# Essential genes from Arctic bacteria used to construct stable, temperature-sensitive bacterial vaccines

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**All bacteria share a set of evolutionarily conserved essential genes that encode products that are required for viability. The great diversity of environments that bacteria inhabit, including environments at extreme temperatures, place adaptive pressure on essential genes. We sought to use this evolutionary diversity of essential genes to engineer bacterial pathogens to be stably temperature-sensitive, and thus useful as live vaccines. We isolated essential genes from bacteria found in the Arctic and substituted them for their counterparts into pathogens of mammals. We found that substitution of nine different essential genes from psychrophilic (cold-loving) bacteria into mammalian pathogenic bacteria resulted in strains that died below their normal-temperature growth limits. Substitution of three different psychrophilic gene orthologs of *ligA*, which encode NAD-dependent DNA ligase, resulted in bacterial strains that died at 33, 35, and 37 °C. One *ligA* gene was shown to render *Francisella tularensis*, *Salmonella enterica*, and *Mycobacterium smegmatis* temperature-sensitive, demonstrating that this gene functions in both Gram-negative and Gram-positive lineage bacteria. Three temperature-sensitive *F. tularensis* strains were shown to induce protective immunity after vaccination at a cool body site. About half of the genes that could be tested were unable to mutate to temperature-resistant forms at detectable levels. These results show that psychrophilic essential genes can be used to create a unique class of bacterial temperature-sensitive vaccines for important human pathogens, such as *S. enterica* and *Mycobacterium tuberculosis*.**

psychrophile | *Colwellia* | *Francisella* | *Mycobacterium*

All organisms have a group of genes that are indispensible for their viability. Members of the domain Bacteria have a minimal set of about 100 essential genes that they all share, with a few exceptions. Historically, the effort to define essential genes relied on the painstaking process of attempting to delete an individual gene with and without a genetic complement, and showing that a deletion was only possible when another copy of the gene was present. Recently the availability of highly efficient transposon mutagenesis techniques, together with microarray analysis, has accelerated the discovery of candidate essential genes (1–5). Furthermore, the explosion in the number of sequenced bacterial genomes has augmented the identification of probable essential genes by allowing the identification of highly conserved genes that are retained in almost all bacteria (6).

The amino acid sequences of the products of essential genes are highly conserved across all of the bacteria, including homologs found in extremophiles, such as thermophiles (heat-loving) and psychrophiles (cold-loving). Although it is generally assumed that psychrophiles descended from warm-loving relatives, there may have been cold environments on the Earth that harbored psychrophiles for as long as 2 billion years (7). Hence, psychrophilic proteins, including essential proteins, have probably been adapting to cold environments for millions to billions of years. The observed increased heat sensitivity of psychrophilic enzymes may be a result of the changes that lead to enhanced activity at lower temperatures or may be because of changes that were introduced by genetic drift in the absence of selective pressure for thermal stability (8–10); both factors may contribute to the thermal lability of many psychrophilic proteins. Overall, any change that leads to lower enzyme stability, such as fewer salt bridges between protein domains, can be found in psychrophilic enzymes.

The introduction of mutations that make an essential gene product temperature-sensitive (TS) renders the host organism TS. This approach has been used for decades to create TS viruses that are often used as live vaccines (11). Typically, a virus is repeatedly passed in eggs or tissue culture at low temperature, and after several passages a TS form is isolated and used as a vaccine. Prominent examples of TS viral vaccines include the Sabin polio vaccine (12) and the FluMist influenza vaccine (13, 14). Although there are live vaccines for bacterial diseases, most notably the antituberculosis (bacillus Calmette–Guérin) vaccine, these are usually generated by attenuating the virulence of a pathogen by passaging in vitro or by some form of mutagenesis.

Most of the gene substitutions performed in this work were done in the Gram-negative bacterium *Francisella tularensis* subsp. *novicida* (*F. novicida*). This strain of *F. tularensis* is highly virulent in mice but is essentially avirulent in humans. However, most *F. tularensis* strains are highly infectious to humans and cause the potentially lethal, zoonotic disease tularemia (15). *F. tularensis* is a facultative intracellular pathogen that can grow in several cell types, and is frequently found within cells of the monocytic cell lineage in infected animals. Although antibody plays a role in immune protection against *F. tularensis*, it is thought that cell-mediated immunity plays the dominant role in the immune clearance of this organism (16).

