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Survival of freeze-dried bacteria

Yukie Miyamoto-Shinohara,* Junji Sukenobe, Takashi Imaizumi, and Toro Nakahara

International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305–8566, Japan

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The aim of this study was to investigate the survival of freeze-dried bacterial species stored at the International Patent Organism Depository (IPOD) and to elucidate the characteristics affecting survival. Bacterial strains were freeze-dried, sealed in ampoules under a vacuum (<1 Pa), and stored in the dark at 5°C. The survival of a variety of species following storage for up to 20 years was analyzed. The survival of freeze-dried species was analyzed in terms of two stages, freeze-drying and storing. Nonmotile genera showed relatively high survival after freeze-drying. Motile genera with peritrichous flagella showed low survival rates after freeze-drying. *Vibrio* and *Aeromonas*, which produce numerous flagella, showed very low survival rates. In *Lactobacillus*, non-trehalose-fermenting species showed better survival rates after freeze-drying than did fermenting species, and those species with teichoic acid in the cell wall showed lower survival rates during storage than species with teichoic acid in the cell membrane. Human pathogenic species of *Corynebacterium*, *Bacillus*, *Streptococcus*, and *Klebsiella* showed lower survival rates during storage than nonpathogenic species within the same genus. Among *Pseudomonas* species, *P. chlororaphis*, the only species tested that forms levan from sucrose, showed the lowest survival rate during storage in the genus. Survival rates of Gram-negative species during storage tended to be lower than those of Gram-positive species, though *Chryseobacterium meningosepticum* had stable survival during storage. The conclusion is that smooth cell surfaces (i.e., no flagella) and lack of trehalose outside the cytoplasm improved survival rates after freeze-drying. Because desiccation is important for survival during storage, the presence of extracellular polysaccharides or teichoic acids is disadvantageous for long-term survival. The lower survival rates of freeze-dried Gram-negative bacteria compared with those of Gram-positive bacteria may be attributed to the thinner peptidoglycan layer and the presence of lipopolysaccharides on the cell wall in the former species.

Key Words——bacteria; extracellular polysaccharide; freeze-drying; lipopolysaccharide; pathogenic species; survival curve; teichoic acid

Introduction

Freeze-drying is one of the most common methods used to store microbial culture collections. Although freeze-drying is applicable to many bacteria, it cannot be used with some, such as *Helicobacter pylori* and *Clostridium botulinum*, because of difficulties in obtaining adequate predrying growth (Rudge, 1991). Survival rates after freeze-drying and during storage vary

* Address reprint requests to: Dr. Yukie Miyamoto-Shinohara, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, 1–1–1, Higashi, Tsukuba, Ibaraki 305–8566, Japan.

Tel: 81–29–861–6075 Fax: 81–29–861–6689

E-mail: y.miyamoto@aist.go.jp

across bacterial species and genera (Lapage et al., 1970).

Many freeze-dried microbial strains have been deposited with IPOD, and their survival rates have been tested periodically for up to 20 years. The deposited strains are of great taxonomic variety. We have tried to establish the survival rates of different microbes in an effort to improve our methods of freeze-drying. At IPOD, freeze-dried microorganisms are sealed in ampoules under a vacuum ($<$1 Pa) and stored in the dark at 5°C; such conditions allow the microorganisms to survive for a longer time during storage than does sealing under approximately 7 Pa (Antheunisse, 1973; Miyamoto-Shinohara et al., 2006). Because the deposited strains represent a broad range of taxa, we have been evaluating the survival rates after freeze-drying and the survival rates during storage for each species in the collection (Miyamoto-Shinohara et al., 2000, 2006).

The survival of freeze-dried strains has been analyzed in terms of rehydration conditions (Abadias et al., 2001; Costa et al., 2000; Tsvetkov and Brankova, 1983), storage conditions (Israeli et al., 1974, 1975; Sinskey et al., 1967), and growth conditions (Hino et al., 1990; Israeli et al., 1993; Sampedro et al., 1998). In this study, we plotted survival curves for freeze-dried bacteria at IPOD and compared the survival patterns of different species.

To better understand the mechanisms underlying the improved survival rates that we have obtained, we analyzed the survival of bacterial species in greater detail, referring to previous reports of the species' microbial characteristics.

Materials and Methods

Strains and media. All the strains tested, with the exception of recombinant ones, were stored at IPOD. Each strain was cultured on agar medium until the beginning of the stationary phase, the conditions of which complied with the depositor's instructions. For *Bacillus* we generally used the following culture media: antibiotic medium 3, brain heart infusion, Luria-Bertani agar (LB), nutrient agar, trypticase soy agar; for *Corynebacterium*: nutrient agar; for *Streptococcus* and *Enterococcus*: brain heart infusion, Rogosa agar, trypticase soy agar; for *Lactococcus*: brain heart infusion, deMan Rogosa Sharpe (MRS), nutrient agar; and for *Lactobacillus*: MRS, Rogosa, tomato juice agar. For Gram-negative species we used nutrient agar, LB agar, or trypticase soy agar (The numbers of strains examined for each species and genus are listed in Tables 1 and 2).

Freeze-drying and recovery. The method of freeze-drying was as described previously (Kawamura et al., 1995; Miyamoto-Shinohara et al., 2000, 2006). Cells on a slant culture were homogenously suspended in suspension medium (10% skim milk, 1% sodium glutamate) and dispensed at 0.2 ml into glass ampoules (7 mm$\times$150 mm). The ampoules were immersed in ethanol at -60°C–-70°C for 2 to 10 min, connected to a manifold-type freeze-dryer (FreezeVac-4C, Tozai Tsusho, Tokyo, Japan; FreezeMobile, Virtis, Gardiner, NY, USA) for 4 to 20 h under a vacuum of $<$1 Pa, and sealed by heating to maintain the vacuum. The sealed ampoules were stored at 5°C in the dark. For the recovery of freeze-dried microbes, ampoules were opened and 0.2 ml sterilized water or physiological saline (0.9 w/v% NaCl) was added aseptically and mixed well. The suspension was then serially diluted in sterilized water, inoculated onto agar plates with a spiral plater (Model D, Spiral System Instrument, Bethesda, MD, USA; Autoplate Model 3000, Spiral Biotech, Bethesda, MD, USA), and cultured under the same conditions as those used before freeze-drying.

