the percentage of filaments in media with no added NaCl (Student's *t* test, two-tailed,  $P < 0.01$ ). Filaments longer than 30  $\mu\text{m}$  (B) and filaments without septa (C) were quantified for strains 4539H, M-09, and E40 grown on TSA-6NaCl (white bars), TSA-7NaCl (light-gray bars), or TSA-8NaCl (dark-gray bars). Student's *t* tests (two-tailed) were performed to identify whether filaments on TSA-7NaCl or TSA-8NaCl were significantly different from those generated on TSA-6NaCl ( $\dagger$ ,  $P < 0.05$ ;  $\dagger\dagger$ ,  $P < 0.01$ ;  $\dagger\dagger\dagger$ ,  $P < 0.001$ ). Filaments on TSA-7NaCl and TSA-8NaCl were similar. In panels A to C, results are presented as mean values from three independent experiments. Error bars represent the standard deviations (SD) of the means. (D) Representative images of strain 4539H on TSA (i, iv), TSA-6NaCl (ii, v), and TSA-8NaCl (iii, vi) stained with crystal violet (i to iii) or FM4-64 (iv to vi). Arrows point to septa. The bar in panel D represents 10  $\mu\text{m}$ .

inhibition of bacterial replication or division induced filamentous growth during osmotic stress.

Filaments on TSA-7NaCl were also examined and were morphologically similar to those on TSA-8NaCl (Fig. 1B and C, Table 1). Of these filaments grown on TSA-7NaCl, 80% were aseptate and 40 to 50% were longer than 30  $\mu\text{m}$ . Statistically, the frequency of filamentation on TSA-7NaCl was similar to that on TSA-8NaCl (Student's *t* test, two-tailed,  $P > 0.05$ ). In subsequent studies, filaments generated on TSA-7NaCl were studied because they were morphologically similar to filaments generated on TSA-8NaCl and because greater numbers of cells could be collected from TSA-7NaCl than from TSA-8NaCl. Additionally, we chose to study *S. Agona* strain S-12 rather than LT2. S-12 both grew and filamented better than did LT2 on TSA-7NaCl (Fig. 1Aiv, Table 1).

**Populations of filaments and control cells had similar amounts of protein and DNA.** *E. coli* exhibits filamentous growth

when DNA (21, 23, 44) replication (21, 23, 44) or protein synthesis is inhibited (23, 27, 44, 52). The concentrations of proteins from membranes or WCLs and of chromosomal DNA in populations of filaments and control cells were measured to address whether deficiencies in the synthesis of protein or DNA contributed to the osmotic-induced filamentation. Control or filamentous populations of strains E40, M-09, S-12, and 4539H were generated on either TSA or TSA-7NaCl, respectively (Table 1). The concentrations of proteins and chromosomal DNA were similar in filamented and control suspensions of cells for each strain when bacterial mass was normalized by OD<sub>600</sub>. Therefore, filamentation of *S. enterica* during osmotic stress is not associated with defects in the synthesis of protein or DNA.

It was possible that filamentation was induced by the SOS response. If the SOS response were induced, osmotic stress would be expected to induce DNA damage. Chromosomal DNA isolated from *S. enterica* grown on TSA or TSA-7NaCl was not sensitive to

TABLE 1 A comparison of macromolecules isolated from *S. enterica* grown on TSA or TSA-7NaCl

