Temperature Sensitivity Conferred by *ligA* Alleles from Psychrophilic Bacteria upon Substitution in Mesophilic Bacteria and a Yeast Species

Jarosław A. Pankowski, Stephanie M. Puckett, Francis E. Nano

Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada

We have assembled a collection of 13 psychrophilic *ligA* alleles that can serve as genetic elements for engineering mesophiles to a temperature-sensitive (TS) phenotype. When these *ligA* alleles were substituted into *Francisella novicida*, they conferred a TS phenotype with restrictive temperatures between 33 and 39°C. When the *F. novicida* *ligA* hybrid strains were plated above their restrictive temperatures, eight of them generated temperature-resistant variants. For two alleles, the mutations that led to temperature resistance clustered near the 5' end of the gene, and the mutations increased the predicted strength of the ribosome binding site at least 3-fold. Four *F. novicida* *ligA* hybrid strains generated no temperature-resistant variants at a detectable level. These results suggest that multiple mutations are needed to create temperature-resistant variants of these *ligA* gene products. One *ligA* allele was isolated from a *Colwellia* species that has a maximal growth temperature of 12°C, and this allele supported growth of *F. novicida* only as a hybrid between the psychrophilic and the *F. novicida* *ligA* genes. However, the full psychrophilic gene alone supported the growth of *Salmonella enterica*, imparting a restrictive temperature of 27°C. We also tested two *ligA* alleles from two *Pseudoalteromonas* strains for their ability to support the viability of a *Saccharomyces cerevisiae* strain that lacked its essential gene, *CDC9*, encoding an ATP-dependent DNA ligase. In both cases, the psychrophilic bacterial alleles supported yeast viability and their expression generated TS phenotypes. This collection of *ligA* alleles should be useful in engineering bacteria, and possibly eukaryotic microbes, to predictable TS phenotypes.

One aspect of synthetic biology is the development of genetic elements that can be used for genome engineering (1). These elements include promoters (2, 3), transcriptional enhancers (4), transcriptional stop elements (5), riboswitches (6), or site-specific recombinases (7). Some include reporter genes that encode fluorescent proteins, pigments, or odors (8). Others (9, 10) have proposed that essential genes could be a useful class of genetic elements that might be widely used to engineer the limits of viability of a variety of microbes under a specified restrictive condition, such as high temperature. One application of temperature restriction of growth could be in creating bacterial pathogens that are identical to the wild type in every trait except for growth above a defined temperature, such as 35°C. Such pathogens could be used in research, teaching, and diagnostic antigen preparation with minimal chance of causing invasive disease in humans. Another application is in creating attenuated vaccines, where temperature sensitivity is already a well-established approach for attenuation.

Essential genes are defined as those that are required for the viability of an organism under all growth conditions (11–13). Biologists try to determine an organism's complement of essential genes for the inherent interest in knowing which minimal set of genes are needed for the functioning of a cell or for the practical goal of knowing which gene products are good targets for lethal chemicals, such as antibiotics.

Because essential genes are required for growth, it is difficult to prove that any one gene is essential, since proof of essentiality requires establishing the impossibility of deleting a particular gene. High-throughput approaches, such as saturation mutagenesis (14, 15), have eased the task of identifying essential genes: those genes that fail to tolerate an insertion are suspected of being essential. As well, comparative genomics help identify highly conserved essential genes through their evolutionary retention index (ERI) (16, 17). Finding that a gene is present in all bacteria (ERI of 1) or in a large proportion of bacteria is considered evidence that

a gene is essential. Using this approach, biologists have identified about 600 genes with high ERI values that are good candidates for essential genes (16). Further experiments with single open reading frame (ORF) deletions have refined this to 300 putative essential genes (11, 12). These genes are thought to be essential in nearly all bacteria.

One of the essential genes found in every living organism is *ligA*, which encodes DNA ligase (18, 19). This enzyme is responsible for catalyzing the formation of a phosphodiester bond between the 5' phosphate of one nucleotide and the 3' hydroxyl group of another in the backbone of a DNA strand. The loss of DNA ligase function causes quick formation of double-strand breaks in the DNA structure, followed by exonucleolytic degradation of the fragmented DNA (20). For this reason, DNA ligase has a crucial role in DNA replication, recombination, and repair.

There are two general classes of DNA ligases, the ATP-dependent and the NAD-dependent enzymes (18, 21). The ATP-dependent ligases are a more diverse group that usually are the essential ligases of *Eukarya* and *Archaea*. They are also commonly found in the genomes of viruses and bacteriophages. On the other hand, the NAD-dependent ligases are much more conserved and are almost

Received 2 December 2015 Accepted 8 January 2016

Accepted manuscript posted online 15 January 2016

Citation Pankowski JA, Puckett SM, Nano FE. 2016. Temperature sensitivity conferred by *ligA* alleles from psychrophilic bacteria upon substitution in mesophilic bacteria and a yeast species. *Appl Environ Microbiol* 82:1924–1932. doi:10.1128/AEM.03890-15.

Editor: R. M. Kelly, North Carolina State University

Address correspondence to Francis E. Nano, fnano@uvic.ca.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03890-15>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

uniquely found among *Bacteria*. Usually a bacterial species possesses a single NAD-dependent DNA ligase, but some bacteria also have ATP-dependent ligases (22). However, these are considered to be nonessential, since experiments have shown that the native bacterial ATP-dependent ligases cannot substitute for the loss of the NAD-dependent ligase (22, 23).