Data analysis. All the strains examined had been deposited in our Patent Depository as patented microorganisms. We therefore had only the genus and species name, or even the genus name without the detailed characteristics of each strain. In light of this absence of detailed information, we tried to cover the uncertainty by grouping strains and processing these groups statistically to find factors affecting the survival of the bacteria.

The survival rate of each strain is expressed as the number of CFUs/ml after freeze-drying as a percentage of the number before freeze-drying (100%). Survival rates of the same species freeze-dried for the same number of years are expressed as mean values with standard deviations, and the rates are designated Y_D (up to a week after freeze-drying), Y_1 (at 1 year after freeze-drying), and so on. In this research, Y_D represents the survival rate of species in response to freeze-drying, whereas the other parameters represent the survival rates of freeze-dried species in response to storage.

The survival rates from 1 to 20 years (Y_1 to Y_{20}) and the standard deviations calculated with real numbers

Table 1. Survival rates of Gram-positive genera, after freeze-drying, and related mobility characteristics.

Genus	No. species	No. strains	$Y_G \pm SD$	Mobility	Flagella
Caseobacter	1	1	96.7	−	
Sarcina	1	2	93.3	−	
Planococcus	1	2	92.1	+	polar
Enterococcus	8	29	90.6±6.5	−	
Listeria	1	2	87.6	±	peritrichous
Cellulomonas	4	12	86.9±14.5	±	polar
Aureobacterium	1	7	85.4	±	
Micrococcus	5	19	85.3±10.4	−	
Rhodococcus	5	29	84.9±9.3	−	
Nocardia	3	7	84.2±7.6	−	
Microbacterium	3	14	81.7±18.2	±	polar
Brevibacterium	6	19	80.2±9.8	−	
Arthrobacter	12	70	79.3±13.3	±	polar
Propionibacterium	1	8	77.7	−	
Staphylococcus	5	16	76.7±19.3	−	
Pediococcus	6	26	76.4±14.6	−	
Sporosarcina	1	1	75.1	+	polar
Leuconostoc	3	11	74.8±5.9	−	
Corynebacterium	12	358	74.6±17.2	−	
Lactococcus	1	14	70.7	−	
Kurthia	1	4	64.3	+	peritrichous
Bacillus	18	346	62.8±16.6	+	peritrichous
Streptococcus	9	50	59.8±21.2	−	
Lactobacillus	20	84	58.5±27.2	±	peritrichous

Symbols: +, present, −, absent, ±, differs among species. See text for details. Data on characteristics are from Sneath et al. (1984) and Holt et al. (1994).

were plotted on a semi-logarithmic scale, representing the curve of microorganism inactivation. By using the least-squares method, the plots were satisfactorily fitted to the following linear equation:

$$\mathrm{Log}_{10}\, Y = A + BX \qquad (1)$$

where Y represents the number of predicted survivors, X represents the number of storage years, A corresponds to the intercept on the y-axis, and B corresponds to the slope, and reflects the decrease in logarithmic survival per year.

The characteristics of each genus and species, including fermentation, hydrolysis, production, growth condition, morphology, DNA base composition, and cell-wall components, were obtained from *Bergey's Manual of Systematic Bacteriology*, vol. 1 (Krieg and Holt, 1984), vol. 2 (Sneath et al., 1984), or *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994).

Results

1. *Survival of genera after freeze-drying*

All the genera of Gram-positive bacteria and Gram-negative bacteria that we tested are listed in Tables 1 and 2, along with the numbers of species and strains; unspecified species (sp.) were grouped as one species class. No significant differences were observed among the survival rates of species (Y_D) in the same genus. The survival rate of each genus after freeze-drying was therefore calculated from the mean value for each species (Y_D) in that genus and is shown as Y_G. The genera in Tables 1 and 2 are listed in descending order of Y_G; the motility and type of flagella are also listed for each genus.

Presence or absence of diamino acid among the cell wall fatty acids, oxygen requirement, catalase reaction, type of major menaquinones, presence or absence of oxidase and catalase, or production of acid from glucose (Holt et al., 1994) was not related to genus-level

Table 2. Survival rates of Gram-negative genera after freeze-drying, and related characteristics.

Genus	No. species	No. strains	$Y_G \pm SD$	Motility	Flagella
Flavobacterium[a]	5	25	87.7±17.1	−	—
Agrobacterium	4	22	72.3±15.7	+	peritrichous
Proteus	3	6	61.0±3.0	+	peritrichous
Citrobacter	3	13	59.8±29.4	+	peritrichous
Enterobacter	4	32	59.7±6.5	+	peritrichous
Acinetobacter	6	43	58.2±25.0	+	polar
Acetobacter	3	12	56.2±19.9	±	peritrichous
Erwinia	3	25	56.0±17.7	+	peritrichous
Klebsiella	5	32	54.8±8.9	−	—
Morganella	1	1	53.3	+	peritrichous
Xanthomonas	3	31	53.1±6.1	+	polar
Serratia	4	32	49.9±3.6	+	peritrichous
Rhizobium	3	9	47.0±22.5	+	peritrichous
Escherichia	3	146	43.4±24.6	±	peritrichous
Alcaligenes	6	46	40.8±23.1	+	peritrichous
Alteromonas	2	5	34.1	+	polar
Pseudomonas[b]	16	330	32.1±13.0	+	polar
Xanthobacter	1	2	30.6	±	peritrichous
Aeromonas	5	15	15.9±18.3	±	polar
Vibrio	2	8	10.7	+	polar
Azotobacter	1	1	8.0	+	peritrichous

Symbols: +, present, −, absent, ±, differs among species. Data on characteristics are from Krieg and Holt (1984) and Holt et al. (1994).

[a] Species of *Chryseobacterium* formerly classified as *Flavobacterium* are contained.

[b] Species of *Burkholderia* and *Comamonas* formerly classified as *Pseudomonas* are contained.

survival rates after freeze-drying (data not shown). Among the Gram-positive bacteria, some genera and species are motile but many are nonmotile (Holt et al., 1994). On the other hand, among the Gram-negative bacteria, of which many genera and species are motile (Holt et al., 1994), the nonmotile genera *Flavobacterium*, *Acinetobacter*, and *Klebsiella* showed relatively high survival rates after freeze-drying (54.8–87.7%), whereas the motile genera *Vibrio* and *Aeromonas* had the lowest survival rates (Table 2). *Vibrio* and *Aeromonas* have polar flagella and are reported to form numerous peritrichous flagella in young cultures on solid media (Baumann et al., 1984; Popoff, 1984; Shinoda and Okamoto, 1977); these conditions were characteristic of our freeze-drying studies.

