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Effects of temperature on the viability, growth and gene profile of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* inoculated in milk

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ABSTRACT

This study was aimed at deciphering the viability and growth of *Yersinia pseudotuberculosis and Yersinia enterocolitica* in milk at different temperatures, and at identifying the temperature-dependent changes in gene expression in *Yersinia*. Fresh *Yersinia* culture was suspended either in pasteurized or in autoclaved milk and subjected to different temperature ranges. Colony forming units (CFU) were determined from inoculated milk after one and two weeks of storage using direct plating technique. In both one and two weeks of storage, growth of *Y. pseudotuberculosis* and *Y. enterocolitica* increased significantly at 4 °C and 24 °C ($P < 0.05$). Furthermore, gene expression profile and DNA microarray analyses were conducted. After one-week storage, the growths of *Y. pseudotuberculosis* and *Y. enterocolitica* were optimal at 4 °C and 24 °C. Remarkably, at 37 °C there was no detectable level of CFU from both *Yersinia* spp inoculated in pasteurized milk, whereas they grew well at 37 °C in autoclaved milk. The NotI-profile of *Y. pseudotuberculosis* grown at 24 °C generated different banding patterns from other treatment groups when compared to FseI and XbaI-PFGE pulso-type. Microarray interrogation of 4038 genes of *Y. pseudotuberculosis* revealed that 38 genes were upregulated by ≥8 fold and 237 genes exhibited ≥2 fold downregulation (at 95% significant level) after temperature shift from 4 °C to 24 °C. The findings of this study highlight the survival potential of *Y. pseudotuberculosis* and *Y. enterocolitica* in milk under different temperatures and the associated gene expression patterns, which may be important in the processing and safety of milk and dairy products.

1. Introduction

Yersinia pseudotuberculosis and *Yersinia enterocolitica* are well-established foodborne pathogens of human concern that survive mainly in moist environments and food matrices. These pathogens are transmitted via ingestion that causes gut-associated diseases such as enteritis, mesenteric lymphadenitis and diarrhea (Koornhof, Smego, & Nicol, 1999; Mikula, Kolodziejczyk & Goldman, 2013). They are most frequently associated with a variety of foods like raw milk, chocolate milk, dairy cream, ice cream, and vegetables like carrots, tomatoes, lettuce and celery (Hanifian & Khani, 2012; Lambertz, Nilsson, & Hallanvuo, 2008). Temperature is one of the most crucial environmental signals sensed by pathogens to adjust the expression of their virulence factors and host survival programs after entry from a cold external environment into a warm-blooded host. Temperature changes enable the bacterial adaptation to diverse hosts and environments (Annamalai & Venkitanarayanan, 2005; Pearce et al., 2012). Refrigerated foods are potential vehicles for growth and transmission of these organisms since *Yersinia* can grow at low temperatures. These factors have led to a renewed interest to identify the fate of foodborne pathogens at pre and post harvest of food items.

In recent years, microarrays have increasingly been used for bacterial genotyping (Bodrossy & Sessitsch, 2004; Gui & Patel, 2011). Microarray data have been published for the identification of highly pathogenic bacteria such as *Yersinia pestis and Y. pseudotuberculosis* for the detection of resistance genes (Chain et al., 2004; Wang et al., 2007). However, the biological role of genes modulated

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at different temperatures and in diverse food matrices is currently not well understood. The objective of this study was aimed at deciphering the thermal effect on *Yersiniae* inoculated in milk and subjected to different temperature stresses.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Y. pseudotuberculosis (NR-804) and *Y. enterocolitica* (NR-214) were generous gifts from Biodefense & Emerging infections Research Resources Repository (BEI Resources; Manassas, VA). *Y. pseudotuberculosis* (29838) and *Y. enterocolitica* biotype 1, serotype 8 (27729) were purchased from the American Type Cultural Collection (Manassas, VA). Yersinia was cultured on *Yersinia*-selective agar base media (BD Difco Laboratories, Sparks, MD) prepared with *Yersinia* antimicrobic supplement, Cefsulodin-Novobiocin (CN) and incubated at room temperature (~24 °C). Prior to treatment, the milk sample was cultured to ensure the absence of *Yersinia* in the milk samples. Fresh *Yersinia* culture was adjusted to one optical density at 660 nm using a NanoDrop Spectrometer (NanoDrop, Wilmington, DE). The corresponding CFU/ml was also simultaneously estimated by broth dilution counting. Ten μl of the bacterial suspension was inoculated in 1 ml of pasteurized milk and autoclaved milk. The inoculated milk samples were placed at different temperatures −80 °C, −20 °C, 4 °C, 24 °C and 37 °C for one and two weeks. Furthermore, additional set of inoculated milk samples were subjected to 67 °C for 30 min or 72 °C for 15 s and stored at 4 °C for one and two weeks.

