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Survival of Frozen Mycoplasmas

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Cooling to −70 C killed a higher percentage of *Acholeplasma laidlawii* and *Mycoplasma mycoides* var. *capri* cells than cooling to −20 C. However, to preserve cell viability for prolonged periods storage at −70 C was much more preferable. The percentage of cells surviving freezing could be increased by increasing the initial cell concentration or by the addition of dimethyl sulfoxide or glycerol as cryoprotective agents. In the presence of 1.5 M of any one of these agents survival rates of up to 100% could be obtained. The optimal cooling rates for maximal survival of *A. laidlawii* under the experimental conditions tested were 11 C/min for cooling to −20 C and about 15 C/min for cooling to −70 C. Increasing the warming rate during thawing from 0.6 to 67 C/min increased survival by 3 log. Oleic acid enrichment of *A. laidlawii* membrane lipids, or reduction in the cholesterol content of *M. mycoides* var. *capri* membranes, increased the percentage of organisms surviving freezing. Hence, the composition of membrane lipids appears to have a marked influence on the susceptibility of mycoplasmas to freezing injury.

The ability to recover viable mycoplasmas from frozen cultures makes possible the preservation of stock cultures by frozen storage and the provision of readily available standard inocula for physiological studies (1, 5, 6, 14). Yet, until now no detailed investigation has been made of the factors affecting the survival of mycoplasmas during the freezing process and the following storage in the frozen state. The practical advantages to defining the conditions leading to maximal recovery of viable mycoplasmas from the frozen state are obvious. Moreover, this investigation can provide a basis for a study of a more theoretical nature, in which mycoplasmas would be used as models for studying the mechanism of freezing injury. Evidence is steadily mounting (8, 11, 21) to the effect that the cell membrane is the chief target of freezing injury. The fact that mycoplasma cells are bounded by a single lipoprotein membrane, the lipid composition of which can be systematically altered, makes these organisms attractive objects for studying problems concerning the interrelationship of membrane composition and freezing injury.

The present communication reports the effects of several freezing parameters, such as rates of cooling and thawing, initial cell concentration, and cryoprotective agents, on the survival of mycoplasmas. The effect of alterations in membrane lipid composition on the susceptibility of the organisms to death by freezing is also reported.

MATERIALS AND METHODS

Organisms and growth media. *Acholeplasma laidlawii* (oral strain) and *Mycoplasma mycoides* var. *capri* (PG3) were the test organisms. The organisms were grown in a modified Edward medium (16) consisting (per liter) of: heart infusion broth (Difco), 13 g; peptone (Difco), 5 g; yeast extract (Difco), 7 g; glucose, 5 g; NaCl, 2.5 g; K_2HPO_4, 2.4 g; sodium deoxyribonucleate, 0.02 g; PPLO serum fraction (Difco), 20 ml; penicillin G, 300,000 U; and thallium acetate, 0.25 g. The pH of the medium was adjusted to 8.5. The fatty acid composition of *A. laidlawii* cell membranes was modified by growing the organisms in Edward medium in which 0.5% fatty acid-poor bovine serum albumin (19) replaced the PPLO serum fraction. The medium was supplemented with 20 μg of either oleic or palmitic acid per ml, added to the medium as ethanolic solutions. The effect of the membrane cholesterol content on the susceptibility of the cells to death by freezing was tested with *M. mycoides* var. *capri*. The strain of *M. mycoides* var. *capri* which Rottem et al. (20) adapted to grow with low cholesterol concentrations was grown in the albumin-containing Edward medium supplemented with cholesterol (0.1 μg/ml) and with palmitic and oleic acids (5 μg/ml of each) to provide cholesterol-poor organisms. Organisms with a high cholesterol content were obtained by growing the nonadapted native strain of *M. mycoides* var. *capri* in the same medium, but with 10 μg of cholesterol/ml.

Preparation of cell suspensions for freezing.

Organisms grown for 18 to 24 h at 37 C were harvested by centrifugation and resuspended in basal Edward medium (containing all the ingredients except for the PPLO serum fraction) or in β-buffer [0.15 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane, 0.01 M β-mercaptoethanol in deionized water, adjusted to pH 7.0 with HCl]. In some experiments the cryoprotective agents glycerol or dimethyl sulfoxide (ME_2SO) were added to the cell suspensions to a final concentration of 1.5 M. The suspensions were placed in cotton-plugged, 1.2-ml Cryules (catalogue no. 12742, Wheaton Glass Co., Millville, N.J.).