Strain	NaCl (%)	% of filamented cells	Amt of protein ( $\mu\text{g}$ )			Amt of chromosomal DNA ( $\mu\text{g}$ )		No. of chromosomes (copies/CFU) <sup>a</sup>
			Isolated from membrane		In the whole-cell lysate (per OD <sub>600</sub> )	Per OD <sub>600</sub>	Per 10 <sup>8</sup> CFU	
			Per OD <sub>600</sub>	Per 10 <sup>8</sup> CFU				
E40	0	<0.2	16.9 $\pm$ 13.3	0.7 $\pm$ 0.1	193 $\pm$ 16	23.8 $\pm$ 5.5	1.6 $\pm$ 0.2	1.9 $\pm$ 0.1
	7	43.1 $\pm$ 9.3 <sup>b</sup>	14.1 $\pm$ 8.4	9.1 $\pm$ 5.1 <sup>b</sup>	229 $\pm$ 41	27.8 $\pm$ 6.8	10.4 $\pm$ 2.9 <sup>c</sup>	12.1 $\pm$ 3.4
M-09	0	<0.2	17.3 $\pm$ 2.9	0.9 $\pm$ 0.4	175 $\pm$ 8	18.8 $\pm$ 16.4	1.6 $\pm$ 0.8	1.9 $\pm$ 1.1
	7	41.3 $\pm$ 4.4 <sup>b</sup>	14.8 $\pm$ 2.9	12.3 $\pm$ 1.2 <sup>b</sup>	239 $\pm$ 41	24.0 $\pm$ 4.2	12.4 $\pm$ 1.3 <sup>b</sup>	14.3 $\pm$ 2.0
S-12	0	<0.1	17.1 $\pm$ 2.9	0.9 $\pm$ 0.5	174 $\pm$ 13	ND <sup>c</sup>	ND	ND
	7	29.7 $\pm$ 2.8 <sup>b</sup>	9.5 $\pm$ 1.6 <sup>d</sup>	4.7 $\pm$ 1.3 <sup>e</sup>	192 $\pm$ 9	ND	ND	ND
4539H	0	<0.2	20.9 $\pm$ 7.0	1.2 $\pm$ 0.6	188 $\pm$ 19	11.0 $\pm$ 1.0	1.1 $\pm$ 0.2	1.3 $\pm$ 0.1
	7	63.1 $\pm$ 12.4 <sup>b</sup>	16.1 $\pm$ 0.8	21.2 $\pm$ 5.2 <sup>e</sup>	272 $\pm$ 27 <sup>d</sup>	18.9 $\pm$ 2.7 <sup>e</sup>	22.3 $\pm$ 2.1 <sup>b</sup>	25.8 $\pm$ 2.7

<sup>a</sup> The chromosome of *S. enterica* is  $4.6 \times 10^6$  bp.

<sup>b</sup>  $P < 0.001$ .

<sup>c</sup> ND, not determined.

<sup>d</sup>  $P < 0.05$ .

<sup>e</sup>  $P < 0.01$ .

digestion with BAL 31 and migrated similarly, but DNA from a  $\Delta dps \Delta recA$  mutant of *E. coli* O157:H7 was sensitive to digestion with BAL 31 after the strain was incubated in LB broth acidified to pH 2.0 (data not shown).

**Filaments were multiple viable units.** Filamented populations of strains E40, M-09, and 4539H generated on TSA-7NaCl had  $12.1 \pm 3.4$ ,  $14.3 \pm 2.0$ , and  $25.8 \pm 2.7$  chromosomes/CFU, respectively, while control cells generated on TSA had less than 2 chromosomes/CFU (Table 1). These calculations indicated that filaments were in fact comprised of multiple viable units. Therefore, we predicted a single filamentous cell would produce more than 10 CFU after septation and division.

Each filament of *S. enterica* forms a single CFU on an agar plate (34). As a result, enumerating CFU of populations of *S. enterica* containing filaments can underestimate the number of individual, viable bacterial cells. In order to enumerate individual cells within a filament-containing population, a reproducible method was developed for filaments to septate and divide prior to the replication and division of control cells. Suboptimal temperatures and culture medium, which decrease the rate of replication, were employed.

