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Transient Loss of Plasmid-Mediated Mercuric Ion Resistance After Stress in *Pseudomonas Aeruginosa*

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After freezing and thawing, *Pseudomonas aeruginosa* harboring a drug resistance plasmid (Hg^{2+r}, Str^r), became acutely sensitive to mercuric ions but not to streptomycin in the plating medium, whereas its sensitivity to both agents became more pronounced indicating a synergistic effect. This freeze-thaw-induced sensitivity was transient and capable of being repaired in a simple salts medium. Transient outer and cytoplasmic membrane damage was also observed in frozen and thawed preparations. From kinetics studies, repair of cytoplasmic membrane damage superseded repair of outer membrane damage and damage measured by mercuric ions and mercuric ions plus streptomycin. Osmotically shocked cells were also sensitive to mercuric ions, mercuric ions plus streptomycin, and sodium lauryl sulfate, but not to sodium chloride or streptomycin alone. This sensitivity was again transient and capable of repair in the same simple salts medium. Active transport of a non-metabolizable amino acid, α-amino isobutyric acid, was sensitive to mercuric ions and became more so after freezing and thawing. A freeze-thaw-resistant mercuric ion-dependent reduced nicotinamide adenine dinucleotide phosphate oxidoreductase was localized in the cytoplasm of this organism. This enzyme and an intact outer membrane appear to be required for mercuric ion resistance in this strain.

Freezing and thawing is a stress which can have profound effects both physiologically and biochemically on populations of bacteria. The effects observed include membrane and wall damage, loss of enzyme activity, structural alteration, and loss of viability (4, 16, 19). The precise factor(s) which causes death in bacteria or other cells on freezing and thawing is not properly understood (4, 16, 19).

In this laboratory, we have been interested in investigating the stability of the bacterial genome and its subsequent expression after stresses such as freezing and thawing. Since many strains of *Pseudomonas* sp. contain a wide range of plasmids with a variety of interesting functions (8), this has offered us an opportunity to study the stability of plasmids and their replication and expression after stress. The plasmid used in this study (pPLI) is of the P2 compatibility group, carrying genes for both streptomycin and mercuric ion resistance (8). Although the mechanism for conferring resistance is not known for the plasmid, mercuric ion resistance is usually conferred by the presence of a mercuric ion degrading enzyme (3, 8, 20). The mercuric ions are usually detoxified in bacteria by reduction, generally by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH), to volatile Hg^0 (3, 8, 12, 20). There is little evidence in the literature for alternative mechanisms. Streptomycin resistance appears to be conferred, at least by plasmid mediation, by streptomycin degradation systems or altered transport mechanisms (3, 8, 9). There appears to be no evidence for altered targets or permeability barrier-mediated resistance due to plasmids (3, 8, 9).

In this paper, we report on the stability of expression of a drug resistance plasmid after two stresses, freezing-thawing and osmotic shock. The effect of freeze-thaw on mercuric ion sensitivity of active transport is also reported. An Hg^{2+}-dependent NADPH oxidoreductase was also detected, and its cellular location was determined.

MATERIALS AND METHODS

Growth and cultural conditions. *Pseudomonas aeruginosa* (PAO1.pPL1) was a generous gift of Philip Lehrbach, Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia (14). The organism was maintained on nutrient agar supplemented with $HgCl_2$ (0.5 mM) and streptomycin (200 μg/ml) and grown aerobically at 37°C to late log-early stationary phase in nutrient broth (partially induced for mercuric ion resistance) or in nutrient broth supplemented with $HgCl_2$ and streptomycin (fully induced). It was harvested by centrifugation at 3,000 ×

g in a Sorvall GLC 1 centrifuge at room temperature and resuspended in water or 0.85% saline at approximately 5×10^9 cells per ml.

Freezing and thawing conditions. Slow (approximately 1 to 2°C/min), rapid (approximately 100°C/min), and ultrarapid freezing (approximately 6,000°C/min) were attained as described before (6). After 10 min at −196°C, samples were either thawed rapidly (greater than 100°C/min) by warming in a 37°C water bath or slowly (about 5 to 10°C/min) by leaving the tube at room temperature.