The complex eukaryotic cell requires several specialized types of DNA ligases (24). Of these, the ATP-dependent DNA ligase I is the one that has a role most similar to that of the NAD-dependent ligase in bacteria. It is conserved in all eukaryotes and is involved in nuclear DNA replication, repair, and recombination. In *Saccharomyces cerevisiae* and other simple eukaryotes, DNA ligase I is also involved in mitochondrial DNA replication and repair. In vertebrates, DNA ligase III aids in nuclear DNA repair but has its primary function in the mitochondria. The enzyme DNA ligase IV is conserved among all eukaryotes and plays a role in nuclear DNA repair. Because of these specialized roles, ATP-dependent ligases are more diverse than NAD-dependent ligases.

The enzymes found in psychrophilic (cold-loving) bacteria have evolved to function at cold temperatures, and one consequence of this adaptation is that many psychrophilic enzymes are more heat labile than their mesophilic counterparts (25). Predictably, these cold adaptation and heat lability properties extend to essential proteins. In our previous work, we have found that psychrophilic forms of DNA ligase are inactivated in a temperature range appropriate for engineering mesophilic bacterial to temperature-sensitive (TS) forms that can be used for live vaccines (26). In this work, we have discovered new alleles of *ligA* conferring psychrophilic properties and show that they impart a range of TS phenotypes in bacteria. Two alleles encoding NAD-dependent DNA ligase were also shown to confer temperature sensitivity in yeast.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* DH10B (Invitrogen) was used as the host for DNA manipulation experiments. The different *ligA* alleles (designated, e.g., C1, P3, S6) were tested for the phenotype that they confer in a *Francisella novicida* restriction-negative strain (27). To test the full-length *ligA* C1 allele, we used *S. enterica* serovar Typhimurium strain LT2 TT18389 (28) (a gift from John Roth) as the host for recombinant *ligA* C1 carried by a chloramphenicol-resistant plasmid pBC SK+ (Agilent Technologies); the TT18389 strain has a chromosomal *ligA* partial deletion. The *Saccharomyces cerevisiae* YBSΔL1 strain with a deleted CDC9 gene and plasmids expressing CDC9 were the kind gift of Steward Shuman (29).

The *ligA* alleles C2, P6, and S1 and the corresponding *F. novicida* strains carrying these alleles have been described previously (26) and were isolated from the ocean psychrophilic strains *Colwellia psychrerythraea* 34H (C2), *Pseudoalteromonas haloplanktis* TAC125 (P6), and our isolate of *Shewanella frigidimarina* (S1) (26). The other *ligA* alleles were isolated from bacteria found in ocean waters collected at the following global positioning system (GPS) coordinates: C1, 82°32.04N, 62°45.86W; P3, 82°32N, 62°46.4W; S2, 48°20.26N, 123°36.24W; S3, 45°55.24N, 129°59.48W; P4, 35°52.16N, 114°39.73W; P5, 82°32N, 62°46.43W; P7, 64°47.9N, 168°36W; S4, 48°20.26N, 123°36.24W. P1 and P2 were collected from the North Pacific Ocean at an unknown location. The known maximal growth temperatures for the psychrophiles that served as the source of the *ligA* alleles are as follows: C1, 12.5°C; C2, 18°C; P4/P5, ~30°C; P6, 18°C; S1, 27°C; S2, 22°C; and S4, 24°C.

E. coli recombinants were grown at 37°C in Luria broth (30) supplemented with kanamycin (Km; 50 µg/ml) as needed. *F. novicida* strains were grown in tryptic soy broth supplemented with 0.1% (wt/vol) L-cys-

teine (TSBC) and kanamycin (15 µg/ml) or sucrose (10%, wt/vol) as needed. *F. novicida* strains harboring *ligA* alleles from psychrophilic organisms were routinely grown at 30°C. *Saccharomyces cerevisiae* VL648 and YBSΔL1 were grown in yeast extract-peptone-dextrose plus adenine (YPAD) (31) medium at 30°C. For selection of recombinants, *S. cerevisiae* strains were plated on synthetic complete –Ura dropout medium (i.e., without uracil) or synthetic complete –Trp dropout medium (i.e., without tryptophan) (31).

Cloning and insertion of psychrophilic *ligA* alleles into the *F. novicida* chromosome. A partial genomic sequence was available for several unidentified psychrophilic bacteria, and this allowed the identification of some of the *ligA* alleles. For strains with no available genomic sequence data, we assumed that many of our psychrophilic isolates were in the *Colwellia*, *Pseudoalteromonas*, and *Shewanella* genera, and we designed primers to amplify the *ligA* alleles from these bacteria. The PCR products of the appropriate size were partially sequenced to identify those that contained the *ligA* ORF. To prepare *ligA* alleles for insertion into *F. novicida*, we needed to join them to DNA that corresponded to regions of the *F. novicida* chromosome that flank *ligA*. Each *ligA* allele was amplified with primers that had overlapping regions to the *F. novicida* flanking regions, and PCR products corresponding to the *ligA* allele, the flanking regions, and a yeast cloning vector were seamlessly assembled using transformation-assisted recombination in *S. cerevisiae* VL648 according to the procedure described by Geitz and Schiestl (32). The vector used for this assembly was a derivative of pRS426 (33) that had the ampicillin marker replaced with genes encoding kanamycin resistance and sensitivity to sucrose (*sacB*). This plasmid can replicate in *S. cerevisiae* and *E. coli* but not in *F. novicida* (see the plasmid map in Fig. S1 in the supplemental material).