In this work we have used the substitution of essential genes from psychrophiles into mesophilic ("moderate temperature-loving") pathogens to create stable, TS strains of bacterial pathogens. We show that these strains can grow in cool sites in the body but fail to disseminate to internal organs. One TS strain was shown to provide protection against a subsequent challenge by a virulent form of the pathogen.

## Results

We chose to use the pathogenic mesophile *F. novicida* as the host for essential genes from the psychrophile *Colwellia psychrerythraea.* The genomic DNA of these Gram-negative bacteria have similar G+C contents (17, 18) of 32.5% (Fn) and 38.0% (Cp), and we thought that this similarity should facilitate heterologous protein expression. The maximum growth temperature of *F. novicida* is about 45 °C and that for *C. psychrerythraea* is about 19 °C.

Author contributions: B.N.D., M.O., D.R.R., C.M.B., and F.E.N. designed research; B.N.D., M.O., C.L.S., D.R.R., and C.M.B. performed research; B.N.D., M.O., C.M.B., and F.E.N. analyzed data; and F.E.N. wrote the paper.

Conflict of interest: A provisional patent application has been filed that covers much of the material in this article. F.E.N. is the inventor and he has assigned rights to the University of Victoria.

This article is a PNAS Direct Submission.

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Data deposition: The sequences of the psychrophilic genes as they appear in the *F. novicida* chromosome have been deposited in the GenBank database (accession nos. HM003397, HM003389, HM003396, HM003395, HM003399, HM003391, HM003398, HM003392, HM003393, HM003394, HM003390, HM003400).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004119107/-/DCSupplemental.

To maximize the chances that the foreign psychrophilic gene would express identically to the homolog that it replaced, we integrated the psychrophilic genes into the *F. novicida* chromosome so that transcription was driven by the host promoter, and translation of the mRNA was directed by the host ribosome binding site (Fig S1 *A–C*). About 60% of the attempts to substitute psychrophilic essential genes into *F. novicida* resulted in strains with a TS phenotype. The precise success rate is difficult to assess, as false-negative results were common and difficult to prove. In some cases the *C. psychrerythraea* gene integrated into the *F. novicida* chromosome in such a way as to form a hybrid between the psychrophilic and mesophilic homologs (Fig. S1 *D* and *E*). The conservation of essential genes and the close G+C content of the DNA of the two bacteria made these events more likely. The temperature inactivation phenotype imparted on *F. novicida* varied with the nine different genes and three orthologs of *ligA* (Table 1).

TS proteins engineered for research purposes often differ from the wild-type form by a single amino acid, and these can revert to temperature-resistance by a change in the variant amino acid or through an amino acid change elsewhere in the protein. Because marine psychrophilic bacteria have been adapting to the cold for at least several million years (7), we reasoned that some essential genes encoding TS products would not be able to mutate to a variant expressing a temperature-resistant product without changing several codons. Supporting this idea, we found that of the nine TS strains that we could test, five were unable to generate temperature-resistant forms at detectable levels (Table 1). Some strains readily yielded temperature-resistant forms, suggesting that many alternative amino acid changes in the psychrophilic gene could lead to temperature resistance. This phenomenon is illustrated for $ligA_{Sf}$, which was able to change to a temperature-resistant form by several different amino acid changes in the N-terminal adenylation domain (8) (Fig. S2). However, for eight independent temperature-resistant mutants of a strain carrying $pyrG_{Cp}$, we were unable to detect any changes to the $pyrG_{Cp}$ gene, suggesting that one or more extragenic suppressor mutations was responsible for the temperature-resistant phenotype. Thus, it is possible that $pyrG_{Cp}$ too is unable to mutate to temperature resistance at a detectable level.