Repair and viability determination. Stressed samples were dispersed in a repair buffer (0.5% K_2HPO_4, 0.04% $MgSO_4$, pH 7.7) at approximately 10^9 cells per ml and incubated aerobically at 37°C (7). Before repair and at intervals during repair, samples were withdrawn and serially diluted in 2 mM $MgSO_4$ before surface plating on nutrient agar and nutrient agar supplemented with streptomycin (200 μg/ml) plus mercuric ions (0.5 mM), mercuric ions (0.5 mM), sodium lauryl sulfate (0.2%), or sodium chloride (0.5 M). The plates were incubated at 37°C to constant count.

Active transport assay. A membrane filtration method was used to assay active transport (7). Routinely, cells (approximately 200 μg/ml) were added to a reaction mixture [0.85% saline, 20 mM tris(hydroxymethyl) aminomethane-hydrochloride buffer (pH 7.4), 20 mM sodium succinate] and incubated at 37°C for 2 min. At time 0, α-^{3}H-labeled amino isobutyric acid was added to 1 mM (1 μCi/μmol), and the samples were incubated at 37°C. After various times, samples were removed and filtered through membrane filters (Gelman GA-6, 0.45 μm; Gelman Instrument Co., Ann Arbor, Mich.) and washed with 5 volumes of saline-tris(hydroxymethyl)aminomethane at room temperature. The filters and adhering cells were placed in vials and dried with a heating lamp. Scintillation fluid (5 ml) was added (3 liters of toluene, 1 liter of Triton X-100, 16.5 g of diphenyl oxazole, 0.5 g of diphenyl oxazolyl benzene), and the radioactivity was counted with a scintillation spectrophotometer (Isocap 300; New England Nuclear Corp.). Transport rates (nanomoles per minute per milligram of protein) were determined with reference to the amount of cell protein added to the reaction mixture as measured by the Folin-Ciocalteau method (15) with bovine serum albumin (fraction V) as the standard. Uptake of amino isobutyric acid was shown to proceed by active processes since pentachlorophenol (an uncoupler) at 10^{-5} M and KCN (a respiratory inhibitor) at 10 mM abolished greater than 95% of the activity.

Osmotic shock and spheroplast formation. Whole cells were osmotically shocked exactly by the method of Neu and Heppel (18), except that ethylenediaminetetraacetate at 0.1 mM (pH 7.0) instead of 1 mM was used. The concentration was decreased to minimize the lytic effect of this chemical on the organism. After osmotic shock, the cells were disrupted with a French pressure cell, and the extract was ultracentrifuged to yield supernatant and particulate fractions as described before (11). Spheroplasts of the organism were prepared as described by Mizuno and Kageyama (17), except that the ethylenediaminetetraacetate (pH 7.0) concentration was lowered to 0.1 mM to minimize lysis of the cells. The resultant spheroplasts were disrupted, and the extract was fractionated into supernatant and particulate fractions as described before (11).

Enzyme assays. Isocitrate dehydrogenase, NADH oxidase, 2′,3′-cyclic phosphodiesterase, and Hg^{2+}-NADPH dependent oxidoreductase were determined spectrophotometrically as described by Knowles et al. (11), Weston and Knowles (21), Bhatti et al. (1, 2), and Komura et al. (12). An international unit was defined as the amount of enzyme required to convert 1 μmol of substrate to product per min. All enzyme assays were performed at room temperature (22 to 24°C), except cyclic phosphodiesterase (37°C).

RESULTS

We have previously shown that this drug resistance plasmid can be cured from the bacterium by curing agents such as mitomycin C and sodium lauryl sulfate. However, freezing and thawing will not effectively cure this plasmid (P. H. Calcott, D. Wood, and L. Anderson, 2nd International Symposium on Microbial Ecology, University of Warwick, England, 1980). Since the plasmid appeared to be relatively stable to the stress of freeze-thaw, we decided to determine whether its expression was also stable.