Once the *ligA* alleles were joined to the *F. novicida* flanking regions and the sequence was verified, the recombinant plasmids were amplified in *E. coli*, isolated, and used to transform chemically competent *F. novicida* (34). The exchange of the psychrophilic *ligA* for the *F. novicida* homologue was accomplished via a Campbell-like integration followed by excision as previously described (26). In essence, the transformation step was used to select for the integration of the plasmid; after coinfectants were identified, the merodiploids were cultured in the presence of 10% sucrose and plated on agar medium with sucrose (see schematic in Fig. S2 in the supplemental material). Colonies were screened for sensitivity to Km and temperature sensitivity. The region including approximately 0.5 kb upstream and downstream from the substituted *ligA* allele was PCR amplified and sequenced.

Determination of restrictive temperatures of *F. novicida ligA* strains. We defined the restrictive temperature as the lowest temperature that failed to support the formation of isolated colonies on agar medium. To determine this temperature, we spread (“streaked”) each culture on agar plates, which were buried in aluminum beads in order to buffer the temperature fluctuations of the incubator. The variation of the temperature of the beads was monitored with a Fluke 53II thermometer, which is accurate to 0.2°C. Even with all of our efforts to control the temperature of the petri plates, our stated restrictive temperatures should be considered accurate to about ±0.5°C.

Reversion frequency determination. TS *F. novicida* strains were grown in TSBC at 30°C until the A_{600} was greater than 2. The cultures were plated without dilution on agar medium, which was incubated for 48 h at a temperature 3°C above the restrictive temperature of the strain being studied. In parallel, the culture was diluted to determine the number of CFU of the culture at its permissive temperature. The reversion frequency was calculated from the number of colonies that appeared at the restrictive temperature divided by the number of CFU in the cultures. These determinations were done at least three times with independent cultures.

Temperature shift experiments. *F. novicida* strains were grown at 25°C in TSBC overnight, diluted 1:50 (4 µl in 196 µl of medium), and inoculated in quadruplicate into 96-well plates. A BioTek ELx808IU microplate reader was used to incubate and agitate the plates at different

FIG 1 Structure of typical substitution of gene conferring psychrophilic properties into the *F. novicida* chromosome. The example here shows the P1 *ligA* allele. Lowercase letters represent the *F. novicida* DNA sequence, and uppercase letters represent the *ligA* gene sequence conferring psychrophilic properties. The start and stop codons are shown in bold letters. The putative ribosome binding site is underlined. Numbers indicate codon positions in the *ligA* ORF.

temperatures and automatically record changes in the optical density. Cultures were initially incubated at 26°C for 2 h, after which the temperature was raised to a target restrictive temperature.

Yeast growth experiments. The yeast strains carrying different alleles of DNA ligase (CDC9, P2, P7, and *F. novicida* ligase) were grown in synthetic complete dropout (–Trp) medium overnight. Cultures were then diluted in fresh medium in order to generate an A_{600} of 0.2 and incubated with shaking at selected temperatures for 12 h. Every 2 h, the A_{600} of the cultures was measured. For each strain, three independent cultures were analyzed at each temperature.

Primers. The oligonucleotide primers used in this work are described in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The sequences of the *ligA* alleles conferring psychrophilic characteristics as they exist in the *F. novicida* chromosome have been submitted to GenBank and been assigned accession numbers as follows: C1, KR154478; C2, HM003389; P1, KR154476; P2, KR154477; P3, KR154481; P4, KR154482; P5, KR154483; P6, HM003396; P7, KR154484; S1, HM003397; S2, KR154479; S3, KR154480; S4, KR006256; S5, KR864903. The C1 gene formed a hybrid when inserted into the *F. novicida* chromosome; thus, the native C1 complete allele was submitted separately and assigned accession number KR818907.

RESULTS AND DISCUSSION

Isolation of *ligA* alleles from psychrophilic bacteria. We created several DNA constructs designed to replace the native DNA ligase gene in the chromosome of the mesophilic bacterium *Francisella novicida* with *ligA* alleles from psychrophilic bacteria. We had previously observed that *F. novicida* is a good host for the expression of alleles conferring psychrophilic characteristics, and this is likely due to its low G+C content (33%), which is close to the G+C content of chromosomes of the psychrophilic organisms that served as the source of the *ligA* alleles. All of our *ligA* gene substitutions were designed to use the native *F. novicida* promoter and ribosome binding site (Fig. 1). To minimize a change of the mRNA structure in the region of the ribosome binding site (RBS) while substituting the maximal amount of the psychrophile-derived *ligA* gene, we utilized the first three codons of the *F. novicida* *ligA* ORF in place of the codons from the psychrophile-derived *ligA* gene. The N-terminal domain is responsible for recognition of the NAD cofactor, which is necessary for the DNA nick adenylation process. However, the first several amino acids in the sequence are not highly conserved and seem to not play an important role. In fact, only conserved residues present in the middle of the Ia domain were proven to be important for the correct enzymatic activity (35). The placement of three first codons from mesophilic DNA ligase in the psychrophilic sequence would serve to standardize the sequence near the RBS and increase the likelihood of a proper expression level. As well, it should not have any impact on the activity of the complete enzyme.

All of the *ligA* alleles derived from the genera *Colwellia*, *Pseudoalteromonas*, and *Shewanella* functioned in *F. novicida*, and

FIG 2 The inability to form isolated colonies defines the restrictive temperature of *F. novicida*-P2. (A) Inoculated agar plate incubated at the maximal permissive temperature of 32.5°C. (B) Inoculated agar plate incubated at the restrictive temperature of 33°C.

their substitutions for the native *ligA* gene resulted in temperature-sensitive (TS) *F. novicida* strains with restrictive temperatures ranging from around 33 to 39°C (Fig. 2 and Table 1). When we subjected the *ligA* region to sequence analysis, we found that all but one of the strains (see discussion of C1 *ligA* allele below) incorporated the complete psychrophile-derived allele, including the intentionally altered first three codons. In addition, to rule out the possibility that the TS phenotype was due to a misfolded psychrophilic DNA ligase interfering with the native enzyme expressed from a cryptic chromosomal location, we reintroduced the *F. novicida* *ligA* gene to form merodiploids of alleles P1, P2, C1, S2, S3, P3, P4, P5, P7, and S4 that harbored both the native and psychrophile-derived *ligA* genes. In all cases, the merodiploids grew well above the restrictive temperature of the cognate TS strain; Fig. S3 in the supplemental material shows representative results for three merodiploids.

