Involvement of outer membrane proteins in freeze–thaw resistance of *Escherichia coli*

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Two families of *Escherichia coli* mutants with altered outer membrane protein components were examined for sensitivity to freezing and thawing and other stresses. A mutant unable to make the lipoprotein (*lpo*) was extremely sensitive to freezing and thawing in water or saline and to challenge with detergent, while the mutant unable to make the porin proteins (*omp*B) was more resistant than the isogenic wild type; strains unable to make the *tsx* and *omp*A proteins were slightly more sensitive to the stresses. Similarly, the *lpo* deficient strain exhibited more and the *omp*B less wall and membrane damage than the wild-type strains. Little difference in the extent of wall damage, but more membrane damage, was seen for the two *tsx* and the *omp*A strains when compared with the wild-type strain. The roles of the specific proteins in determining sensitivity to freeze–thaw are discussed.

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Deux familles de mutants d'*Escherichia coli* possédant des altérations au-niveau des protéines de la membrane externe ont été étudiées pour leur sensibilité à la congélation et à la décongélation et à d'autres stress. Un mutant incapable de produire la lipoprotéine (*lpo*) était extrêmement sensible à la congélation–décongélation en suspension dans l'eau ou dans un soluté salin ainsi qu'à l'action de détergents alors qu'un mutant incapable de produire les protéines (*omp*B) des porines s'avérait plus résistant que la souche sauvage isogénique. Les souches incapables de produire les protéines *tsx* et *omp*A étaient légèrement plus sensibles aux stress. De la même façon, la souche déficiente en *lpo* et la souche déficiente en *omp*B avaient respectivement plus et moins de dommages au-niveau de la paroi et de la membrane comparativement aux souches sauvages. Pour les deux souches *tsx* et *omp*A, il y avait peu de différence dans l'étendue des dommages de la paroi mais les dommages membranaires étaient plus importants comparativement à la souche de type sauvage. On discute le rôle de ces protéines spécifiques dans la sensibilité à la congélation–décongélation.

[Traduit par le journal]

Introduction

The stress of freezing and thawing can result in many profound alterations in microbial cells including loss of ability to divide (viability), loss of cytoplasmic and outer membrane integrity, inactivation of the cell's metabolic machinery, e.g., active transport and protein synthesis, damage to deoxyribonucleic acid, and disruption of cell morphology (Bank and Mazur 1973; Beuchat 1978; Calcott, 1978; Calcott, Steenbergen *et al.* 1979; MacLeod and Calcott 1976; Ray and Speck 1973; Williams and Calcott 1982). In particular, damage to the outer membrane has been detected in Gram-negative bacteria as evidenced by release of periplasmic proteins (Calcott and MacLeod 1975*a*), loss or alteration of lipopolysaccharide (Ray and Speck 1973; Ray *et al.* 1976), loss of outer membrane proteins (Ghani and Calcott 1980 and increases in the permeability of the wall to certain enzymes (MacLeod and Calcott 1976), dyes (Ray and Speck 1973), detergents (Calcott 1978; Ray and Speck 1973), and large molecular weight material (Calcott and MacLeod 1975*b*). This type of damage appears to play a role in the difficulty of enumerating Gram-negative bacteria in environmental samples, particularly stressful ones (see Bennett *et al.* (1981) for a review).

Recently, the outer membrane of a variety of Gram-negative bacteria has been studied at the biochemical, physiological, and genetic levels (DiRienzo *et al.* 1978; Hall and Silhavy 1981). This, together with the availability of mutants deficient in the major components of this structure (DiRienzo *et al.* 1978; Hall and Silhavy 1981), has enabled us to ascertain the role played by these molecules in determining cell resistance to freeze–thaw. In a previous paper, we have examined a family of *Salmonella typhimurium* strains with altered lipopolysaccharide (LPS) structure for the role played by this molecule in resistance to freeze–thaw (Bennett *et al.* 1981). In this paper, we have examined two sets of *E. coli* mutants deficient in the porin proteins, matrix proteins, and lipoprotein and determined the role played by these three major classes of protein in determining resistance to freeze–thaw and to other stresses.