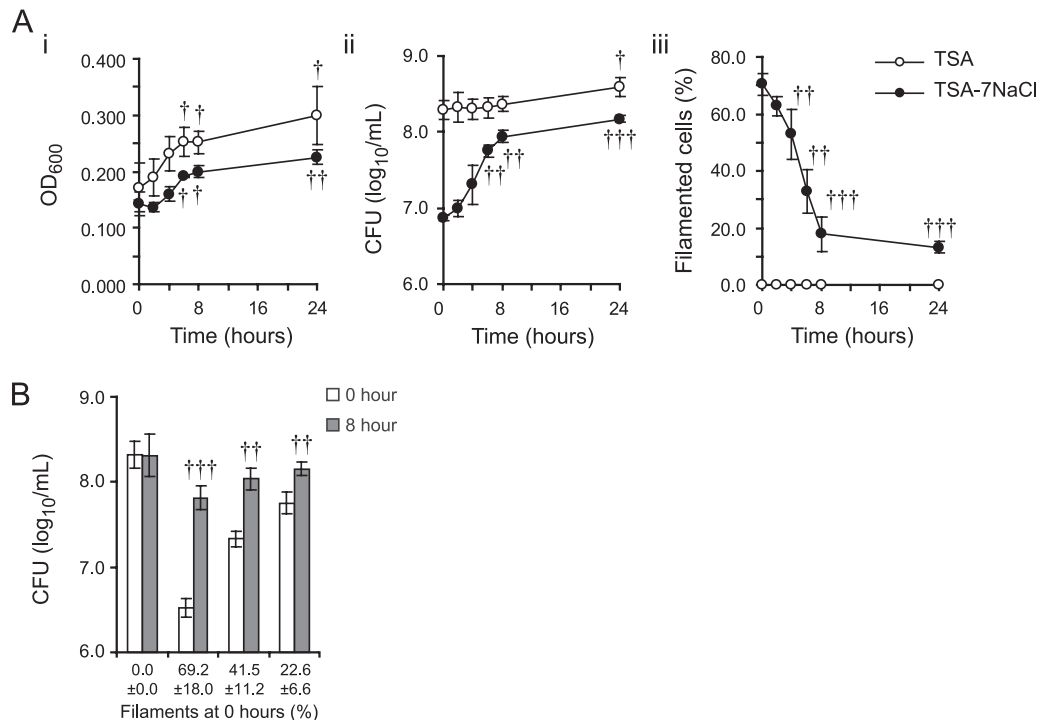
Control and filamented populations of *S. Tennessee* strain 4539H were generated on TSA or TSA-7NaCl, respectively. Harvested cells were adjusted to an OD<sub>600</sub> of 0.2 and incubated in 0.2LB broth at 23°C under static conditions (Fig. 2A). This concentration of LB was chosen because concentrations greater than  $0.2\times$  resulted in replication and division of control cells during incubation (data not shown). In the developed assay, the number of CFU of control cells remained unchanged during 8 h of incubation, although the OD<sub>600</sub> of the bacterial suspension increased 1.4-fold (Fig. 2Ai and ii). Control cells did appear elongated (4 to 6  $\mu\text{m}$ ) after 8 h of incubation in 0.2LB but were not long enough to be considered filaments (Fig. 2Aiii). The elongation of the control cells likely explains the increase in the OD<sub>600</sub> of the control cultures. In contrast, the concentration of CFU in the filamented population increased 1.1 log<sub>10</sub>, while the percentage of filaments in culture decreased from 70.5  $\pm$  3.9% to 17.8  $\pm$  6.1% (Fig. 2Aii and iii). These data indicated that the conditions were favorable for the septation of filaments but not the replication of nonfila-

mentous control cells during 8 h of incubation. Similar results were obtained for strains E40, M-09, and S-12 (data not shown). The percentages of filaments were similar after 8 and 24 h of incubation. By 24 h, the concentration of control cells had doubled (Fig. 2Aii).

Next, the sensitivity of this method was tested to detect increases in the number of viable cells in filamented populations containing fewer than 70% filaments. Suspensions of control cells and filaments were mixed in various proportions to yield cultures that had different percentages of filaments (Fig. 2B). These percentages correlated inversely with the starting concentration of CFU. Significantly, the concentration of each filament-containing culture increased after 8 h of incubation ( $P < 0.05$ ) and reached concentrations similar to that of the control culture. For example, the concentration of CFU increased by 0.4 log<sub>10</sub>/ml when 23% of the initial population was filamented. Consistently, the percentage of filaments decreased by 75% after 8 h of culturing in 0.2LB broth in all strains assayed (Fig. 2Aiii and data not shown).

**Osmotic stress did not affect the amount of FtsZ or PBP3.** Proteins of the Fts family are required for the division of enteric bacteria but not cellular growth or replication of DNA (1, 3, 7, 10, 46, 62). The amounts of FtsZ and PBP3 (FtsI) were quantified via immunoblot to address whether the levels of individual Fts proteins differed in osmotically stressed and unstressed cells. The levels of FtsZ and total PBP3 were unaffected ( $P > 0.05$ ) by the osmolarity of the media after culturing for 4 days (data not shown). Therefore, filamentous growth during osmotic stress is not caused by changes in the expression of FtsZ or PBP3.

