Temperature-Sensitive *Salmonella enterica* Serovar Enteritidis PT13a Expressing Essential Proteins of Psychrophilic Bacteria

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Synthetic genes based on deduced amino acid sequences of the NAD-dependent DNA ligase (*ligA*) and CTP synthetase (*pyrG*) of psychrophilic bacteria were substituted for their native homologues in the genome of *Salmonella enterica* serovar Enteritidis phage type 13a (PT13a). The resulting strains were rendered temperature sensitive (TS) and did not revert to temperature resistance at a detectable level. At permissive temperatures, TS strains grew like the parental strain in broth medium and in macrophage-like cells, but their growth was slowed or stopped when they were shifted to a restrictive temperature. When injected into BALB/c mice at the base of the tail, representing a cool site of the body, the strains with restrictive temperatures of 37, 38.5, and 39°C persisted for less than 1 day, 4 to 7 days, and 20 to 28 days, respectively. The wild-type strain persisted at the site of inoculation for at least 28 days. The wild-type strain, but not the TS strains, was also found in spleen-plus-liver homogenates within 1 day of inoculation of the tail and was detectable in these organs for at least 28 days. Intramuscular vaccination of White Leghorn chickens with the PT13a strain carrying the psychrophilic *pyrG* gene provided some protection against colonization of the reproductive tract and induced an anti-*S. enterica* antibody response.

Salmonella enterica is a widespread pathogen that causes disease in humans and in both wild and domesticated animals. In poorer regions of the world, the human-specific serovars *S. enterica* serovar Typhi and *S. Paratyphi* cause about 27 million infections per year (1, 2). Worldwide, the nontyphoidal forms of *S. enterica* cause more than 90 million infections, resulting in 155,000 deaths, per year (3).

In the United States, *Salmonella* has remained a significant foodborne illness agent and is the second most common cause of intestinal infection. Contaminated poultry and their products are widely accepted as the primary source of human *Salmonella* infections (4, 5). According to scientists at the Centers for Disease Control and Prevention (CDC), there are approximately 40,000 cases of *Salmonella* reported in the United States each year, equating to roughly 16.2 cases per 100,000 people (6). However, milder cases are often not reported, and the CDC estimates that the actual number of infections is more likely to be about 1 million (6). In the European Union, approximately 100,000 cases of *S. enterica* infections from food sources occur annually (7).

One approach to diminish the number of *S. enterica* infections in humans is to lower the rate of transmission from food sources to humans. In this effort, farmers in most developed regions of the world vaccinate poultry against *S. Typhimurium* and *S. Enteritidis* in an effort to reduce the carriage rate, tissue burden, or vertical transmission to eggs or progeny (8, 9). Currently, poultry vaccination programs primarily utilize a combination of killed and live attenuated vaccines. Killed vaccines are usually able to stimulate the production of antibodies but are ineffective at stimulating T helper 1 and cytotoxic T cells (8, 10). Attenuated live *Salmonella* vaccine candidates have received considerable attention due to the strong mucosal, humoral, and cellular immune responses they provide. Prolonged exposure of the immune system to antigens results in the production of long-lasting memory cells and provides better cross protection within serogroups.

Live vaccines are often developed on the principle of attenua-

tion through generation of metabolic drift mutations, modification of metabolic functions and virulence factors, or creation of auxotrophic double-marker mutants obtained through chemical mutagenesis (8, 9, 11). Some modern vaccine candidates have been produced by successive passages in low-nutrition media, creating genetic deletions, producing susceptibility to low or high temperature, or requiring specific supplemental ingredients for growth. Ideally, a live vaccine should be able to proliferate in the host long enough to elicit a strong immune response but not long enough to result in transmission to eggs or progeny or to revert to virulence.

In this work, we tested the effectiveness of a newly developed technology for rational construction of temperature-sensitive (TS) live attenuated bacterial vaccines. We substituted synthetic genes expressing essential proteins deduced from psychrophilic bacteria for their native homologues in *S. Enteritidis* strain PT13a. These substitutions created PT13a strains in a predictable and controlled manner, with TS phenotypes that are more stable than those found in classic TS mutants.

MATERIALS AND METHODS

Bacterial growth. The bacterial strains and plasmids used for this study are listed in Table 1. Bacteria were grown in medium devoid of animal

