Repair of Wall Damage in *Escherichia coli* Recovered from an Aerosol

By P. HAMBLETON

Microbiological Research Establishment, Porton, Salisbury, Wiltshire

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SUMMARY

Escherichia coli B organisms recovered from aerosols had a changed envelope as manifested by the bacteria becoming sensitive to lysozyme. Certain di- and trivalent cations added to suspensions of bacteria recovered from aerosols mitigated this sensitivity. Repair required a carbon-energy source, but not synthesis of protein, ribonucleic acid or mucopeptide. Components of the envelope were released from the aerosolized organisms. These results support the hypothesis that aerosolization caused changes in the outer wall structure of bacteria that resemble changes induced by ethylenediaminetetraacetic acid.

INTRODUCTION

Aerosolization profoundly affects the permeability of some Gram-negative bacteria. Micro-organisms recovered from aerosols lack control of ion transport (Anderson, Dark & Peto, 1968) and certain cytoplasmic constituents leak out (Benbough, 1967; Cox, 1969; Webb, 1969; J. E. Benbough, P. Hambleton & R. E. Strange, unpublished results). In addition there is a change in the outer layers of the cell envelope as shown by the development of sensitivity to certain hydrolytic enzymes, in particular lysozyme (Hambleton, 1970). Permeability changes resulting from aerosolization may not be lethal in themselves but are related to subsequent survival of the airborne bacteria (Anderson *et al.* 1968; Hambleton, 1970).

The exact nature of the lesions resulting in permeability changes is unclear. In the case of sensitivity to lysozyme, however, a number of treatments induce susceptibility in Gram-negative bacteria previously resistant to the enzyme (Salton, 1957; Noller & Hartsell, 1961; Leive, 1968; Heppel, 1969). Susceptibility to the action of lysozyme may arise by conformational or chemical changes in the wall structure, possibly with concomitant release of a specific wall component such as lipopolysaccharide (Leive, 1968; Leive, Shovlin & Mergenhagen, 1968; Rogers, Gilleland & Eagon, 1969) so that the mucopeptide wall layer becomes accessible to the enzyme. Aerosolization appears to result in a similar lesion of the envelopes of coliform bacteria.

The recovery of aerosolized *Escherichia coli* B, shown by a return of resistance to lysozyme, was studied to determine the conditions that induced maximum repair. Also, materials released from *E. coli* B by aerosolization or ethylenediamine-tetraacetic acid (EDTA) were compared immunologically. The information obtained might indicate the nature of the wall lesion and the similarity, or otherwise, of the effects of aerosolization and other treatments upon the Gram-negative cell envelope.

METHODS

Preparation of spray suspensions. A stock culture of *Escherichia coli* strain B was maintained as frozen pellets at -70° (Cox, 1968). Cultures for experiments were prepared as described previously by Hambleton (1970). Organisms were harvested in a bench centrifuge (3000 g, 10 min.), washed twice with distilled water and finally resuspended in distilled water (about 10^{10} viable bacteria/ml.).

Generation and collection of aerosols. Aerosols were generated using a 3-jet Collison spray (Green & Lane, 1957) and mixed with a secondary air supply of controlled relative humidity (RH) in a Henderson (1952) apparatus. Samples of aerosols aged for 1 sec. were collected by raised impingers (May & Harper, 1957) containing 10 ml. collecting fluid. The collecting fluids were 20 mM-sodium phosphate buffer, pH 7.0, 1 M-sucrose in 20 mM-phosphate buffer and a chemically defined growth medium (GM 2) containing (w/v): glycerol (1 %); ammonium citrate (0.5 %); $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ (1 %); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 %); NaCl (0.05 %); and ferric ammonium citrate (0.0005 %); the pH value was 7.0.

The sensitivity of bacteria to lysozyme. Suspensions (5×10^7 organisms/ml.) of bacteria recovered from aerosols and diluted unsprayed suspensions were assayed for sensitivity to lysozyme (50 $\mu\text{g.}/\text{ml.}$) by the procedure described by Hambleton (1970). Numbers of viable organisms were determined before and after incubation with lysozyme.

Wall repair by aerosolized bacteria was assessed by the recovery of resistance to lysozyme as follows. Suspensions of bacteria recovered from aerosols were incubated in the collecting fluid for 1 h. at 37° and the sensitivity of the bacteria to lysozyme tested at intervals. In some experiments, the following compounds were added singly to the suspension of aerosolized organisms in medium GM 2 before incubation: chloramphenicol (50 $\mu\text{g.}/\text{ml.}$), actinomycin D (10 $\mu\text{g.}/\text{ml.}$), penicillin G (1000 units/ml.), and 2,4-dinitrophenol (5 mM).