Materials and methods

Bacterial cultures, growth and harvesting

Escherichia coli strains obtained from Drs. P. Manning, Max-Planck-Institut fur Molekulare Genetik, Berlin, West

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TABLE 1. Genetic characteristics of strains of *E. coli*

Strain	Origin	Genetic characteristics	Wall deficiency
P400	P.M.	F⁻ *thr*, *leu*, *pro*A, *arg* E, *thi*, *ara*, *xyl*, *mtl*, *galK*, *lac*Y, *sup* E, *str*, λ⁻	WT
P407	P.M.	P400, *tsx* 200	No tsx protein
P460	P.M.	P400, *omp*A-1	No 3A protein
P1578	P.M.	P400, *omp*B-105	No 1*a*, 1*b*, proteins
JE5506	M.I.	F⁻ *pps*, *his*, *pro*A, *arg* E, *thi*, *gal*, *lac*, *xyl*, *mtl*, *tsx*	No tsx protein
JE5505	M.I.	JE5506, Δ*lpo*	No tsx or lipoprotein

NOTE: P.M., culture from Dr. P. Manning, Max-Planck Institut fur Molekulare Genetik, Berlin, West Germany; M.I., culture from Dr. M. Inouye, Department of Biochemistry, SUNY at Stonybrook, NY, U.S.A. The origin and genetic characteristics of these strains are described in papers by Manning and Reeves (1979) and Hirota *et al.* (1977). See DiRienzo *et al.* (1978) for nomenclature of outer membrane proteins. WT, wild type.

Germany, and M. Inouye, Department of Biochemistry, State University of New York (SUNY) at Stony Brook, NY, U.S.A., are described in Table 1. The origin and full genetic characteristics of the strains used in this study are described elsewhere (Hirota *et al.* 1977; Manning and Reeves 1979). Both series of mutants were derived from *E. coli* K-12. Organisms were grown to early stationary phase in nutrient broth at 37°C before harvesting at room temperature by centrifugation from and resuspension in 0.85% saline. Organisms were resuspended in the freezing menstrua, water or 0.85% saline, at $1-3 \times 10^9$ organisms/mL.

Freezing and thawing regimen and viability determination

Bacterial suspensions were frozen slowly (1−2°C/min) or rapidly (approximately 100°C/min) to at least −70°C before thawing at a slow rate (1−2°C/min) as described previously (Calcott and MacLeod 1974). The population were frozen and thawed up to three times. Portions of the stressed and unstressed population were serially diluted in sterile 2 m*M* $MgSO_4$ to approximately 10^3 viable cells/mL before surface plating on nutrient agar plates. After drying, the plates were incubated at 37°C to constant count. Viability of a stressed population was calculated as described before (Calcott, Steenbergen *et al.* 1979). In some experiments, stressed populations were surface plated onto nutrient agar supplemented with sodium chloride or sodium lauryl sulfate. The concentrations of these two compounds were the maximal that allowed greater than 90% of unstressed populations to form colonies.

Lysis of resting cultures

Washed bacterial cultures were diluted into 50 m*M* Tris-(hydroxy)aminomethane (Tris−HCl) buffer (pH 7.0) with or without 1 m*M* ethylene diamine tetraacetic acid (EDTA) to an initial absorbance of 0.4−0.7 at 540 nm with a 1-cm light path cuvette. Absorbance was followed continuously before and after challenge of the culture with 0.05 or 1.0% (final concentration) sodium lauryl sulfate. From the tracing and the initial absorbance, a rate of decreases in absorbance (lysis) was calculated and expressed as percent decrease of initial absorbance per minute as described before (Calcott, Zaborowski *et al.* 1979).

Chemicals

All chemicals were purchased from Fisher Scientific Co., Cincinnati, OH, or Sigma Chemical Co., St. Louis, MO. Glass-distilled water was used throughout.