To test the possibility that the TS phenotype of the strains with

TABLE 1 Origin of *ligA* alleles^a conferring psychrophilic characteristics and restrictive temperature and reversion frequencies in *F. novicida* strains harboring these alleles

<i>ligA</i> allele	Origin	Restrictive temp (°C)	Reversion frequency
P1	<i>Pseudoalteromonas</i> sp.	33.0	1.8×10^{-8}
P2	<i>Pseudoalteromonas</i> sp.	33.0	4.1×10^{-9}
C1 ^b	<i>Colwellia</i> sp.	33.0	4.0×10^{-8}
S1	<i>Shewanella frigidimarina</i>	33.0	4.0×10^{-6}
S2	<i>Shewanella</i> sp.	33.0	3.7×10^{-8}
S3	<i>Shewanella</i> sp.	33.5	2.8×10^{-4}
P3	<i>Pseudoalteromonas</i> sp.	34.5	6.7×10^{-9}
C2	<i>Colwellia psychrerythraea</i> 34H	35.0	$<1.2 \times 10^{-10}$
P4	<i>Pseudoalteromonas</i> sp.	35.0	$<4.2 \times 10^{-10}$
P5	<i>Pseudoalteromonas</i> sp.	36.0	$<2.3 \times 10^{-7}$
P6	<i>Pseudoalteromonas haloplanktis</i> TAC125	37.0	$<1.5 \times 10^{-10}$
P7	<i>Pseudoalteromonas</i> sp.	38.0	$<3.0 \times 10^{-10}$
S4	<i>Shewanella</i> sp.	38.5	6.8×10^{-10}
S5	<i>Shewanella oneidensis</i> MR1	>44.0	ND ^c

^a All of these *ligA* alleles were PCR amplified from chromosomal DNA, except the P6 *ligA* allele, which was made synthetically as a codon-optimized version suitable for expression in *F. novicida*.

^b When the full C1 allele supported the growth of an *S. enterica* Δ *ligA* strain, the restrictive temperature was 27°C and the reversion frequency was 1.5×10^{-8} /cell.

^c ND, not determined.

FIG 3 Phylogram of the amino acid sequences of the deduced product of *ligA* alleles postulating their evolutionary relatedness. The temperature of inactivation of the *F. novicida* strain harboring the *ligA* alleles is listed next to each allele. S5 is from a mesophilic *Shewanella* species. The phylogram was generated using the neighbor-joining method in Geneious version 6.0.6. The wild-type *ligA* sequence from *F. novicida* was used as an outgroup.

ligA gene substitutions was due to mutations outside the *ligA* alleles, we returned the *F. novicida* *ligA* homologue to each of the TS strains. The *F. novicida* strain used in these studies allows a high level of transformation with linear DNA destined for integration into the chromosome. We PCR amplified the wild-type *F. novicida* *ligA* allele along with approximately 1 kb of flanking DNA regions. This amplicon was used to transform each of the TS *F. novicida* strains carrying *ligA* alleles conferring psychrophilic properties. After plating the transformation mixtures at restrictive temperatures, a lawn of putative transformants was found for each transformation (see Fig. S4 in the supplemental material). Parallel experiments without added DNA yielded no growth at the restrictive temperatures. DNAs from isolates from three of the transformation experiments were used as the templates for PCR amplification of the *ligA* regions, and these amplicons were subjected to DNA sequence analysis. All of these showed the presence of the *F. novicida* *ligA* gene and the absence of any of the genes conferring psychrophilic properties.

Another possibility is that the introduction of a foreign gene or gene product induces a TS phenotype through an unknown mechanism. While this possibility is difficult to test directly, we approached this indirectly by introducing a foreign mesophilic strain *ligA* gene. The mesophilic bacterium *Shewanella oneidensis* MR-1 is closely related to the three psychrophilic genera used as the source of the *ligA* alleles (Fig. 3). Indeed, the DNA ligase of *S. oneidensis* has 74.5%, 73.5%, 74.6%, and 73.5% amino acid identity with the products of the S1, S2, S3, and S4 *ligA* alleles, respectively. When the *S. oneidensis* *ligA* allele was substituted into the *F. novicida* chromosome, the resulting strain had a temperature pro-

file indistinguishable from that of the parental *F. novicida* (Fig. 4). This demonstrated that the mere introduction of a foreign *ligA* allele does not induce a TS phenotype.

The gene substitution of the *ligA* C1 allele into *F. novicida* (giving “*F. novicida*-C1”) yielded unusual results. The *F. novicida*-C1 strain carried a hybrid *ligA* gene, with the first 457 codons from the *F. novicida* *ligA* gene and the last 223 codons from the *Colwellia* *ligA* gene (Fig. 5). Since the source bacterium of the *Colwellia* sp. *ligA* gene has a maximal growth temperature of 12.5°C, we suspected that the *ligA* gene product may not function at the 30°C that was used to select recombinants. Further experiments to isolate an *F. novicida* carrying the C1 *ligA* allele at lower temperatures failed, and thus we do not know the reason why a complete C1 allele substitution was not found.

