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Skim Milk Enhances the Preservation of Thawed -80°C Bacterial Stocks

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Abstract

The results from bacterial strain recovery efforts following hurricanes Katrina and Rita are reported. Over 90% of strains frozen in 10% skim milk were recovered whereas various recovery rates were observed for glycerol-stored stocks (56% and 94% of *E. coli*, depending upon the laboratory). These observations led to a viability comparison of *Streptococcus pyogenes*, *Campylobacter jejuni*, *Borrelia burgdorferi*, *Salmonella enterica* subsp. *Typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli* strains stored in glycerol or skim milk. In all bacteria examined, 10% skim milk resulted in significantly longer viability after thawing than 15% glycerol solutions currently used in most laboratories.

1. Introduction

The freezing of bacterial cultures is a common method of preserving strains in the laboratory. As early as 1913 it was observed that an additive, such as sugar, milk or glycerol, protected bacteria from cell death following repetitive freeze/thaw cycles necessary in most bacteriological research (Keith, 1913). Today glycerol is one of the most common cryoprotective agents utilized. Conventional methods for freezing a culture include suspension of bacterial cultures in 15% glycerol (F.M. Ausubel, 1993, Baker, 1998). Indeed, such methods allow for the long-term viability of strains, if temperature can be maintained and multiple freeze thaw cycles avoided (Gruft *et al.*, 1968, Hollander & Nell, 1954, Linscott & Boak, 1960, Nakamura *et al.*, 1962, Howard, 1956). Alternatively, skim milk, a common freeze-drying protectant (Barbaree *et al.*, 1982), can be used as a freezing solution (Takahashi *et al.*, 1982, Essiain & Flournoy, 1986, Sinha *et al.*, 1974, Hasegawa *et al.*, 1967), as is regularly used in our laboratory to store *Pseudomonas aeruginosa*. A literature review indicates that the predominant studies of cyroprotectants focus on sensitivity to freeze/thaw cycles (Takahashi

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et al., 1982, Aulet de Saab *et al.*, 2001) with few comparisons of viability over time at elevated temperature (Gruft et al., 1968, Essiain & Flournoy, 1986). While recovering our bacterial strains from 31 days at ambient temperatures (in this case, sustained temperatures of ~30°C) due to power loss as a result of hurricanes Katrina and Rita, we observed an increase in recovery of strains that were stored in 10% skim milk versus 15% glycerol. Here we report that use of a 10% skim milk solution (wt/vol) enhanced the viability of several bacterial species after thawing and prolonged incubation at 30°C. These data suggest that a 10% skim milk freezing solution will provide greater protection against loss of viability if a freezer fails and may increase long-term survival of stocks when maintained at -80°C.

2. Materials and Methods

2.1 P. aeruginosa strain storage

The Schurr lab stored *P. aeruginosa* strains in the -80°C freezer after being cultured on *Pseudomonas* Isolation Agar (PIA, Difco) plates at 37°C. Sterile cotton swabs were used to collect bacteria from the plates and to put the organisms in 2 ml 10% skim milk (Difco). Cryovials (2.5 ml, Nalgene) containing the organisms in 10% skim milk solution were subsequently stored at -80°C. All strains frozen at -80°C from the Nickerson lab were cultured on LB agar plates at 37°C and transferred to an 85% LB/15% glycerol stock solution.

2.2 E. coli strain storage

The Schurr Lab stored *Escherichia coli* strains in the -80°C freezer after growth in Luria-Bertani (LB) broth to an $OD_{600\ nm}$ of 0.375. The broth culture (1 ml) was mixed with 1 ml of 65% glycerol stock solution (containing 0.1 M $MgSO_4$ and 0.025 M Tris-HCl, pH 8.0) and stored at -80°C (F.M. Ausubel, 1993, Baker, 1998). All strains frozen at -80°C from the Nickerson lab were cultured on LB agar plates at 37°C and transferred to an 85% LB/15% glycerol stock solution.