Results

When the *Escherichia coli* strains used were subjected to cycles of rapid freeze−thaw in water and saline, the viability of the population decreased with increasing number of cycles of stress (Fig. 1). As shown before, survival was higher when water was the freezing menstruum compared with saline (Calcott 1978; MacLeod and Calcott 1976). Although not shown, similar data were obtained when the strains were frozen and thawed using a slow freeze−thaw protocol where loss of viability might proceed by a different mechanism (Calcott 1978; MacLeod and Calcott 1976). Mutants unable to synthesize the proteins (see DiRienzo *et al.* (1978), Hall and Silhavy (1981) for reviews) designated 3A (*omp*A) (a matrix protein that spans the outer membrane) or *tsx* (associated with nucleoside uptake) were more susceptible to freeze−thaw in water and extremely sensitive to freeze−thaw in saline, while a mutant deficient in proteins 1*a* and 1*b* (*omp*B) (a peptidoglycan-linked transmembrane porin protein complex) was more resistant to freezing and thawing in water or saline than the isogenic wild type (Manning and Reeves 1979). The deletion of the gene for lipoprotein (a peptidoglycan-linked lipoprotein exposed on the outer side of the outer membrane) biosynthesis caused a dramatic increase in susceptibility of the strain to freeze−thaw in water or saline over the lipoprotein-proficient strain. These last two strains (JE5505 and JE5506) were also deficient in *tsx* protein. Although the two sets of mutants were obtained from different laboratories and a *tsx* proficient strain was not available for comparison for the JE series, the two families of mutants were derived originally from *E. coli* K-12. Since the response of the two *tsx* deficient strains (JE5506 and P407) were remarkably similar, this indicated that any genetic change that had occurred in the two laboratories had not influenced the freeze−thaw

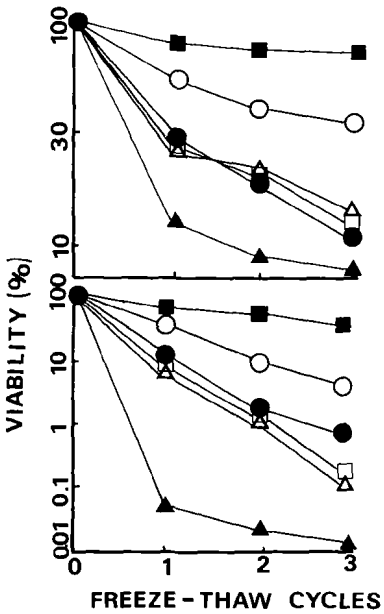

FIG. 1. Freeze–thaw response of a variety of *E. coli* strains. Various *E. coli* strains were grown to stationary phase aerobically in nutrient broth at 37°C, washed and resuspended in water (upper) or 0.85% saline (lower), and subjected to three cycles of rapid freezing and thawing. Samples were removed and assayed for viability as described in Materials and methods. ○, P-400 (WT); ●, P-460 (*omp*A); □, P407 (*tsx*); ■, P1578 (*omp*B); △, JE5506 (*tsx*); ▲, JE5505 (*tsx*, Δ*lpo*). The responses of strains P460, P407, P1578, JE5506, and JE5505 were statistically different from P400 ($p < 0.05$), while the responses of JE5506 and JE5505 were statistically different ($p < 0.01$). The data represent an average of three experiments.

resistance of the parent strains.

Not only was freeze–thaw survival influenced by mutations affecting proteins in the outer membrane, but the extent of wall and membrane damage was influenced by the stress. Using a plating procedure (MacLeod and Calcott 1976; Ray and Speck 1973), the effect of these mutations on the proportion of survivors not exhibiting wall or membrane damage as evidenced by inability to form colonies was determined. The concentration of sodium lauryl sulfate and sodium chloride included in the plating medium to measure the proportion of the population exhibiting wall and membrane damage, respectively, was determined as previously described (MacLeod and Calcott 1976; Ray and Speck 1973) and is shown in Table 2. As can be seen, there was very little effect of the mutations on the resistance of the strains to NaCl, although the two *tsx*, *omp*B, and the *tsx*, Δ*lpo* deficient strains were more susceptible to the detergent in the plating medium. In confirmation of previous reports, the proportion of survivors exhibiting wall damage was higher than those exhibiting membrane damage (Table 3). The mutant unable to make *lpo* gene product was more prone to both wall and membrane damage, while *omp*A strain exhibited more membrane damage after freeze–thawing in water or saline; the mutant unable to make the porin proteins (*omp*B) was less prone when compared with appropriate isogenic proficient strains. The two mutants unable to make *tsx* protein (P407 and JE5506) exhibited comparable amounts of damage to wall but more damage to the membrane than the wild-type strain. The same trends were observed for organisms frozen slowly in water or saline and thawed (data not presented).