2. Survival of Corynebacterium, Bacillus, Streptococcus, and Klebsiella

The logarithmic decrease in survival per year during storage is expressed by B (Eq.(1)), the slope of the survival curve. In some genera, the survival rates of pathogenic species during storage were lower than those of nonpathogenic species.

Table 3 lists species of *Corynebacterium* in descending order of B values. Species that are pathogenic to humans, such as *C. pseudodiphtheriticum*, *C. striatum*, and *C. urealyticum*, showed the lowest survival rates during storage. *Corynebacterium sepedonicum*, a species pathogenic to plants, had a lower survival rate than the nonpathogenic *Corynebacterium* species but a higher survival rate than the species pathogenic to humans. Characteristics other than pathogenesis, such as varieties of utilizable sugars (glucose, arabinose, xylose, rhamnose, fructose, galactose, mannose, lactose, maltose, sucrose, trehalose, raffinose, salicin, dextrin, and starch); occurrence of hydrolytic reactions (e.g., of esculin, hippurate, urea, tyrosine, or casein), production of phosphatase, pyrazinamidase, and methyl red; and reduction of nitrate to nitrite (Collins and Cummins, 1984; Holt et al., 1994) were not correlated with survival rate during storage (data not shown).

Table 3. Survival rates of *Corynebacterium* species during storage, and related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Human pathogen	Plant pathogen
C. liquefaciens	3	16	1.8±0.1	0.005±0.007	9	−	−
C. melassecola	3	15	1.9±0.0	−0.001±0.004	5	−	−
C. glutamicum	222	15	1.9±0.0	−0.003±0.003	15	−	−
C. ammoniagenes	7	10	2.0±0.1	−0.009±0.006	5	−	−
C. acetoacidophilum	14	15	1.9±0.1	−0.015±0.015	10	−	−
C. sepedonicum	2	20	0.6±0.2	−0.028±0.021	6	−	+
C. pseudodiphtheriticum	2	5	1.7±0.1	−0.037±0.026	3	+	−
C. striatum	1	5	1.8±0.0	−0.066±0.009	3	+	−
C. urealyticum	1	4	0.8±0.3	−0.349±0.121	3	+	−

For definitions of *A* and *B*, see the explanation of Eq.(1). $\mathrm{Log}_{10}\ Y=A+BX$ in the text.

X is the number of storage years and *N* is the number of plotted points on the graph of the equation. Human pathogenic species were classified according to Ezaki and Kawamura (2001) and plant pathogens according to the Phytopathological Society of Japan (2000). Symbols: + pathogenic; − not pathogenic.

Table 4. Survival rates of *Bacillus* species during storage and related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Human pathogen	Plant pathogen	phospho-lipase	Growth with lysozyme present
B. brevis	5	5	1.6±0.2	0.012±0.009	3	−	−	−	±
B. stearothermophilus	6	10	1.2±0.3	0.007±0.062	7	−	−		−
B. circulans	4	6	1.5±0.2	0.007±0.057	6	−	−	−	±
B. pumilus	11	10	1.6±0.0	0.003±0.008	7	−	−	−	±
B. macerans	1	10	2.0±0.0	0.000±0.000	4	−	−	−	−
B. licheniformis	6	5	1.9±0.1	0.000±0.019	3	−	−	−	±
B. badius	1	6	1.9±0.1	0.000±0.034	4	−	−		−
B. sphaericus	6	10	1.8±0.1	−0.009±0.024	6	−	−	−	±
B. polymyxa	2	10	1.6±0.2	−0.010±0.030	4	−	+	−	±
B. megaterium	4	5	1.7±0.1	−0.026±0.023	3	−	−	−	−
B. coagulans	3	6	1.7±0.5	−0.032±0.107	4	−	−	−	−
B. subtilis	132	10	1.8±0.0	−0.032±0.008	7	−	+	−	±
B. thuringiensis	2	10	1.8±0.1	−0.034±0.017	5	−	−	±	+
B. cereus	3	6	2.0±0.1	−0.094±0.017	6	+	−	+	+

See Table 3 for details. Characteristic data are from Claus and Berkely (1984).

Table 4 lists *Bacillus* species in descending order of *B* values. Among the species, *B. cereus* showed the greatest decrease in logarithmic survival per year, although the survival rate after freeze-drying (Y_D=79.7±12.4) was higher than the average value for *Bacillus* (Y_G=62.8±16.6; see Table 1). *Bacillus cereus* was the only *Bacillus* species that we examined that was pathogenic to humans (Ezaki and Kawamura, 2001); this species makes extracellular products, including hemolysin, soluble toxin, enzymes lytic for bacterial cells, proteolytic enzymes, phospholipase C (Claus and Berkely, 1984), and poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acid copolymer (Ramsay et al., 1990). *Bacillus thuringiensis* had a lower survival rate during storage than some others in the genus. This species is reported to be pathogenic to the Lepidoptera and is closely related to *B. cereus* (Claus and Berkely, 1984). The two species pathogenic to plants, *B. subtilis* and *B. polymyxa*, had fairly low survival rates during storage; these species form extracellular polysaccharides

Table 5. Survival rates of *Streptococcus*, *Enterococcus*, and *Lactococcus* species during storage and related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Human pathogen	Extracellular polysaccharide
E. avium	2	21	2.0±0.0	−0.005±0.003	7	+	−
L. lactis	13	20	1.6±0.1	−0.006±0.008	6	−	−
E. casseliflavus	2	10	1.8±0.3	−0.012±0.044	4	−	
E. faecium	4	16	2.0±0.1	−0.016±0.007	6	+	−
S. equi	9	20	1.8±0.1	−0.025±0.007	7	+	−
S. mutans	16	21	1.7±0.1	−0.027±0.007	11	+	+
E. faecalis	4	15	2.0±0.1	−0.031±0.010	5	+	−
E. hirae	1	5	1.9±0.0	−0.049±0.005	3	+	
S. gordonii	1	5	1.3±0.2	−0.061±0.056	3	+	+[a]
S. salivarius	7	21	1.7±0.1	−0.064±0.010	6	+	+
S. sanguinis	2	21	1.1±0.3	−0.093±0.024	7	+	+
S. mitis	2	21	1.3±0.4	−0.108±0.035	7	+	+[b]

All species were formerly classified into *Streptococcus*. See footnote to Table 3 for details. Data on characteristics are from Hardie (1984) and Holt et al. (1994). [a] from Haisman and Jenkinson (1991), [b] from Matsushita et al. (1995).