Freezing, thawing, and frozen storage. The cell suspensions were cooled at different rates to −20 or −70 C by means of liquid nitrogen in a BF-4 freezer (Linde Co., Tonawanda, N.Y.). Temperatures of each set of samples during freezing were recorded continuously, using 24-gauge copper constantan thermocouples connected to a Telrad model 702 recorder. The point of measurement was in the middle of the volume to be frozen. The frozen suspensions were stored in electrically operated freezers at −20 C (General Electric freezer) or at −70 C (Revco freezer). The suspensions were usually thawed at room temperature until the last ice crystal disappeared. Different thawing rates were achieved using water baths at different temperatures. Rate of thawing was measured by the same means used for measuring rate of freezing.

Viable cell counts. The number of viable organisms was determined by the colony-count method of Butler and Knight (4). Several 10-fold dilutions of the cell suspensions were prepared in basal Edward medium. Samples (0.01 ml) of the diluted suspensions were placed on the surface of Edward medium plates solidified with 2% (wt/vol) agar. After the drops had been absorbed into the medium the plates were inverted and placed in cans with wet cotton wool to provide a humid atmosphere. The plates were incubated at 37 C for 5 to 7 days. Dilutions yielding 30 to 300 colonies were counted by means of a stereomicroscope. Results were expressed as colony-forming units (CFU) per milliliter of suspension.

Analytical procedures. Protein was determined according to Lowry et al. (7) with bovine serum albumin as standard. Cholesterol in membrane lipids extracted with chloroform-methanol (2:1, by volume) was determined by the $FeCl_3$ method (23). Methyl esters of the fatty acids were prepared and subjected to gas-liquid chromatography (17).

RESULTS

A much lower percentage of *A. laidlawii* and *M. mycoides* var. *capri* cells survived freezing to −70 C than survived freezing to −20 C. However, the number of viable organisms in cell suspensions stored at −20 C for prolonged periods decreased most markedly with time. The decrease was much smaller at a storage temperature of −70 C (Table 1). On the whole, *A. laidlawii* survived freezing better than *M. mycoides* var. *capri*, and in most cases the survival was higher in β-buffer than in Edward medium.

Table 2 demonstrates the dependence of the survival on the initial cell concentration of the frozen suspension. The higher the initial cell concentration the higher the percentage of survivors.

The effect of the cooling rate on the viability of frozen mycoplasmas is shown in Fig. 1. The curve shows the existence of an optimum cooling rate for maximal survival, different for cooling to −20 C and −70 C. The rate of thawing of the frozen cell suspensions was also found to influence survival; the faster the thawing the higher the number of survivors (Table 3).

The addition of ME_2SO or glycerol to the suspending media very effectively protected the organisms from freezing injury, in many cases enabling total recovery of the viable organisms, even when frozen under the unfavorable conditions of slow cooling (Table 4). ME_2SO appears to be preferable to glycerol as a cryoprotective agent for *A. laidlawii*, whereas glycerol appears to be more effective than ME_2SO in the cryoprotection of *M. mycoides* var. *capri*.

The effects of controlled alterations in the membrane lipid composition on the susceptibil-

TABLE 1. *Effect of storage temperature and storage period on the survival of mycoplasmas in Edward medium*[a]

Temp of storage (C)	Storage period (weeks)	*A. laidlawii* (CFU/ml)	*M. mycoides* var. *capri* (CFU/ml)
−20	0	1.3×10^9	8.3×10^7
	3	2.4×10^4	6.3×10^3
	12	1.0×10^2	2.0×10^2
−70	0	2.1×10^7	2.8×10^6
	3	2.7×10^5	2.0×10^6
	12	4.0×10^5	4.0×10^4

[a] The initial counts before cooling were 1.4×10^9 colony-forming units (CFU)/ml for *A. laidlawii* and 2.8×10^9 CFU/ml for *M. mycoides* var. *capri*. Cooling rate was 13 C/min when suspensions were cooled to −20 C and 18 C/min when cooled to −70 C.

TABLE 2. *Effect of the initial cell concentration on the survival of frozen A. laidlawii cells*[a]

Initial cell concn (CFU/ml)[b]	No. of survivors (CFU/ml)[b]	% Survival
2.2×10^{10}	1.8×10^{10}	82.0
2.2×10^9	6.5×10^6	0.3
3.2×10^8	2.5×10^5	0.08
3.0×10^7	8.8×10^3	0.03

[a] The cell suspensions in Edward medium were frozen down to −70 C at a cooling rate of 18 C/min.
[b] CFU, Colony-forming units.