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype and/or characteristics ^a	Reference or source
Strains		
<i>S. enterica</i> subsp. <i>enterica</i> serovar Enteritidis PT13a	Wild-type <i>S. Enteritidis</i>	Zoetis strain collection
<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium DT104	Wild-type <i>S. Typhimurium</i>	Zoetis strain collection
PT13aΩ <i>ligA</i> -37	<i>S. Enteritidis</i> PT13a containing an allelic replacement with the codon-optimized <i>ligA</i> gene <i>ligA</i> _{Ph(37°)} from <i>P. haloplanktis</i> ; at 37°C (restrictive temperature) or higher, this strain does not form individual colonies on agar media	This work
PT13aΩ <i>ligA</i> -38	<i>S. Enteritidis</i> PT13a containing an allelic replacement with the codon-optimized <i>ligA</i> gene <i>ligA</i> _{L17(38°)} from Arctic Ocean isolate L17 (<i>Pseudoalteromonas</i> species); restrictive temperature of 38.5°C	This work
PT13aΩ <i>pyrG</i> -39	<i>S. Enteritidis</i> PT13a containing an allelic replacement with the hybrid, codon-optimized <i>pyrG</i> gene <i>pyrG</i> _{Cp(39°)} from <i>C. psychrerythraea</i> 34H (see Fig. 1); restrictive temperature of 39°C	This work
PT13aΩ <i>ligA</i> Ω <i>pyrG</i> -37	<i>S. Enteritidis</i> PT13a containing allelic replacements with <i>ligA</i> _{Ph(37°)} and <i>pyrG</i> _{Cp(39°)} ; restrictive temperature of 37°C	This work
PT13aΩ <i>ligA</i> Ω <i>pyrG</i> -38	<i>S. Enteritidis</i> PT13a containing allelic replacements with <i>ligA</i> _{L17(38°)} and <i>pyrG</i> _{Cp(39°)} ; restrictive temperature of 38°C	This work
DT104Ω <i>ligA</i> -37	<i>S. Typhimurium</i> DT104 containing an allelic replacement with <i>ligA</i> _{Ph(37°)} ; restrictive temperature of 37°C	This work
DT104Ω <i>ligA</i> -38	<i>S. Typhimurium</i> DT104 containing an allelic replacement with <i>ligA</i> _{L17(38°)} ; restrictive temperature of 38.5°C	This work
DT104Ω <i>pyrG</i> -39	<i>S. Typhimurium</i> DT104 containing an allelic replacement with <i>pyrG</i> _{Cp(39°)} ; restrictive temperature of 39°C	This work
DT104Ω <i>ligA</i> Ω <i>pyrG</i> -37	<i>S. Typhimurium</i> DT104 containing allelic replacements with <i>ligA</i> _{Ph(37°)} and <i>pyrG</i> _{Cp(39°)} ; restrictive temperature of 37°C	This work
<i>S. Typhimurium</i> TT18389	<i>S. Typhimurium</i> LT2 strain that has a disrupted chromosomal copy of <i>ligA</i> and is supported by a copy of <i>Salmonella ligA</i> cloned into pBR313 (Ap ^R)	John Roth, UC Davis
<i>E. coli</i> S17-1	Plasmid-mobilizing strain	24
Plasmids		
pKNOCK-Km	Km ^r R6K ori; suicide vector	23
pKS	pKNOCK-Km with a <i>sacB</i> cassette and <i>Salmonella</i> promoter	This work
pKS:: <i>ligA</i> _{Ph(37°)}	pKS with essential gene fusions	This work
pKS:: <i>ligA</i> _{L17(38°)}	pKS with essential gene fusions	This work
pKS:: <i>pyrG</i> _{Cp(39°)}	pKS with essential gene fusions	This work
pHSG415	TS variant of pSC101; Cm ^r Km ^r Ap ^r	22
pHSG415S	Reduced version of pHSG415; Cm ^r	This work
pRS416	<i>Saccharomyces-E. coli</i> shuttle vector used for transformation-assisted recombination; Ap ^r	19
pBRINT-Gm	ColE1-based plasmid; gentamicin resistant	25

^a The gene subscripts indicate the sources of the psychrophilic essential genes and the temperatures of inactivation of the gene products. *Ph*, *Pseudoalteromonas haloplanktis*; *Cp*, *Colwellia psychrerythraea* 34H; L17, unidentified Arctic bacterium that is presumably related to *P. haloplanktis*.

products and consisting of 1% “veggie peptone” (Novagen), 0.5% yeast extract (Difco), and 0.5% NaCl.

Sources of psychrophilic essential genes. The *ligA* gene encodes the homodimer NAD-dependent DNA ligase, and *pyrG* encodes the homotetramer CTP synthase. The deduced amino acid sequences of the *ligA* allele *ligA*_{Ph(37°)} (gene ID 3710245; accession no. NC_007481.1), from *Pseudoalteromonas haloplanktis* TAC125 (12), and the *pyrG* allele *pyrG*_{Cp(39°)} (gene ID 3520478; accession no. NC_003910.7), from *Colwellia psychrerythraea* 34H (13), were used to design codon-optimized versions appropriate for *S. enterica*. Both *P. haloplanktis* and *C. psychrerythraea* are marine psychrophilic bacteria with maximal growth temperatures of about 18°C.

The *ligA*_{L17(39°)} gene was discovered in this work. The unidentified source bacterium, designated L17, was isolated from an ocean water sample collected at 64°47.9’N, 168°36’W, from 55 m below the surface, on 12 October 1999. Briefly, the primers LigA-Bam-Pseudo-2 (TAGTGGATCC TATGTTTATTGAGGTTTAAATGTCTAGCAG) and LigA-Xho-Pseudo-2 (CAATACTCGAGCTAACCATTATGCTTTTGAAGCAGC) were designed

to amplify the *ligA* gene from *Pseudoalteromonas*-like strains. DNA fragments that corresponded to the estimated size of the target gene were cloned as BamHI-XhoI fragments into plasmid pBC SK+ (Stratagene), a chloramphenicol-resistant (Cm^r) derivative of pBluescript. Cloned inserts were sequenced, and clones with full-length *ligA* genes were transformed into *S. Typhimurium* LT2 strain TT18389 (14) (a gift from John Roth, UC-Davis), which has a disrupted chromosomal copy of *ligA* and is supported by a copy of *Salmonella ligA* on pBR313 (ampicillin resistant [Ap^r]). Cm^r transformants were subcultured repeatedly at 30°C in LB broth containing 30 µg/ml of Cm until the original Ap^r plasmid was lost and the strain’s viability was dependent on *ligA*_{L17(39°)}. The temperature growth maximum for the resulting recombinant was determined to be 38.5°C. The native gene sequence of *ligA*_{L17(39°)} has been submitted to GenBank, but in this study, we used a codon-optimized synthetic version of the gene with a G+C content of 57%.

Synthetic gene design and genetic manipulations. The deduced amino acid sequences encoded by *ligA*_{Ph(37°)}, *ligA*_{L17(39°)}, and *pyrG*_{Cp(39°)} were used to design synthetic genes that had codon compositions appro-

priate to *S. enterica*. Proprietary algorithms used by the synthetic DNA supplier (GenScript or BioBasic) were used for codon optimization, and the resulting codon adaptation index (CAI) (15) was close to 1. If possible, the first three codons of the *S. enterica* homologue were incorporated into the synthetic gene to keep the translation initiation rate close to that of the native *S. enterica* gene (16). In all cases, the strengths of the ribosome binding sites (RBSs) were the same between the synthetic gene and the *S. enterica* homologue, within the margin of error inherent in the predictive algorithm (17, 18).

