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Relation of Lipopolysaccharide and Fatty Acid Ester Release to the Ethylenediaminetetraacetic Acid Alteration of Permeability in *Enterobacteriaceae*

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Escherichia coli subjected to cold osmotic shock released 30 to 40% of their fatty acid esters and 42% of their cellular hexosamine. In contrast, *Enterobacter*, although they released 40% of fatty acid esters, release only 25% of hexosamine. *Proteus* released less than 15% of either fatty acid esters or hexosamine. These differences are taken to explain the differences among the *Enterobacteriaceae* in releasing surface enzymes after osmotic shock. It is felt that the release of additional lipopolysaccharide after osmotic shock is necessary for the release of surface enzymes that are not freed by ethylenediaminetetraacetic acid-tris(hydroxymethyl)aminomethane exposure.

Numerous studies in recent years have investigated the effect of ethylenediaminetetraacetic acid (EDTA) on altering permeability of various *Enterobacteriaceae* (7, 12, 13). Leive has shown that many coliform bacteria could be made permeable to small molecules by brief exposure to EDTA in a tris(hydroxymethyl)aminomethane (Tris) buffer (8). However, the exit of large molecules, such as enzymes and transport proteins, occurs only if the cells are subjected to a cold osmotic shock (5, 14). Studies in this laboratory have demonstrated that members of the *Klebsielleae* release only 50 to 60% of their content of so-called surface enzymes (14). *Proteus* and *Providencia* do not release nucleotidases which in vivo experiments suggest are surface enzymes (14). Electron microscopic studies of alkaline phosphatase (3) and of the 5'-nucleotidases also have demonstrated that these are located on the surface of the cell (15).

One of the biochemical changes produced by EDTA in coliforms is the release of cell surface lipopolysaccharide (9). Similar studies from Eagon's laboratory (16) have shown that EDTA releases a protein-lipopolysaccharide complex from *Pseudomonas* strains. Leive has suggested that release of surface lipopolysaccharide and increased permeability may not have a direct cause and effect relationship since the two phenomena can be dissociated under certain conditions (7).

We examined various *Enterobacteriaceae* to determine whether the process of osmotic shock released a significantly greater amount of lipopolysaccharide, protein, or fatty acids than brief exposure to EDTA, which would account for the release of surface enzymes by osmotic shock. Similarly we studied the cell wall materials released by osmotic shock from *Proteus* strains which do not release their surface enzymes.

MATERIALS AND METHODS

Bacteria. *E. coli* K37, *Enterobacter aerogenes* (Yale) and *Proteus vulgaris* are strains previously described (14).

Growth and osmotic shock. The high-phosphate medium of Neu and Chou (14) or the Tris-based medium (14) was used. Carbon source was 0.5% glucose. Organisms were incubated at 35 C on a rapid rotary shaker, and growth was followed by change in optical density (OD) at 600 nm in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Osmotic shock was performed by published methods (14). The only modification was that all centrifugation prior to the cold shock was performed at 23 C. Control bacteria were disrupted prior to any treatment by sonic disintegration at 0 C using 15 sec bursts for 2 min.

Chemical and biochemical assays. The bacterial cell pellet was extracted by the method of Ames (1), and the organic phase was taken to dryness on a rotary vacuum evaporator and then suspended in alcohol-ether (3:1). Analysis of this material for fatty acid esters was done by the method of Stern and Shapiro. Fatty acids esterified to the complex lipids were measured colorimetrically as the ferric complex of the hydrox-

amic acids formed from the esters with hydroxylamine (17). Methyl stearate was used as the standard. Release of lipid containing fatty acid esters was determined by the difference in this value before and after the stages of treatment by EDTA-Tris and osmotic shock. Hexoseamine assay was performed by the modified Elson-Morgan technique (6), DNA by the method of Dische (2), and RNA by the orcinol method of Mejbaum (11). 5'-Nucleotidase was assayed as described previously (14).

RESULTS AND DISCUSSION

No significant differences in the amount of RNA (measured by orcinol) or protein released in the EDTA exposure stage or cold shock stage among the three bacteria could be demonstrated. This is of particular significance since 97% of the 5'-nucleotidase was released from the *E. coli* and 70% from *Enterobacter* but less than 1% from *Proteus*.