When cells were frozen slowly or rapidly in water or saline, survival was not influenced by thawing rate (Table 1). However, slow thawing decreased cryosurvival for preparations frozen ultrarapidly when compared with those thawed rapidly. This has been shown previously for

TABLE 1. *Effect of freezing and thawing rates on freeze-thaw-induced susceptibility of fully induced P. aeruginosa*

Freezing menstruum	Plating medium	Rate of freezing[a]	% Survival after thawing[b]	
			Slow	Rapid
Water	NA[c]	S	65.0	70.3
		R	49.1	58.0
		U	8.6	49.0
	NA + Hg^{2+} (0.5 mM)	S	0.06	1.2
		R	0.07	1.0
		U	0.015	0.8
	NA + Hg^{2+} + streptomycin (200 μg/ml)	S	0.002	0.45
		R	0.0015	0.32
		U	0.00012	0.05
0.85% NaCl	NA	S	10.1	12.5
		R	9.7	15.5
		U	1.3	8.5
	NA + Hg^{2+}	S	0.002	0.3
		R	0.0008	0.25
		U	0.00008	0.08
	NA + Hg^{2+} + streptomycin	S	0.0013	0.12
		R	0.0006	0.08
		U	0.000004	0.003

[a] Freezing rates were: slow (S), 1 to 2°C/min; rapid (R), ~100°C/min, or ultrarapid (U), ~6,000°C/min.

[b] Thawing was slow (5 to 10°C/min) or rapid (~100°C/min).

[c] NA, Nutrient agar.

other microorganisms (4, 6, 16, 19). When subjected to these various freeze-thaw conditions, the surviving cells, whether fully induced (Table 1) or partially induced (data not shown) for Hg^{2+} resistance, always gave lower counts when plated on mercuric ion-based agar media when compared with nonsupplemented media. This indicated that a portion of the survivors became sensitive to mercuric ions. In addition, lower counts were always obtained on the mercuric ion-plus-streptomycin agar media when compared with the mercuric ion media. This would indicate that streptomycin acted in a synergistic manner with the mercuric ions since frozen and thawed cells did not show an increased sensitivity to streptomycin (Table 2).

The freeze-thaw-induced sensitivity to mercuric ions and the synergistic effect of streptomycin was seen whether cells were frozen slowly, rapidly, or ultrarapidly in water or saline and thawed slowly or rapidly (Table 1). These two phenomena were most obvious for cells frozen ultrarapidly in saline and then thawed slowly. This freeze-thaw-induced mercuric ion sensitivity or injury could be related to wall or membrane damage (or both) which is known to occur in frozen and thawed bacteria (4, 16, 19).

To test whether this sensitivity to mercuric ions and mercuric ions plus streptomycin was reversible, frozen and thawed populations of partially induced bacteria were incubated at 37°C for various times in a salts-repair medium previously shown to support repair of damage to *Escherichia coli* (7). Viable counts were followed on nutrient agar and on nutrient agar supplemented with mercuric ions, mercuric ions plus streptomycin, sodium lauryl sulfate, or sodium chloride. The sodium lauryl sulfate and sodium chloride were included to measure the proportion of the survivors exhibiting outer membrane and cytoplasmic membrane damage, respectively (16, 19). From the data presented in Fig. 1, it is evident that partially induced frozen and thawed cells not only exhibited injury measured by a decrease in counts on mercuric ion-plus streptomycin-supplemented media, but also exhibited outer and cytoplasmic membrane damage. However, many more cells showed sensitivity to mercuric ions and mercuric ions plus streptomycin than showed sensitivity to sodium lauryl sulfate or sodium chloride.

Incubation in the repair medium did not cause an increase in the viable count on nutrient agar, indicating that growth was not occurring (Fig. 1). Thus, it is clear that the repair medium facilitated an increase in resistance of the surviving cells to mercuric ions, mercuric ions plus streptomycin, sodium lauryl sulfate, and sodium chloride, irrespective of the freezing and thawing

TABLE 2. *Effect of streptomycin on survival of fully induced P. aeruginosa after freezing and thawing*

Freezing rate[a]	Freezing menstruum	Viability (%) on nutrient agar	
		No streptomycin	With streptomycin
Slow	Water	65.2	58.5
	0.85% NaCl	12.3	11.9
Rapid	Water	68.3	64.7
	0.85% NaCl	18.1	17.3
Ultrarapid	Water	49.3	39.7
	0.85% NaCl	10.1	9.7

[a] Slow, 1 to 2°C/min; rapid, approximately 100°C/min; and ultrarapid, approximately 6,000°C/min. All samples were thawed rapidly.