In order to introduce the psychrophilic essential gene into the PT13a chromosome via homologous recombination, the codon-optimized synthetic versions of the psychrophilic genes were fused to PT13a chromosomal DNA so that about 0.5 to 1 kb of PT13a DNA flanking the native, essential gene target was on both sides of the exchanged psychrophilic essential gene. These gene fragments were assembled by PCR amplifying the synthetic psychrophilic gene and the PT13a flanking regions and seamlessly assembling them into plasmid pRS416 (19), using transformation-assisted recombination in *Saccharomyces cerevisiae* (20).

To introduce psychrophilic genes into PT13a, the synthetic gene and native flanking regions were cloned as an XhoI-XhoI fragment into a derivative of the TS plasmid pHSG415 (21, 22). Prior to our cloning experiment, pHSG415 was digested with PvuII and religated, generating pHSG415S. These steps removed the kanamycin and ampicillin resistance genes and reduced the plasmid size. Once an essential gene construct was introduced at the unique XhoI site, recombinant pHSG415S was electroporated into PT13a. Transformants were cultured at 44°C in the presence of Cm selection to promote a Campbell-like integration of recombinant pHSG415S into the PT13a chromosome. Individual colonies were screened for the absence of an autonomously replicating plasmid by using PCR and standard plasmid preparations and for the presence of the psychrophilic essential gene by using PCR detection. Appropriate isolates were subcultured four times in broth medium at 30°C, diluted, and plated, and individual colonies were screened for the loss of antibiotic resistance and for temperature sensitivity indicative of plasmid excision via homologous recombination. Approximately 10% of the colonies had lost the integrated plasmid, and about 20% (*ligA* alleles) or 0.1% [*pyrG*_{Cp(39°)} allele] had excised the plasmid, leaving the psychrophilic essential gene in place of the native PT13a gene.

We also sought to construct strains with two gene substitutions. However, plasmid pHSG415S could not be used to introduce a second TS essential gene because the high-temperature selection step would kill the original TS PT13a strain. For this purpose, the pKNOCK-Km suicide plasmid (23) was modified by inserting a *sacB* gene at the BamHI site. A PCR amplicon containing the psychrophilic essential gene with PT13a flanking regions was cloned into the XhoI site of the pKNOCK-*sacB* plasmid, and this was conjugated from the plasmid-mobilizing strain *Escherichia coli* S17-1 (24) to PT13a. In order to enable selection against the donor strain, the recipient PT13a strain was transformed with pBRINT-Gm (25), and after conjugation, the plasmid was cured by growing the strain in the absence of gentamicin. The recombinant pKNOCK plasmid was rescued in the recipient by integration into the PT13a chromosome. Selection for excision of the plasmid was carried out by subculturing the strain three times in broth containing 10% sucrose for 48 h and then plating the cultures on agar plates with 10% sucrose. To verify that a true gene exchange had occurred, the region of the chromosome containing the targeted substitution, together with about 300 bp of flanking regions, was amplified by PCR, and the amplicon was sequenced. The resulting TS PT13a strains, derived from the TS genes *ligA*_{Ph(37°)}, *ligA*_{L17(39°)}, and *pyrG*_{Cp(39°)}, were named PT13a Ω *ligA*-37, PT13a Ω *ligA*-38, and PT13a Ω *pyrG*-39, respectively. Double mutants incorporating both *ligA* and *pyrG*_{Cp(39°)} alleles were also created and were named PT13a Ω *ligA* Ω *pyrG*-37 and PT13a Ω *ligA* Ω *pyrG*-38.

Determination of the restrictive temperatures of TS strains. The TS PT13a strains were inoculated onto agar medium and incubated at different temperatures to test the temperature limits of growth. The plates were

immersed in about 1 kg of aluminum pellets to buffer temperature fluctuations, and the maximum and minimum temperatures were recorded by a Fluke 53II thermometer ($\pm 0.05\%$ accuracy). The restriction temperature assigned to each strain was designated as 1°C higher than the highest temperature that permitted formation of individual colonies following 48 h of incubation.

Intramacrophage and broth growth. Intracellular growth assays were performed essentially as described previously (26), using the mouse macrophage-like cell line J774. Growth of bacterial strains and temperature shifts were done as previously described (27). PT13a strains were grown in microtiter plates, and the A_{595} was measured every 15 min with a BioTek EL808 plate reader.

Rate of mutation to temperature resistance. Reversion rate experiments were performed essentially as previously described (27), and reversion rates were calculated, in essence, by dividing the number of CFU that appeared at the restrictive temperature by the number of CFU that arose at a permissive temperature.

Viability staining and flow cytometry. A combination of stains allows one to assess the relative proportions of live and dead bacteria in a culture. TS and control strains of *S. enterica* were grown at 30°C to mid-exponential phase. Each strain was then diluted 1:20 in duplicate in LB broth, with one duplicate incubated at 30°C and the other incubated at 39°C or 42°C, with shaking, for 12 h. After growth, each culture was diluted to an A_{595} of 1.0 and subsequently serially diluted to approximately 1×10^6 cells/ml. Next, 1 ml of each strain was centrifuged, washed, and resuspended in 1 ml of 0.85% NaCl solution. The fluorescent dyes DMAO and EthD-III were used alone or in combination (Biotium Viability/Cytotoxicity Assay for Bacteria Live & Dead Cells kit) according to the manufacturer's instructions.

After staining, bacteria were analyzed with a FACSCalibur flow cytometry system (BD Biosciences) equipped with an argon laser (488 nm) at 15 mW. Green fluorescence (live bacteria) was collected in the FL1 (fluorescein isothiocyanate [FITC]) channel, and red fluorescence (dead bacteria) was collected in the FL2 (phycoerythrin [PE]) channel. All parameters were collected as logarithmic signals. Population concentrations were estimated using CellQuestPro and FloJo Ver 9.6.3 software.

Dissemination of TS PT13a in mice. All mouse experiments followed the guidelines of the Canadian Council on Animal Care. Seven-week-old female BALB/cByJ mice were inoculated with PT13a strains subcutaneously at the base of the tail. At various times postinoculation, mice were euthanized and tissues or organs harvested to determine bacterial burdens as previously described (27).

