

Filament Formation by *Salmonella* spp. Inoculated into Liquid Food Matrices at Refrigeration Temperatures, and Growth Patterns When Warmed

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ABSTRACT

In this study, the formation of multicellular filamentous *Salmonella* cells in response to low temperatures was investigated by using isolates of *Salmonella enterica* serovar Enteritidis PT4 and *S. enterica* serovar Typhimurium DT104 as the inocula. The formation of filamentous cells in two liquid food matrices at the recommended maximum temperature for refrigeration (8°C) was monitored and compared with that in tryptone soya broth. Giemsa staining was performed to locate nuclear material within the filaments. Single filaments were warmed on agar at 37°C, and the subsequent rate of septation was quantified. For all strains tested, >70% of the *Salmonella* cells inoculated had become filamentous after 4 days in media at 8°C, indicating that filamentation could occur during the shelf life of most refrigerated foods. Strains with impaired RpoS expression were able to form filaments at 8°C, although these filaments tended to be shorter and less numerous. All strains also formed filamentous cells at 8°C in retail milk or chicken meat extract. Filaments often exceeded 100 µm in length and appeared straight-sided under the microscope in media and in foods, and Giemsa staining demonstrated that regularly spaced nucleoids were present. This phenotype indicates that an early block in cell septation is probably responsible for filamentation. When filaments were warmed on agar at 37°C, there was a rapid completion of septation, and for one filament, a >200-fold increase in cell number was observed within 4 h. There are clear public health implications associated with the filamentation of *Salmonella* in contaminated foods at refrigeration temperatures, especially when the possibility of rapid septation of filamentous cells upon warming is considered.

Low-temperature storage to either prevent or restrict microbial growth is central to food preservation and is becoming ever more important as consumers demand less processed goods and more fresh foods (9). The maximum recommended refrigeration temperature is 8°C (5), but in a recent study, 55% of domestic and 32% of retail store refrigerators were found to operate at temperatures of ≥9°C (25). These data highlight the importance of gaining a better understanding of how foodborne pathogens, including *Salmonella*, behave at this upper limit of refrigeration.

Even in the 1920s, it was understood that *Salmonella* could grow in foods at temperatures of <15°C (4). *Salmonella* are now thought to be capable of growth in terms of increasing CFU per milliliter at refrigeration temperatures (≤8°C) under specific conditions. For example, *Salmonella enterica* serovars Heidelberg, Derby, and Typhimurium have been reported to grow in laboratory broth at 5.9 to 7.5°C, and these and four other serovars have been reported to grow on agar at 5.5 to 6.5°C (19). In addition, growth in terms of CFU has been demonstrated in chicken dishes at 2 and 7°C (4, 6). Other reports, however, describe an absence of *Salmonella* growth at refrigeration tempera-

tures, for example, at 7 to 8°C on beef (18) and at 4 to 10°C in custard and on ham salad (4). It therefore appears that food type can influence the ability of *Salmonella* to grow at low temperatures. Growth at refrigeration temperatures tends to be much slower than that at higher temperatures with an increased lag period (17) (U.S. Department of Agriculture Pathogen Modeling Program, version 5.1). Even when growth at low temperatures is not observed, *Salmonella* are reported to survive for up to 1 year at 7°C (1).

It is understood that cells incubated at low temperatures differ from those grown at higher temperatures because of the expression of proteins involved in the cold shock response (7), among other factors. Prior cold storage has been demonstrated to increase the lethality of a subsequent stress such as heat or acid in *S. enterica* serovar Enteritidis phage type (PT) 4, suggesting that cells are injured by cold stress (12, 14). On the other hand, *S. enterica* serovar Enteritidis PT4 and *Escherichia coli* are also reported to “grow” in terms of an increase in biomass without a concurrent increase in CFU at 4 and 8°C, respectively, producing long filaments in laboratory media (24, 28). Phillips et al. (24) also found that another PT4 isolate formed shorter and less-numerous filaments at 4°C, and this isolate was subsequently identified as a *rpoS* mutant (14). *Salmo-*

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nella cell morphology returned to the typical small rod shape upon incubation at 37°C, but whether this development was due to the completion of septation in filamentous cells or to the regrowth of the remaining population of small cells was unclear (24). Recent work examining the responses of *S. enterica* serovar Enteritidis PT4 and *S. enterica* serovar Typhimurium definitive type (DT) 104 to low water activity showed that filamentation occurred irrespective of the RpoS status of the *Salmonella* cells (20). Filamentation of rod-shaped bacteria has also been demonstrated at elevated incubation temperatures (26, 29) and at high concentrations of carbon dioxide (22).

