FUNCTIONAL RECONSTITUTION OF EDTA-TREATED ESCHERICHIA COLI

David P. Brunner, Ross A. Caputo and Ronald W. Treick

Department of Microbiology, Miami University, Oxford, Ohio 45056

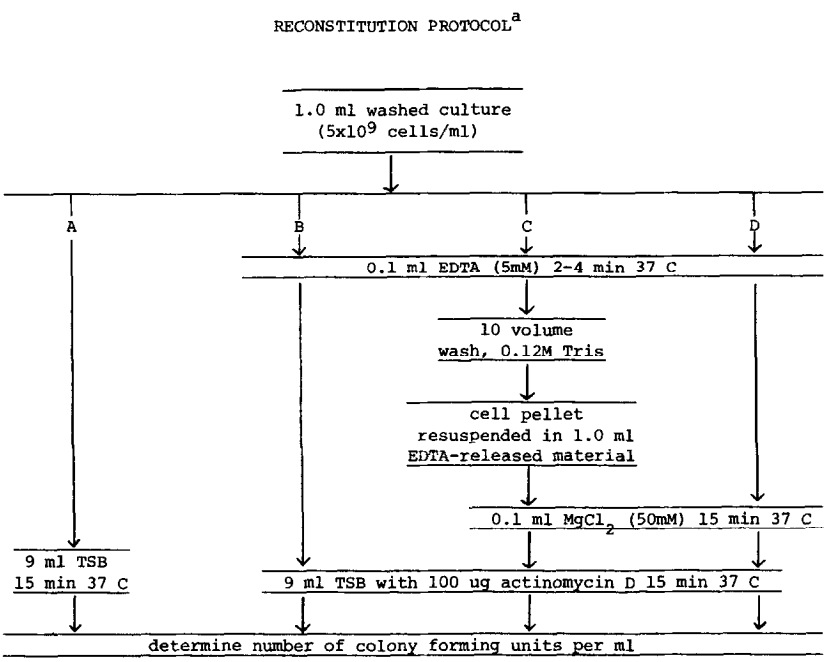
Received November 19,1976

SUMMARY: Strains of Escherichia coli K-12 were treated with EDTA and reconstituted with EDTA-released outer envelope material. Reconstitution was accomplished by reversing the effect of EDTA with the addition of $MgCl_2$ in the presence of outer envelope material. Following reconstitution actinomycin D sensitive cells were found to be resistant to the lethal effect of the drug. Mix-Match Reconstitution experiments suggested that the reassembly of the outer envelope material with EDTA-treated cells was not strain specific. Further data obtained in Fraction Reconstitution experiments indicated that the interplay of both F1 and F2 components of the EDTA-released material was more important than the individual role of either component.

INTRODUCTION.

Disruption of the E. coli cell envelope with EDTA results in the release of 30-50% of the lipopolysaccharide (LPS). This correlates with an increased susceptibility to many antibacterial compounds (1). The EDTA-released material was further studied by Leive et al., who distinguished two separate fractions by ultracentrifugation (2). The more rapidly sedimenting fraction, F1, was composed of LPS associated with protein and lipid while the lighter, F2, fraction was composed solely of LPS and thought to originate from the outer envelope surface. A mutant which did not display increased sensitivity to actinomycin D upon treatment with EDTA was shown to release F1 complex but not F2. This suggested that the release of F2 and not F1 was associated with the permeability change caused by EDTA (3).

To determine if the increased permeability exhibited by EDTA-treated cells was due to the release of the envelope components, we have accomplished a functional reconstitution of the cell's permeability barrier. Reconstitution being defined as the reassociation of EDTA-released material with a significant number of bacterial cells treated with EDTA, thus preventing the lethal effect of actinomycin D.



[a]The percent survival and percent reconstitution values were determined from the CFU/ml data as follows:

% survival = treated (B)/untreated (A)

% reconstitution = treated (C or D)/untreated (A)

Fig 1. Diagrammatic representation of the protocols used in reconstitution experiments. Cultures were grown to log phase in 0.12M Tris based TSB (pH 8.0). Cells were then washed twice in Tris buffer and resuspended to $5x10^9$ cells/ml. Aliquots of the culture were then treated as shown. Depicted is the untreated control (protocol A), treated control (protocol B), Native Reconstitution (protocol D) and both Mix-Match and Fraction Reconstitution formats (protocol C).