Oral and i.m. injections of chickens with PT13a Ω *pyrG*-39. All experiments involving chickens were carried out with the oversight of the Zoetis institutional animal care and use committee and in compliance with national legislation. Each treatment consisted of 60 Charles River specific-pathogen-free (SPF) line 22 White Leghorn chickens that were either not vaccinated or injected intramuscularly (i.m.) in the right breast muscle or orally gavaged in the crop with 0.1 ml of 10^8 -CFU/dose PT13a Ω *pyrG*-39. After vaccination, 15 birds each were placed in 4 replicated positive-pressure isolators per treatment. For assessment of antibody production, a subset of 1 bird/isolator (4 birds/treatment) was bled at 9, 12, and 16 weeks.

At 16 weeks, birds were challenged with 0.1 ml 10^8 -CFU/dose PT13a in the crop. To assess colonization, 10 g of each organ, including muscle (~ 5 g each of the left and right breasts), the organ pool (consisting of ~ 3.3 g each of liver, spleen, and kidney), the reproductive tract (consisting of the ovaries and oviduct), the intestinal pool (consisting of ~ 3.3 g each of the duodenal loop, the ileum and jejunum at Meckel's diverticulum, and the cecal tonsils), and cecal contents, was sampled and plated on agar medium for viable count determinations. To determine reductions in colonization, all plate counts and results were compared to those for non-vaccinated birds. Statistical analysis of log-transformed colonization counts was determined using a mixed linear model with repeated measures with fixed effects.

FIG 1 Diagrammatic representation of allelic replacements of the psychrophilic essential genes *ligA_{Ph}(37°C)* (A), *ligA_{L17}(39°C)* (B), and *pyrG_{Cp}(39°C)* (C) in the *S. enterica* chromosome. Flanking regions of the *S. enterica* essential gene were fused to the psychrophilic essential gene at various points within the native gene. A single underline indicates the *S. enterica* sequence, and a double underline indicates the psychrophilic gene and deduced amino acid sequences. Numbers indicate codon positions.

Nucleotide sequence accession numbers. The native gene sequence of *ligA_{L17}(39°C)* was submitted to GenBank under accession no. [KP101613](#); the codon-optimized synthetic version of the gene was submitted under GenBank accession no. [KP101610](#). The sequences of the TS gene insertions and their immediate flanking regions were deposited in GenBank under accession numbers [KP101611](#), [KP101610](#), and [KP101612](#).

RESULTS AND DISCUSSION

Isolation of a *ligA* allele expressing a TS product. We had previously isolated three *ligA* alleles from psychrophilic bacteria that produced products that were inactivated in the range of 33 to 37°C (27). Since one potential application of this research is to construct a vaccine for poultry, which have body temperatures in the range of 39 to 41°C, we wanted to identify a *ligA* allele that expressed a product that was inactivated at temperatures above 37°C. To accomplish this, we screened several DNA samples from psychrophilic bacteria collected from the Arctic and Pacific Oceans, using PCR amplification of putative *ligA* alleles. We designed primers against the *ligA* genes of *Moritella*, *Pseudoalteromonas*, *Colwellia*, and *Vibrio* species and used these for PCR. Several amplicons of the appropriate sizes were cloned and transformed into an *S. enterica* strain with a deletion of the essential *ligA* gene, which was complemented by plasmid-borne *S. enterica* *ligA* (14). After repeated subculturing of transformants, with antibiotic selection for the plasmid carrying the putative psychrophilic *ligA* gene, strains were screened for temperature sensitivity. We found four TS clones, but only one was inactive at temperatures above 37°C, and this was designated *ligA_{L17}(39°C)*. This gene expressed a deduced product of 673 amino acids, which is 1 amino acid longer than the *S. enterica* homologue and 1 amino acid shorter than the *P. haloplanktis* *ligA* product. The proteins encoded by *ligA_{L17}(39°C)* and *ligA_{Ph}(37°C)* show 92% identity, suggesting a close relationship between the bacteria that served as the source of these *ligA* alleles. Both deduced products of the psychro-

philic alleles showed 61% amino acid identity to the *S. enterica* homologue.

Construction of TS PT13a strains. We had previously shown that substitution of essential genes from psychrophilic bacteria for the homologues in the *Francisella novicida* chromosome often resulted in strains that were TS for growth and unable to revert at their restrictive temperatures (27). We applied this approach to construct TS PT13a strains, although in this study we used synthetic, codon-optimized genes to encode psychrophilic gene products.

To create strains expressing an essential gene product, we designed a synthetic DNA fragment that included the psychrophilic essential gene and surrounded the essential gene with *S. enterica* sequences that naturally flank the *S. enterica* homologue. For the two psychrophilic *ligA* genes used in this study, we designed the juncture between the upstream *S. enterica* region and the *ligA* open reading frame (ORF) so that the native *S. enterica* transcription and translation control elements would govern expression of the substituted essential genes (Fig. 1). We calculated the strengths of the RBS before and after insertion of the psychrophilic essential genes and found that they were the same, within the margin of error generated by the calculation algorithm (18). The regions of the *ligA* ORFs corresponding to the psychrophilic genes were designed to have codon usage patterns optimized for *S. enterica*.

For the *S. enterica* strains carrying the *pyrG* gene from *Colwellia psychrerythraea*, we modified our design to include the first 72 codons of the *S. enterica* *pyrG* gene. In some bacteria, *pyrG* is regulated by transcriptional attenuation in addition to the usual transcription initiation control (28, 29). When we previously attempted to introduce the *pyrG_{Cp}* gene into *F. novicida*, we repeatedly found that the only viable recombinant had created a hybrid *pyrG* gene that incorporated 72 codons of *F. novicida* *pyrG* (27). Thus, we deliberately created a similar hybrid *Salmonella-Col-*

FIG 2 Restrictive temperature of PT13a Δ pyrG-39. Both PT13a and the TS mutant PT13a Δ pyrG-39 were streaked onto a single agar plate and immersed in 1 kg of aluminum pellets at 40.7°C for 48 h. The TS mutant PT13a Δ pyrG-39 showed growth in the heavy part of the streak but did not produce individual colonies.

wellia *pyrG* gene in PT13a. The portion of the hybrid gene derived from *Colwellia* was codon optimized for *S. enterica*.