(levan or dextran) from sucrose (Claus and Berkely, 1984). Claus and Berkely (1984) reported several characteristics that differ among *Bacillus* species, including growth conditions, gelatin hydrolysis, tyrosine degradation, nitrate reduction to nitrite, acid formation from various carbohydrates, and deamination of phenylalanine. None of these characteristics, however, was related to survival rate during storage (data not shown). Because spores of *B. subtilis* have higher survival rates than vegetative cells after freeze-drying (Fairhead et al., 1994), future studies should examine spore formation in cultures of *Bacillus* species prepared for freeze-drying.

Species of *Enterococcus* and *Lactococcus* were previously classified as belonging to the genus *Streptococcus* (Hardie, 1984); Table 5 lists these three genera in descending order of *B* values. Species showing the greatest decrease in logarithmic survival per year tended to be orally pathogenic to humans and to produce extracellular polysaccharides. The common characteristic of orally pathogenic *Streptococcus* is the production of extracellular polysaccharides from sucrose, a mechanism that is considered to be helpful in colonization of the mouth (Colman and Ball, 1984; Hardie, 1984). Species not producing polysaccharides (*E. avium*, *L. lactis*, *E. faecium*, *S. equi*, and *E. faecalis*; Fig. 1(a)–(c)) showed higher survival rates during storage than many of the species producing extracellular polysaccharides (*S. mutans*, *S. gordonii*, *S. salivarius*, *S. sanguinis*, and *S. mitis*; Fig. 1(d)–(f)). Growth conditions, varieties of utilizable sugars (inulin, lactose, mannitol, raffinose, ribose, salicin, sorbitol, and trehalose), and hydrolytic reaction of, for example, arginin, hippurate, esculin, starch, and urea vary greatly among *Enterococcus*, *Lactococcus*, and *Streptococcus* species (Hardie, 1984; Holt et al., 1994), but

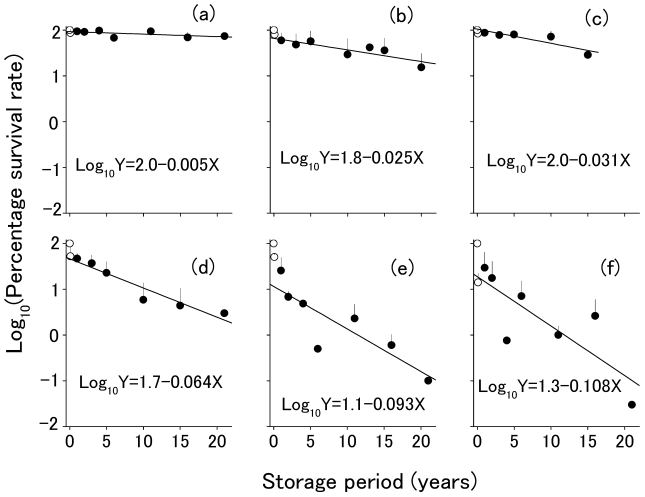

Fig. 1. Survival curves of *Enterococcus* and *Streptococcus* species, (a) *E. avium*, (b) *S. equi*, (c) *E. faecalis*, (d) *S. salivarius*, (e) *S. sanguinis*, (f) *S. mitis*.

(○–) and (●–) show means and standard deviations calculated with real numbers of survival rate each year, respectively. (○), freeze-drying effect; (●), storage effect. Only the upper standard deviations are shown. The values during storage were fitted to the equation $\mathrm{Log}_{10}Y=A+BX$ by the least-squares method (see data in Table 5).

Table 6. Survival rates of *Klebsiella* species during storage, and the related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Pathogen
K. planticola	6	10	1.5±0.1	−0.031±0.015	8	−
Klebsiella sp.	6	15	1.8±0.1	−0.070±0.012	9	
K. terrigena	1	15	1.8±0.1	−0.079±0.010	5	−
K. oxytoca	9	10	1.5±0.1	−0.120±0.015	4	+
K. pneumoniae	10	15	2.0±0.2	−0.148±0.027	5	+

Pathogen: +, pathogenic; −, nonpathogenic. For definition of *A* and *B*, see Eq.(1) in the text.

none of these characteristics was related to survival during storage in the species studied here (data not shown).

Table 6 lists species of the nonmotile Gram-negative genus *Klebsiella* in descending order of *B* values. *Klebsiella planticola* (Fig. 2(a)) and *K. terrigena* had more stable survival rates during storage than did *K. oxytoca* (Fig. 2(b)) and *K. pneumoniae* (Fig. 2(c)). The characteristic difference between these species is that the former two are nonpathogenic species and the latter two are pathogenic (Ezaki and Kawamura, 2001; Orskov, 1984). The two pathogenic species contain large polysaccharide capsules and form large mucoid colonies, especially on carbohydrate-rich media (Holt et al., 1994); these types of media were used in our freeze-drying study.

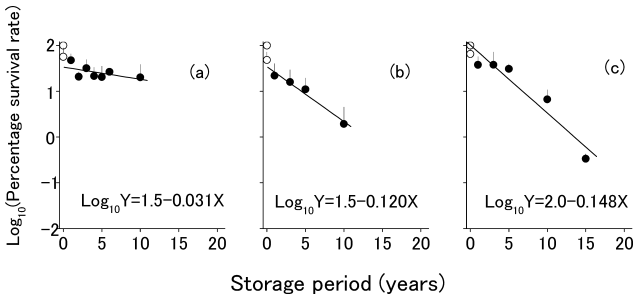

Fig. 2. Survival curves of *Klebsiella* species, (a) *K. planticola*, (b) *K. oxytoca*, (c) *K. pneumoniae*.

(○–) and (●–) show means and standard deviations calculated with real numbers of survival rate each year, respectively. (○), freeze-drying effect; (●), storage effect. Only the upper standard deviations are shown. The values during storage were fitted to the equation $\mathrm{Log}_{10}Y=A+BX$ by the least-squares method (see data in Table 6).

3. Survival of Lactobacillus

Table 7 lists species of *Lactobacillus* and their patterns of carbohydrate fermentation (Holt et al., 1994; Kandler and Weiss, 1984) in descending order of survival after freeze-drying (Y_D). Gluconate- and ribose-fermenting species had higher Y_D, and sorbitol- or trehalose-fermenting species had lower Y_D.

