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TRANSIENT LOSS OF PLASMID MEDIATED MERCURIC ION RESISTANCE AFTER FREEZING AND THAWING OF *PSEUDOMONAS AERUGINOSA*[1]

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ABSTRACT. After freezing and thawing, *Pseudomonas aeruginosa* harboring a drug resistance plasmid (Hg^{2+R}, $Strep^{R}$), became acutely sensitive to mercuric ions but not to streptomycin in the plating medium. Its sensitivity to both agents in the plating medium became more pronounced, suggesting a synergistic effect of the mercuric ions with streptomycin. This freeze-thaw-induced sensitivity was transient and capable of being repaired in a simple salts medium ($0.5\%K_2HPO_4+0.04\%MJSO_4$ pH7.7). Transient wall and membrane damage were also observed in frozen-thawed preparations. From kinetics studies, repair of membrane damage preceded repair of wall damage and damage measured by mercuric ions and mercuric ions plus streptomycin. Osmotically shocked cells also were sensitive to mercuric ions, mercuric ions plus streptomycin and sodium lauryl sulfate, but not to sodium chloride or streptomycin alone. This sensitivity was transient and capable of repair in the same simple salts medium. Active transport of a non-metabolisable 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 NADPH oxido-reductase was localized in the cytoplasm, and the enzyme and an intact outer membrane appear to be required for mercuric ion resistance in this strain.

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INTRODUCTION

Freezing and thawing is a stress that 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. The precise factor(s) that causes death in bacteria or other cells on freezing and thawing is not properly understood (Calcott 1978, MacLeod and Calcott 1976, Ray and Speck 1973).

We have been investigating the stability of the bacterial genome and its subsequent expression following stresses such as freezing and thawing. Since many strains of *Pseudomonas sp.* contain a wide range of plasmids with a variety of interesting functions an opportunity to study the stability of plasmids and their replication and expression after stress was provided. The plasmid used in this study (pPLI) is of the P2 compatibility group carrying genes for both streptomycin and mercuric ion resistance (Chakrabarty 1976). The plasmid is cured from the strain (PAO1) by mitomycin C, heat and sodium lauryl sulfate, but not by freezing and thawing to any degree (Calcott *et al* 1980). While 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 (Brown 1975, Summers and Silver 1978). The mercuric ions are usually detoxified in bacteria by reduction, generally by NADPH or NADH, to volatile Hg (Brown 1975, Chakrabarty 1976, Komura *et al* 1971). There is little evidence in the literature for alternative mechanisms. Streptomycin resistance appears to be conferred, at least by plasmid mediation,

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by streptomycin degradation systems or altered transport mechanism (Brown 1975, Chakrabarty 1976, Davies and Smith 1978). There appears to be no evidence for altered targets or a permeability barrier mediated resistance due to plasmids.

In this paper, the effect of freezing and thawing and osmotic shock on Hg^{2+} sensitivity and the effect of freeze-thaw on Hg^{2+} sensitivity of active transport was studied. Hg^{2+} dependent NADPH oxidoreductase was detected and its cellular location determined.

METHODS AND MATERIALS

Pseudomonas aeruginosa (PAO1.pPL1) (generous gift of Philip Lehrbach, Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia) contained a plasmid coding for resistance to streptomycin, gentamicin, sulfonamide, Hg^{2+} ions and UV light (Lehrbach *et al* 1977). It was grown to late log-early stationary phase aerobically at 37 °C in nutrient broth, harvested by centrifugation at 3000 x g in a Sorvall GLC 1 centrifuge at room temperature, and resuspended in water or 0.85% saline at approximately 5 x 10^9 cells/ml.

FREEZING AND THAWING. Slow (approximately 1 to 2 °C/min), rapid (approximately 100 °C/min) and ultrarapid freezing (approximately 6000 °C/min) were attained as described by Calcott and MacLeod (1974). 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 5-10 °C/min) by leaving the tube at room temperature.

REPAIR AND VIABILITY. Stressed samples were dispersed in a repair buffer (0.5% K_2HPO_4, 0.04% $MgSO_4$, pH 7.7) at approximately 1 x 10^9 cells/ml and incubated aerobically at 37 °C. 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) and/or mercuric ions (0.5mM) or sodium lauryl sulfide (0.2%) or sodium chloride (0.5M). The plates were incubated at 37 °C to a constant count.