To prevent interference with subsequent viability assays these compounds were removed by centrifuging (5 min. at 3000 g) and washing the bacterial suspensions twice with the appropriate collecting fluid, before testing for sensitivity to lysozyme.

Estimation of bacterial numbers. Total numbers of bacteria were determined with a Thoma counting chamber. Viable bacteria were estimated by plating samples (0.25 ml.) of the suspensions, diluted in the appropriate collecting fluid, on to tryptone agar plates; colonies were counted on four replicate plates after incubation for 20 h. at 37° .

Effect of cations and inhibitors on lysozyme. *Micrococcus lysodeikticus* from the laboratory collection was grown overnight in nutrient broth (Oxoid no. 2 nutrient broth, 25 g./l.; Oxoid L 21 yeast extract, 4 g./l., pH 7.4) at 37° with shaking. Organisms were harvested by centrifuging (3000 g, 10 min.), washed once in 20 mM-phosphate buffer, pH 7.0, and resuspended in phosphate buffer ($E_{540 \text{ nm.}}$ 1.0). Lysozyme was added to the suspension and lysis of the bacteria at room temperature observed as a decrease in turbidity at 540 nm. This procedure was repeated with suspensions of *M. lysodeikticus* to which were added singly Mn^{2+} , Ca^{2+} , Fe^{3+} , Zn^{2+} , Mn^{2+} (each 10 mM); chloramphenicol (50 $\mu\text{g.}/\text{ml.}$); actinomycin D (10 $\mu\text{g.}/\text{ml.}$); penicillin G (1000 units/ml.); and 2,4-dinitrophenol (5 mM).

Preparation of material released by aerosolization or EDTA treatment. A suspension of *Escherichia coli* B (10^{11} viable organisms/ml.) was aerosolized and a sample of the cloud collected into phosphate buffer (20 ml.) after it had travelled along the exposure tube for 1 sec. This process was continued for 30 min. and resulted in recovery of a thick suspension (about 10^{10} organisms/ml.). Bacteria were removed by centrifuging and the supernatant fluid was sterilized by filtration through a membrane filter (Oxoid, grade 0.45, Oxoid Ltd, Southwark Bridge Road, London). The filtrate was freeze-dried and the residue taken up in

2.0 ml. of distilled water, dialysed for 8 h. against distilled water (to remove salts), again freeze-dried and finally dispersed in 0.5 ml. of distilled water.

Escherichia coli B was treated with EDTA as described by Leive *et al.* (1968) and the released material isolated as described for aerosolized cells.

Immunological tests. Materials released from *Escherichia coli* B by aerosolization or EDTA treatment were tested for immunological activity by the agar-gel double diffusion technique (Ouchterlony, 1953). Both preparations were allowed to diffuse against rabbit anti-*E. coli* B serum.

Materials. Lysozyme and 2,4-dinitrophenol were obtained from B.D.H. Chemicals Ltd, Poole, Dorset. Chloramphenicol was from Parke Davis & Co Ltd, Hounslow, Middlesex, and penicillin G (Crystapen) from Glaxo Laboratories Ltd, Greenford, Middlesex. Actinomycin D was a gift from Dr A. Andrew, Merck, Sharp & Dohme Ltd, Hoddesdon, Hertfordshire.

Fig. 1. Wall repair by aerosolized *Escherichia coli* B organisms. Bacteria were sprayed from distilled water and held in an aerosol at 75 % RH for 1 sec. Samples were collected into and incubated for 1 h. at 37° in GM2 medium (○); GM 2 medium without glycerol and citrate (△); GM 2 medium without Mg²⁺ and Fe³⁺ (●); GM 2 medium containing 2,4-dinitrophenol (5 mM, ▲) and their sensitivity to lysozyme assayed at intervals.

RESULTS

Before aerosolization, *Escherichia coli* B organisms were resistant to the bactericidal effect of lysozyme. Even after incubation of a diluted suspension of unsprayed bacteria (5×10^7 organisms/ml.) with the enzyme (50 µg./ml.) for 1 h. at 37° the maximum decrease in viability observed was always less than 2 %. In all the experiments described below the aerosols of *E. coli* B were aged for 1 sec. at 75 % RH before sampling. The viability of bacteria (in the absence of lysozyme) recovered from such aerosols generally exceeded 85 % in all the collecting fluids used (Table 1) but considerable proportions of the bacteria were sensitive to lysozyme (Fig. 1).