Replacement of the *S. enterica* homologue with the synthetic psychrophilic gene was confirmed by genotyping the resulting strain, using PCR analysis and DNA sequencing of the affected region of the chromosome. Our sequencing results confirmed the predicted gene substitutions (Fig. 1) and showed that there was no residual sequence from the replaced PT13a gene or the cloning plasmid. The successful single- and double-gene replacements are listed in Table 1.

The same series of TS gene substitutions were made in *S. enterica* serovar Typhimurium DT104 (Table 1), but these strains were not studied beyond genotyping and temperature phenotyping. Because the DT104 strain is multiply drug resistant, the TS strains may be useful as safe research or diagnostic strains.

TS phenotypes imparted by psychrophilic gene substitutions. In order to clearly define a restrictive temperature, we used the inability to form colonies on agar plates at a particular temperature as the criterion for viability (Fig. 2 and data not shown). Using this approach, we found that the TS *S. enterica* strains PT13a Δ ligA-37, PT13a Δ ligA-38, and PT13a Δ pyrG-39 had restrictive temperatures of 37°C, 38.5°C, and 39°C, respectively (Table 1).

Strains carrying psychrophilic *ligA* alleles had clearly defined restrictive temperatures as assessed by growth on petri plates. As expected from previous experience using *ligA* alleles in *F. novicida*, both PT13a Δ ligA-37 and PT13a Δ ligA-38 had a clear growth arrest at their restrictive temperatures, but strains carrying the *pyrG*_{CP(39°)} allele had a less pronounced temperature cutoff point (Fig. 2). Agar plates inoculated with PT13a Δ pyrG-39 showed strong growth in areas of the plate that were heavily inoculated but did not produce individual colonies.

In addition to determining the viability of TS strains of *S. enterica* through their ability to form colonies on agar medium at different temperatures, we wanted to assess their presumed viability at different temperatures through an independent approach. To do this, we used a combination of fluorescent nucleic acid dyes, one of which stains both dead and live bacteria (green fluorescence) and the other of which stains only bacteria with damaged cell membranes (red fluorescence), which are presumed to be

dead. A flow cytometer was used to quantify the proportions of bacteria that were stained with one or both dyes. When these dyes were used to stain TS *S. enterica* strains that had been cultivated for 12 h at either a permissive or restrictive temperature, we obtained the results shown in Fig. 3. Normally one would expect healthy cells to generate a pattern where most fluorescence events are placed in the lower right quadrant of the fluorescence sorter dot plot, and the results shown in Fig. 3 appear to be skewed such that an unusual number of events are recorded toward the lower left quadrant. We suggest that the PT13a strain, as a recent clinical isolate, has protective outer structures, such as O antigen and capsule, that inhibit the penetration of the normally permeant green fluorescent dye into the cell. Furthermore, we posit that when the cell wall structures are damaged, both the permeant green dye and the nonpermeant red dye can enter the cell more easily, resulting in a staining pattern with most of the fluorescence events lying in the upper right quadrant.

In the control experiment using the parental *S. enterica* PT13a strain, we found the same, overwhelming proportion of cells staining with the green dye, indicating that they were viable (Fig. 3A and B). However, for the TS strains PT13a Δ ligA-37 and PT13a Δ ligA-38, we found that the majority of cells were stained only with the green dye (alive) when these strains were grown at 30°C but that the majority were stained with both dyes (dead) when these strains were grown at 39°C, a restrictive temperature for both strains (Fig. 3C to F). Interestingly, the reaction of the PT13a Δ pyrG-39 strain with the dyes suggested that the majority of the bacteria did not have damaged cell membranes even after being cultivated at a restrictive temperature for 12 h (Fig. 3G and H), and this was consistent with our observation that the PT13a Δ pyrG-39 strain seemed to die slowly (see Fig. 4C). Experiments were also performed in which no dye was added to the PT13a parental strain (Fig. 3I), the green dye alone was added to PT13a (Fig. 3J), or the red dye alone was added to heat-killed (80°C, 90 min) PT13a (Fig. 3K). While the green dye-stained PT13a strain yielded a fluorescence distribution pattern similar to those for the other experiments performed with cells grown at permissive temperatures, the red dye appeared to stain the heat-killed cells poorly, suggesting that the mechanism of cell death affects the penetration of the dye into the cell.

Stability of the TS phenotype. For a live vaccine to be safe, the attenuated phenotype cannot revert to the wild-type form at an appreciable level. To test the genetic stability of the psychrophilic essential genes, we measured the appearance of colonies on agar plates at restrictive versus permissive temperatures. For strains PT13a Δ ligA-37 and PT13a Δ ligA-38, we did not detect any “revertants” that grew at a restrictive temperature, and we calculated the theoretical reversion rates for these strains to be less than 1.6×10^{-10} and 1.4×10^{-10} , respectively. However, the PT13a Δ pyrG-39 strain displayed an unusual phenomenon. When cells were plated at a high density and incubated at a restrictive temperature, there was approximately 1 colony per 1×10^7 cells plated. However, when colonies of presumptive temperature-resistant revertants were spread (“streaked”) on agar medium, they showed a phenotype identical to that of the parental, TS strain (Fig. 2). Similarly, when these colonies were inoculated into broth cultures, they behaved like the parental strain. Thus, we were unable to find any colonies that behaved as temperature-resistant mutants, and sequencing of the *pyrG* allele revealed a DNA sequence identical to that of the temperature-sensitive parental strain, PT13a Δ pyrG-39.

FIG 3 Distribution of presumed live (green dye staining) and dead (green and red dye staining) *TS. enterica* cultures grown at permissive (A, C, E, and G) and restrictive (B, D, F, and H) temperatures. (I) PT13a with no staining. (J) PT13a stained with the green dye only. (K) Heat-killed (80°C, 90 min) PT13a stained with the red dye only. PT13a killed with either heat or isopropyl alcohol (not shown) took up the red dye poorly.