2.3 Strain Recovery

For recovery, all strains were taken from room temperature freezers (~30°C) at the Tulane University Health Sciences Center in New Orleans, transported to Texas (Houston for Nickerson lab and Dallas for Schurr lab), and 1 ml of each suspension was streaked on the LB agar plates with appropriate antibiotics, if necessary. Recovery was scored as positive if at least one colony was visible on the plates after incubation overnight at 37°C, however, most strains yielded hundreds of colonies on the recovery plate using this method.

2.4 Glycerol vs. skim milk survival comparison

Streptococcus pyogenes (SF370 (Ferretti *et al.*, 2001) and JRS4 (Scott *et al.*, 1986)), *Campylobacter jejuni* 81-176 (Black *et al.*, 1988), and *Borrelia burgdorferi* 297 (Xu & Johnson, 1995) cultures were obtained from colleagues hosting the displaced Schurr laboratory at the University of Texas Southwestern Medical Center. *Salmonella enterica* subsp. *Typhimurium* cultures [χ3339 (Gulig & Curtiss, 1987), JW322 (Honer zu Bentrup *et al.*, 2006), JW67 (Wilson & Nickerson, 2006)] were obtained from the Nickerson laboratory and *P. aeruginosa* [PAO1 (Holloway, 1955) and PAO568 (Fyfe & Govan, 1980)] and *E. coli* (DH-5α and DH-5α λPIR) strains from the Schurr laboratory were tested. *P. aeruginosa*, *E. coli*, and *Salmonella enterica* serovar *Typhimurium*, *S. pyogenes* and *C. jejuni* strains were grown and frozen in 10% skim milk or 15% glycerol (see Figure 1 legend). All stock suspensions were frozen at -80°C for three days and then incubated at 30°C for nine weeks, or until the glycerol stock strains were no longer viable. Serial dilutions were performed and CFUs counted every 7 days for *P. aeruginosa*, *E. coli* and *S. enterica* serovar *Typhimurium*, and every 24 hours for *S. pyogenes* and *C. jejuni*. *Borrelia burgdorferi* strain 297 (Xu & Johnson,

1995) (50 μl of a 1.5 ×10^7 *Borrelia/ml* culture) was frozen at -80°C in 100 μl aliquots of either 5% Difco skim milk suspensions (1:1 *Borrelia* culture : 10% milk solution) or 30% glycerol (final concentration) in BSK-II growth medium (Pollack *et al.*, 1993). Two freezer vials of milk-preserved and two vials of glycerol-preserved *Borrelia* were placed at 30°C for 1 hr, 17 hrs, 24 hrs and 48 hrs. At the end of the incubation period, the four cultures (two milk-preserved and two glycerol-preserved) were each resuspended in 1 ml of BSK-II medium and the *Borrelia* allowed to recover at 34°C. Dark field examination of each culture was performed at set time points to assess spirochete survival and 10 microscopic fields per culture (at 400X magnification) were counted for bacterial enumeration.

3. Results and Discussion

Due to power failure, flooding and inability to enter New Orleans as a result of hurricanes Katrina and Rita, bacterial strains from the Schurr and Nickerson laboratories (as well as many other investigators in New Orleans) were kept at ambient temperature for 31 days in non-functional -80°C freezers. The exact ambient temperature of our laboratories was unknown, however reasonable approximations can be gathered from the National Climatic Data Center (http://www.ncdc.noaa.gov/oa/ncdc.html). Temperatures for Aug 29 to Sept 1 are unavailable and official measured temperatures are only available after Sept 5th. According to this data the mean outside temperature in New Orleans was 28.8 °C (Fig. 1). This temperature (~30°C) was used to examine bacterial viability in thawed glycerol and skim milk cryopreservation solutions over time (see below).