To determine the public health implications of cold-induced filamentation in *Salmonella* spp., isolates of PT4 and DT104 were held at 8°C in foods and laboratory media. The septation of filamentous cells following transfer to 37°C was quantified. Cold-induced filamentation was observed in all *Salmonella* strains tested, including *rpoS* mutants, within 4 days at 8°C in foods and laboratory media. The rapid septation of filaments to form a large number of daughter cells further highlights the potential public health risk.

MATERIALS AND METHODS

Bacterial strains and preparation of cultures. In this study, four strains of *S. enterica* serovar Enteritidis PT4 (E, I, LA5, and EAV54) and two strains of *S. enterica* serovar Typhimurium DT104 (30 and 10) were used. Strains E, LA5, and 30 exhibit normal RpoS expression (14). Strains I and 10 are naturally occurring isolates with impaired RpoS expression (14, 15), and strain EAV54 is an isogenic *rpoS* mutant (3). Strain LA5 was originally isolated from a chicken (3), strain 30 was isolated from cattle feces (30), strains 10 and E were human isolates (30), and strain I was from a chicken carcass.

Salmonella strains were recovered from storage at -20°C on Protect beads (Mast Diagnostics, Merseyside, UK). A bead was streaked onto 5% horse blood agar and incubated at 37°C for 24 h. Stationary-phase cultures were prepared by inoculating 9 ml of tryptone soya broth (TSB; Oxoid, Hampshire, UK) and incubating it at 37°C. After 3 h, 1 µl of this broth was transferred into 9 ml of TSB before incubation at 37°C for 15 h, by which time the cultures had reached the early stationary phase (13).

Preparation and inoculation of foods for filamentation studies. To prepare the chicken meat extract, 600 g of chicken breast was cut into small pieces, heated in 2.5 liters of deionized water until the water boiled, and held for 2 h. The broth was cooled for 3 h, the meat was removed, and the liquid was drained through cotton wool to remove some of the fat. The chicken meat extract was autoclaved at 110°C for 10 min, and 15-ml aliquots were prepared. The skimmed milk was autoclaved at 100°C for 10 min and aseptically divided into 15-ml volumes. The chicken meat extract and milk samples were inoculated with 150 µl of the six stationary-phase cultures in triplicate such that the starting cell density was approximately 10⁷ CFU/ml before chilling.

Viable counts and microscopy of cultures held at refrigeration temperatures. The inoculated cultures were held at 8°C for 4 days. The United Kingdom Institute of Food Science and Technology states that chilled foods should be held at -1 to 8°C (5). Therefore, 8°C was chosen to simulate worst-case scenarios, particularly since past studies have shown that refrigerators often operate at or above the maximum recommended temperature for

refrigeration (27). During incubation at 8°C, microscopy was carried out and viable counts were determined. For cell number determination, dilutions were made in maximal recovery diluent (Oxoid, Basingstoke, UK), and viable cells were enumerated by the method of Miles and Misra (23) with plating onto blood agar and incubation for 48 h at 37°C to ensure optimal recovery of injured cells that may have prolonged lag periods (21). Filament number and size was quantified by counting the proportion of cells that were <4, 4 to 10, and >10 times typical small cell lengths (approximately 2 µm) in three microscope fields of view.

The cultures were then held at 8°C for a further 10 days, and the longest visible filaments were measured to obtain an indication of the maximal filament length likely to occur during refrigeration. Photographic computer images of the filaments under the microscope were captured, and accurate length measurements were calculated for individual cells after 14 days at 8°C with image analysis software (Scion Image LG5, available from zippy.nimh.nih.gov).

Single-cell analysis system. The single-cell analysis system comprised an aluminum microscope slide attached to a temperature-controlled heated stage. A hole of approximately 20 mm in diameter was cut in the aluminum, and a glass coverslip was glued flush with the underside. This setup was fully autoclavable. As required, 200-µl aliquots of molten agar (buffered peptone water [Oxoid] supplemented with 13 g of agar base per liter) were pipetted into the well and immediately covered with a flame-sterilized coverslip. The coverslip was removed when the agar had dried, and approximately 10 to 20 µl of cultures held at 8°C for 14 days were inoculated onto the surface of the agar. The coverslip was replaced, and the slide was mounted on a stage unit that was preheated to and maintained at 37°C. Cells were visualized with the ×100 oil immersion objective with transmitted light unless the cells were highly elongated, in which case the ×40 objective was employed. Computer images were captured at regular intervals (approximately every 30 min) for up to 24 h.