In previous work (27), we noticed that the *F. novicida* strain carrying the psychrophilic *pyrG* gene generated pseudo-temperature-resistant mutants, similar to what we observed with PT13a Ω pyrG-39. Interestingly, a similar observation was recently made by Gallagher et al. (30), who found that *E. coli* strains with tightly controlled essential genes generated colonies on nonper-

missive agar medium. Close examination of the presumed mutants revealed that they were a form of phenotypic persisting cells that retained the genotype and phenotype of the original cells when regrown. These authors speculated that these cells might be analogous to bacterial cells that persist after being exposed to antibiotic selection (31).

FIG 4 Growth characteristics of TS *S. enterica* strains. (A to C) Growth of TS *S. enterica* strains in bacteriological broth before and after the shift to a restrictive temperature at the 4-h time point. The small differences in the growth characteristics of PT13a parent strain and PT13a Δ pyrG-39 for growth at 43°C (inset in panel C). Unpaired two-way analysis of variance (ANOVA) for comparisons of the parent *S. enterica* strain and the TS strains yielded *P* values of <0.0001 for panels A and B, <0.001 for panel C, and 0.001 for the inset in panel C (43°C). (D to F) Growth of TS *S. enterica* strains in J774A.1 macrophage-like cells before and after the shift to a restrictive temperature at the 6-h time point. Separate *P* values were generated for comparisons of the graphs for data points from 0 through 6 h and 6 through 12 h and are as follows: for panel D, 0.58 and 0.002; for panel E, 0.27 and 0.02 (11-h time point only; calculated by the Student *t* test); and for panel F, 0.09 and 0.53. Error bars in all panels (not always visible) indicate standard errors of the means (SEM).

Growth of TS *S. enterica* strains in broth. We had previously observed that TS *F. novicida* strains carrying a psychrophilic *ligA* substitution abruptly stopped growth at their restrictive temperatures, whereas those carrying a psychrophilic *pyrG* allele continued to grow (increased culture turbidity) well after a shift to a nonpermissive temperature. We found the same pattern with *S. enterica* strains carrying psychrophilic *pyrG* alleles. When broth cultures of PT13a Δ ligA-37 or PT13a Δ ligA-38 were shifted from 30°C to a restrictive temperature (39°C), growth ceased quickly (Fig. 4A and B). However, when PT13a Δ pyrG-39 was shifted from 30 to 39°C, the culture turbidity still increased significantly, although growth was slow compared to that of wild-type *S. enterica* (Fig. 4C). A more pronounced slowing of growth of the

PT13a Δ pyrG-39 strain was observed when it was grown at 43°C, but even at this temperature, some residual growth occurred (Fig. 4C, inset). Indeed, our data make it difficult to differentiate between live and dead forms of PT13a Δ pyrG-39 at temperatures above 39°C; thus, we relied on the inability of this strain to form colonies to indicate that it was “dead,” even though it may have been metabolically active for many hours at 39°C or higher temperatures.

Growth of *S. enterica* strains in J774 macrophage-like cells. In an infected animal, *S. enterica* is found both extracellularly and intracellularly. Since the intracellular environment is an important niche for *S. enterica*, we tested the growth of TS strains before and after a shift to a restrictive temperature. The number of viable

FIG 5 Dissemination of TS *S. enterica* strains in mice. BALB/c mice were injected subcutaneously at the base of the tail. Bacterial burdens were then determined both at the site of injection and in the liver and spleen homogenate to analyze both bacterial persistence and dissemination at various time points ($n = 4$ mice per time point). The limit of detection was 50 CFU, and error bars (not always visible) indicate SEM.

bacterial cells was assayed by plating lysed macrophage cultures on agar medium at 30°C. Both TS strains PT13a Ω ligA-37 and PT13a Ω ligA-38 showed rapid declines in viability after a shift to 39°C. By 6 h, viability was about 1,000-fold less than that of the parental strain (Fig. 4D and E). The temperature shift had no effect on the number of viable PT13a Ω pyrG-39 cells, consistent with the growth phenotype of broth cultures (Fig. 4F).

Survival and dissemination of TS *S. enterica* in mice. A desirable property of TS live vaccines is an ability to grow sufficiently to induce a protective immune response without causing morbidity in the host. A potential advantage of a TS vaccine is that it can be engineered to grow in tissue at a cool site in the body but not be able to grow in internal organs in the warm body core. To examine how TS *S. enterica* strains would survive and grow in a host animal, we infected BALB/c mice at the base of the tail and measured dissemination to the liver and spleen. At the tail site of injection, the number of wild-type PT13a cells declined about 4-fold over the first 4 days and then increased about 5-fold by day 7. Bacteria were detectable to the last time point, 28 days after injection. The

bacteria disseminated quickly to the liver and spleen, where they showed a growth and persistence pattern (Fig. 5A) similar to that found for the tail injection site. When the PT13a Ω ligA-37 strain was inoculated in the same fashion, it was eliminated from the site of infection within 1 day and was never detectable in the liver-plus-spleen homogenate (Fig. 5B; note the different time scale). The points on the graph at 50 CFU (the limit of detection) represent no detectable bacteria. The PT13a Ω ligA-38 strain, which has a restrictive temperature about 1.5°C higher than that of the PT13a Ω ligA-37 strain, was detectable at 4 days, but not 7 days, postinoculation (Fig. 5C). It was never found in the liver-plus-spleen homogenate. In contrast, the PT13a Ω pyrG-39 strain was detectable at the cooler tail site of inoculation for up to 21 days, and we detected low levels of this strain in the liver-plus-spleen homogenate on days 21 and 28 postinoculation (Fig. 5D). On day 28, one of four mice had detectable levels of PT13a Ω pyrG-39 in the liver-plus-spleen homogenate. A strain with two psychrophilic gene substitutions, PT13a Ω ligA Ω pyrG-37, had a pattern of persistence similar to that of the strain with just the *ligA*_{Ph(37°)} substi-

TABLE 2 Efficacy of vaccination with PT13a Ω pyrG-39 in reducing colonization with *Salmonella*, measured at 1 week postchallenge

Treatment	% of birds colonized ^a			
	Organ pool	Ceca	Intestinal tract	Reproductive tract
Placebo	100	100	100	92.9
PT13a Ω pyrG-39 (1×10^8 CFU/dose; i.m.)	92.9	96.4	96.4	28.6*
PT13a Ω pyrG-39 (1×10^8 CFU/dose; oral)	100	100	96.4	57.1*

^a *, significantly different ($P \leq 0.05$) from the placebo treatment.

tution (Fig. 5E). While it is uncertain whether the subtle differences in persistence of the PT13a Ω ligA-37 and PT13a Ω ligA-38 strains are biologically significant, it is clear that the PT13a Ω pyrG-39 strain persists substantially longer in infected mice than either of the strains with gene substitutions at the *ligA* locus.