Most of the TS *F. novicida* strains harboring psychrophilic essential genes grew identically to the wild-type strain in broth cultures (Fig. 1 *A–C*). However, as *F. novicida* is a facultative intracellular pathogen, the more important and stringent phenotype is the growth rate in infected macrophages. When the *F. novicida* strains with substitution of $ligA_{Cp}$, $ligA_{Ph}$, and $dnaK_{Cp}$ were used to infect the J774 macrophage-like cell line, we found that the *F. novicida* carrying the psychrophilic *ligA* genes grew like the wild-type strain at permissive temperatures, but that the *F. novicida* strain carrying $dnaK_{Cp}$ grew poorly (Fig. 2 *D–F*). The decline in the number of viable TS *F. novicida* in the macrophages after the shift to the restrictive temperature is a result of the phenotype of the bacterium and not because of death of the macrophages. Microscopic examination of the J774 cells infected with the TS *F. novicida* strains and reacted with a viability stain showed that they were healthy before and after the shift to the higher temperature. However, J774 cells infected with the wild-type *F. novicida* strain showed progressively fewer viable cells as the intracellular growth experiment continued (Fig. S3).

The temperature of the human body is dependent on a number of variables, but there is a significant difference between the temperature of the skin (~32–36 °C) and the body core (~37–40 °C) (19, 20). TS viral vaccines, such as FluMist, take advantage of the anatomical differences in human body temperatures (11, 13, 14).

**Table 1. Psychrophilic genes that induce TS phenotype on Mesophiles**

| Gene symbol* | Temp (°C)† | Mutation rate‡ | Product function |
|---|---|---|---|
| $ligA_{Sf}$ | 33 | $4.0 \times 10^{-6}$ | DNA ligase |
| $ligA_{Cp}$ | 35 | $<1.2 \times 10^{-10}$ | DNA ligase |
| $ligA_{Ph}$ | 37 | $<1.5 \times 10^{-10}$ | DNA ligase |
| $hemC_{Cp}$ | 37 | $<2.5 \times 10^{-10}$ | Heme biosynthesis |
| $pyrG_{Cp}$ | 37 | $8.5 \times 10^{-8}$ § | CTP synthetase |
| $dnaK_{Cp}$ | 38 | $<3.2 \times 10^{-10}$ | Molecular chaperone |
| $murG_{Cp}$ | 38 | $2.6 \times 10^{-4}$ | Muramic acid metabol. |
| $dnaK_{Sf}$ | 39 | $<3.1 \times 10^{-10}$ | Molecular chaperone |
| $fmt_{Cp}$ | 41 | $1.8 \times 10^{-9}$ | Methionyl-tRNA formyltransferase |
| $ftsZ_{Cp}$ | 42 | ND | Cell division protein |
| $cmk_{Cp}$ | 43 | ND | Cytidylate kinase |
| $tyrS_{Cp}$ | 44 | ND | Aminoacyl-tRNA synth. |

ND, Not determined. The restrictive temperature of these strains was too close to that of the wild-type strain to allow an accurate determination of the mutation rate.

*Subscripts indicate bacterial source of psychrophilic gene: CP: *C. psychrerythraea*; PH: *Psuedoalteromonas haloplanktis*; SF: *Shewanella frigidimarina*.

†Indicates restrictive temperature which is the lowest temperature at which no growth is observed.

‡Rate at which mutants arise that are resistant to temperatures 3 °C higher than the restrictive temperature. Note that strains carrying $ligA_{Cp}$ do not generate temperature-resistant strains at 37 °C at a detectable level.

§Mutations that were examined (8/8) were outside of the $pyrG_{Cp}$ gene.