Giemsa nucleic acid stain. Giemsa stain was used to visualize the polynucleated regions within elongated cells in cultures held at 8°C for 14 days. A 0.4% (wt/vol) solution of Giemsa (Sigma Aldrich, Dorset, UK) in buffered methanol was applied to heat-fixed cells for 2 min. The resulting solution was mixed with deionized water on the glass slide and allowed to stand for a further 3 min, flushed again with deionized water, air dried, and viewed using transmitted light. Images were captured as described above.

Statistical analysis. All chilling work was carried out in triplicate with different original cultures. Data analysis was performed with Microsoft Excel 97. Statistical significance was calculated with a two-sample *t* test (assuming equal variance).

RESULTS

Filament formation at 8°C. All *Salmonella* strains tested formed long filaments in TSB over 4 days of incubation at 8°C (Table 1), including PT4 strains that could not produce RpoS and DT104 strains, for which chilling-induced filamentation has not previously been reported. After 4 days, >70% of the cells observed were filamentous (>4 cell lengths) for each *Salmonella* culture. In cultures of the three RpoS-expressing strains (E, LA5, and 30), >90% of cells were filamentous (100% in LA5 cultures), whereas the cultures of *rpoS* mutants always contained fewer filaments (*P* = 0.005; Table 1). The proportion of very

TABLE 1. Filamentation of *S. enterica* serovar Enteritidis PT4 strains E, I, LA5, and EAV54 and *S. enterica* serovar Typhimurium DT104 strains 30 and 10 after 4 days of incubation at 8°C

Salmonella serotype	Strain	Filamentation (%)					
		TSB		Chicken broth		Skimmed milk	
		Filaments	>10 cell lengths	Filaments	>10 cell lengths	Filaments	>10 cell lengths
Enteritidis	E	97 ± 3	13 ± 3	100 ± 0	6 ± 6	61 ± 6	3 ± 3
	I ^a	72 ± 5	11 ± 3	80 ± 1	4 ± 4	40 ± 6	0 ± 0
	LA5	100 ± 0	45 ± 10	93 ± 7	17 ± 12	94 ± 6	12 ± 3
	EAV54 ^a	82 ± 10	15 ± 3	81 ± 10	12 ± 6	61 ± 9	6 ± 6
Typhimurium	30	95 ± 3	30 ± 9	97 ± 3	28 ± 7	85 ± 8	19 ± 5
	10*	91 ± 5	0 ± 0	93 ± 7	13 ± 4	48 ± 6	0 ± 0

^a Unable to express RpoS.

long filaments (>10 cell lengths) was usually larger in the RpoS-expressing strains ($P = 0.006$) and accounted for 45% of cells in LA5 cultures after 4 days. At the other extreme, DT104 strain 10 exhibited no filaments of >10 cell lengths after 4 days at 8°C. After 14 days of incubation, the longest measured filaments were approximately 150 μm.

All strains formed filamentous cells in chicken meat extract and skimmed milk when held for 4 days at 8°C. The filaments in the foods were identical in appearance to those in laboratory media and had a similar size distribution. In chicken broth, as in laboratory media, a larger proportion of the *Salmonella* population had become filamentous in cultures of the RpoS-expressing strains than in cultures of the *rpoS* mutant strains. Overall, similar proportions of filamentous cells were found in chicken broth and TSB. There were generally fewer filamentous cells in skimmed milk than in TSB. Again, the RpoS-expressing strains usually formed more and larger filaments.

Growth patterns of *S. enterica* serovar Enteritidis PT4 and *S. enterica* serovar Typhimurium DT104 isolates after extended storage at 8°C. A stationary-phase culture of *S. enterica* serovar Enteritidis PT4 isolate E was incubated at 8°C for 14 days. Filaments were then spread on agar and incubated at 37°C. All filamentous cells were observed to septate rapidly, and a small number of cells were monitored closely to illustrate this observation. Images of two filaments of isolate E and their progeny were captured, and cell numbers were determined; one of these filaments was 35 μm long, and the other was 20 μm long

TABLE 2. Increase in cell numbers from single filaments of *S. enterica* serovar Enteritidis PT4 isolate E at 37°C following 14 days of incubation at 8°C

Time at 37°C (h)	No. of CFU of isolate E (length in μm)	
0	1 (35)	1 (20)
1	1	1
2	16	9
3	68	23
4	210	95

(Table 2). After 3 h at 37°C, these two filaments had produced 68 and 23 daughter cells, respectively, and individual daughter cells were clearly visible by microscopy (Fig. 1).