Wall repair was estimated by the increase (within 1 h.) of the number of micro-organisms resistant to lysozyme treatment. The increase was not due to multiplication of lysozyme

resistant organisms during the incubation period because cell division during the first hour of incubation was insignificant (Table 1). Some cell division did occur during the second hour of incubation in medium GM 2 whereas viability decreased when Mg^{2+} and Fe^{3+} were not present in the medium.

Table 1. *Viability of Escherichia coli B sprayed from distilled water, stored in an aerosol at 75 % RH for 1 sec. and sampled into various collecting fluids*

Bacterial suspensions were incubated at 37° for up to 2 h.; viable and total bacterial counts were determined at intervals.

Collecting fluid	Incubation time (min.)	Total count (ml. ⁻¹ × 10 ⁻⁷)	Viable count (ml. ⁻¹ × 10 ⁻⁷)	Ratio viable/total
20 mM-Sodium phosphate buffer, pH 7.0	0	6.8	5.3	0.78
	30	6.8	5.9	0.86
	60	7.2	6.1	0.84
	90	7.2	5.6	0.78
	120	6.8	5.6	0.82
1 M-Sucrose in 20 mM-phosphate buffer	0	7.2	9.6	1.3
	30	6.4	7.5	1.2
	60	7.2	6.5	0.9
	90	7.4	7.9	1.1
	120	6.8	6.8	1.0
GM 2* without Mg^{2+} and Fe^{3+}	0	7.2	6.5	0.9
	30	5.6	4.2	0.75
	60	7.2	3.7	0.52
	90	6.4	2.5	0.39
	120	5.2	2.7	0.52
GM 2*	0	7.2	6.6	0.87
	30	6.8	6.6	0.97
	60	7.6	7.1	0.93
	90	8.4	8.4	1.0
	120	10.6	10.1	0.95

* See the Methods section.

Table 2. *Effect of certain cations on the sensitivity of aerosolized Escherichia coli B suspensions to lysozyme (50 µg./ml.)*

Bacteria were sprayed from water, stored in an aerosol at 75 % RH for 1 sec. and collected into 20 mM-sodium phosphate buffer pH 7.0. Portions of the suspension were incubated for 1 h. at 37° with one of the cations (10 mM). Sensitivity of the bacterial suspensions to lysozyme was tested before and after incubation for 1 h. In the absence of lysozyme viable counts remained constant during incubation.

Cation (10 mM)	Viable organisms resistant to lysozyme (%)	
	Time of incubation	
	0 min. (no added cation)	60 min. (added cation)
None	18	16
Mg^{2+}	21	51
Ca^{2+}	24	20
Fe^{3+}	26	49
Zn^{2+}	21	63
Mn^{2+}	26	35

Di- and trivalent cations and wall repair. The structure and organisation of the cell walls of some micro-organisms appears to be partially dependent upon the binding of divalent cations (Asbell & Eagon, 1966; Leive, 1968). Some effects of di- and trivalent cations on the sensi-

tivity of aerosolized *Escherichia coli* B organisms are shown in Table 2. Immediately after sampling only about 20 % of the viable bacteria were resistant to the action of lysozyme. After incubation for 1 h. at 37° in the presence of Mg^{2+} , Fe^{3+} or Zn^{2+} (10 mM) 51 %, 49 % and 63 % respectively of the population were resistant; Mn^{2+} (10 mM) was rather less effective (35 % resistant after 1 h.) and Ca^{2+} (10 mM) had no effect. None of these cations affected the rate of lysis of *Micrococcus lysodeikticus* by lysozyme (50 μ g./ml.), showing that they did not inhibit the enzyme. As wall repair is complete only in the presence of a carbon-energy source (Fig. 1) these results imply that the cations might be protecting the damaged wall from lysozyme attack, rather than leading to repair.

Fig. 2. Agar-gel diffusion pattern of material released from *Escherichia coli* B organisms by aerosolization (1) and by EDTA treatment (2) against rabbit anti-*E. coli* B serum (Ab).

Metabolism and wall repair. When aerosolized *Escherichia coli* B organisms were collected into and incubated in GM 2 medium, maximum wall repair occurred within 1 h. (Fig. 1). Omission of the carbon-energy source from the medium profoundly decreased the extent of wall repair. The omission of Fe^{3+} and Mg^{2+} from the medium limited the repair process showing (Fig. 1) that a cation requirement exists for the recovery of maximum resistance to lysozyme. Hence wall repair by aerosolized bacteria is a process requiring the presence of some multivalent cations coupled with active metabolism by the micro-organisms.