Table 8 lists *Lactobacillus* species (all nonpathogenic to humans) in descending order of *B* values. The survival rate during storage appeared to be related to plant pathogenesis and serological differentiation. The species showing the greatest decrease in logarithmic survival per year, *Lactobacillus delbrueckii*, is pathogenic, but only to plants (Kandler and Weiss, 1984). Serological studies of *Lactobacillus* have shown that these species can be assigned to several groups on the basis of specific antigenic characteristics, including production of cell wall polysaccharides, cell wall teichoic acids, and membrane teichoic acids (Knox and Wicken, 1973; Sharpe, 1981). Teichoic acids are components of the outer layers of Gram-positive bacteria; they function like extracellular polysaccharides in processes such as antigen activity, adhesion, and biofilm formation (Birdsell et al., 1975; Doyle et al., 1975; Graham and Beveridge, 1994; Knox and Wicken, 1973; Neuhaus and Baddiley, 2003).

Those species with glycerol teichoic acid (GTA) in the cell wall and cell membrane (*L. helveticus*) and in the cell membrane (*L. fermentum*) had higher survival rates during storage than species that had GTA in the cell wall (*L. brevis* and *L. delbrueckii*) (Fig. 3(a), (b), (e), (f)). Among these species, *L. helveticus* also has a surface-layer protein that confers hydrophobic properties on the cell surface (Schär-Zammaretti and Ubbink, 2003; Steen et al., 2003; Ventura et al., 2000). In regard to antigens in the cell wall, species with ribitol teichoic acid (RTA; *L. plantarum*) or polysaccharides (*L. casei* and *L. salivarius*) showed higher survival rates during storage than species with GTA (Fig. 3(c)–(f)). Like extracellular polysaccharides, teichoic acids in the cell wall appear to decrease survival rates during storage, with species having GTA in the cell wall showing

Table 7. Survival rates of *Lactobacillus* species after freeze-drying, and related characteristics.

Species	No. strains	Y_D±SD	Fermented carbohydrate			
			Gluconate, ribose	Sorbitol	Sucrose	Trehalose
L. confusus	5	92.3±12.6	+	−	+	−
L. reuteri	3	90.7±4.6	+	−	+	−
L. animalis	1	89.8	−	+	+	−
L. casei	15	72.2±20.8	±	±	±	±
L. fermentum	8	66.7±24.8	+	−	+	±
L. brevis	4	65.8±35.0	+	−	±	−
L. plantarum	4	63.5±24.2	+	+	+	+
L. acidophilus	8	62.5±24.5	−	+	+	±
L. alimentariu	1	53.0	+	−	+	−
L. delbrueckii	3	45.0±21.3	−	+	±	±
L. amylovorus	1	43.0	−	+	+	+
L. salivarius	5	33.2±20.2	−	+	+	+
L. helveticus	1	26.0	−	+	−	±
L. gasseri	5	22.2±17.0	−	+	+	±

Symbols: +, 90% or more strains positive; −, 90% or more strains negative; ±, 11–89% strains positive. Data on carbohydrate fermentation are from Kandler and Weiss (1984).

Table 8. Survival rates of *Lactobacillus* species during storage, and related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Plant pathogen	Serological classification	
							Antigen	Location
L. helveticus	2	10	0.8±0.1	−0.005±0.024	4	−	GTA	cell membrane, cell wall
L. fermentum	7	16	1.5±0.1	−0.018±0.016	7	−	GTA	cell membrane
L. plantarum	4	5	1.7±0.0	−0.025±0.014	3	−	RTA	cell wall
L. casei	15	15	1.8±0.1	−0.035±0.009	6	−	Polysaccharide	cell wall
L. salivarius	5	21	1.3±0.2	−0.078±0.019	7	−	Polysaccharide	cell wall
L. brevis	4	16	1.9±0.2	−0.108±0.023	5	−	GTA	cell wall
L. delbrueckii	2	15	1.7±0.5	−0.183±0.063	5	+	GTA	cell wall

See Table 3 for details. Characteristic data are from Kandler and Weiss (1984).

lower survival rates than those with polysaccharides.

4. Survival of Pseudomonas

Table 9 lists species of *Pseudomonas*, a motile genus, and two species formerly classified as *Pseudomonas* in descending order of *B* values, along with their physiological characteristics (Holt et al., 1994; Palleroni, 1984). In this genus, survival rates during storage did not appear to be related to pathogenesis. *Pseudomonas pseudoalcaligenes* and *P. alcaligenes*, the two species that cannot utilize glucose as an energy source, showed relatively low survival rates during storage. Although trehalose is an effective protectant for freeze-dried cells (Conrad et al., 2000; Crowe et al., 1992), trehalose utilization was not correlated with survival rate during storage in this study. In regard to polysaccharide formation, *P. chlororaphis* (Fig. 4(c)), one of two species that can form levan from sucrose, had the lowest survival rate. Three of five *P. fluorescens* biovars are also reported to form levan (Holt et al., 1994; Palleroni, 1984), but in this study the average survival rate of the species was high, with no

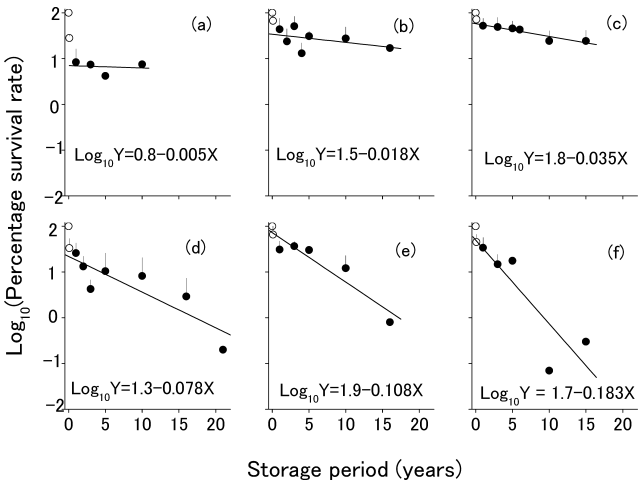

Fig. 3. Survival curves of *Lactobacillus* species, (a) *L. helveticus*, (b) *L. fermentum*, (c) *L. casei*, (d) *L. salivarius*, (e) *L. brevis*, (f) *L. delbrueckii*.