**Osmotic stress correlated with changes in the activity of PBP2.** Filaments of *S. enterica* on TSA-7NaCl and TSA-8NaCl occasionally branched, and their width appeared greater than that of control cells (Fig. 1Diii and vi). PBPs regulate the synthesis of the bacterial cell wall, and unbalanced synthesis can affect cellular morphology (11, 40, 41, 47). PBPs from all four strains of *S. enterica* cultured on TSA or TSA-7NaCl were active *in vitro* as determined by their binding to BOCILLIN-FL, a fluorescent derivative of penicillin V (Fig. 3A). To identify which of the fluorescent bands were PBPs, we incubated membrane proteins with increas-



**FIG 2** Filaments of *S. Tennessee* were viable and septated when osmotic stress was removed. (A) *S. Tennessee* strain 4539H grown on TSA (open circles) or TSA-7NaCl (closed circles) was inoculated into 0.2LB broth and incubated at 23°C. The OD<sub>600</sub> of the cells in suspension (i), log<sub>10</sub> CFU/ml (ii), and percent filaments (iii) were calculated over time. (B) Mixed populations of filaments and control cells with various percentages of filaments were incubated at 23°C in 0.2LB broth and the log<sub>10</sub> CFU/ml of each was measured at 0 h (white bars) and 8 h (gray bars) of incubation. Filaments were enumerated via fluorescence microscopy with DAPI (1 μg/ml). In both panels A and B, mean values from three independent experiments are reported. Error bars represent the SD of the means. Student's *t* tests (two-tailed) were performed to identify significant changes of each culture relative to measurements taken at 0 h of incubation (†, *P* < 0.05; ††, *P* < 0.01; †††, *P* < 0.001).

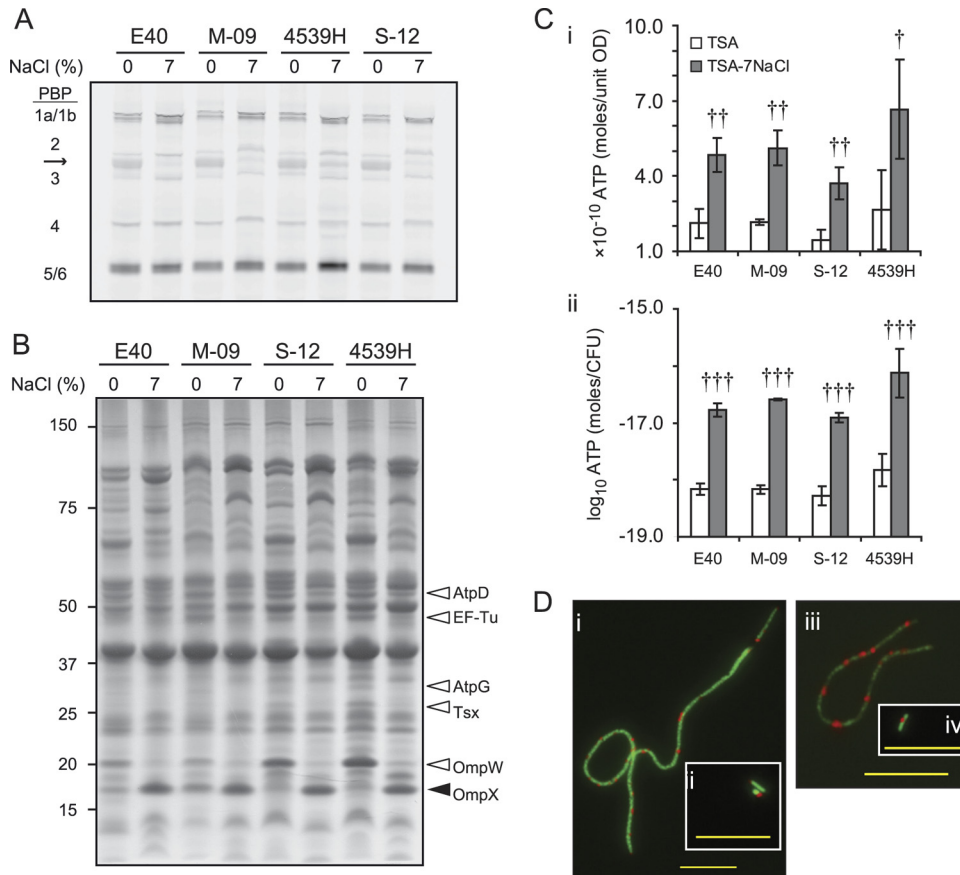
ing concentrations of unlabeled penicillin G and then counterlabeled with BOCILLIN-FL (data not shown). All bands but one (arrow in Fig. 3A) decreased in intensity as the concentration of unlabeled penicillin G increased, confirming their identities as PBPs.