The diminished persistence of the TS *S. enterica* strains in mice could be due to their temperature sensitivity, a reduced fitness separate from the TS phenotype, or a combination of these two properties. With all of the TS strains, we were able to detect bacteria at the site of injection in the tail, a cool body site, longer than we were able to detect bacteria in the liver and spleen, which are maintained at about 37°C. The parental PT13a strain persisted equally long at the tail injection site and in the liver and spleen. Cumulatively, these data suggest that the TS phenotype played a significant role in the elimination of the TS PT13a strains, but we cannot rule out an effect of a possible reduced fitness of these strains that is independent of the TS phenotype.

Vaccination of chickens with PT13a Ω pyrG-39. Our mouse studies suggested that only the *Salmonella* strain with the *pyrG*_{CP(39°)} allele persisted for a significant period and therefore was the best candidate for inducing a robust immune response. We thus used the PT13a Ω pyrG-39 strain to vaccinate chickens to test if this would reduce the level of colonization of different organs when chickens were challenged with wild-type PT13a. When a 10^8 -CFU inoculum of PT13a Ω pyrG-39 was used in an i.m. or oral vaccination regimen, neither route reduced the colonization of the ceca or the intestinal tract by a statistically significant amount (Table 2). However, i.m. and oral vaccinations reduced the reproductive tract colonization by 64% and 36%, respectively (Table 2). Chickens vaccinated by either route or chickens vaccinated with phosphate-buffered saline (PBS) did not show anti-*Salmonella* reactive antibodies at 9 or 12 weeks, but 4% of placebo-vaccinated and 86% of i.m. vaccinated chickens showed reactive antibodies 16 weeks following the inoculations. The PT13a Ω pyrG-39 vaccine strain was not detectable at the site of inoculation by agar plating 16 weeks after injection, but it may have persisted elsewhere in the chickens' bodies.

In this work, we showed that we could create TS strains of *S. enterica* clinical isolates by substituting synthetic genes based on the deduced amino acid sequences of essential genes of psychrophilic bacteria for the native homologues. Furthermore, we showed that the TS phenotype imparted by a single gene substitution was extremely stable, with theoretical rates of reversion to temperature resistance calculated to be below 10^{-10} per generation. We also succeeded in creating TS forms of *S. enterica* that

incorporated two psychrophilic essential gene substitutions, and their rate of reversion to temperature resistance is presumably the product of the reversion rates for the two individual psychrophilic genes.

The TS strains of *S. enterica* described in this work were derived from the PT13a parent, which is a common phage type of *S. enterica* associated with human infection in North America (32). Although it was not discussed in detail, we also created three strains with a single psychrophilic gene substitution and one strain with two psychrophilic gene substitutions in the *S. Typhimurium* DT104 background. DT104 strains are associated with epidemics in animals and humans and are multiply drug resistant (33). Thus, the collection of TS *S. enterica* strains that we have generated are based on clinically important strains and can be used as safe strains in research or diagnostic laboratories or tested for their value in stimulating a protective immune response.

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F. E. Nano owns the intellectual property associated with the psychrophilic gene technology, and B. N. Duplant is the owner of Duvax Vaccines and Reagents Inc., which has been granted a license for this technology.

REFERENCES

- Buckle GC, Walker CLF, Black RE. 2012. Typhoid fever and paratyphoid fever: systematic review to estimate global morbidity and mortality for 2010. *J Glob Health* 2:010401. <http://dx.doi.org/10.7189/jogh.02.010401>.
- Crump JA, Luby SP, Mintz ED. 2004. The global burden of typhoid fever. *Bull World Health Organ* 82:346–353.
- Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis* 50:882–889. <http://dx.doi.org/10.1086/650733>.
- Lister SA. 1994. *Salmonella* enteritidis in broiler breeders. *Vet Rec* 134:659.
- DuPont HL. 2007. The growing threat of foodborne bacterial enteropathogens of animal origin. *Clin Infect Dis* 45:1353–1361. <http://dx.doi.org/10.1086/522662>.
- Rabsch W, Tschäpe H, Bäumler AJ. 2001. Non-typhoidal salmonellosis: emerging problems. *Microbes Infect* 3:237–247. [http://dx.doi.org/10.1016/S1286-4579\(01\)01375-2](http://dx.doi.org/10.1016/S1286-4579(01)01375-2).
- European Food Safety Authority (EFSA). 2011. *Salmonella*. www.efsa.europa.eu/en/topics/topic/salmonella.htm.
- Cerquetti MC, Gherardi MM. 2000. Vaccination of chickens with a temperature-sensitive mutant of *Salmonella enteritidis*. *Vaccine* 18:1140–1145. [http://dx.doi.org/10.1016/S0264-410X\(99\)00377-1](http://dx.doi.org/10.1016/S0264-410X(99)00377-1).
- Karasova D, Sebkova A, Vrbas V, Havlickova H, Sisak F, Rychlik I. 2009. Comparative analysis of *Salmonella enterica* serovar Enteritidis mutants with a vaccine potential. *Vaccine* 27:5265–5270. <http://dx.doi.org/10.1016/j.vaccine.2009.06.060>.
- Babu U, Scott M, Myers MJ, Okamura M, Gaines D, Yancy HF, Lillehoj H, Heckert RA, Raybourne RB. 2003. Effects of live attenuated and killed *Salmonella* vaccine on T-lymphocyte mediated immunity in laying hens. *Vet Immunol Immunopathol* 91:39–44. [http://dx.doi.org/10.1016/S0165-2427\(02\)00265-9](http://dx.doi.org/10.1016/S0165-2427(02)00265-9).
- Nandre RM, Matsuda K, Chaudhari AA, Kim B, Lee JH. 2012. A genetically engineered derivative of *Salmonella* Enteritidis as a novel live vaccine candidate for salmonellosis in chickens. *Res Vet Sci* 93:596–603. <http://dx.doi.org/10.1016/j.rvsc.2011.11.005>.
- Médigue C, Krin E, Pascal G, Barbe V, Bernsel A, Bertin PN, Cheung F, Cruveiller S, D'Amico S, Duilio A, Fang G, Feller G, Ho C, Mangelot S, Marino G, Nilsson J, Parrilli E, Rocha EPC, Rouy Z, Sekowska A, Tutino ML, Vallenet D, von Heijne G, Danchin A. 2005. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudo-*