ACTIVE TRANSPORT ASSAY. The membrane filtration method was used to assay active transport exactly as described by Calcott *et al* (1979) except that the cells were energized by 20 mM sodium succinate. Transport rates (n moles/min/mg protein) were determined with reference to the amount of cell protein added to the reaction mixture as measured by the Folin-Ciocalteau method (Lowrey *et al* 1951) with bovine serum albumin (Fraction V) used as standard. Uptake of α-amino isobutyoric acid (AIB) 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 SPHEROPLASTS. Whole cells were osmotically shocked according to the method of Neu and Heppel (1965) except that 0.1 mM EDTA (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 ultracentrifuged to yield supernatent and particulate fractions as described by Knowles *et al* (1974). Spheroplasts of the organism were prepare as described by Mizuno and Kageyama (1978), except that the EDTA (pH 7.0) concentration was lowered to 0.1 mM to minimize lysis of the cells. The resultant spheroplasts were disrupted and the extract fractionated into supernatent and particulate fractions as described by Knowles *et al* (1974).

ENZYME ASSAYS. Isocitrate dehydrogenase, NADH oxidase, 2′,3′ cyclic phosphodiesterase and Hg^{2+}-NADPH dependent oxido-reductase were determined spectrophotometrically as described by Knowles *et al* (1974), Weston and Knowles (1974), Bhatti *et al* (1976). A unit is defined as that amount of enzyme required to convert 1μ mole substrate to produce/min. All enzyme assays were performed at room temperature (22-24 °C) except cyclic phosphodiesterase (37 °C).

RESULTS

Since the plasmid appears to be relatively stable to freeze-thaw (Calcott *et al* 1980), it was decided to determine whether expression of the plasmid was stable to this stress. After freezing and thawing under a number of conditions, the surviving cells always gave a lower count when plated on Hg^{2+} or streptomycin plus Hg^{2+} agar media as compared to the non-supplemented samples (table 1). This indicated that a portion of the survivors was sensitive to Hg^{2+} ions and an ever larger portion was sensitive to streptomycin plus Hg^{2+} ions. Mercuric ion sensitivity occurred when the cells were frozen in water or saline and then thawed but no increased susceptibility was observed for frozen-thawed cells when plated on streptomycin agar (table 2).

The extent of damage, was dependent on the freezing and thawing conditions used. Routinely, thawing rates did not influence survival of the populations after freezing slowly or rapidly; ultra-rapidly frozen cells were very susceptible to slow thawing (table 1). Injury, as measured by inability to grow on Hg^{2+} or Hg^{2+} plus streptomycin agar media, however, was affected dramatically by thawing rate. The slower

TABLE 1

Effect of thawing rate on freeze-thaw induced susceptibility to mercury and mercury plus streptomycin in Pseudomonas aeruginosa.

Plating Medium	Freeze Rate*	Thawing % Survival**	
		Slow*	Rapid
FROZEN IN WATER			
Nutrient Agar (N.A.)	S*	62	85
	R	70	75
	U	10.5	85
NA+Hg^{2+}(0.5 mM)	S	$6x10^{-3}$	$2x10^{-1}$
	R	$2x10^{-4}$	$3x10^{-1}$
	U	$1x10^{-4}$	$3.5x10^{-1}$
Na+Hg^{2+}+Strep (200 µg/ml)	S	$3x10^{-5}$	$5x10^{-2}$
	R	$2x10^{-5}$	$2.8x10^{-5}$
	U	$1x10^{-5}$	$5x10^{-2}$
FROZEN IN 0.85% SALINE			
NA	S	10.8	11.5
	R	6.25	7.05
	U	2.5	28
NA+Hg^{2+}	S	$1.7x10^{-5}$	$8.5x10^{-3}$
	R	$7.8x10^{-5}$	$5x10^{-1}$
	U	$4.2x10^{-5}$	$5x10^{-1}$
Na+Hg^{2+}+Strep	S	$5x10^{-6}$	$9.5x10^{-3}$
	R	$5x10^{-7}$	$1.3x10^{-4}$
	U	$3x10^{-6}$	$1.1x10^{-2}$

*Freezing rates were slow=S (1-2 °C/min), rapid=R (~100 °C/min) or ultra.-rapid=U (~6000 °C/min).
**Thawing was slow (5-10 °C/min) or rapid (~100 °C/min).

the rate, the higher the fraction of injured cells. The relationship between cooling and warming rates and death and injury was similar for preparations frozen in water or saline (table 1).