While there exists in the literature reports of the reassembly of the LPS material, no reference could be found which dealt with a functional reconstitution of the cell's permeability barrier, as described above (4,5,6).

MATERIALS AND METHODS.

The bacterial strains used in this study were either Escherichia coli K12 P678 or mutants derived from this parental strain. Mutant strains were selected for their increased sensitivity to dyes and were found to have varying degrees of outer membrane alterations.

EDTA-released material was extracted according to the procedure of Leive (2). The bacteria were grown in 0.12M Tris HCl (pH 8.0)-based LB medium (7) supplemented with either 0.4% glucose or 0.4% glucose with D-[U-^{14}C] glucose (40 μCi/mole).

TABLE 1

DISTRIBUTION OF F1 and F2[a]

Strain	Total CPM	F1	F2	F2/F1
P678	1648	278	1282	4.6
MU71	1655	819	678	0.8
MU71-H-1	1650	1150	338	0.3

[a]CPM (counts per minute) were obtained from rate-zonal centrifugation experiments as described in Methods. The F1 and F2 components were represented by the radioactivity of fractions corresponding to the 10 - 80 S and 5 to 5.5 S region of the gradient respectively (2).

Distribution of F1 and F2 EDTA-released fractions was determined by rate-zonal ultracentrifugation. Linear sucrose gradients were prepared with a Beckman Density Gradient Former from 8% to 20% (w/v). RNase free sucrose was prepared in 0.01M Tris HCl (pH 8.0) with 0.5mM EDTA. The material was layered in a 0.1 ml volume on the top of the gradient and centrifuged at 234,000 g for 10 h in a SW 50.1 Spinco rotor. Ten equal fractions were collected from the bottom of the tube. Sedimentation coefficients were estimated according to the method of McEwen (8). Radioactivity was monitored by appropriate liquid scintillation techniques.

The susceptibility of test strains to actinomycin D was determined by a modification of the procedure of Leive (1,9). Strains were examined before and after EDTA treatment (Fig 1). Colony forming units (CFU) were determined by plating duplicate samples of each of three dilutions on Trypticase Soy Agar (TSA) plates. In all susceptibility tests and reconstitution studies, 0.12M Tris HCl-based Trypticase Soy Broth (TSB) was used for culture growth and diluent.

In Native Reconstitution experiments (Fig 1, protocol D), cells which had been treated with EDTA (0.5mM final concentration) for 15 min at 37 C were then incubated with $MgCl_2$ (5mM final concentration) for 15 min at 37 C. Actinomycin D (10 ug/ml in TSB) was then added and the incubation continued for 15 min at 37 C. Samples were then plated to determine CFU.

In the Mix-Match Reconstitution experimental protocol (Fig 1, protocol C), cells were washed with 10 volumes 0.12M Tris HCl to remove envelope material released by EDTA treatment. Cell pellets were then resuspended in EDTA-released material from a similar number of cells of each strain type.

Since the sedimentation velocity of the F1 and F2 components of the EDTA-released material were known, each fraction could be obtained by rate-zonal ultracentrifugation. Individual F1 and F2 components, as well as an F1 and F2 mixture, were used in Fraction Reconstitution experiments (Fig 1, protocol C). It should be noted that in the Fraction Reconstitution experiments, there exists a 4-fold concentration of material added to the EDTA treated cells.

TABLE 2

FREQUENCY OF RECONSTITUTION[a]

Reconstitution protocol	EDTA-released material	Reconstitution test strains P678	MU71	MU71-H-1
Native[b]	self	11.2	8.7	3.1
Mix-match[c]	P678	6.0	7.4	0.8
	MU71	8.0	1.9	0.8
	MU71-H-1	8.9	1.3	0.8
Fraction[d]	F1	0.4	0.9	3.2
	F2	6.4	5.3	0.2
	F1+F2	12.1	9.5	3.6

[a]Treated controls showed less than 0.1% survival (Fig 1, protocol B), while untreated controls showed 100% survival (Fig 1, protocol A).

[b]refers to Fig 1, protocol D.

[c]refers to Fig 1, protocol C.

[d]refers to Fig 1, protocol C.

RESULTS AND DISCUSSION.

The EDTA-released material was subjected to rate-zonal ultracentrifugation on a linear sucrose gradient (8-20%, w/v). The distribution of F1 and F2 fractions expressed as a ratio of F2 vs F1 is presented in Table 1. The data illustrate that both mutant strains release less F2 and more F1 material than the parent.