TABLE 2. Sensitivity of various *E. coli* strains to NaCl and sodium lauryl sulfate

Strain	Genetic characteristics	Concn. of agent allowing >90% of cells to form colonies	
		NaCl (*M*)	Sodium lauryl sulfate (%)
P400	WT	0.5[a]	0.25
P460	*omp*A	0.5	0.2
P407	*tsx*	0.5	0.1*
P1578	*omp*B	0.5	0.05*
JE5506	*tsx*	0.75	0.15*
JE5505	*tsx*, Δ*lpo*	0.65	0.01**

NOTE: * Indicates values that were significantly different from the value for the wild type (WT) ($p < 0.05$); ** indicates value that was significantly different from the value of strain JE5506 ($p < 0.01$).

[a]Maximum concentration of agent that allows >90% of viable cells of an unstressed population to form visible colonies in 48 h. The values represent an average of three experiments.

Since the mutations appeared to influence the extent of wall damage after freeze–thaw and the ability of organisms to grow in the presence of detergent, we have examined the effect of EDTA and detergent on cell integrity in resting suspension of the various strains. Table 4 shows the effect of the presence of EDTA and both low and high levels of detergent on cell integrity. In the absence of EDTA, the 3A (*omp*A) and two *tsx* deficient strains were as resistant to both levels of detergent as the wild type. While the porin-deficient strain (*omp*B) was more resistant, the lipoprotein-deficient strain (*tsx*, Δ*lpo*) was extremely sensitive to both low and high levels of deterent. The presence of EDTA did not dramatically alter the response of most of the strain to detergent; however, the lipoprotein-deficient strain (*tsx*, Δ*lpo*) showed a drastic increase in sensitivity to the detergent in the presence of EDTA.

Discussion

Deletion of specific proteins from the outer membrane of *Escherichia coli* by mutation caused a decrease in stability of the structure as evidenced by a decreased ability of the mutants to grow in the presence of higher

TABLE 3. Extent of wall and membrane damage caused by freeze–thaw on a variety of *E. coli* strains

Strain	Genetic characteristics	Freezing menstruum	% of survivors not exhibiting damage to: wall	membrane
P400	WT.	Water	2.1	46
		Saline	4.7	59.6
P460	*omp*A	Water	0.6	34.4*
		Saline	2.8	37.2*
P407	*tsx*	Water	1.6	19.7*
		Saline	6.2	18.6*
P1578	*omp*B	Water	9.2*	71.1*
		Saline	20.0*	53.0*
JE5506	*tsx*	Water	1.6	25.0*
		Saline	5.3	29.3*
JE5506	*tsx*, Δ*lpo*	Water	0.05**	0.15**
		Saline	0.25**	0.15**

NOTE: Washed *E. coli* suspensions in water or saline (0.85%) were subjected to rapid freezing and thawing as described in the text. The populations were surface plated after appropriate dilution in 2 m*M* $MgSO_4$ onto nutrient agar, with or without the supplements NaCl or sodium lauryl sulphate at the appropriate concentration (see Table 2). The proportion of survivors (of those growing on nutrient agar) capable of growing in the presence of sodium chloride and sodium lauryl sulfate were considered to be exhibiting no membrane and wall damage, respectively (MacLeod and Calcott 1976; Ray and Speck 1973). The experiments were performed three times and the values are averages. * Indicates values that were significantly different from the values for strain P400 ($p < 0.05$); ** indicates values that were significantly different from the values for strain JE5506 ($p < 0.01$).