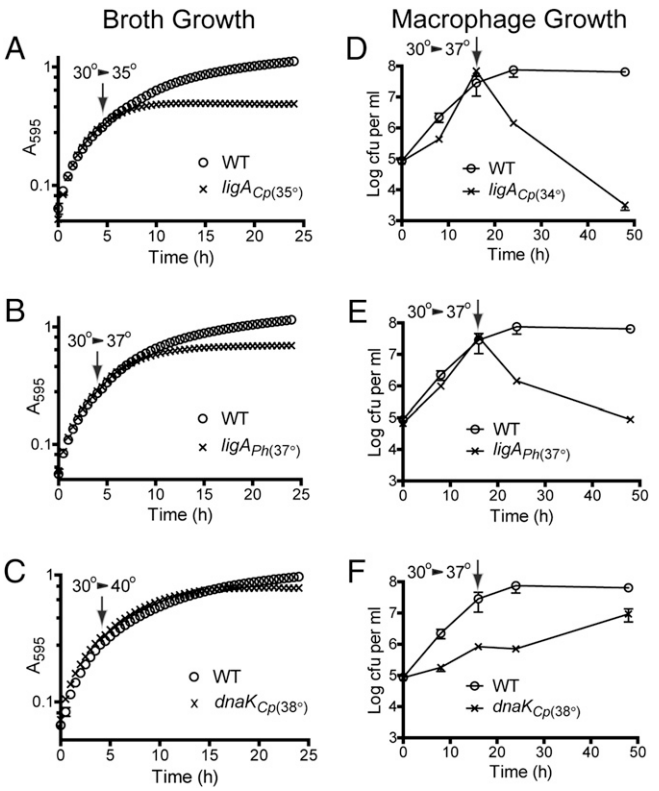


**Fig. 1.** Growth of *F. novicida* strains carrying TS essential genes. (*A–C*) Growth of strains in bacteriological broth before and after shift to restrictive temperatures. The apparent increase in growth rate of the hybrid strain seen in *C* was observed in all three repetitions of this experiment. (*D–F*) Growth of TS strains in J774 macrophage-like cells before and after a shift to restrictive temperature. The *F. novicida* strain harboring the TS *dnaK* always grew poorly in J774 cells at all temperatures tested. Error bars (not visible in some panels) indicate SEMs.

One of the possible uses of a TS pathogenic bacterial strain is as a vaccine that is able to grow in cool tissue near the body surface but unable to proliferate in the warmer body core. To test if our TS *F. novicida* strains had this property, we injected TS strains s.c. at the base of the tail in rats and mice. Rats are able to support high body burdens of *F. novicida* without any apparent morbidity (21), and thus allowed injection of larger numbers of the wild-type strain and strains with higher restrictive temperatures. Following a s.c. injection in rat or mouse tails, those TS *F. novicida* strains with restrictive temperatures at or below 37 °C failed to appear in the spleen, whereas those with restrictive temperatures above 37 °C could be detected in the spleen (Table 2). All strains could be easily found at the site of infection 3 d after injection. Similar results were seen when a TS *F. novicida* strain was injected into the fleshy part of the mouse ear (Fig S4). In contrast, wild-type *F. novicida* appeared in the spleen in large numbers, indicating that it spread from the skin site of injection. These results show that the distribution of TS *F. novicida* in a mammal is determined by the restrictive temperature of the bacterium, and strains with lower restrictive temperatures persist at a cool site in the body.

The persistence of TS *F. novicida* strains at skin sites suggests that they may be able to induce a protective immunity. In the mouse, *F. novicida* is extraordinarily infectious and lethal, with the $LD_{50}$ below 10 CFU (22). To test if TS *F. novicida* strains can induce a protective immune response, we vaccinated mice s.c. at the base of the tail and challenged mice with an intranasal infection 21 d after vaccination. In one experiment, we examined the immunity induced by three TS strains compared with a mock vaccination. Using organ bacterial burden as a measure of protection, we found that TS *F. novicida* strains carrying $ligA_{Cp}$, $ligA_{Ph}$, or $dnaK_{Cp}$ all provided protection against a challenge with wild-type *F. novicida* (Fig. 2 *A* and *B*). Although the *F. novicida* strain carrying $dnaK_{Cp}$ gave the most dissemination, it grew the poorest in macrophages (Fig. 1), and induced the least amount of immunoprotection. These results suggest that dissemination does not necessarily enhance immune stimulation. Because *F. novicida-*$ligA_{Ph}$ appeared to provide the best protection, we used it in a second, larger vaccine-protection study. Once again, s.c. vaccination with this strain provided clear protection against a lethal intranasal challenge (Fig. 2 *C* and *D*), as evidenced by the lower bacterial organ burdens, morbidity, and weight loss afforded to vaccinated mice.