To test whether this sensitivity ot Hg^{2+} ions and Hg^{2+} ions plus streptomycin was reversible, frozen-thawed preparations were incubated at 37 °C for various times in a salts repair medium previously shown to support repair of damage to *E. coli* (Calcott *et al* 1979). Viable counts were followed on nutrient agar, nutrient agar supplemented with Hg^{2+} ions, Hg^{2+} 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 wall and membrane damage respectively (Calcott 1978, MacLeod and Calcott 1976). From figure 1, it is evident that frozen-thawed cells knot only exhibit injury measured by a decrease in counts on mercuric ion plus streptomycin supplemented media but also display wall and membrane damage. Many more cells showed sensitivity to Hg^{2+} ions and Hg^{2+} ions plus streptomycin than sensitivity to sodium lauryl sulfate or sodium chloride. The same trends were seen for populations frozen in saline, except that survival was much lower.

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 and time facilitated an increase in resistance of the surviving cells to Hg^{2+}, Hg^{2+} ions plus streptomycin, sodium lauryl sulfate and sodium chloride, irrespective of the freezing and thawing conditions. The kinetics of appearance of resistance to each agent were not similar. Resistance to sodium chloride was regained rapidly (complete in ½ hr), indicating that membrane damage was repaired rapidly. Wall damage (as measured by sensitivity to sodium lauryl sulfate) was repaired less rapidly and was usually complete after 1 to 1.5 hr. Repair of damage measured by Hg^{2+} ions and Hg^{2+} ions plus streptomycin sensitivities took between 1 and 2 hrs, indicating that the outer membrane might play a role in mercuric ion resistance in this organism.

TABLE 2

Effect of streptomycin in the plating media on survival of Pseudomonas aeruginosa after freezing and thawing.

Freezing*	Frozen in	% Viability on Nutrient Agar**	
		No Strep	Streptomycin
Slow	H_2O	57.8	45.1
	0.85% NaCl	18.3	15.9
Rapid	H_2O	85.0	73.2
	0.85% NaCl	15.9	16.9
Ultra-rapid	H_2O	46.0	37.0
	0.85% NaCl	8.6	10.1

*Slow corresponded to 1-2 °C/min; rapid approximately 100 °C/min and ultra rapid approximately 6000 °C/min. All samples were thawed rapidly.
**No Strep=no Streptomycin. Streptomycin=200 µg/ml Streptomycin.

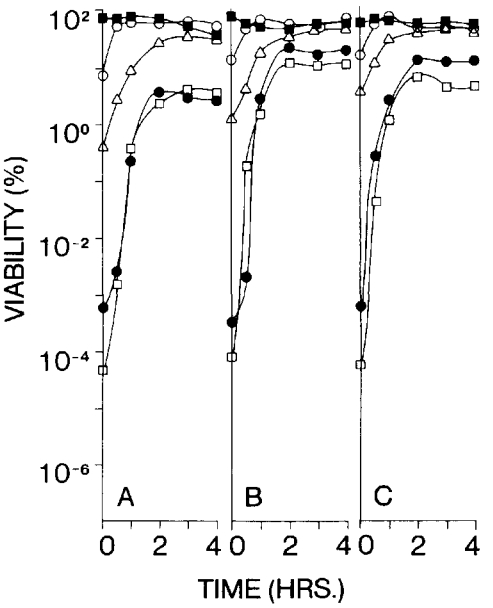

FIGURE 1. Repair of structural damage measured by sensitivity to sodium lauryl sulfate, sodium chloride, mercuric ions and mercuric ions plus streptomycin in cells frozen and thawed in water. Stationary phase *Pseudomonas aeruginosa* (PAO1. pPL1) was washed and suspended in water before freezing slowly (1-2 °C/min), rapidly (approx 100 °C/min) or ultra-rapidly (approximately 6000 °C/min) before thawing slowly (5-10 °C/min). The populations were suspended at approximately 10^9 cells/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 in nutrient agar (■) with or without the supplements of 0.2% sodium lauryl sulfate (△), 0.5M sodium chloride (○), 0.5 mM mercuric ions (●), and 0.5 mM mercuric ions plus 200 μg/ml streptomycin (□). Colonies were counted after 24 hr incubation at 37 °C.