The activity of each PBP was measured by its binding to BOCILLIN-FL and normalized to the total amount of protein in each sample as determined by Coomassie stain (Table 2, Fig. 3B). Of all the PBPs that were assayed, PBP2 was the only one whose activity was consistently greater in cells cultured on TSA-7NaCl than in cells cultured on TSA (Table 2). The activity of PBP2 increased 3- to 5-fold in filamentous populations of strains M-09, S-12, and 4539H relative to that in control cells of each strain, and these increases were statistically significant (*P* < 0.05). A 1.5-fold increase in the activity of PBP2 was also observed in filamentous populations of strain E40 relative to that in control cells, but this increase was not significant (*P* > 0.05). The activities of PBPs 1, 4, and 5/6 were largely similar in filamentous and control populations. The activity of PBP3 increased in filaments of strains E40 and M-09 but remained unchanged in filaments of strains S-12 and 4539H relative to that in control cells. These data suggest that PBP2 is the only candidate of the identified PBPs to contribute to the osmotic-induced filamentation of *S. enterica*.

**The levels of ATP synthase in *S. enterica* correlated inversely and ATP levels directly with osmotic stress.** To identify other proteins that may contribute to the filamentation of salmonel-

lae during osmotic stress, membrane proteins from four strains of *S. enterica* were separated via SDS-PAGE and stained with Coomassie G-250 (Fig. 3B). Six proteins correlated either directly or inversely with osmotic stress in all strains tested. These proteins were identified via MALDI-TOF mass spectrometry (Table 3). The levels of two outer membrane proteins (OmpW and Tsx), the translation factor, EF-Tu, and the F<sub>0</sub>F<sub>1</sub>-ATP synthase subunits β (AtpD) and γ (AtpG) were less in filamented cells than in control cells. In contrast, the amount of OmpX was greater in filamented cells than in control cells. While five of the proteins were sequenced from samples of strain 4539H that had been grown on TSA or TSA-7NaCl, OmpX could be sequenced only from samples grown on TSA-7NaCl. Another protein not homologous to OmpX—identified as peptidoglycan-associated outer membrane lipoprotein—migrated similarly to OmpX in samples isolated from unstressed cells (Fig. 3B). OmpX was not the major constituent of this band, indicating that the fold difference in the amount of OmpX is likely greater than what was observed (Fig. 3B, Table 3).

The amount of both AtpD and AtpG in bacterial membranes decreased by 50 to 90% during osmotic stress (Fig. 3B, Table 3). We hypothesized that the decreased levels of these two proteins would negatively affect the amount of ATP in filaments compared to that in control cells and that this difference contributed to filamentation. To test these predictions, the levels of ATP in control and filamented populations of *S. enterica* were quantified (Fig. 3C). The levels of ATP in E40, M-09, S-12, and 4539H were 3-fold greater in filaments than in control cells