TABLE 4. Susceptibility of various strains of *E. coli* to detergent and EDTA in resting suspension

Strain	Genetic characteristics	Presence of 1 m*M* EDTA	Rate of lysis (% loss of initial absorbance/min) in presence of detergent at: 0.05%	1.0%
P400	WT	−	3.45	11.4
		+	8.3	13.4
P460	*omp*A	−	2.7	11.9
		+	2.8	7.4
P407	*tsx*	−	4.3	7.7
		+	5.9	11.6
P1578	*omp*B	−	0.3*	2.1*
		+	0.5*	<0.1*
JE5506	*tsx*	−	2.0	6.2
		+	10.5	12.3
JE5505	*tsx*, Δ*lpo*	−	28*	>60*
		+	>60*	>100*

NOTE: Washed suspensions of organism in Tris–HCl in the presence or absence of 1 m*M* EDTA were challenged with 0.05 or 1.0% sodium lauryl sulfate (final concentration). Lysis rates were calculated by traces of absorbance versus time with reference to the initial absorbance. In no situation was a culture lysed by the presence of EDTA and Tris–HCl. These values represent the average of three experiments. * Indicates values that were significantly different from the responses of strain P400 and JE5506 ($p < 0.01$).

levels of sodium lauryl sulfate, a detergent whose entry into the cell is limited by the permeability of the outer membrane (MacLeod and Calcott 1976). Although these mutations altered the resistance of the strain to a detergent, they were without effect on the cell resistance to sodium chloride, indicating that the permeability of the cytoplasmic membrane was relatively unimpaired (MacLeod and Calcott 1976). This decreased stability of the outer membrane was further evidenced when sensitivity of the strains to freeze–thaw was com-

pared. Irrespective of the rate of cooling or the freezing menstruum, strains carrying mutations in the *tsx*, *omp*A, or *lpo* genes were more sensitive to the stresses involved than the appropriate proficient strains. This would indicate that these proteins, directly or indirectly, play structural roles as well as certain transport or receptor functions (DiRienzo *et al.*1978; Hall and Silhavy 1981). That the strain defective in porin synthesis (*omp*B) was more resistant to freeze–thaw than the corresponding wild type would indicate that the function of this protein in the wall, although possibly structural, was very much different. The gene products controlled by the *omp*B gene form a specific hydrophilic channel connecting the exterior of the cell to the periplasmic space which allows passage of small molecular weight (less than 650 for *E. coli*) material, e.g., sugars, amino acids, and certain antibiotics (DiRienzo *et al.* 1978). Thus, it is attractive to postulate that the presence of the hydrophilic porin channel in the outer membrane is lethal to the cells undergoing freezing and thawing since it allows a free passage for external ice to seed across the outer membrane. Although not proven, this idea is supported since the porin-deficient strain (*omp*B) exhibited less outer membrane and cytoplasmic membrane damage than wild-type strain, whereas destabilizing the outer membrane in the other mutants (*omp*A and *tsx*) resulted in increased damage to both membrane structures. The same trends were seen when outer membrane stability was determined in the various strains by challenge in resting suspension with detergent in the presence or absence of EDTA.

In a previous paper, we presented evidence for the involvement of lipopolysaccharide in determining resistance to freeze–thaw in the Gram-negative bacterium, *Salmonella typhimurium* (Bennett *et al.* 1981). We postulated that the exposed phospholipids were the site of action of the stress of freezing and thawing and that stress-induced alteration of the phospholipid domains caused disruption of lipid–protein and lipid–lipopolysaccharide bonds that resulted in release of these two macromolecules. In addition, the long chains of the lipopolysaccharide probably acted to shield the exposed phospholipids. In this study, we would like to add that the role of the proteins in determining resistance to freeze–thaw is more active and that in addition to damage to the phospholipid bilayer, the nature of the protein, e.g., porin versus nonporin and its interaction with other structures in and around the wall are equally important. The structural *lpo* protein which is securely anchored to the underlying peptidoglycan and embedded in the outer membrane bilayer (Hall and Silhavy 1981) plays an important role in stabilizing the outer layers against the stresses of freeze–thaw at least in the absence of a functional *tsx* gene product. Although not examined, it is likely that *lpo* mutants would be particular sensitive to osmotic shock, cold shock, drying, and lyophilization; all processes that cause extensive cell membrane and wall damage (Gray and Postgate 1976). Clearly, another approach that could be utilized in our studies would be to examine the interaction of lipopolysaccharide structure with outer membrane protein complement in determining resistance to freeze–thaw, in particular that between decreased lipopolysaccharide chain length (that causes decreased resistance) and loss of porin proteins (that causes an increase).