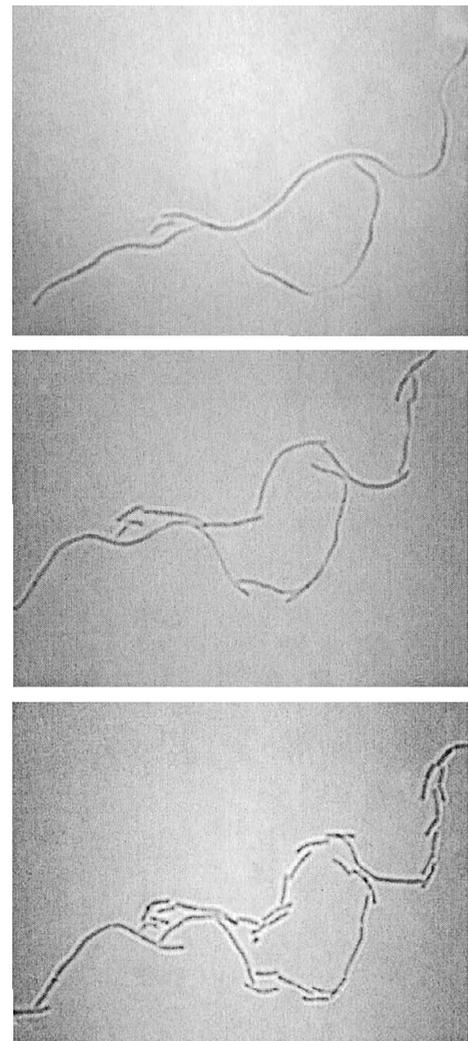


FIGURE 1. Septation of filamentous cells of *S. enterica* serovar Typhimurium DT104 isolate 30 at 37°C following filament formation over 14 days at 8°C. The light micrographs show images captured after 1 h (top), 4 h (middle), and 6 h (bottom) on agar at 37°C.

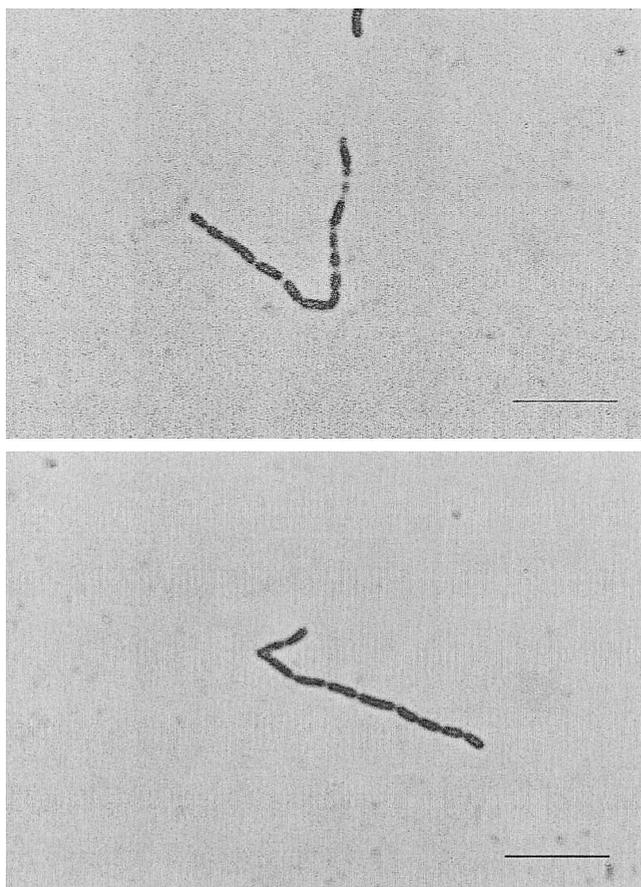


FIGURE 2. Giemsa-stained filamentous cells of *S. enterica* serovar Typhimurium DT104 isolate 30. Bar = 10 μm .

By 4 h, the respective *Salmonella* populations comprised 210 and 95 cells (Table 2).