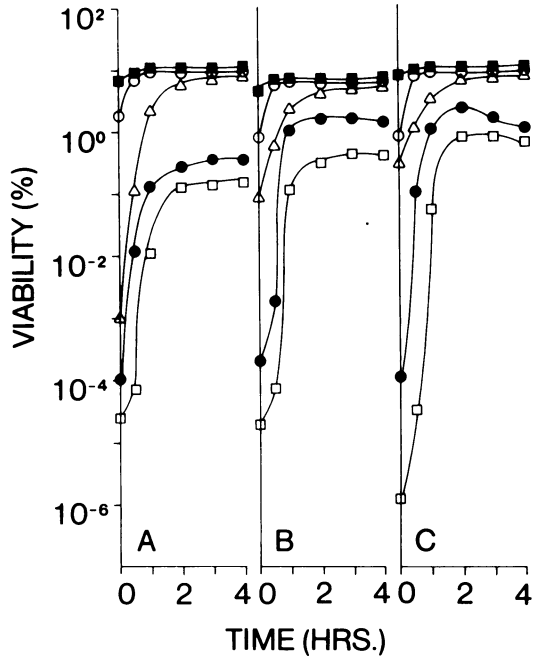

FIG. 1. *Repair of damage in partially induced frozen and thawed cells. Partially induced P. aeruginosa (PAO1.pPL1) cells were washed and suspended in 0.85% saline, frozen (A) slowly (1 to 2°C/min), (B) rapidly (approximately 100°C/min), or (C) ultrarapidly (approximately 6,000°C/min), and thawed slowly (5 to 10°C/min). The populations were then suspended at approximately 10^9 cells per ml in a repair buffer (0.5% K_2HPO_4, 0.04% $MgSO_4$, pH 7.7) and incubated aerobically at 37°C. At various times, samples were removed and diluted in 2 mM $MgSO_4$ and surface plated onto nutrient agar (■) or nutrient agar supplemented with 0.2% sodium lauryl sulfate (△), 0.5 M sodium chloride (○), 0.5 mM mercuric ions (●), or 0.5 mM mercuric ions plus 200 μg of streptomycin per ml (□). Colonies were counted after at least 24 h of incubation at 37°C.*

conditions. However, the kinetics of appearance of resistance to each agent were not similar. Resistance to sodium chloride was regained rapidly (complete in 0.5 h), indicating rapid repair of cytoplastic membrane damage. Outer membrane damage (as measured by sensitivity to sodium lauryl sulfate) was repaired less rapidly and was usually complete after 1 to 1.5 h, whereas establishment of resistance to mercuric ions and mercuric ions plus streptomycin took between 1 and 2 h. This would indicate that the outer membrane might play a role in mercuric ion resistance in this organisms.

To determine whether the level of induction of the mercuric ion resistance played a role in the freeze-thaw-induced loss of mercuric ion resistance or the synergistic effect of streptomycin and mercuric ions, fully induced bacteria were frozen and thawed and then allowed to repair cell damage under the same conditions to which the partially induced cells were exposed. Figure 2 shows that fully induced cells showed not only sensitivity to mercuric ions but also the synergism between streptomycin and mercuric ions. Thus, these two effects were independent of the level of induction of the mercuric ion resistance. However, in fully induced populations many more cells were able to withstand the mercuric ions after freeze-thaw than in partially induced populations (Fig. 1 and 2). This would indicate that the level of induction played a role in determination of mercuric ion resistance after stress. If these stressed cells were then incubated in the repair buffer, cytoplasmic membrane damage was repaired rapidly (0.5 to 1 h), whereas outer membrane damage was repaired more slowly (2 h). Resistance to mercuric ions in the presence or absence of streptomycin was regained in 2 to 3 h. However, the extent of repair was more complete in the fully induced than in the partially induced cells (Fig. 1 and 2). Thus, the level of induction played an important role in the regeneration of mercuric ion resistance.