Since freezing and thawing is a complex stress, we also 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 *Pseudomonas* population (fig. 2). In the survivors, membrane damage, as measured by inability to plate on sodium chloride supplemented agar, was not evident. As expected, wall damage was detected by a decrease in plating efficiency on sodium lauryl sulfate supplemented agar (fig. 2). Similar decreases in plating efficiencies were recorded on Hg^{2+} ion and Hg^{2+} ion plus streptomycin supplemented media; however, cells retained their ability to plate on streptomycin supplemented agar (fig. 2). 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 Hg^{2+} ion resistance. Further support for this can be obtained by studying the cell energy requiring process, active transport. If the outer membrane was 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

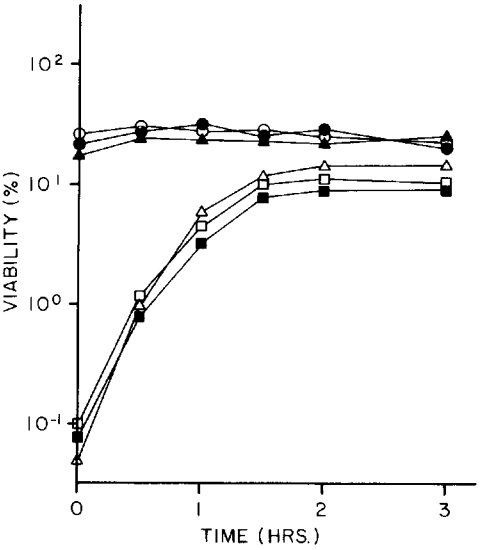

FIGURE 2. Repair of osmotic shock-induced sensitivity to mercuric ions and sodium lauryl sulfate in *Pseudomonas aeruginosa*. Stationary phase *P. aeruginosa* washed in 2mM magnesium sulfate and then osmotically shocked. The population was suspended in repair buffer (0.5% K_2HPO_4, 0.05% $MgSO_4$, pH 7.7) at approximately 10^9 cells/ml and incubated at 37 °C aerobically. At various times, samples were removed, diluted in 2mM $MgSO_4$ and surface plated onto nutrient agar (●), with or without the supplements of 0.2% sodium lauryl sulfate (△), 0.5M sodium chloride (○), 0.5mM mercuric chloride (□), 200 μg/ml streptomycin (△), and mercuric ions plus streptomycin (■). Colonies were counted after at least 24 hr incubation at 37 °C.

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 Hg^{2+} 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 (Chakrabarty 1976, Komura *et al* 1971, Summers and Silver 1978), we have assayed for and detected a Hg^{2+} dependent NADPH oxido-reductase in this strain. This enzyme was present in the strains bearing the plasmid that had been maintained on nutrient agar slants supplemented with streptomycin and Hg^{2+} ions (our routine method) and then sub-cultured into nutrient broth. If the culture was maintained on nutrient agar, the enzyme was undetectable after 3 subcultures, even though the plasmid was maintained in greater than 99.9% of the population. Under these circumstances, cells plated with less than 10^{-5} efficiency on Hg^{2+} ion agar. An isogenic strain lacking the plasmid also showed no enzyme activity and did not grow in the presence of Hg^{2+} (Calcott 1980). Since an intact outer membrane 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 used two techniques for localization, osmotic shock and spheroplast formation. With both methods, we determined the distribution of a periplasmic enzyme (2′3′ cyclic phosphodiesterase), a cytoplasmic enzyme (isocitrate dehydrogenase) and a membrane bound enzyme (NADH oxidase). Using both techniques, we released the periplasmic enzyme quantitatively and in the case of osmotic shock none of the membrane bound enzyme (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 and would locate the enzyme as soluble and cytoplasmic, as in *E. coli* studies (Komura *et al* 1971, Summers and Silver 1978). Although this enzyme was neither released by 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 Hg^{2+} resistance. The enzyme in whole cell or in cell-free extract appears to be stable with less than 20% loss in activity after freeze-thaw. Thus, loss of mercury resistance cannot be explained by release or inactivation of the enzyme.

TABLE 3

Active transport rates of α-amino isobutyric acid (AIB) into unfrozen, and frozen and thawed Pseudomonas aeruginosa.

Cell Treatment	Reaction Medium (mM Hg^{2+})	Rate of AIB accumulation (nm/min/mg prot) (nm)	% unfrozen	% Inhib.
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	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

*Unfrozen or frozen-thawed cells (all preparations were thawed slowly) were incubated in a reaction mixture (.85% NaCl+20 mM Tris, pH 7.4+20 mM succinate) with and without Hg^{2+}. At time 0, ^{3}H-AIB 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 table 2.