To define more clearly the metabolic processes contributing to repair, the effects of some substances of known inhibitory activity (P. Hambleton, unpublished results) were studied. Chloramphenicol (50 μ g./ml.), an inhibitor of protein synthesis in bacteria, did not affect the rate of wall repair by aerosolized *Escherichia coli* B. Inhibition of RNA synthesis by actinomycin D (10 μ g./ml.), to which aerosolized *E. coli* B organisms are sensitive (P. Hambleton, unpublished results), only slightly impaired the rate of wall repair. Similarly the presence of penicillin G (1000 units/ml.) did not affect repair showing the process to be independent of cell wall mucopeptide synthesis. Medium GM 2 containing 1 M-sucrose was used to stabilize osmotically sensitive organisms during incubation with penicillin G. The sucrose was removed with the penicillin before testing for sensitivity of the bacteria to lysozyme.

One compound that did prevent wall repair was 2,4-dinitrophenol (5 mM). After incubation of aerosolized *Escherichia coli* B with this substance very little repair occurred during the time taken by control organisms fully to recover resistance to lysozyme (Fig. 1). This indi-

cates that metabolic pathways leading to energy production are essential to the repair process in aerosolized bacteria. All the potential inhibitors had no effect on the rate of lysis of *Micrococcus lysodeikticus* by lysozyme.

Immunological studies. The Ouchterlony agar-gel double diffusion technique was used to compare the immunological activity of material released from *Escherichia coli* B by aerosolization and by EDTA treatment. Both preparations were allowed to diffuse in agar gel against rabbit anti-*E. coli* B serum. The material released from aerosolized bacteria contained two components both of which differed immunologically from the one component released from EDTA treated organisms (Fig. 2).

DISCUSSION

The permeability of *Escherichia coli* B to certain enzymes (Hambleton, 1970) is markedly affected by aerosolization. Other effects on permeability control have been observed (Anderson *et al.* 1968; J. E. Benbough, P. Hambleton & R. E. Strange, unpublished results) which indicate that the effects of aerosolization on the cell envelope may be non-specific. The experiments described here shown that the repair of wall damage by aerosolized bacteria is similar to the restoration of permeability control which occurs in organisms recovering from EDTA treatment (Leive, 1968).

A number of treatments that apparently alter the bacterial surface cause Gram-negative bacteria to become sensitive to lysozyme (Salton, 1957; Voss, 1967; Work, 1967; Wilkinson, 1968). Since cell-surface lipopolysaccharide (LPS) is external to the mucopeptide substrate of lysozyme a change in the structure of LPS could result in organisms becoming susceptible to this enzyme. Release of LPS from Gram-negative bacteria by treatments known to render the organisms sensitive to lysozyme has, in fact, been demonstrated (Leive *et al.* 1968; Rogers *et al.* 1969; Winshell & Neu, 1970).

The relationship between permeability and the structure and organization of the envelope has been extensively studied with Gram-negative bacteria treated with EDTA (Repaske, 1958; Asbell & Eagon, 1966; Leive, 1968; Rogers *et al.* 1969; Winshell & Neu, 1970). Concomitant with changes in permeability, some wall LPS was released into the surrounding medium. Leive (1968) has demonstrated that the permeability changes induced in *Escherichia coli* B by EDTA can be reversed and that energy utilization is essential. The results described here show clearly that aerosolized *E. coli* B organisms can recover resistance to lysozyme under certain conditions.

Maximum wall repair only took place when bacteria were incubated in a complete nutrient medium. In the absence of multivalent cations recovery of resistance to lysozyme was incomplete. Minimum repair was achieved by organisms incubated in GM 2 medium lacking a carbon-energy source. The role of divalent cations in the maintenance of the structure and organization of the outer lipopolysaccharide-lipoprotein layers of the Gram-negative cell envelope has been discussed by Asbell & Eagon (1966). Partial recovery of resistance to lysozyme by suspensions of aerosolized *Escherichia coli* B was mediated by Mg^{2+} , Zn^{2+} and Fe^{3+} (10 mM); these ions presumably exert their effect by stabilizing the stressed outer wall layers.

Wall repair is apparently independent of the functioning of a number of synthetic pathways in aerosolized bacteria. For example, when protein or RNA synthesis was inhibited by chloramphenicol or actinomycin D respectively, wall repair still occurred. Similarly, repair did not depend on synthesis of wall mucopeptide. Metabolism of a carbon-energy source is, however, essential for the restoration of resistance in aerosolized bacteria to lysozyme, as shown by the inability of the micro-organisms to repair in the presence of 2,4-dinitrophenol

which uncouples oxidative phosphorylation (Gel'man, Lukoyanova & Ostrovskii, 1967). These results show a basic similarity between the conditions necessary for wall repair by aerosolized bacteria and for the restoration of the permeability barrier by EDTA-treated bacteria (Leive, 1968), suggesting that the wall lesions resulting from both stresses are similar.