Giemsa-stained micrographs. Giemsa staining revealed the presence of multiple copies of nuclear material, and stained cells exhibited a beaded appearance where the stain had bound to the DNA (Fig. 2; actual color blue). Nonnucleoid regions remained unstained. Similar “beaded” cells were observed with epifluorescence microscopy where the fluorochrome failed to stain the cell wall, and therefore bacteria appeared to be separated by a gap (data not shown).

DISCUSSION

In this study, the filamentation of six strains of *Salmonella* at refrigeration temperatures was investigated. To investigate the potential public health implications, the ability of *Salmonella* to form filaments rapidly in foods at refrigeration temperatures and the subsequent speed of septation into multiple daughter cells was investigated. The strains studied included *rpoS* mutants, reported to form shorter and less-numerous filaments at 4°C (24), and strains of *S. enterica* serovar Typhimurium DT104, a serotype for which chilling-induced filamentation has not been reported to date.

Filament formation occurred at low temperatures in all six strains of the two major disease-causing serovars tested. In addition, 25 further strains of Enteritidis and Typhimu-

rium and 1 strain each of 18 other serovars have been tested for filamentation at refrigeration temperatures at the PHLS Food Microbiology Research Unit, Exeter, UK, and all strains of all serovars tested were able to form filaments (unpublished data). Thus, internationally important *Salmonella* serovars have the ability to elongate and form multinucleate filaments at the chill temperatures achieved by many domestic refrigerators. Previous work on filamentation by isolates of *S. enterica* serovar Enteritidis PT4 at 4°C have revealed that the ability to express RpoS was important (24). In this study, we demonstrated that RpoS expression was not required for filamentation at 8°C, although there were some observed differences between wild-type and *rpoS* mutant strains in terms of the size and number of filaments formed. The poor filamentation of *rpoS* mutants at 4°C may be related to their increased sensitivity to chill temperatures (2, 16).

Extended storage at 8°C resulted in the formation of very long filaments (up to 150 μm in length). Filamentous cells formed at 8°C were subsequently allowed to septate by incubation at 37°C on agar. A 210- or 95-fold increase in cell number was observed in 4 h, without the observed increase in biomass that would be expected if the increase in cell number were entirely due to binary fission. This finding was also reported for *Salmonella* filaments formed at low water activity and rehydrated, although the resulting daughter cells were not quantified (20). The presence of multiple copies of nuclear material in the filamentous cells suggests that the failure to divide was a result of incomplete cell septation rather than a failure of DNA segregation (8). This result also indicates that after rapid septation, the resulting daughter cells are likely to contain complete genomes and could be infectious. It remains to be determined whether filamentous *Salmonella* are virulent. Presumably, a single filament represents a single infectious unit unless septation is completed prior to the point of invasion for *Salmonella* in the human intestine wall, through warming either after a food has been chilled or after a chilled food has been ingested. In this study, we have shown that septation of the filaments is complete in 4 h, after which time the number of infectious units has increased approximately 150-fold, and this development may boost *Salmonella* numbers sufficiently for infection to occur.

All of the *Salmonella* isolates used in this study were able to form filaments in either skimmed milk or a chicken meat extract at 8°C. These filaments were as numerous in the chicken meat extract as in a laboratory medium but were less frequent and shorter in skimmed milk. This finding could be due to restricted growth in milk, perhaps due to the lack of an essential nutrient or the activity of an antibacterial agent.

The temperature of domestic and retail refrigerators is known to often exceed the maximum recommended temperature for chilled foods (8°C (25, 27)). Thus, filamentation of *Salmonella* (if present) is almost certainly occurring in refrigerated retail foods at temperatures of 8°C and possibly at lower temperatures (24).

There has been much discussion about the infectious dose of *Salmonella* in chilled foods (10, 11). In outbreak

investigations, the infectious dose is usually derived from direct enumeration carried out soon after the removal of a food from cold storage, as is usual practice in microbiology (at least in the United Kingdom). If *Salmonella* were present as filamentous cells, such methods could underestimate the bacterial biomass and, consequently, the potential infectious dose present in the food.

Given the importance of refrigerated foods as vehicles for *Salmonella* infections (10, 11), filamentation may be of practical and public health significance. If risk assessment is based on data expressed as CFU per milliliter, then we may be underestimating the risk of salmonellosis. Further research is required in order to identify a way to prevent filamentation of *Salmonella* in chilled foods.

In conclusion, there are clear public health implications associated with the filamentation of *Salmonella* at refrigeration temperatures, since such filamentation occurs in selected *Salmonella* serovars and in retail foods and the filaments can septate rapidly when warmed, resulting in a ~150-fold increase in CFU per milliliter.

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