Since freezing and thawing is a complex stress, we have evaluated the effect of a simple stress, osmotic shock, on the cell's resistance to mercuric ions and streptomycin. Unlike the situation in *E. coli*, osmotic shock caused a decrease in viability in the *P. aeruginosa* population (Fig. 3). In the survivors, cytoplasmic membrane damage (measured by sodium chloride sensitivity) was not evident, whereas outer membrane damage (decrease in resistance to sodium lauryl sulfate) was detected (Fig. 3). Similarly, decreases in plating efficiencies were recorded on mercuric ion-supplemented media and on mercuric ion-plus-streptomycin-supplemented media, though no synergistic effects were seen. Stressed cells retained their ability to grow on streptomycin-supplemented agar (Fig. 3). By incubating osmotically shocked cells in the simple salts medium used to allow repair of freeze-thawed cells, resistance to mercuric ions was regained. These experiments strengthen the requirement for an intact outer membrane in this strain for expression of mercuric ion resistance.

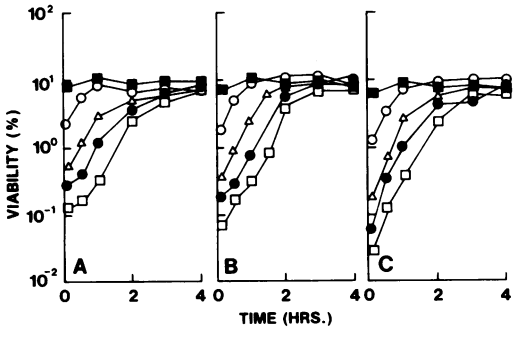

FIG. 2. *Repair of damage in fully induced frozen and thawed cells. The symbols and methods employed were exactly as in Fig. 1, except that fully induced cells of P. aeruginosa PA01 (pPL1) were used.*

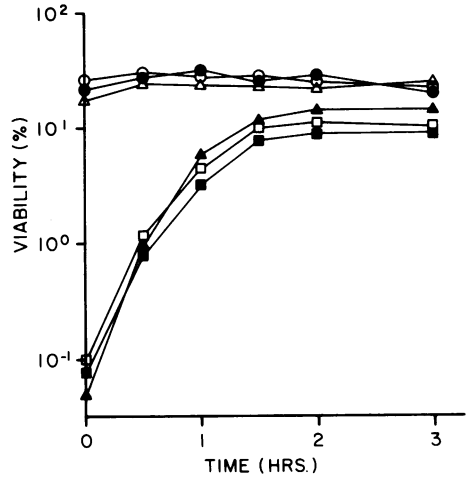

FIG. 3. *Repair of osmotic shock-induced sensitivity in P. aeruginosa. Partially induced P. aeruginosa cells were washed in 2 mM magnesium sulfate and then osmotically shocked as described in the text. The population was suspended in repair buffer (0.5% K_2HPO_4, 0.04% $MgSO_4$, pH 7.7) at approximately 10^9 cells per ml and incubated at 37°C aerobically. At various times, samples were removed, diluted in 2 mM $MgSO_4$, and surface plated onto nutrient agar (●), or nutrient agar supplemented with 0.2% sodium lauryl sulfate (□), 0.5 M sodium chloride (○), 0.5 mM mercuric chloride (■), 200 μg of streptomycin per ml (△), or mercuric ions plus streptomycin (▲). Colonies were counted after at least 24 h incubation at 37°C.*

Further support for this was obtained by studying the cell energy-requiring process, active transport. If the outer membrane were acting as a selective barrier to mercuric ions then after freezing and thawing the effect of the ion should be increased. As can be seen in Table 3, unfrozen cells were sensitive to mercuric ions. Freezing and thawing decreased the overall transport activity and also made the cells hypersensitive to mercuric ions.