The immunological activity of the LPS released by EDTA-treatment of *Escherichia coli* has been examined by Leive *et al.* (1968). If the permeability changes in EDTA-treated micro-organisms results, at least in part, from a release of surface LPS (Leive, 1968; Winshell & Neu, 1970) one might expect material of similar immunological specificity to be released from aerosolized bacteria. At least two antigens were released from aerosolized *E. coli* B organisms but the immunological reactivity of both differed from that of the single component released after EDTA treatment of the bacteria. Nevertheless, this is the first evidence that the development, by aerosolized bacteria, of susceptibility to lysozyme may be associated with the release of components from the cell envelope.

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REFERENCES

- ANDERSON, J. D., DARK, F. A. & PETO, S. (1968). The effect of aerosolization upon survival and potassium retention by various bacteria. *Journal of General Microbiology* **52**, 99–105.
- ASBELL, M. A. & EAGON, R. G. (1966). The role of multivalent cations in the organisation and structure of bacterial cell walls. *Biochemical and Biophysical Research Communications* **22**, 664–671.
- BENBOUGH, J. E. (1967). Death mechanisms in airborne *Escherichia coli*. *Journal of General Microbiology* **47**, 325–333.
- COX, C. S. (1968). A method for the routine preservation of micro-organisms. *Nature, London* **220**, 1139.
- COX, C. S. (1969). The cause of loss of viability of airborne *Escherichia coli* K12. *Journal of General Microbiology* **57**, 77–80.
- GEL'MAN, N. S., LUKOYANOVA, M. A. & OSTROVSKII, D. N. (1967). *Respiration and Phosphorylation of Bacteria*. New York: Plenum Press.
- GREEN, H. L. & LANE, W. R. (1957). *Particulate Clouds: Dusts, Smokes and Mists*. London: E. and F. N. Spon.
- HAMBLETON, P. (1970). The sensitivity of Gram-negative bacteria, recovered from aerosols, to lysozyme and other hydrolytic enzymes. *Journal of General Microbiology* **61**, 197–204.
- HENDERSON, D. W. (1952). An apparatus for the study of airborne infection. *Journal of Hygiene, Cambridge* **50**, 53–68.
- HEPPEL, L. A. (1969). The effect of osmotic shock on release of bacterial proteins and on active transport. *Journal of General Physiology* **54**, 95–109.
- LEIVE, L. (1968). Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. *Journal of Biological Chemistry* **243**, 2373–2380.
- LEIVE, L., SHOVLIN, V. K. & MERGENHAGEN, S. E. (1968). Physical, chemical and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediaminetetraacetate. *Journal of Biological Chemistry* **243**, 6384–6391.
- MAY, K. R. & HARPER, G. J. (1957). The efficiency of various liquid impinger samplers in bacterial aerosols. *British Journal of Industrial Medicine* **14**, 287–297.
- NOLLER, E. C. & HARTSELL, S. E. (1961). Bacteriolysis of *Enterobacteriaceae*. 1. Lysis by four lytic systems utilizing lysozyme. *Journal of Bacteriology* **81**, 482–491.
- OUCHTERLONY, O. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta pathologica et microbiologica scandinavica* **32**, 231–240.
- REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of versene. *Biochimica et biophysica acta* **30**, 225–232.

- ROGERS, S. W., GILLELAND, H. E. JUN. & EAGON, R. G. (1969). Characterization of a protein-lipopoly-saccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. *Canadian Journal of Microbiology* **15**, 743-748.
- SALTON, M. R. J. (1957). The properties of lysozyme and its action on micro-organisms. *Bacteriological Reviews* **21**, 82-99.
- VOSS, J. G. (1967). Effects of organic cations on the Gram-negative cell wall and their bactericidal activity with EDTA and surface active agents. *Journal of General Microbiology* **48**, 391-400.
- WEBB, S. J. (1969). The effect of oxygen on the possible repair of dehydration damage by *Escherichia coli*. *Journal of General Microbiology* **58**, 317-326.
- WILKINSON, S. G. (1968). Studies on the cell walls of *Pseudomonas* species resistant to EDTA. *Journal of General Microbiology* **54**, 195-213.
- WINSHELL, E. B. & NEU, H. C. (1970). Relation of lipopolysaccharide and fatty acid ester release to the ethylenediaminetetraacetic acid alteration of permeability in Enterobacteriaceae. *Journal of Bacteriology* **102**, 537-539.
- WORK, E. (1967). Factors affecting the susceptibility of bacterial cell-walls to the action of lysozyme. *Proceedings of the Royal Society B* **167**, 446-447.