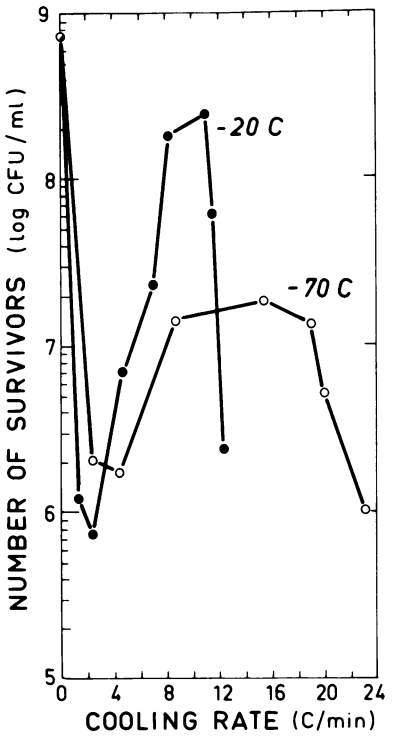

FIG. 1. *Effect of the cooling rate on the survival of A. laidlawii cells suspended in β-buffer and cooled to −20 C or to −70 C.*

TABLE 3. *Effect of warming rate on the survival of frozen A. laidlawii*

Warming rate (C/min)	No. of survivors[a] (CFU/ml)
0.6	6.0×10^2
29.0	3.9×10^3
67.0	5.4×10^5
80.0	5.3×10^5

[a] The initial cell concentration was 3.0×10^9/ml suspended in β-buffer and cooled to −70 C at 0.9 C/min. CFU, Colony-forming units.

ity of the cells to death by freezing is shown in Tables 5 and 6. The marked increase in the oleic acid content of *A. laidlawii* membrane lipids was accompanied by a significant increase in the survival of frozen organisms (Table 5). The reverse can be said with regard to cholesterol. The marked decrease in the cholesterol content of *M. mycoides* var. *capri* membranes, brought about by the adaptation of this organism to grow with very little cholesterol, appears to increase the resistance of the organisms to death by freezing (Table 6).

DISCUSSION

Our results show that, for prolonged periods, storage at −70 C is preferable to storage at −20 C, even though more mycoplasmas are killed during the freezing process itself when cooling is to −70 C. The better preservation of mycoplasmas at −70 C has already been reported by Kelton (6) and by Addey et al. (1). According to Mazur (10) death of bacterial cells during frozen storage may be caused by one or more of the following factors: starvation, recrystallization of intracellular ice, and prolonged exposure to the concentrated extra- and intracellular solutes in the residual unfrozen solution. Prolonged storage at −70 C may be preferable to storage at −20 C since at −70 C all detectable liquid water disappears (22), minimizing exposure of the cells to concentrated solutes. Moreover, at −70 C metabolic processes are completely arrested so that starvation may be discounted as a damaging factor. At −20 C, on the other hand, metabolic processes may continue, though at a very slow pace. Thus, the membrane-associated phospholipase of *Mycoplasma hominis* exhibited its activity during prolonged storage of the membranes at −20 C, but not at −70 C (18). Hence, starvation and autolytic degradation may be added to the factors contributing to the slow death of mycoplasmas kept at −20 C.

TABLE 4. *Effect of cryoprotective agents on the survival of mycoplasmas during freezing*[a]

Cryoprotective agent added (1.5 M)	% Survival							
	A. laidlawii				*M. mycoides* var. *capri*			
	Edward medium		β-Buffer		Edward medium		β-Buffer	
	−20 C	−70 C	−20 C	−70 C	−20 C	−70 C	−20 C	−70 C
ME_2SO	100	100	60	100	87.5	0.19	5.7	0.64
Glycerol	100	6.7	10.8	2.8	53.1	4.4	100	82.1
None	0.1	0.1	1.6	0.002	15.6	0.014	0.002	0.001

[a] The initial count was about 2×10^9 colony-forming units/ml for both organisms. The suspensions were cooled to −20 C at 0.6 C/min and to −70 C at 1.2 C/min.

TABLE 5. *Effect of variations in the fatty acid composition of membrane lipids on the susceptibility of A. laidlawii to death by freezing*[a]

Cooling conditions[b]	% Survival	
	Organisms grown with PPLO serum fraction[c]	Organisms grown with albumin and oleate[d]
Slow cooling to −20 C	1.2	1.5
Rapid cooling to −20 C	10.0	55.0
Slow cooling to −70 C	0.1	2.2
Rapid cooling to −70 C	3.6	33.0

[a] The cooling medium was β-buffer.