Thus, altered structure of the outer membrane, both in the lipopolysaccharide and the protein components, can have profound effects on the resistance of the host bacterium to freeze–thaw and most probably to other stresses. This might explain the variability in sensitivity of Gram-negative bacteria in stressful environments such as water bodies, frozen foods, and other locales where organisms with injured walls can be detected.

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BANK, H., and P. MAZUR. 1973. Visualization of freezing and damage. J. Cell. Biol. **57**: 729–742.

BENNETT, G. M., A. SEAVER, and P. H. CALCOTT. 1981. Effect of defined lipopolysaccharide core defects on resistance of *Salmonella typhimurium* to freezing and thawing and other stresses. Appl. Environ. Microbiol. **42**: 843–849.

BEUCHAT, L. R. 1978. Injury and repair of Gram-negative bacteria, with special consideration of the involvements of the cytoplasmic membrane. Adv. Appl. Microbiol. **23**: 219–243.

CALCOTT, P. H. 1978. Freezing and thawing microbes. Patterns of progress. Microbiology Ser. PPM 14. Merrow Publishing, Shilden, England.

CALCOTT, P. H., and R. A. MACLEOD. 1974. Survival of *Escherichia coli* from freeze–thaw damage: a theoretical and practical study. Can. J. Microbiol. **20**: 671–681.

——— 1975*a*. The survival of *Escherichia coli* from freeze–thaw damage: permeability barrier damage and viability. Can. J. Microbiol. **21**: 1724–1732.

——— 1975*b*. The survival of *Escherichia coli* from freeze–thaw damage: the relative importance of wall and membrane damage. Can. J. Microbiol. **21**: 1960–1968.

CALCOTT, P. H., 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 Microbiol. Lett. **6**: 267–272.

CALCOTT, P. H., C. ZABOROWSKI, W. L. LEVINE, and N. H. TRUONG. 1979. Drug resistance plasmid (pPL1) mediated changes in the susceptibility of *Pseudomonas aeruginosa* to stress. FEMS Microbiol. Lett. **7**: 75–80.

DIRIENZO, J. M., K. NAKAMURA, and M. INOUYE. 1978. The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly and functions. Annu. Rev. Biochem. **47**: 481–532.

GHANI, B. A., and P. H. CALCOTT. 1980. Protein synthesis in *Escherichia coli* recovering from freezing and thawing damage. Ohio J. Sci. **80**: 69.

GRAY, T. R. G., and J. R. POSTGATE. 1976. The survival of vegetative microbes. Symp. Soc. Gen. Microbiol. **26**: 1–414.

HALL, M. N., and T. J. SILHAVY. 1981. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Annu. Rev. Genet. **15**: 91–142.

HIROTA, Y., H. SUZUKI, Y. NISHIMURA, and S. YASUDA. 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. U.S.A. **74**: 1417–1420.

MACLEOD, R. A., and P. H. CALCOTT. 1976. Cold shock and freezing damage to microbes. Symp. Soc. Gen. Microbiol. **26**: 81–109

MANNING, P. A., and P. REEVES. 1979. Outer membrane protein 3B of *Escherichia coli* K-12: effects of growth temperature on the amount of the protein and further characterization on acrylamide gels. FEMS Microbiol. Lett. **1**: 275–278.

RAY, B., and M. L SPECK. 1973. Freezing-injury in bacteria. CRC Crit. Rev. Clin. Lab. Sci. **4**: 161–213.

RAY, B., M. L. SPECK, and W. J. DOBROGOSZ. 1976. Cell wall lipopolysaccharide damage in *Escherichia coli* due to freezing. Cryobiology, **13**: 153–160.

WILLIAMS, D. L., and P. H. CALCOTT. 1982. Role of DNA repair genes and an R plasmid in conferring cryoresistance on *Pseudomonas aeruginosa*. J. Gen. Microbiol. **123**: 215–218.