To further test the properties of the *ligA* C1 allele, we introduced it on the chloramphenicol-resistant (Cm^r) plasmid pBC SK⁺ into an *S. enterica* strain with an inactivated chromosomal copy of *ligA* that was supported by a pBR313-borne (ampicillin-resistant [Ap^r]) copy of the *S. enterica* *ligA*. After growth in broth with chloramphenicol but without ampicillin, we found isolates that had lost the Ap^r plasmid. These isolates had a restrictive temperature of 27°C (see Fig. S5 in the supplemental material), and DNA sequencing of the plasmid present in the cell showed that it carried the C1 *ligA* allele. These findings suggest that the C1 *ligA* gene product is inactivated at about 27°C.

Comparative analysis of the *ligA* deduced amino acid sequences. In order to ascertain if there are discernible amino acid differences that correlate with the restrictive temperatures in-

FIG 4 Growth characteristics of wild-type *F. novicida* (A) and *F. novicida*-MR-1 (S5) that harbors a *ligA* gene from *S. oneidensis* MR-1 (B). The maximal growth temperature for *F. novicida* is about 45°C. Error bars (standard errors of the means [SEM]) are presented but are usually too small to be discerned.

duced by the *ligA* alleles, we analyzed the sequences using multiple alignments (see Fig. S6 to S8 in the supplemental material). We found that large segments of the deduced amino acid sequences are highly similar even between *ligA* alleles of psychrophilic and mesophilic origin. This is especially true in some of the conserved motifs such as motif I and motif III (see Fig. S6 in the supplemental material). Yet, while most *ligA* deduced amino acid sequences with high identity have similar restrictive temperatures, there are cases of closely similar alleles with different phenotypes, for example the S2 and S4 alleles (Fig. 3; see also Fig. S6 to S8 in the supplemental material). Thus, a simple analysis of any amino acid sequence differences does not allow a prediction of the inactivation temperature of the *ligA* gene product.

Growth properties of *F. novicida* harboring psychrophilic strain *ligA* alleles. We wanted to assess if the substitution of a foreign *ligA* gene affected the growth rate of the hybrid strains of *F. novicida*. Thus, we analyzed their growth in liquid medium over a range of temperatures below and above the restriction temperatures of the different strains (Fig. 6). In all of these experiments, 30°C was used as the lowest incubation temperature and served as a baseline for the other growth temperature conditions. For the wild-type strain, the generation time did not change appreciably with incubation temperatures up to 42°C. However, each of the TS variants of *F. novicida* grew more slowly at incubation temperatures that were 2°C to 3°C below their restrictive temperatures. Progressively higher incubation temperatures correlated with earlier time points when growth slowed or stopped.

When substituting the *ligA* alleles into *F. novicida*, we preserved the *F. novicida* transcriptional control, and we tried to minimize disruption of mRNA structure surrounding the RBS regions. However, the fusion of the *F. novicida* region distal to the *ligA* promoter to the psychrophilic strain *ligA* ORF inevitably resulted in a new mRNA and a new RBS environment. Our calculations (see Table S2 in the supplemental material) of the predicted strengths of the RBSs for the psychrophilic strain *ligA* alleles showed that most of them were within a factor of two from the original, *F. novicida*, *ligA* RBS. The substitution of the S1, S3, and

C2 alleles substantially lowered the strength of the RBSs, but in general, there is not an apparent relationship between the predicted strength of the RBSs lying in front of the *ligA* alleles conferring psychrophilic properties and the growth rates of the strains harboring those alleles (see Table S2 and Fig. S9 in the supplemental material).

Frequencies of mutations conferring temperature resistance in *F. novicida* strains carrying *ligA* alleles. One of the potential advantages of using naturally occurring essential genes from psychrophiles is that millions of years of evolutionary adaptation may have generated some proteins that cannot be converted to a temperature-stable form through a single amino acid change resulting from a single nucleotide change. Of the 13 TS *F. novicida* strains, four (with alleles C2, P4, P6, and P7) failed to generate colonies when more than 10¹⁰ CFU were plated on agar medium and incubated 3°C above their restrictive temperatures (Table 1). For the P5 strain, we did not find temperature-resistant colonies, but this strain generated only low CFU (~10⁷) at the permissive temperature, so it is impossible to predict a meaningful mutation frequency to temperature resistance of the P5 *ligA* allele. The *F. novicida* strains harboring the S3 *ligA* allele had an unusually high frequency of mutation to temperature resistance, but the rest of the alleles that generated temperature-resistant strains mutated at frequencies typical for point mutations. In every case, we found the temperature-resistant phenotypes to be stable when reinoculated onto agar plates.

Mutations that convert TS *ligA* gene products into temperature-resistant forms. To determine the nature of the mutations leading to temperature resistance, we analyzed the DNA sequence of the *ligA* region from at least two temperature-resistant isolates from each strain that generated such mutants. Seven of eight strains had a single nucleotide alteration that caused a change in the encoded amino acid in the *ligA* gene product (examples are shown in Table 2 and Fig. 7). However, all of the three analyzed *F. novicida* strains carrying *ligA* P2 had no changes in the *ligA* coding sequence or in the nearest upstream and downstream regions. Additionally, while we found three mutated forms of the S3 *ligA* allele, we have also isolated four temperature-resistant *F. novicida*-S3 strains with no changes in their *ligA* ORF (Table 2), indicating that extragenic suppressors altered their maximal growth temperatures.