(○–) and (●–) show means and standard deviations calculated with real numbers of survival rate each year, respectively. (○), freeze-drying effect; (●), storage effect. Only the upper standard deviations are shown. The values during storage were fitted to the equation $\mathrm{Log}_{10}Y=A+BX$ by the least-squares method (see data in Table 8).

further information about the biovars of the strains tested.

5. Survival of Flavobacterium

Table 10 lists *Flavobacterium aquatile* and two species of *Chryseobacterium* formerly classified as *Flavobacterium* in descending order of *B* values, along

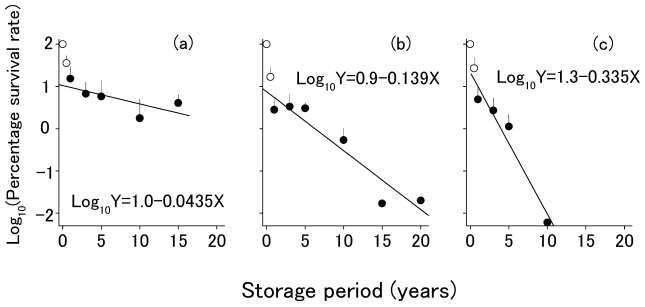

Fig. 4. Survival curves of *Pseudomonas* species, (a) *P. aeruginosa*, (b) *P. solanacearum*, (c) *P. chlororaphis*.

(○–) and (●–) show means and standard deviations calculated with real numbers of survival rate each year, respectively. (○), freeze-drying effect; (●), storage effect. Only the upper standard deviations are shown. The values during storage were fitted to the equation $\mathrm{Log}_{10}Y=A+BX$ by the least-squares method (see data in Table 9).

Table 9. Survival rates of *Pseudomonas* species, *Burkholderia* species and *Comamonas* species during storage, and the related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Pathogen		Utilization			Levan formation from sucrose
						Human	Plant	Glucose	Geraniol	Trehalose	
P. aeruginosa	10	15	1.0±0.2	−0.044±0.024	5	+	+	+	+	−	−
P. fluorescens	28	20	1.0±0.5	−0.053±0.048	8	−	+	+	−	+	+ or −
P. putida	45	16	0.9±0.4	−0.078±0.043	12	−	−	+	−	−	−
P. gladioli	1	16	0.9±0.3	−0.079±0.041	6	−	+	+	−	+	
P. cichorii	1	15	1.8±0.1	−0.118±0.012	6	−	+	+	−	−	−
B. cepacia[a]	17	15	1.1±0.4	−0.136±0.045	8	+	+	+	−	+	
P. stutzeri	8	15	1.5±0.4	−0.139±0.049	5	−	−	+	−	−	−
P. solanacearum	4	20	0.9±0.3	−0.139±0.023	6	−	+	+	−	+	−
C. testosteroni[a]	4	11	0.6±0.4	−0.151±0.069	7	+	−	+	−		−
P. pseudoalcaligenes	3	5	1.3±0.3	−0.203±0.089	3	−	+	−	−	−	−
P. aureofaciens	3	10	1.0±0.1	−0.219±0.013	4	−	−	±	−	±	
P. alcaligenes	1	5	0.7±0.3	−0.246±0.074	3	+	−	−	−	−	−
P. pickettii	2	5	1.7±0.2	−0.290±0.059	3	−	−	+	−	−	−
P. chlororaphis	4	10	1.3±0.3	−0.335±0.058	4	−	−	+	−	+	+

[a] These species were formerly classified into *Pseudomonas*. Symbols: +, 90% or more strains positive; −, 90% or more strains negative; ±,11–89% of strains positive; + or −, difference among biovar. For definitions of *A* and *B*, see Eq.(1) in the text.

Table 10. Survival rates of *Flavobacterium* species and *Chryseobacterium* species during storage and related characteristics.

Species	No. strains	*X*	*A*±SD	*B*±SD	*N*	Pathogen	Acid produced aerobically from: mannitol, trehalose, glycerol	Urease production	Growth at 42°C	Starch hydrolysis	ONPG hydrolysis
C. meningosepticum[a]	2	15	1.7±0.1	−0.001±0.017	5	+	±	±	±	−	+
C. balustinum[a]	1	5	1.5±0.4	−0.093±0.103	3	−	−	−	−	−	−
F. aquatile	3	15	1.6±0.1	−0.174±0.011	5	−	−	−	−		−

[a] These species were formerly classified into *Flavobacterium*. For symbols, see Table 9. For definition of *A* and *B*, see Eq.(1) in the text.

with their physiological characteristics (Holmes et al., 1984; Holt et al., 1994). Survival of *C. meningosepticum* was very stable during storage, whereas the survival rates of *C. balustinum* and *F. aquatile* were much less so (Fig. 5). Among these nonmotile species, only *C. meningosepticum* is pathogenic (Ezaki and Kawamura, 2001); its high survival rate is opposite to that of some *Corynebacterium*, *Bacillus*, *Streptococcus* and *Klebsiella* and many Gram-positive bacteria. *Chryseobacterium meningosepticum* differs from the other two species in the ability of some strains to aerobically produce acid from several sugars, produce urease, and grow at 42°C (as shown by ± in Table 10). Neither *C. meningosepticum* nor *C. balustinum* has the ability to hydrolyze starch, and the reaction of *F. aquatile* is not known. *Chryseobacterium meningosepticum* can hydrolyze ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and commonly produces β-galactosidase, unlike the other two species (Holmes et al., 1984; Holt et al., 1994). In contrast to *C. meningosepticum*, however, other ONPG-positive species of the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Serratia* (Brenner, 1984; Holt et al., 1994) did not show stable survival during storage in this study (data not shown). *Chryseobacterium meningosepticum* also produces oligosaccharide-cleaving enzymes (Elder and Alexander, 1982; Plummer et al., 1991). In addition, electron micrographs have revealed that *C. meningosepticum* has a smoother surface than *F. aquatile*, which has extracellular appendages (Thomson et al., 1981).