The bacterial species recovered from the Schurr and Nickerson laboratories are listed in Table 1. The Schurr lab recovered 236 out of 258 (91.5%) of the *P. aeruginosa* strains stored in 10% skim milk, while only 92 out of 163 (56.4%) of *E. coli* strains stored in glycerol survived. The Nickerson lab, which uses 85% LB/15% glycerol for storage medium of all bacterial stocks, observed a 60% (6/10) survival rate of *P. aeruginosa* strains while recovering 94% of *E. coli* strains that were frozen. The Nickerson lab observed an 87.1% survival rate of *Salmonella enterica* Serovars Typhimurium and Typhi, and *Salmonella bongori*, collectively; a 40% survival rate of *Pseudomonas putida* stocks; and a 100% survival rate of *Agrobacterium tumefaciens*, *Novosphingobium capsulatum*, *Rhodobacter sphaeroides* and *Klebsiella pneumoniae* (Table 1).

There were differing recovery results for *E. coli* strains between the Nickerson (94%) and Schurr (56.4%) laboratories despite similar methods of cryopreservation (85% LB/15% glycerol vs. 50% LB/32.5% glycerol) for these strains. The lower *E. coli* survival rate observed by the Schurr laboratory may have been a result of the age of these strains as they had been stored at -80°C for 6 or more years. Alternately, the decreased percentage of LB in *E. coli* stocks from the Schurr laboratory may have affected survival after thawing. However for *Pseudomonas* strains, the Nickerson laboratory recovered 60% of their stocks (stored in glycerol) and the Schurr lab recovered 91.5% (stored in skim milk). Additionally, one of the Schurr laboratory personnel stored all of their bacterial stocks in 85%LB/15%glycerol and recovered 53% of their strains while another stored all of their bacterial strains in skim milk and recovered 98% (data not shown). These observations led to experiments to test if 10% skim milk or 15% glycerol was the better reagent for bacterial survival after thawing.

To determine if 10% skim milk or 15% glycerol was the better reagent for viability after thawing, various bacteria were collected. After 49 days incubation at 30°C, *P. aeruginosa* strains PAO1 and PAO568 stored in skim milk had a CFU/ml greater than 1.0×10^9 compared to 1×10^6 in glycerol stocks, demonstrating a 3-log difference in viable cells (Figure 2A). For the same period, *E. coli* strains DH5α and DH5α λPIR stored in skim milk contained 3-logs more viable cells than their glycerol counterparts (Figure 2B). Furthermore, all *Salmonella*

strains stored for the same time period in glycerol were no longer viable while skim milk stored strains all produced greater than 1.0×10^8 CFU/ml (Figure 2C). Glycerol stored strains of *S. pyogenes* and *C. jejuni* both contained non-viable bacteria after 5 days at 30°C. Skim milk storage of these same strains produced CFUs greater than 1.0×10^6 and 1.0×10^7 respectively, at the same time point (Figure 2D & 2E). The *Borrelia* cultures that were frozen in the 5% skim milk solution were viable at all time points, even after incubation for 48 h at 40°C. The *Borrelia* cultures preserved in 30% glycerol did not survive after 1 h at 30°C (data not shown).

Since it appeared that *P. aeruginosa* survived 49 days at relatively high CFUs (Figure 2A), we have attempted to recover 1,088 clinical *P. aeruginosa* Cystic Fibrosis (CF) isolates that have been at room temperature for the past 203 days. Remarkably, to date, 357/364 (98%) of the *Pseudomonas* CF clinical isolates stored in 10% skim milk have been recovered.

4. Conclusions

Freezing cultures at -80°C remains the most common method of preserving cultures over a prolonged period of time. Our data indicate that a 10% skim milk solution is a better cryoprotectant than the widely used 15% glycerol solution if a freezer fails for an extended period of time. Skim milk may be affecting the fatty acid content of the cell membrane, thereby altering membrane fluidity (Carvalho *et al.*, 2004, Annous *et al.*, 1999) or calcium may be contributing to the stability of cellular enzymes (Barach *et al.*, 1976). We recommend the use of a 10% skim milk (wt./vol.) solution for storage to enhance long term viability of cells and protect against cell death during sustained periods of elevated temperatures.