Since it appeared that some of the temperature-resistant isolates derived their phenotypes from extragenic suppressor mutations, we wanted to test if the intragenic *ligA* mutations found in

FIG 5 Integration of the C1 *ligA* allele resulted in a hybrid gene with 456 codons from the *F. novicida* *ligA* gene that encodes the N-terminal region of the 680-amino-acid hybrid protein.

FIG 6 Growth characteristics of *F. novicida* strains harboring *ligA* alleles conferring psychrophilic phenotype. The strains were cultured at 25°C for 16 h, diluted, and then grown at the elevated temperatures. The incubation temperatures are listed in each panel from top to bottom to correspond to the order of the growth curves; this allows the reader to discern the position of each growth curve even when two or more curves overlap. Error bars (SEM) are presented but are usually too small to be discerned. The generation times for the strains growing at a permissive temperature are listed in Table S2 in the supplemental material.

most of the isolates truly generated their temperature-resistant phenotypes. To do this, we PCR amplified the *ligA* regions from five temperature-resistant isolates derived from temperature-sensitive strains of *F. novicida* carrying psychrophilic strain *ligA* substitutions and used the PCR products to transform *F. novicida*-C2 (maximum temperature, 35°C) and selected for growth at 37°C. In all cases, the temperature-resistant transformants carried the

ligA mutations associated with the *ligA* from the temperature-resistant donor strains. The results from one such experiment are shown in Fig. S10 in the supplemental material.

The majority of intragenic temperature-resistant mutations occurred in the C-terminal region of DNA ligase protein, including the zinc binding domain and the helix-hairpin-helix (HHH) region (Fig. 7), both of which are involved in binding DNA. A

TABLE 2 Examples of mutations found in temperature-resistant mutants

Mutant allele	Amino acid change	Codon change	Protein region
P1	A520V	GCA→GTA	HHH domain
P2 ^a	None found	None found	
C1	E475K	GAA→AAA	HHH domain
S2	E433K	GAA→AAA	Zinc binding domain
S3 ^b	M8I	ATG→ATA	N-terminal end
S3 ^b	D9N	GAT→AAT	N-terminal end
P3	A520V	GCT→GTT	HHH domain
S4	D550G	GAT→GGT	HHH domain
S4	E518G	GAA→GGA	HHH domain

^a Presumably, mutations extragenic to *ligA* altered the restrictive temperature.
^b Some temperature-resistant strains carrying the S3 allele had no mutations in the *ligA* gene, and extragenic mutations were presumably responsible for the altered phenotype.

Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2>) structure prediction of temperature-resistant S4 LigA placed both the E518G and the D550G changes in loop regions between two α-helices. The A520V changes found in temperature-resistant variants of LigA-P1 and LigA-P3 were predicted to lie in a portion of the enzyme that has no predicted secondary structure. The E433K mutation in LigA-S2 occurred at the beginning of a predicted α-helix in the zinc binding domain adjacent to one of the cysteines that are involved in binding zinc ion.

Temperature-resistant variants of S3 *ligA* had mutations that introduced changes at the N terminus of the LigA product (Table 2), and we were surprised to find that the mutations were identical to those previously found in temperature-resistant mutants of S1 *ligA* (26). We noted that both of the S1 and S3 alleles had the lowest predicted RBS strengths (86 arbitrary units [AU]) of all of the alleles and wondered if the mutations leading to temperature resistance affected the strengths of the RBSs. We found that indeed both the S1 and S3 predicted RBS strengths were increased at least 3-fold (317 to 1,531 AU) (see Table S3 in the supplemental material) by the mutations found in the temperature-resistant variants. The mRNA of the S1 and S3 alleles is predicted to form a secondary structure that encompasses the beginning of the coding sequence, which could affect the gene translation. These mRNA secondary structures were destabilized by the mutations found at the 5' end of the S1 and S3 *ligA* alleles (see Fig. S11 and Table S4 in the supplemental material). If the temperature resistance phenotype of the S3 variants is due to a change in mRNA structure rather than a change in protein structure, then this type of mutation should be regarded as a form of “RBS strength suppressor” muta-

FIG 7 Location of mutations that converted TS alleles conferring psychrophilic properties into ones that produced temperature-resistant products. Blocks signify the protein domains of bacterial NAD-dependent DNA ligases. Ia, cofactor binding domain; NT, nucleotransferase domain; OB, OB fold domain; Zn, zinc binding domain; HHH, helix-hairpin-helix domain; BRCT, BRCT domain. The double-headed arrow indicates the mutations that occurred at the very N-terminal end of the protein and did not have a consistent character other than to increase the strength of the RBS; the circular arrows indicate the mutations changing glutamic acid into lysine; the single arrow indicates the mutation changing alanine into valine; and the square arrows indicate mutations changing acidic residue into glycine.

FIG 8 *S. cerevisiae* strain supported by the P2 *ligA* allele. Inoculated agar plate incubated at the maximal permissive temperature of 34°C (A) or at the restrictive temperature of 34.5°C (B).

tion analogous to multicopy suppressor mutations that result from having many copies of a particular gene.

Psychrophilic *ligA* alleles that support *S. cerevisiae* viability. The essential genes of different biological kingdoms are represented by widely divergent genes and by different sets of genes. Thus, it was surprising that the *ligA* gene of *E. coli* could support the viability of *S. cerevisiae* that had a knockout in its essential *CDC9* DNA ligase encoding gene (DNA ligase type I) (29), even though the bacterial enzyme is NAD dependent and the yeast enzyme is ATP dependent. Given these results, we speculated that some psychrophilic strain *ligA* genes could support *S. cerevisiae* growth and wondered if a TS phenotype would result in such a hybrid strain.