DISCUSSION

The plasmid (pPL1) appears to be stable in the population as noted by Calcott *et al*

TABLE 4

Localization of Hg^{2+} dependent NADPH oxido-reductase and 3 other enzymes in Pseudomonas aeruginosa PAO1 (pPL1) using osmotic shock and spheroplast formation.

Cell Fraction	Protein		Phospho-diesterase		Isocitrate dehydrogenase		Hg^{2+} Reductase		NADH Oxidase	
	mg	mg/ml	% total	μ/mg	% total	μ/mg	% total	μ/mg	% total	μ/mg
*Experiment I**										
Mg-wash	5.5	1.3	0	<0.001	0	< 0.01	0	<0.01	0	<0.01
Sucrose wash	14.5	2.9	0	<0.001	0.13	0.1	0.83	0.3	0	< .01
Osmotic Shock Fluid	36	3.6	90.5	0.025	6.6	2.1	8.9	1.3	0	< .01
Particulate Fraction from Shocked Cells	41	4.1	9.5	0.002	28.5	8	7.82	1.0	93.6	8.0
Supernatent Fraction from Shocked Cells	45	2.6	0	<0.001	64.8	16.5	86.7	10.1	6.4	0.5
*Experiment II**										
Mg-wash	3.4	1.7	0	<0.001	0	< 0.01	0	< 0.01	—	—
Spheroplast supernatent	9.5	1.9	81.0	0.017	7.4	.89	6.9	.46	—	—
Disrupted spheroplasts	38	7.6	19.0	0.001	92.6	2.8	93.1	1.53	—	—

*In Experiment I, cells were osmotically shocked by the method of Neu and Heppel (1965) and In Experiment II were converted to spheroplasts by the method of Mizuno and Kageyama (1978). Each fraction from the extractions was assayed for protein, phosphodiesterase, isocitrate dehydrogenase, Hg^{2+} dependent NADPH oxido-reductase and NADH oxidase spectrophotometrically as described in the materials and methods.

(1980), and expession of one of its functions, streptomycin resistance, also appeared to be stable from the present data. Frozen and thawed cells showed a transient loss of mercuric ion resistance and this resistance could be restored by incubation of the stressed cells in a simple salts medium. The kinetics of reestablishment of resistance to Hg^{2+} ions and Hg^{2+} ions plus streptomycin showed kinetics similar to those shown for repair of wall damage. Kinetics of these functions were slower than that for repair of cell membrane damage, indicating that resistance to Hg^{2+} ions and Hg^{2+} ions plus streptomycin was not dependent on an intact cell membrane alone but probably required an intact wall or outer membrane. The idea of a requirement for an intact wall or outer membrane was strengthed with the studies of loss of Hg^{2+} 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 oxido-reductase was required for resistance to mercuric ions since cultures devoid of activity plated with an efficiency less than 10^{-5} on Hg^{2+} ion agar (Calcott 1980). Based on experiments locating the Hg^{2+} ion NADPH oxido-reductase and its stability to freezing and thawing, loss of mercuric ion resistance in frozen-thawed cells cannot simply be explained by inactivation or physical loss of the enzyme from the cell. It is apparent that after the stresses imposed on the cell, the enzyme is still present in the cell in an active form. 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, while not affecting the enzyme *per se,* decreased the generation of reducing equivalents and their transfer to the enzyme. This idea was strengthened by two observations. First, respiration, and two energy utilizing processes, active transport and protein synthesis, were susceptible to freeze-thaw (Calcott *et al* 1979, Ghani and Calcott 1980, Lee *et al* 1977), indicating

that generation and utilization of energy were impaired after this stress. Second, osmotic shock caused no detectable membrane damage and the percentage of the population showing injury to the wall was very similar to the percentage showing sensitivity to Hg^{2+} ions and Hg^{2+} ions plus streptomycin. The situation for freeze-thawed cells was different. After this stress, the percentage of cells showing sensitivity to Hg^{2+} 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 mercury 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 (Chakrabarty 1976, Davies and Smith 1978).

Survival of frozen and thawed populations was always lower when plated on mercuric ion plus streptomycin agar than when plated on Hg^{2+} ion agar (table 1). This observation is difficult to explain when one considers that streptomycin alone did not lower the count of stressed populations. It would indicate, however, that the streptomycin acted in a synergistic manner with Hg^{2+} ions. Conceivably, Hg^{2+} ions could interact with specific sulphydryl proteins in and around the cell wall layers, inactivating them and preventing the elimination of the streptomycin from the cell targets.