- alteromonas haloplanktis* TAC125. Genome Res 15:1325–1335. <http://dx.doi.org/10.1101/gr.4126905>.
13. Methé BA, Nelson KE, Deming JW, Momen B, Melamud E, Zhang X, Moulton J, Madupu R, Nelson WC, Dodson RJ, Brinkac LM, Daugherty SC, Durkin AS, DeBoy RT, Kolonay JF, Sullivan SA, Zhou L, Daviden TM, Wu M, Huston AL, Lewis M, Weaver B, Weidman JF, Khouri H, Utterback TR, Feldblyum TV, Fraser CM. 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. Proc Natl Acad Sci U S A 102:10913–10928. <http://dx.doi.org/10.1073/pnas.0504766102>.
 14. Park UE, Olivera BM, Hughes KT, Roth JR, Hillyard DR. 1989. DNA ligase and the pyridine nucleotide cycle in *Salmonella typhimurium*. J Bacteriol 171:2173–2180.
 15. Sharp PM, Li W. 1987. The codon adaptation index—a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res 15:1281–1295. <http://dx.doi.org/10.1093/nar/15.3.1281>.
 16. Kudla G, Murray AW, Tollervey D, Plotkin JB. 2009. Coding sequence determinants of gene expression in *Escherichia coli*. Science 324:255–258. <http://dx.doi.org/10.1126/science.1170160>.
 17. Na D, Lee D. 2010. RBSDesigner: software for designing synthetic ribosome binding sites that yields a desired level of protein expression. Bioinformatics 26:2633–2634. <http://dx.doi.org/10.1093/bioinformatics/btq458>.
 18. Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 27: 946–950. <http://dx.doi.org/10.1038/nbt.1568>.
 19. Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19–27.
 20. Orr-Weaver TL, Szostak JW, Rothstein RJ. 1981. Yeast transformation: a model system for the study of recombination. Proc Natl Acad Sci U S A 78:6354–6358. <http://dx.doi.org/10.1073/pnas.78.10.6354>.
 21. White AP, Allen-Vercos E, Jones BW, DeVinney R, Kay WW, Surette MG. 2007. An efficient system for markerless gene replacement applicable in a wide variety of enterobacterial species. Can J Microbiol 53:56–62. <http://dx.doi.org/10.1139/w06-102>.
 22. Hashimoto-Gotoh T, Franklin F, Nordheim ATK. 1981. Specific-purpose plasmid cloning vectors. I. Low copy number, temperature-sensitive, mobilization-defective pSC101-derived containment vectors. Gene 16:227–235.
 23. Alexeyev M. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. Biotechniques 26:824–827.
 24. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat Biotechnol 1:784–791. <http://dx.doi.org/10.1038/nbt1183-784>.
 25. Balbas P, Alexeyev M, Shokolenko I, Bolivar FV. 1996. A pBRINT family of plasmids for integration of cloned DNA into the *Escherichia coli* chromosome. Gene 172:65–69. [http://dx.doi.org/10.1016/0378-1119\(96\)00028-5](http://dx.doi.org/10.1016/0378-1119(96)00028-5).
 26. Schmerk CL, Duplantis BN, Howard PL, Nano FE. 2009. A *Francisella novicida* *pdpa* mutant exhibits limited intracellular replication and remains associated with the lysosomal marker LAMP-1. Microbiology 155: 1498–1504. <http://dx.doi.org/10.1099/mic.0.025445-0>.
 27. Duplantis BN, Osusky M, Schmerk CL, Ross DR, Bosio CM, Nano FE. 2010. Essential genes from Arctic bacteria used to construct stable, temperature-sensitive bacterial vaccines. Proc Natl Acad Sci U S A 107:13456–13460. <http://dx.doi.org/10.1073/pnas.1004119107>.
 28. Meng Q, Switzer RL. 2001. Regulation of transcription of the *Bacillus subtilis* *pyrG* gene, encoding cytidine triphosphate synthetase. J Bacteriol 183:5513–5522. <http://dx.doi.org/10.1128/JB.183.19.5513-5522.2001>.
 29. Meng Q, Switzer RL. 2002. *cis*-Acting sequences of *Bacillus subtilis* *pyrG* mRNA essential for regulation by antitermination. J Bacteriol 184:6734–6738. <http://dx.doi.org/10.1128/JB.184.23.6734-6738.2002>.
 30. Gallagher RR, Patel JR, Interiano AL, Rovner AJ, Isaacs FJ. 2015. Multilayered genetic safeguards limit growth of microorganisms to defined environments. Nucleic Acids Res 43:1945–1954. <http://dx.doi.org/10.1093/nar/gku1378>.
 31. Vega NM, Allison KR, Khalil AS, Collins JJ. 2012. Signaling-mediated bacterial persister formation. Nat Chem Biol 8:431–433. <http://dx.doi.org/10.1038/nchembio.915>.
 32. Hickman-Brenner FW, Stubbs AD, Farmer JJ, III. 1991. Phage typing of *Salmonella enteritidis* in the United States. J Clin Microbiol 29:2817–2823.
 33. Poppe C, Smart N, Khakhria R, Johnson W, Spika J, Prescott J. 1998. *Salmonella typhimurium* DT104: a virulent and drug-resistant pathogen. Can Vet J 39:559–565.