To test our hypothesis, we cloned two *Pseudoalteromonas ligA* alleles, P2 and P7, into the centromere vector, pYX132, which is based on tryptophan auxotrophy for selection and utilizes the strong constitutive TPI1 promoter to drive the expression of recombinant genes. As a representative of a bacterial mesophilic gene, we cloned the *F. novicida ligA* gene into this vector; and as a positive control, we also cloned the yeast *CDC9* gene. These recombinant plasmids were transformed into the *S. cerevisiae* YBSΔL1 haploid strain, which has a chromosomal knockout of *CDC9* and carries *CDC9* on a plasmid that is based on uracil selection. After transformation and selection for the recombinant pYX132 (Trp-based plasmid), colonies were spread on agar medium containing 5-fluoroorotic acid to counterselect against the uracil selection-based plasmid, which removes the only copy of *CDC9*. The surviving colonies were tested for the presence of the original *CDC9*-bearing plasmid, and those lacking the plasmid were studied further. We found recombinants that lacked any copy of *CDC9* and that bore *ligA* alleles from psychrophilic strains P2 and P7, and we found that all of these isolates were temperature sensitive (Fig. 8). However, the restrictive temperatures for the *S. cerevisiae* strains harboring *ligA* alleles from P2 and P7 were 34.5 and 36.5°C compared to the restrictive temperatures for *F. novicida* strains with these alleles, which were 33 and 38°C. *S. cerevisiae* strains harboring the *F. novicida ligA* allele or strains in which we introduced the *CDC9* gene on the pYX132 plasmid grew in the same temperature range as the parent *S. cerevisiae* strain (Fig. 9).

In this work, we have identified several *ligA* alleles conferring psychrophilic properties that can be used as genetic elements to engineer TS variants of prokaryotes and, for some alleles, a eukaryote microbe. The range of temperature sensitivity induced by

FIG 9 Growth of *S. cerevisiae* strains dependent on psychrophilic bacterial *ligA* alleles. The *S. cerevisiae* strains carrying *ligA* alleles CDC9 (A), wild-type *F. novicida* *ligA* (B), *ligA* P2 (C), and *ligA* P7 (D) were grown for 16 h at 30°C and diluted to an A_{600} of 0.3 to generate new cultures. Triplicate cultures were grown at each temperature, and A_{600} values were measured every 2 h. Error bars represent standard errors of the means.

the alleles, from 27°C to 39°C, allows for robust growth of the TS microbe at temperatures suitable for bioprocess applications and for live vaccines.

ACKNOWLEDGMENTS

Francis E. Nano owns the intellectual property associated with the psychrophilic gene technology. We thank Nicole Little for technical help and Barry Duplantier for helpful discussions.

FUNDING INFORMATION

Gouvernement du Canada | Natural Sciences and Engineering Research Council of Canada (NSERC) provided funding to Francis E. Nano under grant number 41841-2012.

REFERENCES

- Nandagopal N, Elowitz MB. 2011. Synthetic biology: integrated gene circuits. *Science* 333:1244–1248. <http://dx.doi.org/10.1126/science.1207084>.
- Davis JH, Rubin AJ, Sauer RT. 2011. Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Res* 39:1131–1141. <http://dx.doi.org/10.1093/nar/gkq810>.
- McWhinnie RL, Nano FE. 2014. Synthetic promoters functional in *Francisella novicida* and *Escherichia coli*. *Appl Environ Microbiol* 80:226–234. <http://dx.doi.org/10.1128/AEM.02793-13>.
- Amit R, Garcia HG, Phillips R, Fraser SE. 2011. Building enhancers from the ground up: a synthetic biology approach. *Cell* 146:105–118. <http://dx.doi.org/10.1016/j.cell.2011.06.024>.
- Chen Y-J, Liu P, Nielsen AAK, Brophy JAN, Clancy K, Peterson T, Voigt CA. 2013. Characterization of 582 natural and synthetic terminators and quantification of their design constraints. *Nat Methods* 10:659–664. <http://dx.doi.org/10.1038/nmeth.2515>.
- Ceres P, Trausch JJ, Batey RT. 2013. Engineering modular “ON” RNA switches using biological components. *Nucleic Acids Res* 41:10449–10461. <http://dx.doi.org/10.1093/nar/gkt787>.
- Yang L, Nielsen AAK, Fernandez-Rodriguez J, McClune CJ, Laub MT, Lu TK, Voigt CA. 2014. Permanent genetic memory with >1-byte capacity. *Nat Methods* 11:1261–1266. <http://dx.doi.org/10.1038/nmeth.3147>.
- French CE, de Mora K, Joshi N, Elfick A, Haseloff J, Ajioka J. 2011. Synthetic biology and the art of biosensor design. National Academies Press, Washington, DC.
- Mandell DJ, Lajoie MJ, Mee MT, Takeuchi R, Kuznetsov G, Norville JE, Gregg CJ, Stoddard BL, Church GM. 2015. Biocontainment of genetically modified organisms by synthetic protein design. *Nature* 518:55–60. <http://dx.doi.org/10.1038/nature14121>.
- Rovner AJ, Haimovich AD, Katz SR, Li Z, Grome MW, Gassaway BM, Amiram M, Patel JR, Gallagher RR, Rinehart J, Isaacs FJ. 2015. Recoded organisms engineered to depend on synthetic amino acids. *Nature* 518: 89–93. <http://dx.doi.org/10.1038/nature14095>.
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:2006.0008.
- Yamamoto N, Nakahigashi K, Nakamichi T, Yoshino M, Takai Y, Touda Y, Furubayashi A, Kinjo S, Dose H, Hasegawa M, Datsenko KA, Nakayashiki T, Tomita M, Wanner BL, Mori H. 2009. Update on the Keio collection of *Escherichia coli* single-gene deletion mutants. *Mol Syst Biol* 5:335. <http://dx.doi.org/10.1038/msb.2009.92>.
- Zhang R, Lin Y. 2009. DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucleic Acids Res* 37:D455–D458. <http://dx.doi.org/10.1093/nar/gkn858>.
- Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenther D, Bovee D, Olson MV, Manoil C. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 100: 14339–14344. <http://dx.doi.org/10.1073/pnas.012602299>.
- Akerley BJ, Rubin EJ, Novick VL, Amaya K, Judson N, Mekalanos JJ. 2002. A genome-scale analysis for identification of genes required for growth or survival of *Haemophilus influenzae*. *Proc Natl Acad Sci U S A* 99:966–971. <http://dx.doi.org/10.1073/pnas.012602299>.
- Gerdes SY, Scholle MD, Campbell JW, Balazsi G, Ravasz E, Daugherty MD, Somera AL, Kyrpides NC, Anderson I, Gelfand MS, Bhattacharya A, Kapatral V, D'Souza M, Baev MV, Grechkin Y, Meeh F, Fonstein MY, Overbeek R, Barabasi A-L, Oltvai ZN, Osterman AL. 2003. Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. *J Bacteriol* 185:5673–5684. <http://dx.doi.org/10.1128/JB.185.19.5673-5684.2003>.
- Tzamalaki E, Poirazi P, Tollis IG, Reczko M. 2011. A computational exploration of bacterial metabolic diversity identifying metabolic interac-