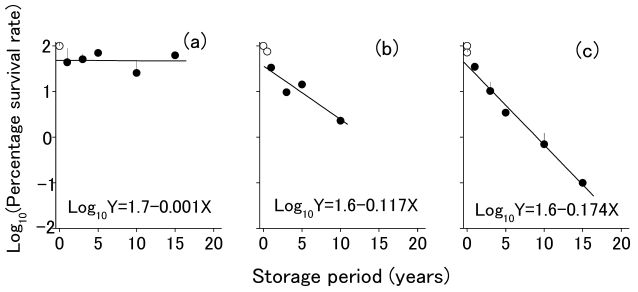

Fig. 5. Survival curves of *Flavobacterium* species and *Chryseobacterium* species, (a) *C. meningosepticum*, (b) *C. balustinum*, (c) *F. aquatile*.

(○–) and (●–) show means and standard deviations calculated with real numbers of survival rate each year, respectively. (○), freeze-drying effect; (●), storage effect. Only the upper standard deviations are shown. The values during storage were fitted to the equation $\mathrm{Log}_{10}Y=A+BX$ by the least-squares method (see data in Table 10).

6. Survival rates of Gram-negative versus Gram-positive bacteria

Survival rates during storage of Gram-negative species tended to be lower than those of Gram-positive species. Figure 6 illustrates the distributions of *B* values for 110 Gram-positive species and 77 Gram-negative species. Most Gram-negative bacteria showed relatively low survival rates during storage, although a few species, such as *C. meningosepticum*, showed stable rates. The mean *B* value for the 77 Gram-negative species was −0.117±0.109, compared with −0.023±0.045 for the 110 Gram-positive species (*t*-test, df=185, $p<0.0001$). A few of the Gram-positive species with *B* values greater than −0.075 were pathogenic species that produce extracellular polysaccharides, or species with teichoic acids on their cell walls.

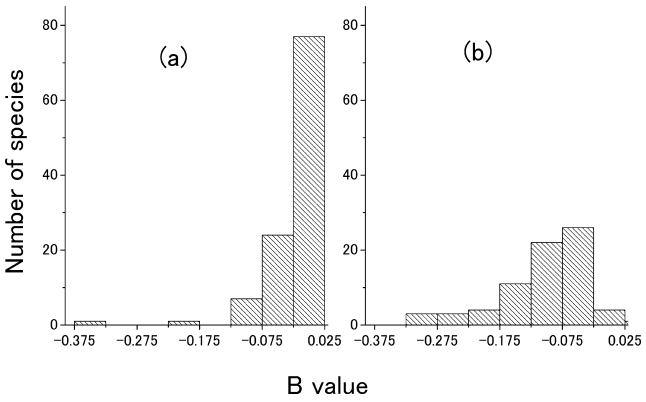

Fig. 6. Frequency distribution of *B* values of (a) Gram-positive species ($n=110$) and (b) Gram-negative species ($n=77$).
For a definition of *B*, see Eq.(1) in the text.

Discussion

1. Survival after freeze-drying is related to cell wall structure

Survival after freeze-drying reflects the ability of the cell to resist the effects of rapid freezing and drying. Differences in survival should reflect structural differences in the cell wall and cell membrane of organisms; the cell wall and membrane of *S. cerevisiae* and *L. acidophilus* are degraded by freeze-drying or drying (Brennan et al., 1986; Deere et al., 1998). In most cases, no significant differences were observed in the survival rates of species (Y_D) of the same genus, with the exception of *Lactobacillus*. At species level, this research suggested that survival in the lactobacilli was related to the presence of trehalose in the cell membrane. In the lactobacilli, carbohydrates are taken up with the help of specific permeases and are phosphorylated in the cytoplasm (Kandler and Weiss, 1984). This phosphorylation is assumed to be important in the regulation of carbohydrate metabolism in Gram-positive bacteria (Viana et al., 2000). Trehalose is a carbohydrate useful for preventing desiccation in bacterial cells, and the presence of trehalose in the cell membrane during dehydration helps to prevent leakage of dry cell membranes during rehydration (Crowe et al., 1992). Bacterial cells freeze-dried with trehalose or sucrose show better survival and lower membrane phase-transition temperatures during drying (Leslie et al., 1995). Trehalose utilization begins with phosphotransferase-mediated uptake delivering trehalose-6-phosphate to the cytoplasm (Boos et al., 1990). The non-trehalose-fermenting species *Lactobacillus confusus*, *Lactobacillus reuteri*, and *Lactobacillus animalis* had high survival rates after freeze-drying (Y_D) (Table 7), suggesting that these species may have trehalose or trehalose-like substances outside the cytoplasm. The relationship between trehalose assimilation and its location in freeze-dried microorganisms should be examined further.

At genus level, the study suggested that survival was related to the presence of flagella. Many Gram-negative bacteria move individually with flagella, whereas many Gram-positive bacteria are nonmotile and form cell clusters. Our results showed that cells with numerous flagella were more sensitive to freeze-drying than those with smooth surfaces. The relationship between type of flagella and survival after freeze-drying needs to be examined further by using co-isogenic mutants of *Bacillus subtilis* with defective flagella (Calvio et al., 2005).

Comparison of Gram-positive bacteria and Gram-negative bacteria suggested that survival was related to the thickness of the cell wall peptidoglycan layer. Survival rates of Gram-negative genera after freeze-drying (Y_G) varied from 8.0% to 87.7% (Table 2), whereas those of Gram-positive genera ranged from 58.5% to 96.7% (Table 1). Gram-negative bacteria have a peptidoglycan layer about 5 to 10 nm thick between the inner and outer plasma membranes, whereas the thickness of this layer in Gram-positive bacteria is about 20 to 80 nm (Alberts et al., 1994; Beveridge, 1999; Cooper, 2000; Gupta, 2002; Lodish et al., 2000; Salton and Kim, 1996). The cell walls of Gram-negative bacteria, with a thinner peptidoglycan layer than those of Gram-positive bacteria, have a much greater tendency to rupture during the processes of desiccation and rehydration (Pembrey et al., 1999).