The data in Table 2 show functional reconstitution of EDTA-treated cells by addition of EDTA-released material. Parental strain, P678, appears to have the highest efficiency for Native Reconstitution and strain MU71-H-1 the lowest.

TABLE 3

DOSE RESPONSE RECONSTITUTION[a,b,c]

Colony forming units per ml ($X10^6$)	% survival increase	EDTA-released material added (ml)
259	0.0	0.00
265	2.3	0.05
270	4.3	0.10
341	31.7	0.25
351	35.5	0.50
376	45.2	0.75
424	63.7	1.00

[a] In this study, $MgCl_2$ was used at a final concentration of 50mM.

[b] Treated controls (Fig 1, protocol B with Tris HCl wash) showed less than 0.1% survival ($5X10^6$ CFU/ml), while untreated controls (Fig 1, protocol A) showed 100% survival ($6.1X10^9$ CFU/ml).

[c] Dose response reconstitution was performed by protocol C, Fig 1.

Extensive controls examining each of the reagents used, alone and in combinations with each other, have been conducted to substantiate the role of the EDTA-released material in reconstitution. The results showed that no other combination of steps outlined (Fig 1) could be responsible for the reconstitution phenomena. The EDTA-released material must reassociate in the presence of $MgCl_2$. Without the EDTA-released material, $MgCl_2$ will not protect against actinomycin D challenge. Binding of actinomycin D to EDTA-released material, thereby rendering less inhibitor availably to the cell, has been ruled out by actinomycin D binding studies utilizing the method of Caputo *et al.* (10).

Results of a dose response study (Table 3) examining the efficacy of reconstitution with varying amounts of EDTA-released material show that reconsti tution is due to the EDTA-released material.

As shown in Table 2, reconstitution was indicated in each combination utilizing a Mix-Match Reconstitution protocol (Fig 1, protocol C). Self-reconstitution occurred at a lower efficiency when compared to the Native Reconstitution data. This lowered efficiency was observed each time the 0.12M Tris HCl wash was performed. Parental strain, P678, again shows the highest reconstitution and MU71-H-1 the lowest. This experimental protocol accomplished reconstitution of cells with EDTA-released material other than their own.

The F1 and F2 components of the EDTA-released material were partially purified and separated by rate-zonal ultracentrifugation. The data in Table 2 show reconstitution of EDTA-treated cells with F1, F2 and F1+F2 components. As can be seen, strains P678 and MU71 were reconstituted more efficiently with the F2 component. Strain MU71-H-1, on the other hand, was reconstituted more efficiently with the F1 material. The results obtained with strains P678 and MU71-H-1 were predictable, since it is reasonable to expect reconstitution to be most efficient with those components which are predominant in the EDTA-released material. In strain MU71, however, the F1 and F2 concentrations are similar and the F2 component is primarily responsible for reconstitution.

This investigation indicates that the interplay of F1 and F2 components is responsible for the most efficient reconstitution of a resistant state of sensitive cells to actinomycin D. The importance of specific components may vary with the inhibitor employed, therefore reconstitution is being investigated with other inhibitors. The data demonstrate that reconstitution of EDTA-treated cells with envelope material allowed a significant portion of the population to survive actinomycin D treatment.

REFERENCES.

1. Leive, L. (1968) J. Biol. Chem. 243, 2373-2380.
2. Leive, L., Shovlin, V. K., and Mergenhagen, S. E. (1968) J. Biol. Chem. 243, 6384-6391.
3. Voll, M. J., and Leive, L. (1970) J. Biol. Chem. 245, 1108-1114.
4. Asbell, M. A., and Eagon, R. G. (1966) J. Bacteriol. 92, 380-387.
5. DePamphilis, M. L. (1971) J. Bacteriol. 105, 1184-1199.

6. Gilleland, Jr., H. E., Stinnett, J. D., Roth, I. L., and Eagon, R. G. (1973) J. Bacteriol. 113, 417-432.
7. Miller, J. H. (1972) Experiments in molecular genetics. p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. McEwen, C. R. (1967) Anal. Biochem. 20, 114-149.
9. Leive, L. (1974) Ann. N.Y. Acad. Sci. 235, 109-129.
10. Caputo, R. A., Treick, R. W., Griffin, C. C., and Farrell, M. P. (1975) Appl. Microbiol. 29, 476-479.