[b] Cooling rates were: 1 C/min to −20 C and 1.6 C/min to −70 C for slow; and 24 C/min to −20 and to −70 C for rapid.

[c] Initial count was 1.7×10^9 colony-forming units (CFU)/ml. Fatty acid composition of membrane lipids: $C_{12:0}$, 6.2; $C_{14:0}$, 26.1; $C_{16:0}$, 42.4; $C_{18:0}$, 5.3; $C_{18:1cis}$, 0.9; unidentified, 19.1.

[d] Initial count was 6.3×10^9 CFU/ml. Fatty acid composition of membrane lipids: $C_{12:0}$, 2.8; $C_{14:0}$, 4.5; $C_{16:0}$, 17.2; $C_{18:0}$, 2.4; $C_{18:1cis}$, 72.1; unidentified, 1.0.

TABLE 6. *Effect of variations in the cholesterol content of the cell membrane on the susceptibility of M. mycoides var. capri to death by freezing*[a]

Cooling conditions[b]	% Survival	
	Adapted strain (cholesterol poor)[c]	Native strain (cholesterol rich)[d]
Slow cooling to −20 C	95.0	0.8
Rapid cooling to −20 C	90.0	28.0
Slow cooling to −70 C	48.0	0.2
Rapid cooling to −70 C	100.0	54.0

[a] The cooling medium was β-buffer.

[b] Cooling rates were: 1.2 C/min to −20 C and 2.5 C/min to −70 C for slow; and 19.8 C/min to −20 C and 24.1 C/min to −70 C for rapid.

[c] Initial inoculum was 2.1×10^8 colony-forming units (CFU)/ml. Cholesterol made up 2.8% of the total membrane lipids.

[d] Initial inoculum was 5.0×10^8 CFU/ml. Cholesterol made up 23% of the total membrane lipids.

We found that the rate of cooling very markedly influences the survival of mycoplasmas subjected to freezing. As with yeasts (2) and gram-negative bacteria (15), maximal survival of frozen mycoplasmas was achieved at a certain optimal range of cooling rates (Fig. 1). According to Bank and Mazur (2), at suboptimal cooling velocities, the loss of viability is caused by the so-called "solution effects." These solution effects are a result of the progressive conversion of extracellular water to ice and include cellular dehydration, pH changes, and concentration and precipitation of solutes (11). Cells cooled at rates above the optimal value have insufficient time to equilibrate osmotically with the frozen external media before freezing internally, so the amount of water lost by the cells decreases and the probability and extent of intracellular ice formation increases. This results in a decrease in cell survival.

The warming rate during thawing of frozen cell suspensions is another factor which determines the percentage of survival of frozen mycoplasmas. Rapid warming is preferable to slow warming, because it apparently prevents the recrystallization of intracellular ice (13).

The cryoprotective agents ME_2SO and glycerol were found to be most effective in protecting mycoplasmas from freezing injury during the cooling process, probably by the reduction of the fraction of cell water frozen (12). Glycerol rapidly penetrates into mycoplasma cells (9). No data are available on the permeation of ME_2SO into mycoplasmas but it is known to penetrate into other cells even faster than glycerol (3).

Our experiments indicate that the composition of membrane lipids quite significantly affects the susceptibility of cells to death by freezing. The increase in the unsaturation of membrane lipids brought about by oleic acid enrichment, and the decrease in the cholesterol content, are known to increase membrane permeability (9, 20). Increased membrane permeability would enable the more rapid flow of water enhancing equilibration between the inside and outside of the cell. This will act to decrease freezing injury according to our current concepts. However, increased membrane permeability by oleic acid enrichment or by cholesterol depletion has been shown to occur only at temperatures above the phase transition temperature of membrane lipids, when the lipids are in the liquid-crystalline phase. Hence, oleic acid enrichment or cholesterol depletion would influence membrane permeability during only that part of the cooling process when membrane lipids are still in the liquid-crystalline phase. Below this temperature, when membrane lipids undergo the transition to the crystalline phase, a nonspecific and dramatic increase in membrane permeability may occur under certain conditions, probably due to increased susceptibility of the cell membrane to brittle fracture (9). Thus, interpreting the ef-

fects of variations in membrane lipid composition on freezing injury of mycoplasmas becomes complicated. Nevertheless, our rather preliminary data encourage a more intensive investigation on the effects of membrane lipid composition on freezing injury, utilizing mycoplasmas as convenient tools for this purpose.

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