Since mercuric ion resistance appears to be mediated by enzymes that convert Hg^{2+} to volatile products, usually at the expense of cellular NADPH or NADH, in other bacteria (8, 12, 20), we assayed for and detected a Hg^{2+}-dependent NADPH oxidoreductase in this strain. This enzyme was present in the plasmid-bearing strains which had been maintained on nutrient agar slants supplemented with streptomycin and mercuric ions and then subcultured into nutrient broth, but only to 10 to 30% of the level of fully induced cultures (5). However, when the culture was maintained on nutrient agar, the enzyme was undetectable after three subcultures, even though the plasmid was maintained in greater than 99.99% of the population. These cells then plated with less than 10^{-5} efficiency on mercuric ion agar. An isogenic strain lacking the plasmid and sensitive to Hg^{2+} also showed no enzyme activity. Thus, the enzyme plays a major role in mercuric ion resistance. Since an intact outer membrane also appeared to be required for resistance, we have examined the enzyme to determine whether it was periplasmic in nature and could have been released or at least disrupted from its site in the wall on stress. We have used two techniques for localization, osmotic shock and spheroplast formation. With both methods, we have determined the distribution of periplasmic (2'3'-cyclic phosphodiesterase), cytoplasmic (isocitrate dehydrogenase), and membrane-bound (NADH oxidase) enzymes (1, 2, 11). Using both techniques, we released the periplasmic enzyme quantitatively, and in the case of osmotic shock none of the membrane-bound enzyme was released (Table 4). The cytoplasmic enzyme was essentially retained by the cells, although a small amount was released indicating lysis of a proportion of the cells (Table 4). The Hg^{2+}-dependent NADPH oxidoreductase was also retained by the cell with loss of a small amount. This would locate this enzyme as soluble and cytoplasmic, as in *E. coli* (12, 20). Although this enzyme was released by neither osmotic shock nor spheroplast formation, it is possible that the harsher freeze-thaw conditions might release the enzyme from the cell. We have been unable to detect more than 5% release of the enzyme after freezing and thawing, even though greater than 99% of the cells showed loss of mercuric ion resistance. In addition, the enzyme in whole cells or in cell-free extract was stable. After freeze-thaw, we could detect less than 20% loss in activity. Thus loss of mercuric ion resistance could not be explained by release or inactivation of the enzyme.

TABLE 3. *Active transport rates of α-amino isobutyric acid into unfrozen, and freeze-thawed P. aeruginosa*

Treatment to cells[a]	Concn of Hg^{2+} in reaction mixture (mM)	nmol of α-amino isobutyric acid accumulated/min/mg of protein (% of unfrozen)	% Inhibition
Unfrozen	0	16.6	
	0.1	4.82	71
	0.2	1.21	92.7
Ultrarapidly frozen	0	6.48 (39)	
	0.1	1.15	82.2
	0.2	<0.01	100
Rapidly frozen	0	6.98 (42)	
	0.1	0.44	93.7
	0.2	0.20	97.2
Slowly frozen	0	2.82 (17)	
	0.1	0.12	95.8
	0.2	0.06	97.7

[a] Unfrozen or freeze-thawed cells (all preparations were thawed slowly) were incubated in a reaction mixture [0.85% NaCl, 20 mM tris(hydroxymethyl)-aminomethane (pH 7.4), 20 mM succinate] with and without mercuric ions. At time 0, [α-^{3}H]amino isobutyric acid was added, and after various times, samples were collected on membrane filters, washed, and counted. Rates were determined from the rate of accumulation of ^{3}H label per unit of cell protein. The rates of freezing were as described in footnote *a* of Table 2.

DISCUSSION

The plasmid under study and the expression of one of its functions, streptomycin resistance, appeared to be stable to stress in this organism. Frozen and thawed cells did show a transient loss of mercuric ion resistance, which could be restored by incubation of the stressed cells in a simple salts medium. The kinetics of reestablishment of resistance to mercuric ions and mercuric ions plus streptomycin were similar to those shown for repair of outer membrane damage. The kinetics of repair of these functions was slower than that for cytoplasmic membrane damage repair. This indicated that resistance to mercuric ions and mercuric ions plus streptomycin was not dependent on an intact cytoplasmic membrane alone, but probably required an

TABLE 4. *Localization of Hg^{2+}-dependent NADPH oxidoreductase and three other enzymes in P. aeruginosa PAO1 (pPL1) using osmotic shock and spheroplast formation*

Expt[a]	Cell fraction	Protein		Phosphodiesterase		Isocitrate dehydrogenase		Hg^{2+} reductase		NADH oxidase	
		mg	mg/ml	% of total	IU/mg	% of total	IU/mg	% of total	IU/mg	% of total	IU/mg
I	$MgSO_4$ wash	7.8	1.6	0	<0.001	0	<0.01	0	<0.01	0	<0.01
	Sucrose wash	15.1	3.1	0	<0.001	0.14	0.15	0.25	0.38	0	<0.01
	Osmotic shock fluid	45.6	3.1	83.3	0.045	5.3	1.8	5.9	2.9	3.5	0.35
	Particulate fraction from shocked cells	51.3	6.5	16.6	0.008	35.1	10.5	17.1	7.5	84.6	7.5
	Supernatant fraction from shocked cells	49.0	2.9	0	<0.001	59.4	18.6	76.8	35.3	11.9	1.1
II	$MgSO_4$ wash	4.1	1.6	0	<0.001	0	<0.01	0	<0.01		
	Spheroplast supernatant fluid	10.5	2.5	80.2	0.050	1.65	1.1	6.4	8.5		
	Disrupted spheroplasts	44.3	9.8	19.8	0.003	98.3	15.5	93.6	29.6		