**FIG 3** Growth of *S. enterica* during osmotic stress correlated with changes in the levels of membrane proteins and ATP. Stationary-phase inocula of strains E40, M-09, S-12, and 4539H were cultured on TSA (white bars) or TSA-7NaCl (gray bars) for 4 days at 30°C to generate populations of control cells and filaments, respectively. (A) PBPs (denoted on the left-hand side of the gel) were bound to BOCILLIN-FL *in vitro* and detected by SDS-PAGE. The arrow points to a nonspecific, autofluorescent band. (B) Membrane proteins were separated by SDS-PAGE and stained with Coomassie blue G-250. Proteins whose levels decreased (open arrowhead) or increased (closed arrowhead) during osmotic stress in all strains tested were identified and are denoted to the right of the arrowheads. The migration of a protein standard is indicated on the left-hand side of the gel. In panels A and B, a representative image of at least three independent experiments is shown. (C) The amount of ATP in salmonellae grown on TSA (white bars) or TSA-7NaCl (gray bars) was quantified and normalized by  $OD_{600}$  (i) or CFU (ii). Bars represent the means from three independent experiments, and error bars represent the SD of the means. Student's *t* tests (two-tailed) were performed to identify significant changes in the amount of ATP in populations of control cells and filaments for each strain ( $\dagger$ ,  $P < 0.05$ ;  $\dagger\dagger$ ,  $P < 0.01$ ;  $\dagger\dagger\dagger$ ,  $P < 0.001$ ). (D) Representative images from two independent experiments of filaments (i, iii) and control cells (ii, iv) of strains 4539H (i, ii) and M-09 (iii, iv) incubated with CTC. The reduced formazan product of CTC is red, and nucleic acids were stained with SYTO-24 (green). The bar in panel D represents 10  $\mu$ m.

when normalized to  $OD_{600}$  (Fig. 3Ci). *S. enterica* grown on TSA-7NaCl had 1.5  $\log_{10}$ /CFU more ATP than cells grown on TSA (Fig. 3Cii). Also, respiration in filaments and control cells was observed microscopically by using CTC as an electron ac-

ceptor. When reduced, CTC forms a fluorescent formazan product (Fig. 3D), which was observed in both filaments and control cells. Therefore, electron transport was not inhibited in filaments, nor was the synthesis of ATP.

**TABLE 2** Activity of PBPs in four strains of *S. enterica* cultured for 4 days on TSA or TSA-7NaCl<sup>a</sup>

Strain	Activity (relative fluorescent units)									
	TSA					TSA-7NaCl				
	PBP1 <sup>b</sup>	PBP2	PBP3	PBP4	PBP5 and PBP6	PBP1 <sup>b</sup>	PBP2	PBP3	PBP4	PBP5 and PBP6
E40	240 $\pm$ 62	34 $\pm$ 36	18 $\pm$ 3.0	48 $\pm$ 27	570 $\pm$ 500	420 $\pm$ 59 <sup>c</sup>	51 $\pm$ 7.6	59 $\pm$ 19 <sup>c</sup>	95 $\pm$ 17	930 $\pm$ 180
M-09	270 $\pm$ 110	19 $\pm$ 13	19 $\pm$ 7.3	61 $\pm$ 17	860 $\pm$ 220	500 $\pm$ 150	64 $\pm$ 9.6 <sup>d</sup>	54 $\pm$ 11 <sup>c</sup>	110 $\pm$ 55	1,100 $\pm$ 200
S-12	220 $\pm$ 97	15 $\pm$ 3.0	44 $\pm$ 52	68 $\pm$ 34	1100 $\pm$ 190	310 $\pm$ 110	46 $\pm$ 10 <sup>d</sup>	43 $\pm$ 16	96 $\pm$ 54	1,100 $\pm$ 418
4539H	290 $\pm$ 27	18 $\pm$ 2.8	110 $\pm$ 40	73 $\pm$ 11	1000	380 $\pm$ 97	100 $\pm$ 83 <sup>c</sup>	90 $\pm$ 67	110 $\pm$ 52	1,400 $\pm$ 270

<sup>a</sup> Activity (in relative fluorescent units) was normalized to the total amount of protein detected with Coomassie stain in each sample and is reported relative to the activity of PBPs 5 and 6 in strain 4539H cultured on TSA, which was given an arbitrary value of 1,000.

<sup>b</sup> Includes PBP1a and PBP1b.

<sup>c</sup>  $P < 0.05$ .

<sup>d</sup>  $P < 0.01$ .