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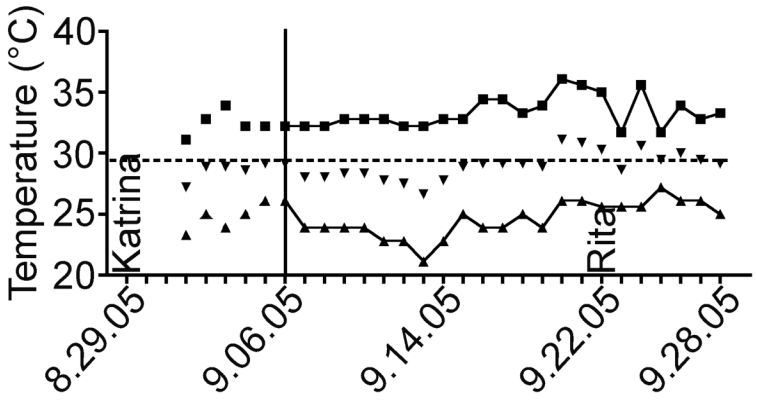

Figure 1.

New Orleans Temperatures from August 29, 2005 to September 28, 2005. High temperatures, boxes; median temperature, inverted triangles; low temperatures, triangles. Temperatures before September 6, 2005 are unofficial and demarcated by a vertical line. These were the temperatures that the Nickerson and Schurr laboratory strains endured before recovery from Tulane University Health Science Center on September 28, 2005.