To test if a psychrophilic *ligA* could function in other Gram-negative bacteria, we introduced the $ligA_{Cp}$ gene into a *Salmonella enterica* strain that had an inactivated chromosomal *ligA* (23). The resulting hybrid strain was TS (Fig. 3*A*), indicating that the $ligA_{Cp}$ gene can function in an enteric Gram-negative bacterium, and is thus likely to function in this large group of bacteria that include numerous important pathogens, such as *Salmonella thyphi*, *Escherichia coli*, and *Yersinia pestis* (Fig S5). We also introduced a codon-optimized version of $ligA_{Cp}$ into *Mycobacterium smegmatis*, a research surrogate for *Mycobacterium tuberculosis*. A codon optimized version of $ligA_{Cp}$ was thought to be necessary because the $ligA_{Cp}$ gene is 40% G+C and the *M. smegmatis ligA* gene is 70% G+C. After deleting most of the *M. smegmatis ligA* gene (24), we found that the resulting strain was TS (Fig. 3*B*), indicating that the $ligA_{Cp}$ gene product can function in a Gram-positive lineage bacterium, providing the potential for constructing a stable TS *M. tuberculosis* strain that can be used as a vaccine.

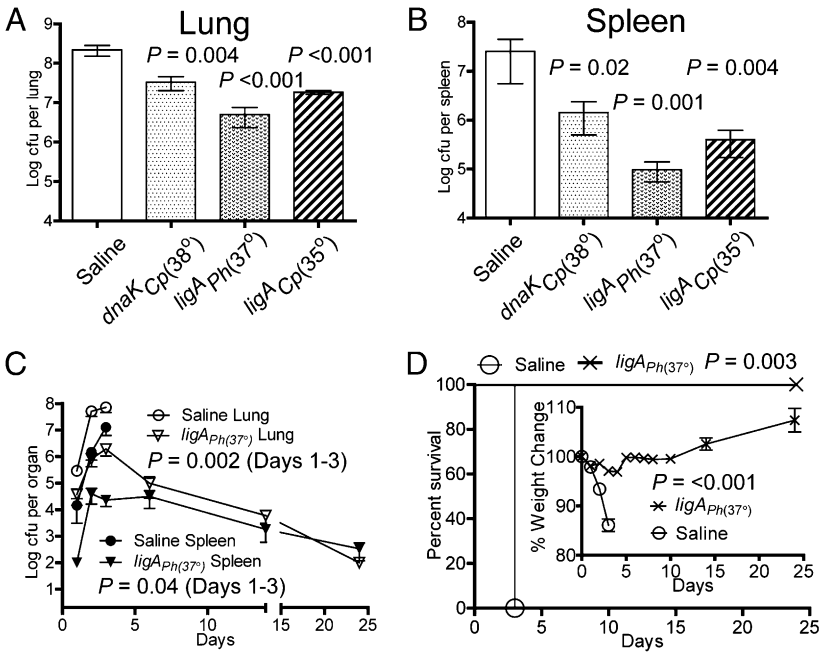


**Fig. 2.** Protective immunity induced by TS *F. novicida* strains. (*A* and *B*) Three strains were tested for their ability to induce a protective immune response, as measured by a reduction in the bacterial burden in the spleen and lungs following a challenge infection. All three strains provided a statistically significant level of protection when compared with a mock vaccination with saline. Vaccinations were via a s.c. injection at the base of the tail, and challenges were via an intranasal instillation of wild-type *F. novicida*. For *A* and *B*, *P* values were calculated with a two-tailed, unpaired *t* test ($n = 3$). (*C*) Protective effect of *F. novicida-*$ligA_{Ph}$ as measured by organ burden vs. time after challenge, as compared with a mock vaccination with saline. Saline vaccinated mice were killed on day 3 postchallenge. On day 24, the lungs of six of six mice yielded no detectable *F. novicida* (limit of detection, 100 CFU per organ). At the same timepoint, one of six spleens had detectable *F. novicida*. *P* values for data plotted for days 1 to 3 were calculated using two-way ANOVA ($n = 4$). (*D*) Survival curve of vaccinated vs. saline-injected mice. Any mouse showing significant morbidity was killed, and none were allowed to die of the effects of the infection. (*Insert*) Weight change of vaccinated vs. saline-injected mice following challenge with wild-type *F. novicida*. *P* values for the survival curve and the weight changes calculated using the Mantel-Cox and the two-way ANOVA test ($n = 4$ for days 1–3), respectively. Data-points for days 6 and 14, $n = 3$; for day 24, $n = 6$.