[a] In experiment I, fully induced cells were osmotically shocked by the method of Neu and Heppel (17), and in experiment II, cells were converted to spheroplasts by the method of Mizuno and Kageyama (10). Each fraction from the extractions were assayed for protein, phosphodiesterase, isocitrate dehydrogenase, Hg^{2+}-dependent NADPH oxidoreductase and NADH oxidase spectrophotometrically as described in the text.

intact wall or outer membrane. The requirement for an intact wall or outer membrane was strengthened with the studies of loss of mercuric ion resistance in cells after osmotic shock and the increase in mercuric ion sensitivity of active transport in cells after freeze-thaw.

The Hg^{2+}-dependent NADPH oxidoreductase was also required for resistance to mercuric ions since cultures devoid of activity plated with very low efficiency on mercuric ion agar (5). From the experiments on location of the mercuric ion NADPH oxidoreductase and its stability on freezing and thawing, loss of mercuric ion resistance in frozen and thawed cells cannot be explained simply by inactivation or physical loss of the enzyme from the cell. It is apparent that after the stresses imposed, the enzyme is still present in the cell in an active form. However, the assays employed in this paper were far from physiological with the reducing equivalents being supplied exogenously. Undoubtedly, the enzyme is associated with an environment in the cell rich in reducing equivalents, possibly the respiratory chain or the Krebs cycle enzymes. It is possible that freezing and thawing, although it did not affect the enzyme per se, decreased the generation of reducing equivalents and their transfer to the enzyme. This possibility is strengthened by two observations. First, respiration and two energy-utilizing processes, active transport and protein synthesis, are susceptible to freezing and thawing (7, 10, 13), indicating that generation and utilization of energy are impaired after this stress. Second, osmotic shock caused no detectable cytoplasmic membrane damage, and the percentage of the population showing injury to the outer membrane was very similar to the percentage showing sensitivity to mercuric ions and mercuric ions plus streptomycin. The situation for frozen and thawed cells was different. After this stress, the percentage showing sensitivity to mercuric ions in the presence or absence of streptomycin was always higher than those showing wall damage. This difference might represent cells with other impairments such as membrane damage or inability to energize their mercuric ion detoxication enzyme.

Resistance to streptomycin appeared to be very stable, indicating that the integrity of the outer or cytoplasmic membrane was not essential for resistance. The precise mechanism of streptomycin in this strain is unknown but could involve an altered target, an inactivating enzyme, or an altered transport mechanism (8, 9).

Survival of frozen and thawed populations was always lower when cells were plated on mercuric ion plus streptomycin agar than when cells were plated on mercuric ion agar. This observation is difficult to explain when one considers that streptomycin alone did not lower the count of stressed populations. This would indicate, however, that the streptomycin acted in a synergistic manner with mercuric ions. The level of induction of the mercuric ion-detoxifying enzymes would appear to play no part, since partially and fully induced cultures showed this phenomenon after freeze-thaw. However, the synergistic effect was not seen in organisms subjected to osmotic shock. Thus, it is likely that the cytoplasmic membrane might play a critical role though the precise mechanism cannot be elucidated with the data presented.

These data serve to illustrate, at least partially, the similarity between stresses such as freezing and thawing and osmotic shock. Both

processes cause loss of periplasmic proteins (4, 16, 18, 19) and cause a transient increase in sodium lauryl sulfate and mercuric ion sensitivity (this paper): freezing and thawing, but not osmotic shock, caused a transient membrane damage. It is attractive to postulate that the cold osmotic shock that occurs on osmotic shock is perhaps a component of the freeze-thaw conditions and that the mechanisms of damage involved are very similar.

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