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 (Calcott 1978, MacLeod and Calcott 1976, Neu and Heppel 1965, Ray and Speck 1973) and transient increases in sodium lauryl sulfate and Hg^{2+} ion sensitivity. Freezing and thawing, but not osmotic shock, caused a transient membrane damage. It is possible that the cold osmotic shock that occurs is perhaps a component of the freeze-thaw condition and that the mechanism of damage involved may be similar.

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LITERATURE CITED

Brown, M. R. W. 1975 Resistance of *Pseudomonas aeruginosa*. John Wiley and Sons, New York.

Bhatti, A. R., I. W. DeVoe and J. M. Ingram 1976 Cell division in *Pseudomonas aeruginosa:* participation of alkaline phosphatase. J. Bacteriol. 126: 400-409.

Calcott, P. H. 1978 Freezing and thawing microbes. *In:* Patters of Progress Microbiology Series PPM 14, Merrow Publishing, Shilden (England).

——— 1980 Induction by streptomycin of mercuric ion resistance in *Pseudomonas aeruginosa*. FEMS Letters. 8: 211-213.

———, D. V. Wood and L. Andersen 1980 Curing of drug resistance plasmids from *Escherichia coli* and *Pseudomonas aeruginosa*. 2nd Inter. Microbiol. Ecol. Symp. Univ. of Warwick, England, Sept. 7-12.

——— and R. A. MacLeod 1974 Survival of *Escherichia coli* from freeze-thaw damage: a theoretical and practical study. Canadian J. Microbiol. 20: 671-681.

———, S. M. Steenbergen and R. S. Petty 1979 The effects of freezing and thawing on the uptake and retention of amino acids by *Escherichia coli*. FEMS Letters. 6: 267-272.

Chakrabarty, A. M. 1976 Plasmids in *Pseudomonas*. Ann. Rev. Genet. 10: 7-30.

Davies, J. and D. Smith 1978 Plasmid-determined resistance antimicrobial agents. Ann. Rev. Microbiol. 32: 469-518.

Ghani, B. A. and P. H. Calcott 1980 Protein synthesis in *Escherichia coli* recovering from freezing and thawing damage. Ohio J. Sci. April Program and Abstracts 80: 69.

Knowles, C. J., P. H. Calcott and R. A MacLeod 1974 Periplasmic CO-Binding c-type cytochrome in a marine bacterium. FEBS Letters 49; 78-83.

Komura, I., T. Funaba and K. Izaki 1971 Mechanism of mercuric chloride resistance in microorganisms. II NADPH-dependent reduction of mercuric chloride by a multiple drug resistance strain of *Escherichia coli*. J. Biochem (Tokyo) 70: 895-901.

Lee, S. K., P. H. Calcott and R. A. MacLeod 1977 Relationship of cytochrome content to the sensitivity of bacteria to NaCl on freezing and thawing. Canadian J. Microbiol. 23: 413-419.

Lehrbach, P., A. H. C. Kung, B. T. O. Lee and G. A Jacoby 1977 Plasmid modification of radiation and chemical mutagen sensitivity in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 98: 167-176.

Lowrey, O. H., N. J. Rosebrough, N. L. Farr and R. J. Randall 1951 Protein measurement with folin-phenol method. J. Biol. Chem. 195: 193-275.

MacLeod, R. A. and P. H. Calcott 1976 Cold shock and freezing damage to microbes, pp. 81-109. *In:* Survival of Vegetative Bacteria, 26th Symp. Soc. Gen. Microbiol, Cambridge University Press, Cambridge.

Mizuno, T. and M. Kageyama 1978 Separation and characterization of the inner and outer membrane of *Pseudomonas aeruginosa*. J. Biochem. (Tokyo). Biol. Chem. 240: 3685-3692.

Neu, H. L. and L. A. Heppel 1965 The release of enzymes from *E. coli* by osmotic shock and during the formation of spheroplasts. J. Biolog. Chem. 240: 3685-3692.

Ray, B. and M. L. Speck 1973 Freezing-injury in bacteria. CRC Crit. Rev. Clin. Lab. Sec. 4: 161-213.

Summers, A. O. and S. Silver 1978 Microbial transformations of metals. Ann. Rev. Microbiol. 32: 637-672.

Weston,. J. A. and C. J. Knowles 1974 The respiratory system of the marine bacterium, *Beneckea natriegens*. Cytochrome composition. Biochim. Biophys. Acta 332: 228-236.

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