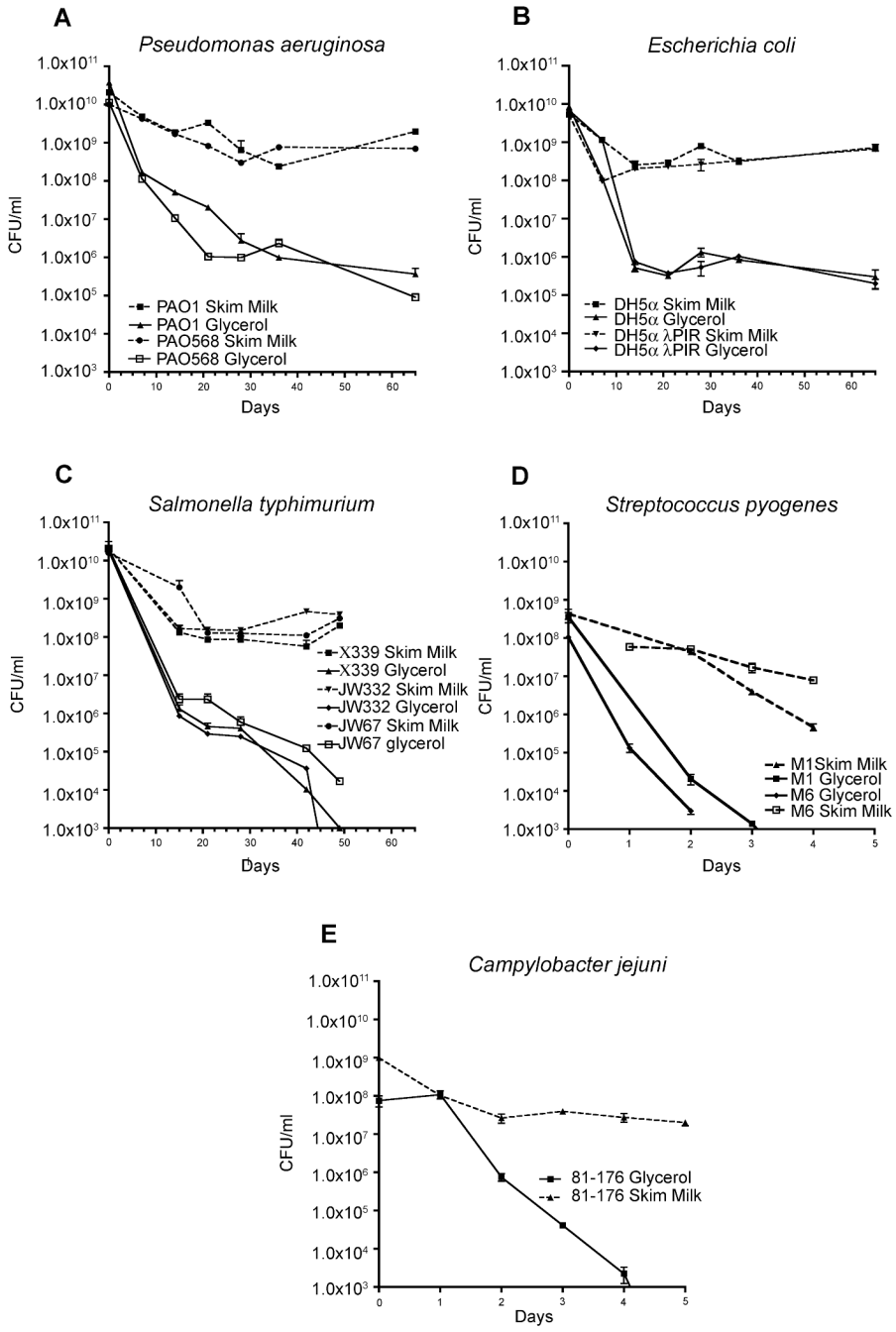

Figure 2.

Thawed bacterial strains survive longer in 10% skim milk than in 15% glycerol. *P. aeruginosa* PAO1 and PAO568 (A), *E. coli* DH-5α and DH-5α λPIR (B), and *Salmonella enterica* subsp. *Typhimurium* (C) were cultured on LB agar plates at 37°C overnight. *S. pyogenes* (D) strains were cultured on Todd-Hewitt supplemented with 2% yeast extract (THY) plates. Bacteria were collected from the plates with sterile cotton swabs and used to put the bacteria in 2 ml of 10% skim milk solution (dashed line) or a 15% glycerol solution (solid lines) in triplicate. *C. jejuni* 81-176 (E) was cultured on Mueller-Hinton agar containing 10 μg/ml trimethoprim. The plates were incubated for 48 h under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37°C. Growth from each plate was collected by sterile cotton

swabs and resuspended in 2.0 ml of 10% skim milk or 85% Mueller-Hinton broth and 15% glycerol. All stock suspensions were frozen at -80°C for three days and then incubated at 30° C for nine weeks, or until the glycerol stock strains were no longer viable. Serial dilutions were performed and CFUs counted every 7 days for *P. aeruginosa*, *E. coli* and *S. enterica* serovar *Typhimurium* and every 24 hours for *S. pyogenes* and *C. jejuni*.

Table 1

Survival of bacterial strains after 31 days at sustained elevated temperatures of ~30°C.

Bacterial Species	# of Strains Examined	# of Strains Recovered	Percent Survival
Schurr Laboratory			
Pseudomonas aeruginosa	258	236	91.5%
Escherichia coli	163	92	56.4%
Nickerson Laboratory			
Pseudomonas aeruginosa	10	6	60%
Escherichia coli sp. strain	100	94	94%
TOP10	52	49	94.2%
DH5a	15	14	93.3%
MG1655	12	12	100%
Other	22	19	86.4%
Salmonella enterica Serovars:	62	54	87.1%
Typhimurium	56	48	85.7%
Typhi	2	2	100%
Salmonella bongori	4	4	100%
Pseudomonas putida	5	2	40%
Agrobacterium tumefaciens	7	7	100%
Novosphingobium capsulatum	3	3	100%
Rhodobacter sphaeroides	1	1	100%
Klebsiella pneumoniae	1	1	100%