**Table 2. Dissemination of TS *F. novicida* strains from tail to spleen in Lewis rats and BALB/c mice**

| | Rats* | | Mice† | |
|---|---|---|---|---|
| *F. novicida* strain | CFU/tail | CFU/spleen | CFU/tail (SD) | CFU/spleen (SD) |
| Wild type (45 °C) | $10^4/7 \times 10^3$ | $4 \times 10^6/2 \times 10^6$ | $1 \times 10^5$ ($10^5$) | $2 \times 10^4$ ($2 \times 10^5$) |
| $ligA_{Cp}$(35 °C) | $5 \times 10^2/3 \times 10^2$ | $<10^2/<10^{2‡}$ | $2 \times 10^3$ ($10^4$) | $<10^2$ |
| $ligA_{Ph}$(37 °C) | $3 \times 10^2/2 \times 10^2$ | $<10^2/<10^2$ | $2 \times 10^4$ ($10^4$) | $<10^2$ |
| $dnaK_{Cp}$(38 °C) | $2 \times 10^4/8 \times 10^4$ | $5 \times 10^2/<10^2$ | $3 \times 10^3$ ($2 \times 10^3$) | $5 \times 10^2$ ($9 \times 10^2$) |
| $fmt_{Cp}$(41 °C) | $5 \times 10^3/2 \times 10^3$ | $4 \times 10^5$/2.1x$10^5$ | Not determined | Not determined |

*Two rats injected, and individual organ burdens listed.
†Average from three mice (SD = SD), each injected with about $10^5$ CFU (except wild type, 35 CFU). Organ burdens were determined on day 3 postinfection.
‡The limit of detection is 100 CFU.

## Discussion

In this study we have exploited the conservation of function of bacterial essential genes and the diversity of the temperature stability of these gene products imposed by environmental adaptation. Furthermore, we have taken advantage of the extensive evolution of the psychrophilic essential genes to identify some which are unable to change to a temperature-resistant form through simple mutational changes. Our work focused primarily on essential genes from one psychrophile, C. *psychrerythraea*, but our testing of a few genes from other psychrophiles yielded some that functioned in the mesophile *F. novicida* and rendered it TS. Among the hundreds of psychrophilic bacterial species, there are undoubtedly numerous essential gene products that are inactivated at temperatures that are relevant to biotechnology applications. Given our finding that about one-half of the essential genes are unable to mutate to temperature resistance, it is likely that a high proportion of newly found TS essential genes will also be stably TS. Furthermore, the functioning of the product of $ligA_{Cp}$ in *Francisella*, *Salmonella,* and *Mycobacterium* suggest that some psychrophilic essential genes will function in a wide variety of human pathogenic bacteria, representing both Gram-negative and Gram-positive species and including bacteria with genomes of widely different G+C contents.

We have highlighted in this work the possibility of using psychrophilic essential genes for constructing live, TS vaccines. Live bacterial vaccines are often considered the most efficacious for protecting against diseases that require cell-mediated immunity for clearance. However, the technology described in this work may have applications in other areas, and the type of psychrophilic essential gene used could be tailored to a particular need. Inactivation of a DNA repair enzyme, like DNA ligase, results in bacterial cells that are quickly and irreversibly inactivated. When grown in large bioreactors, such strains could be easily killed at temperatures that would not damage antigenic molecules that might make up the components of a killed whole-cell or subunit vaccine. TS essential genes could be used in any bacterium whenever there is a need to use heat to kill the bacterium at a moderate temperature.

The current effort to study highly infectious select-agent pathogens is hampered by the need for expensive biocontainment facilities. The availability of TS strains that are fully competent at expressing virulence genes at 35 °C, but relatively harmless to humans because of a TS lesion that prevents growth at 37 °C, would allow most of the biochemical and genetic analysis of dangerous pathogens to be performed without the need for full physical containment.