TABLE 3 Membrane proteins of *S. enterica* identified via MALDI-TOF mass spectrometry whose levels correlated with osmotic stress

GI	Name	Description	E value	% of sequence covered	Change of steady-state levels (TSA-7NaCl:TSA)			
					E40	M-09	S-12	4539H
16767149	AtpD	F <sub>0</sub> F <sub>1</sub> -ATP synthase subunit beta	1.2E-70	67	0.51 ± 0.20 <sup>a</sup>	0.36 ± 0.13 <sup>b</sup>	0.24 ± 0.26 <sup>b</sup>	0.37 ± 0.24 <sup>b</sup>
16767150	AtpG	F <sub>0</sub> F <sub>1</sub> -ATP synthase subunit gamma	4.0E-12	16	0.20 ± 0.09 <sup>b</sup>	0.14 ± 0.13 <sup>b</sup>	0.08 ± 0.07 <sup>a</sup>	0.34 ± 0.24 <sup>b</sup>
16422703	EF-Tu	Elongation factor	9.9E-09	28	0.57 ± 0.25 <sup>a</sup>	0.47 ± 0.20 <sup>c</sup>	0.21 ± 0.11 <sup>b</sup>	0.32 ± 0.30 <sup>b</sup>
16763793	Tsx	Nucleoside channel, receptor of phage T6 and colicin K	3.1E-31	54	0.28 ± 0.20 <sup>c</sup>	0.15 ± 0.12 <sup>c</sup>	0.22 ± 0.21 <sup>c</sup>	0.24 ± 0.21 <sup>b</sup>
16765076	OmpW	Outer membrane protein	3.1E-26	64	0.42 ± 0.23 <sup>a</sup>	0.26 ± 0.05 <sup>b</sup>	0.13 ± 0.01 <sup>b</sup>	0.18 ± 0.08 <sup>b</sup>
16764195	OmpX	Outer membrane protein	9.9E-32	58	3.99 ± 0.86 <sup>c</sup>	3.34 ± 1.43 <sup>a</sup>	3.38 ± 0.84 <sup>c</sup>	2.58 ± 1.37 <sup>a</sup>

<sup>a</sup> *P* < 0.05.<sup>b</sup> *P* < 0.001.<sup>c</sup> *P* < 0.01.

## DISCUSSION

Filamentous growth of rod-shaped bacteria occurs in suboptimal conditions where division, but not growth, is inhibited. Different serovars of *S. enterica* filament during growth in hyperosmotic, acidic, alkali, and refrigerated environments (19, 31, 34–36). Likewise, both *E. coli* and *L. monocytogenes* filament in similar suboptimal conditions (19, 29, 36, 61).

Salmonellae generate filaments at *a<sub>w</sub>* values of 0.92 to 0.96 in medium supplemented with glycerol, sucrose, or NaCl (34, 36). Four strains of *S. enterica* representing different serovars were tested for filamentous growth on agar and in broth medium supplemented with different concentrations of NaCl (Fig. 1A). As the concentrations of NaCl within these media increased, growth decreased, and filaments were observed under conditions permissible for marginal growth. Independent of their concentration during inoculation, strains we examined filamented on TSA and in TSB supplemented with 6 to 8% NaCl (Fig. 1A and D and data not shown). Exponential-phase inocula did not grow or filament when medium was supplemented with 8% or more NaCl, while stationary-phase inocula both grew and filamented under these conditions. Stationary-phase cells did not grow or form filaments in TSB-10NaCl. Therefore, filaments form in hyperosmotic environments that support marginal growth but not septation.

**Filamentous growth and RpoS.** It is known that exponential-phase cells are more sensitive to many types of stress than are stationary-phase cells (20). The difference we observed in cells in these two growth phases with regard to their capacity to filament in response to osmotic stress likely reflects the role of RpoS-regulated genes to enable cells to survive osmotic stress (37). Accordingly, LT2, a strain that poorly expresses RpoS (32), formed filaments in the presence of lower concentrations of NaCl than other strains tested (Fig. 1A). Our finding that RpoS is not directly required for filamentation is consistent with the findings of Mattick and colleagues (34).

**Enumeration of viable units within filaments.** A multichromosomal filament and a single chromosomal control cell both form a single CFU on an agar plate. Yet filaments generated during osmotic stress are viable and can septate when incubated in medium without added humectants (Fig. 2A) (34). This issue must be considered in retrospective analyses of infectious dose and risk assessment of food-borne pathogens, like *Salmonella*. Populations of filamented and control cells had equal amounts of protein and chromosomal DNA when normalized by biomass and so cannot be used for the detection and enumeration of filaments (Table 1).