2. Survival during storage is related to the presence of extracellular polysaccharides or teichoic acid

Survival of freeze-dried microorganisms during storage is expressed by a decrease in the logarithm of survival per year (*B*). The genera *Corynebacterium*, *Bacillus*, and *Streptococcus* include both pathogenic and nonpathogenic species, and the pathogenic species had lower *B* values than the nonpathogenic ones. Pathogenesis is sometimes accompanied by the presence of extracellular polysaccharides, as shown for *Streptococcus* in Table 5. The presence of extracellular polysaccharides appears to be associated with

decreased survival of freeze-dried pathogenic bacteria during storage. Pathogenic species that move with flagella release the flagella and grow a stalk at the pole to adhere to the host cell (Shapiro et al., 2002). In contrast, nonmotile pathogenic species of *Streptococcus* produce extracellular polysaccharides that aid in adhering to and colonizing host cells (Hardie, 1984). Members of the Gram-negative genera *Klebsiella* are nonmotile, and two pathogenic species have polysaccharide capsules (Holt et al., 1994). *Klebsiella pneumoniae* produces more extracellular polysaccharides than does *E. coli* (Jones and Bradshaw, 1996), and in our study the survival rate of *K. pneumoniae* during storage was lower ($B=-0.148\pm0.027$; Table 6) than that of *E. coli* ($B=-0.041\pm0.005$; Miyamoto-Shinohara et al., 2006). Because most pathogenic Gram-negative species are motile, production of extracellular polysaccharides may not be necessary for their survival in natural environments. In motile *Pseudomonas*, the survival rates of species during storage were not related to pathogenesis. The survival rate of *P. chlororaphis*, one of two species that forms levan from sucrose, decreased faster than those of other *Pseudomonas* species; this finding suggests that the presence of extracellular polysaccharides decreases the survival rate during storage of freeze-dried bacteria.

By retaining water in the cells, extracellular polysaccharides help bacteria to survive in desiccant environments (Billi and Potts, 2002; Potts, 1994; Schnider-Keel et al., 2001). Soil bacteria such as *Pseudomonas* spp. and *B. subtilis* produce extracellular polysaccharides when proliferating in dry environments (Hamon and Lazazzera, 2001; Roberson and Firestone, 1992), and *Streptococcus* and *Lactobacillus* strains that produce extracellular polysaccharides increase the moisture levels of cheese (Low et al., 1998; Petersen et al., 2000; Vuyst and Degeest, 1999). Teichoic acid molecules on the cell wall surface are more exposed and hold more water than those in the cell membrane. Thus, species that have teichoic acids within the cell membrane are better able to withstand freeze-drying and have better survival rates during storage. The direct influence of extracellular polysaccharides or teichoic acid on the survival of freeze-dried bacteria should be examined further.

3. Survival during storage is related to the presence of lipopolysaccharides

Freeze-dried Gram-negative bacteria generally had lower survival rates during storage than did freeze-dried Gram-positive bacteria. Among species of Gram-positive bacteria, those with extracellular polysaccharides or teichoic acids showed lower survival rates than other species. Analogous to polysaccharides and teichoic acids, lipopolysaccharides are held by relatively weak bonds in the outer membrane of Gram-negative bacteria, with hydrophilic polysaccharide chains outermost. The outermost polysaccharide region of lipopolysaccharides may prevent the removal of water molecules during the process of the freeze-drying. Most Gram-negative bacteria have lipopolysaccharides on their surfaces; these lipopolysaccharides may trap water molecules in the storage ampoules, causing a decrease in survival rates during long-term storage. Those Gram-negative species with extracellular polysaccharides in addition to lipopolysaccharides would retain even more water molecules, and their survival rates would be lower than those of species having only lipopolysaccharides on the cell wall. Some strains of *Rhizobium etli* (Carlson et al., 1995), *Neisseria gonorrhoeae* (Crooke et al., 1998), and *Helicobacter pylori* (Merkx-Jacques et al., 2004) are reported to have lost the ability to synthesize lipopolysaccharides. These strains without lipopolysaccharide should be studied in future to determine whether or not they have stable survival during storage.

4. Improving the survival of freeze-dried bacteria during storage

Reports of residual moisture in freeze-dried ampoules (Greiff, 1971; Podolsky and Konstantinov, 1980; Seligmann and Farber, 1971) suggest that there is residual air containing water molecules, even in ampoules sealed under vacuum. The water molecules may become trapped in extracellular polysaccharides around the dried cell wall and injure the cell during storage. Complete desiccation is thought to be important for the survival of freeze-dried cells.

All the bacterial species studied here were freeze-dried and stored under the same conditions, but survival rates during storage varied among species. To improve the survival of freeze-dried bacteria during storage, we suggest three strategies: reducing the number of water molecules in an ampoule, reducing the activity of the water molecules, and preventing

bacterial cells from trapping water molecules.

To reduce the number of water molecules in an ampoule, bacteria should be prepared under dry conditions and completely freeze-dried. Ampoules should be sealed under as high a vacuum as possible, which will depend on the capacity of the vacuum pump used and the strength of the glass ampoules.

To reduce the activity of water molecules, ampoules should be stored at low temperature. Freeze-dried bacteria stored at lower than −20°C show better survival rates than those stored at 4°C (Gu et al., 2001), and vacuum-dried specimens stored at 5°C show better survival than those stored at 37°C (Iijima and Sakane, 1973; Sakane and Kuroshima, 1997). The activity of water molecules is reduced at low temperatures and reduced more by freezing. In this research, freeze-dried bacteria were stored at 5°C, allowing water molecules in the ampoules to be somewhat active during the years of storage and likely decreasing bacterial survival as a result.

To prevent bacterial cells from trapping water molecules, a desiccant should be added to the ampoule. A double ampoule within desiccant prepared by the American Type Culture Collection (ATCC) is ideal. When vacuum-drying is used, with a cotton wool plug in the ampoule, the plug functions as a desiccant to improve the survival of the specimen (Iijima and Sakane, 1973). In addition, because extracellular polysaccharides and teichoic acids trap water molecules, ways of inhibiting the formation of those substances should be examined. The marine bacterium *Pelagiobacter* sp. shows higher survival rates when cells are washed with seawater before vacuum-drying (Katsuta and Kasai, 2003), suggesting that polysaccharides around the cells might be removed physically. Because *Lactobacillus rhamnosus* hydrolyzes extracellular polysaccharide by glycohydrolase (Pham et al., 2000), enzymatic removal of those substances might be possible. The production of extracellular teichoic acids by *Lactobacillus salivarius* and *Staphylococcus aureus* is controlled by the addition of Tween 80 in the media used, or by the use of aero-anaerobic conditions (Jacques et al., 1980; Sadovskaya et al., 2005). Thus, to improve the survival rates during storage of these species when freeze-dried, future studies should examine which culture conditions suppress the production of polysaccharides and teichoic acids.

Although extracellular polysaccharides and teichoic acid are important for bacterial pathogenesis and survival in desiccated environments, these compounds may negatively affect the survival of freeze-dried cells.

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