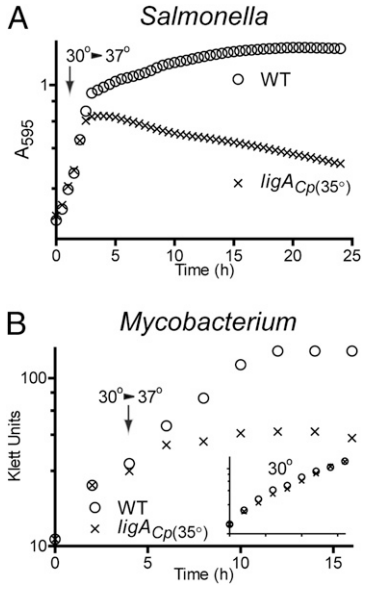


**Fig. 3.** Growth dependence on $ligA_{Cp}$ renders *Salmonella* and *Mycobacterium* TS. (*A*) *S. enterica* ser. Thyphimurium with phage Mu insertion in the chromosomal *ligA* gene showed growth dependence on a plasmid-borne copy of $ligA_{Cp}$, and was TS. Half of the data-points removed for clarity. (*B*) *M. smegmatis* with a deletion of 54% of its *ligA* gene is supported for growth by a plasmid-borne copy of codon-optimized $ligA_{Cp}$.

## Materials and Methods

Bacterial broth growth was done in tryptic soy broth with 0.1% cysteine (TSBC) for *F. novicida*, M7H9 broth with 0.05% Tween-80 for *M. smegmatis*, or in Luria-Bertani broth for *S. enterica*. *F. novicida* mutants were created through the use of overlap PCR to join the psychrophilic homolog gene of interest (18, 25) with the *F. novicida* flanking region. The fused PCR product was ligated to an erythromycin resistant–sucrose hydrolase (*sacB*) cassette (26) and integrated into the *F. novicida* chromosome. Following counter selection with sucrose, erythromycin-sensitive colonies were identified and tested for a TS phenotype. *Salmonella* and *Mycobacterium* TS mutants were created through plasmid complementation of chromosomal insertion or deletion mutants (23, 24). *F. novicida* and *S. enterica* growth curves were performed in the BioTek EL808 plate reader using Gen5 software; *M. smegmatis* growth was measured with a Klett-Summerson colorimeter. Twelve-week-old female BALB/C mice were used for infection experiments, and they were handled in accordance with the guidelines of the Canadian Council on Animal Care. For vaccination, anesthetized mice were injected using a tuberculin syringe s.c. at the base of the tail. Intranasal challenge was via an instillation of 6 μL of bacteria into each nostril; for the experiments in Fig. 2 *A* and *B*, this is equivalent to 88 CFU, and for Fig. 2 *C* and *D* this volume contained 30 CFU. Bacterial organ burden was determined by agar plating of organ homogenates on TSBC agar plates. Intramacrophage growth was assessed by plating infected macrophage lysates on agar medium, as described previously (27) and in *SI Materials and Methods*. The DNA sequences of integrated psychrophilic genes have been submitted to GenBank and have the accession numbers: $ligA_{Sf}$: HM003397; $ligA_{Cp}$: HM003389; $ligA_{Ph}$: HM003396; $hem_{Cp}$: HM003395; $pyrG_{Cp}$: HM003399; $dnaK_{Cp}$: HM003391; $murG_{Cp}$: HM003398; $dnaK_{Sf}$: HM003392; $fmt_{Cp}$: HM003393; $ftsZ_{Cp}$: HM003394;

$cmk_{Cp}$: HM003390; $tyrS_{Cp}$: HM003400. Additional methods are presented in *SI Materials and Methods*.

**ACKNOWLEDGMENTS.** We thank L. S. D. Anthony for assistance with the animal infections, and R. Verrall (Defence Research and Development Canada, Halifax, NS, Canada) for Arctic Ocean samples. This work was supported by Grant 22851 from the Bill and Melinda Gates Foundation Grand Challenges Program, Grant 5R01 AI056212-02 from the National Institutes of Allergy and Infectious Diseases, the Natural Sciences and Engineering Council of Canada, and Grant MOP 89812 from the Canadian Institutes of Health Research (to F.E.N.).

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