Enumerating viable units of filaments was achieved by incu-

bating a population of filaments in 0.2LB broth for 8 h at 23°C. During incubation, filamentous salmonellae were able to septate and divide, while nonfilamentous salmonellae were not (Fig. 2Aii and iii). This assay was sensitive; when 23% of the cells in a population of *S. Tennessee* were filamented, CFU increased 0.4 log<sub>10</sub>/ml (Fig. 2B). The levels of ATP in filaments relative to control cells could in part explain how filaments were able to divide prior to control cells (Fig. 3C). Additionally, control cells had to fully replicate cellular constituents that filaments had already replicated.

**DNA, protein, and levels of proteins in filaments.** Filamentous growth occurs by numerous mechanisms, such as when the replication of DNA is blocked with nalidixic acid or protein synthesis is repressed (17, 23, 27, 44, 52). Filaments of *S. enterica* generated on TSA-7NaCl yielded as much chromosomal DNA and protein as control cells when cell suspensions were normalized by OD<sub>600</sub> (Table 1). Many proteins, including DnaK, in control and filamented populations were detected at similar levels (Fig. 3B and data not shown). The activities of numerous PBP2s were similar in filamentous and control populations *in vitro* as assayed by their ability to bind a fluorescent derivative of penicillin G, BOCILLIN-FL (Fig. 3A, Table 2). Collectively, these findings indicate filaments are not likely caused by the repression of DNA or protein synthesis or the misfolding of PBP2s.

Filamentous growth during osmotic stress likely does not require an intact SOS response. Chromosomal DNAs isolated from filaments and control cells were equally resistant to the exonuclease, BAL 31, indicating that chromosomal DNA retained its integrity in osmotically stressed *S. enterica* (data not shown). Also,  $\Delta$ recA *E. coli* serotype O157:H7 is capable of forming filaments on TSA-6NaCl (A. J. Stasic, C. W. Kaspar, and A. C. L. Wong, unpublished data).

**Potential role of PBP2 in osmotic-induced filaments.** PBP2 was more active in membranes isolated from strains of *S. enterica* cultured on TSA-7NaCl than TSA (Fig. 3A). The increase in its activity was statistically significant for three of the four strains assayed (Table 2). Directed by the MreB cytoskeletal complex, PBP2 inserts peptidoglycan into the bacterial cell wall, resulting in elongation (16, 53, 54, 59). Mutations in *pbpA*, which encodes PBP2, renders *E. coli* sensitive to osmotic stress (8, 45). Therefore, PBP2 is a candidate for future investigation in the filamentous growth of *S. enterica* during osmotic stress.

**Levels of ATP and subunits of the F<sub>0</sub>F<sub>1</sub>-ATP synthase.** *S. enterica* cultured on TSA-7NaCl contained 3-fold more ATP than salmonellae cultured on TSA when normalized by biomass



(Fig. 3Ci). Yet, the amount of the  $F_0F_1$ -ATP synthase subunits  $\beta$  and  $\gamma$  were reduced in filamentous populations relative to control cells (Fig. 3B, Table 3). Both filaments and control cells were capable of oxidative phosphorylation, as measured by the reduction of CTC to a fluorescent formazan product. This assay was qualitative and therefore does not provide information into the rates of oxidative phosphorylation. Furthermore, it does not distinguish between aerobic and anaerobic electron acceptors. During osmotic shock, exponentially growing *E. coli* accumulates ATP via *de novo* synthesis, while respiration decreases by 90% (38, 42). Transcription of the genes encoding the  $F_0F_1$ -ATP synthase is negatively regulated when exponentially growing *E. coli* is osmotically shocked (48, 57).

ATP could contribute to the survival of *S. enterica* during osmotic stress and regulate the intracellular osmolarity of *E. coli* during osmotic shock, thereby protecting cells from plasmolysis (42). ATP could also contribute to the outgrowth of filaments (Fig. 2A). The elevated levels of ATP in filaments could indicate a reduction in the activity of ATP-dependent reactions. For example, inhibiting the ATPase activity of either FtsA or FtsE will induce filamentous growth in *E. coli* (6, 14, 17, 60).

Our findings show that during osmotic stress, *S. enterica* undergoes filamentous growth that is correlated with an increase in PBP2 and ATP levels and a decrease in levels of the  $F_0F_1$ -ATP synthase in the bacterial membrane. How PBP2 and metabolism contribute to these characteristics of osmotically induced filaments is the subject of ongoing research.

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