- tions and growth-efficient strain communities. *BMC Syst Biol* 5:167. <http://dx.doi.org/10.1186/1752-0509-5-167>.
18. Pascal JM. 2008. DNA and RNA ligases: structural variations and shared mechanisms. *Curr Opin Struct Biol* 18:96–105. <http://dx.doi.org/10.1016/j.sbi.2007.12.008>.
 19. Lehman IR. 1974. DNA ligase: structure, mechanism, and function. *Science* 186:790–797. <http://dx.doi.org/10.1126/science.186.4166.790>.
 20. Kouzminova EA, Kuzminov A. 2012. Chromosome demise in the wake of ligase-deficient replication. *Mol Microbiol* 84:1079–1096. <http://dx.doi.org/10.1111/j.1365-2958.2012.08076.x>.
 21. Shuman S. 2009. DNA ligases: progress and prospects. *J Biol Chem* 284:17365–17369. <http://dx.doi.org/10.1074/jbc.R900017200>.
 22. Wilkinson A, Day J, Bowater R. 2001. Bacterial DNA ligases. *Mol Microbiol* 40:1241–1248. <http://dx.doi.org/10.1046/j.1365-2958.2001.02479.x>.
 23. Petit MA, Ehrlich SD. 2000. The NAD-dependent ligase encoded by *yerG* is an essential gene of *Bacillus subtilis*. *Nucleic Acids Res* 28:4642–4648. <http://dx.doi.org/10.1093/nar/28.23.4642>.
 24. Ellenberger T, Tomkinson AE. 2008. Eukaryotic DNA ligases: structural and functional insights. *Annu Rev Biochem* 77:313–338. <http://dx.doi.org/10.1146/annurev.biochem.77.061306.123941>.
 25. Georlette D, Damien B, Blaise V, Depiereux E, Uversky VN, Gerday C, Feller G. 2003. Structural and functional adaptations to extreme temperatures in psychrophilic, mesophilic, and thermophilic DNA ligases. *J Biol Chem* 278:37015–37023. <http://dx.doi.org/10.1074/jbc.M305142200>.
 26. 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>.
 27. Gallagher LA, McKevitt M, Ramage ER, Manoil C. 2008. Genetic dissection of the *Francisella novicida* restriction barrier. *J Bacteriol* 190:7830–7837. <http://dx.doi.org/10.1128/JB.01188-08>.
 28. 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.
 29. Sriskanda V, Schwer B, Ho CK, Shuman S. 1999. Mutational analysis of *Escherichia coli* DNA ligase identifies amino acids required for nick-ligation in vitro and for in vivo complementation of the growth of yeast cells deleted for CDC9 and LIG4. *Nucleic Acids Res* 27:3953–3963. <http://dx.doi.org/10.1093/nar/27.20.3953>.
 30. Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62:293–300.
 31. Murthy MSS, Rao BS, Reddy NMS, Subrahmanyam P, Madhvanath U. 1975. Non-equivalence of YEPD and synthetic complete media in yeast reversion studies. *Mutat Res Mol Mech Mutagen* 27:219–223. [http://dx.doi.org/10.1016/0027-5107\(75\)90080-9](http://dx.doi.org/10.1016/0027-5107(75)90080-9).
 32. Gietz RD, Schiestl RH. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2:31–34. <http://dx.doi.org/10.1038/nprot.2007.13>.
 33. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110:119–122. [http://dx.doi.org/10.1016/0378-1119\(92\)90454-W](http://dx.doi.org/10.1016/0378-1119(92)90454-W).
 34. Anthony LS, Gu MZ, Cowley SC, Leung WW, Nano FE. 1991. Transformation and allelic replacement in *Francisella* spp. *J Gen Microbiol* 137:2697–2703. <http://dx.doi.org/10.1099/00221287-137-12-2697>.
 35. Sriskanda V, Shuman S. 2002. Conserved residues in domain Ia are required for the reaction of *Escherichia coli* DNA ligase with NAD⁺. *J Biol Chem* 277:9695–9700. <http://dx.doi.org/10.1074/jbc.M111164200>.