Table 1 demonstrates that, in both exponential and stationary phases of growth, *E. coli* and *Enterobacter* release from 30 to 40% of their fatty acid esters during the period of exposure to EDTA and subsequent cold osmotic shock. *Proteus* released less than 15% of total fatty acid esters. In the case of *E. coli*, the amount of fatty acid esters released in the shock stage is double that released at EDTA exposure. *Enterobacter*, on the other hand, released equal amounts at both stages.

Hexosamine release, which can be considered to represent lipopolysaccharide release, shows the most significant differences among the three organisms in response to both EDTA exposure and osmotic shock (Table 2). *E. coli* released essentially equal amounts of hexosamine at both stages of treatment. *Enterobacter* released significantly less at the shock stage. *Proteus* released only one-third the hexosamine released by *E. coli* at either stage.

These experiments suggest that release of surface enzymes from members of the *Enterobacteriaceae* could be related to the release of lipopolysaccharide and fatty acid esters that occurs in *E. coli* but not in *Proteus* at both the EDTA stage and the osmotic shock stage.

Evidence that the lipoprotein and lipopolysaccharide components do not form separate layers in *E. coli* cell walls (4), as contrasted with *Proteus mirabilis* (10), would support the concept that the lipopolysaccharide lipoprotein is disrupted by the EDTA and cold shock to release the surface proteins. In the case of *Proteus*, the proteins could remain covalently bound to the lipoprotein.

The fact that EDTA exposure makes the cells permeable to entry and exit of molecules but does not release the surface enzymes does not seem related to fatty acid release since fatty acid release in *E. coli* and *Enterobacter* is similar. However, the release of significantly more lipopolysaccharide by *E. coli* with osmotic shock suggests that the release of additional surface lipopolysaccharide is necessary to free these enzymes from their surface binding sites. Additional investigation may be able to further clarify this point.

TABLE 1. *Fatty acid esters released*[a]

Organism	Growth stage	Fatty acid esters (μg/g)	EDTA stage	Shock stage
			%	%
Escherichia coli	Exponential	28.7[b]	10	23
	Stationary	32.2	18	20
Aerobacter	Exponential	16.0	19	22
	Stationary	22.1	23	20
Proteus vulgaris	Exponential	29.7	3	9
	Stationary	27.8	4	5

[a] Each value is the average value obtained from three separate experiments. Organisms were grown to either exponential or early stationary phase and harvested by centrifugation. The cells were washed twice with 0.03 M NaCl-0.01 M Tris-hydrochloride (*p*H 7.3) at 23 C. A sample of cells was removed as an untreated control to be assayed for fatty acid esters. The cells were resuspended at a ratio of 1 g per 80 ml in 0.03 M Tris-hydrochloride (*p*H 7.3)-0.5 M sucrose containing either 1 mM or 0.1 mM EDTA for stationary or exponential cells, respectively. After 10 min at 23 C, the cells were removed by centrifugation and a portion was assayed for fatty acid esters. The remainder of the cells were suspended in either 0.5 mM $MgCl_2$ (exponential cells) or water (stationary cells) at 4 C for 10 min, at which time cells were again collected and assayed for fatty acid esters. Supernatant fractions at each stage and untreated disintegrated cells were assayed for enzyme released.

[b] Base levels expressed as micrograms per gram of control bacteria.

TABLE 2. *Release of hexosamine upon exposure to EDTA and osmotic shock*

Organism	EDTA stage[a]	Shock stage[a]	Per cent of total	5'-Nucleotidase released
				%
Escherichia coli	440 (23)	368 (19)	42	94
Aerobacter	368 (19)	84 (6)	25	60
Proteus vulgaris	380 (9.7)	180 (4.6)	14.3	0.5

[a] Bacteria were grown to early stationary phase. Treatment was as outlined in Table 1. The sonic extract of whole untreated cells and supernatant fractions after the EDTA and shock stages were extensively dialyzed against 0.15 M NaCl containing 0.015 M sodium citrate and then water. They were lyophilized to dryness, suspended in 4 N HCl, and hydrolyzed for 5 hr. The hydrolysate was processed and assayed for hexosamine as described by Kabat (6).

[b] Expressed as milligrams per gram. Values in parentheses are per cent.

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