2.2. Enumeration of Yersinia

Bacterial counts were documented by serial dilution and plating procedure as reported previously (Abdela, Graham, Tsegaye, Temesgen, & Yehualaeshet, 2011). Ten-fold serial dilution was performed aseptically after each treatment and storage period. Ten μl of the inoculated milk was mixed with 90 μl (10^{-1}) of peptone water and followed by serial dilutions up to 10^{-8}. A direct drop plating of 10 μl on CN supplemented agar plate was used to enumerate the number of colony forming units per ml (CFU/ml). The number of surviving bacteria (CFU/ml) was then compared with the initial CFU/ml count at time-0 (t_0). Plates inoculated with a sample dilution that yield between 30 and 300 colonies were considered for counting and the viable counting method was carried out in triplicates.

2.3. Yersinia growth in pasteurized and autoclaved milk

Yersinia culture was inoculated in both pasteurized and autoclaved milk. The inoculated milk was exposed to the aforementioned thermal treatments (−80 °C, −20 °C, 4 °C, 24 °C and 37 °C) for one and two weeks. After specified time points, the *Yersinia* inoculated milk was cultured and the CFU were counted and documented in $\log_{10}$.

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to the standard method described for molecular sub-typing in the PulseNet, CDC protocol (Barrett, Gerner-Smidt, & Swaminathan, 2006; Gerner-Smidt et al., 2006; Ribot et al., 2006). Slight modification was done and supported by the *in silico* simulation of Molecular Biology Experiments (http://insilico.ehu.es). *Y. pseudotuberculosis* and *Y. enterocolitica* challenged at different temperatures (−80 °C, −20 °C, 4 °C, and 24 °C) were subjected to PFGE to explain the genomic pattern diversity. Fresh culture suspension was prepared in 200 μl of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) containing 10 μl of proteinase K (20 mg/ml stock). The bacterial suspension was saturated to one optical density (OD) at 660 nm. The suspension was then mixed with an equal volume of PFGE-grade melted agarose (Bio-Rad Laboratories, CA) with 1% SDS agarose in TE Buffer (10 mM Tris: 1 mM EDTA, pH 8.0) and the mixture was poured into plug molds (Bio-Rad Laboratories, CA). After 30 min at 4 °C, the plugs were placed into sterile 10 ml tubes. The plugs were ready for the restriction digestion after a series of washes with pre-heated sterile ultrapure water and TE buffer.

Prior to restriction enzyme digestion, all plugs were equilibrated for 30 min in the corresponding restriction buffer. Following digestion at 37 °C with restriction endonuclease XbaI, FesI, SpeI, and NotI, the restriction fragments were resolved into a pattern of discrete bands using PFGE using CHEF-DRIII (Bio-Rad Laboratories, CA). The enzymes selection and possible restriction fragment profiles were initially reported and supported from previous publications (Fredriksson-Ahomaa, Autio, & Korkeala, 1999; Sakai et al., 2005) and the *in silico* program (Bikandi, San Millan, Rementeria, & Garaizar, 2004).

The electrophoretic separation was adjusted for each restriction enzyme as deducted from autoalgorithms programs based on the fragment size. Lambda ladder and low range pulsed-field gel markers (New England Biolabs, AIpswich, MA) were used as molecular weight markers to determine the fragments size. The gels were maintained at 14 °C during electrophoresis and stained with 1 μg/ml GelRed (Biotium, Haryard, CA) for 15–20 min, de-stained with water for 30 min and the DNA bands were checked under UV light. The Gel was finally visualized and documented by Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA). The DNA restriction patterns of the isolates were then compared with other treatment groups to determine their relatedness.

2.5. Microarray

Gene expression microarray was performed using NimbleGen array (Roche NimbleGen, Madison, WI), which interrogates the most comprehensive and up-to-date list of targets for *Y. pseudotuberculosis* (4038 genes). Fresh *Y. pseudotuberculosis* culture was inoculated in milk and placed at 4 °C and 24 °C. Total RNA extraction and cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) and quantified at A260 nm using a NanoDrop Spectrometer. Prior to cDNA synthesis, RNA samples were verified to be of sufficient quantity and purity. The samples were processed in accordance with NimbleGen published protocols. Briefly, the synthesized double stranded cDNA were labeled with Cy-3 and Cy-5. Labeled samples were then hybridized to the NimbleGen arrays. Following hybridization the arrays were washed and scanned. Image data extraction was processed using software provided by the NimbleGen producing data files for downstream analysis.

Arrays were scanned in accordance with the protocols provided by NimbleGen. The intensity data were log-transformed and normalized using a simple median normalization method. An intensity ratio (test DNA normalized intensity/reference DNA normalized intensity) was recorded for each spot and then was converted to log 2.0. Bioinformatics analysis of the microarray was performed using DNASTAR software (DNASTAR Inc., Madison, WI).

2.6. Statistical analysis

All the CFU data were conducted in triplicate and the CFU data were transformed to $\log_{10}$ scales for data normalization and analysis purposes. The mean, standard deviation, standard error of