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Decrease in Ribosomal Density of *Proteus mirabilis* Exposed to Subinhibitory Concentrations of Ampicillin or Cephalothin (38888)

VICTOR LORIAN, L. D. SABATH,[1] AND MAYA SIMIONESCU[2]
(Introduced by Dennis W. Watson)

Division of Microbiology and Epidemiology, Department of Pathology, The Bronx-Lebanon Hospital Center, Bronx, New York 10456, Department of Medical Microbiology, Channing Laboratory, Harvard Medical Unit, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02118, and Rockefeller University, New York, New York 10021

By conventional light microscopy, it has been shown that gram-negative bacilli exposed to subinhibitory concentrations of benzyl-penicillin become very much elongated and appear as filaments and whorls (1–5). Although it had been suggested that cephalosporins have the same modes of action as do penicillins (6), it has been shown (7, 8) that at subinhibitory concentrations penicillins cause a remarkable elongation (up to 93 μm), whereas cephalosporins cause globules and a minimal cell elongation of *Proteus mirabilis* (up to 14 μm).

The present study was undertaken to determine whether transmission electron microscopy would reveal other morphological differences in the effects produced by subinhibitory concentrations of a penicillin (ampicillin) and a cephalosporin (cephalothin) on *P. mirabilis* 6.

Materials and Methods. Exposure to antibiotics. P. mirabilis 6, originally isolated from the urine of a patient at Bronx-Lebanon Hospital, was grown in trypticase soy broth (TSB) (BBL) for 18 hr at 37°. The culture was diluted 1:100 in TSB and 0.1 ml was spread on a filter membrane PHWP09025 (Millipore Corp., Bedford, MA) and placed on the surface of trypticase soy agar (TSA) (BBL) in a petri dish. The plates with the inoculated membranes were incubated at 37° for 90 min, after which membranes were removed and transferred onto other TSA plates containing cephalothin (Lilly) or ampicillin (Bristol) at various concentrations and incubated for another 180 min. The antibiotic concentrations were equal to the minimum inhibitory concentration (MIC) as determined by an agar dilution technique (9) or to 2-fold dilutions of each MIC down to 1:16 MIC. The MIC's were 1.56 μg/ml for ampicillin and 3.1 μg/ml for cephalothin.

Electron microscopy. The processing for electron microscopy was carried out by the one-step fixation staining in block according to Simionescu *et al.* (10). Sections at 60-nm thickness for electron microscopy were cut with a diamond knife on an MT2-B Porter-Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT). The sections were stained for 2 min with uranyl acetate followed by 5 min with lead citrate. Examination was carried out with a Philips 300 electron microscope operated at 80 kV and provided with a 200-μm condensor aperture and a 30-μm objective aperture. Examinations and measurements were done on electron micrographs at final magnifications of 82000.

Enumeration of ribosomes. The number of ribosomes in at least 1 μm^2 were counted on each of 10 electron micrographs at a magnification of 82,000 for each antibiotic concentration tested. The results of this method agreed with another way of determining ribosomes/μm^2; at each concentration the total number of ribosomes in the cross section of a single cell was counted at 82,000 magnification. The cross-sectional area was determined by cutting out a piece of photographic paper congruent to the picture of the cell, weighing it, and dividing that by the weight (1.304 g) of an 8.2 × 8.2-cm piece of Kodak paper 5, corresponding to a 1 μm^2. Thus, it was possible to determine the number of ribosomes per square

[1] Present address: Mayo Memorial Building, Box 219, University of Minnesota, Minneapolis, MN 55455.

[2] Present address: Yale University School of Medicine, Section of Cell Biology, New Haven, CT 06510.

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TABLE I. EFFECT OF ANTIBIOTICS ON RIBOSOME FREQUENCY PER μM^2 OF CROSS-SECTIONAL AREA OF *Proteus mirabilis* 6.

Antibiotic concentration	Ampicillin	Cephalothin
0 (control)	2241 ± 150[a]	2241 ± 150
1:16 MIC[b]	2123 ± 114 (<0.1)	2114 ± 186 (<0.1)
1:8 MIC	1968 ± 242[c] (<0.01)	1740 ± 173[c] (<0.001)
1:4 MIC	1679 ± 239[c] (<0.001)	1348 ± 324[c] (<0.001)
1:2 MIC	1458 ± 211[c] (<0.001)	1250 ± 326[c] (<0.005)
MIC	948 ± 332[c] (<0.001)	1001 ± 672[c] (<0.02)

[a] Mean number ± one standard deviation of ribosomes per square micrometer cross-sectional area in electron micrographs of bacteria. Counts were made on 1 μm^2 in each of 10 different sections at each concentration (except with cephalothin at MIC and 1:2 MIC (five sections each) and ampicillin at MIC (eight sections). Number in parentheses represents value of *P* (from Student *t* test) in comparison with value for control.

[b] MIC = minimum inhibitory concentration, (1.56 μg/ml for ampicillin, and 3.1 μg/ml for cephalothin).

[c] Statistically significant difference from control.

TABLE II. COMPARISON OF RIBOSOME COUNTS (PER μM^2) BY TWO METHODS.[a]

Antibiotic concentration	Ampicillin		Cephalothin	
	1 μm^2 count	Total cross-section counted	1 μm^2 count	Total cross-section counted
0	2356	2114	2356	2114
1:16 MIC	2134	2035	2294	1927
1:8 MIC	1801	1938	1800	1797
1:4 MIC	1946	1358	1384	1485
1:2 MIC	1429	1278	1374	1460
MIC	826	823	584	679

[a] The number of ribosomes in an area 8.2 x 8.2 cm (in the 82,000× enlarged electron micrographs) were counted to get the number of ribosomes per square micrometer, by the first method; for the second method the total number of ribosomes in electron micrographs of a single cell were counted, and divided by the area of that cross-sectional area (determined by weighing a cut-out piece of paper congruent to the cell in which the ribosomes had been counted). The comparison was made at each concentration for only one of the 10 cells counted per concentration to give the mean values presented in Table I.

[b] MIC as in Table I.

nanometer (Table II). All experiments were repeated twice with comparable results.

Results. Both ampicillin and cephalothin caused significant and comparable reductions in the number of ribosomes per square micrometer cross-sectional area in cells exposed to 1:8, 1:4, and 1:2 MIC and to the MIC (Tables I and II, Fig. 1a and b). Cells exposed to ampicillin that became filaments did not show gross changes in cell wall or cytoplasmic membrane at or below 1:2 MIC (Fig. 1a and b).

Discussion and Summary. The finding of reduced ribosomal densities at lower concentrations than those required to stop growth or cause visible defects in the cell wall is in contrast to the current view that the initial lesion produced by penicillins or cephalosporins is a defect in murein synthesis (11). This reduction in ribosomal density could be a primary or secondary effect and might be due to a decreased rate of growth, which has been shown to be associated with a lower ribosome frequency (12, 13) or to less stable ribosomes which disintegrated either spontaneously or as a result of the fixation procedures. The possibility that the decrease in ribosomal frequency was due to dilution (influx of liquid through a defective cell wall and/or cytoplasmic membrane, or efflux of ribosomes) remains, but no gross defect in cell wall was seen in more than 50 sections of cells showing reductions in ribosomal frequency.

These findings suggest that an effect on ribosomes may antecede an effect on the cell wall.

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Address reprint requests to Dr. Victor Lorian,

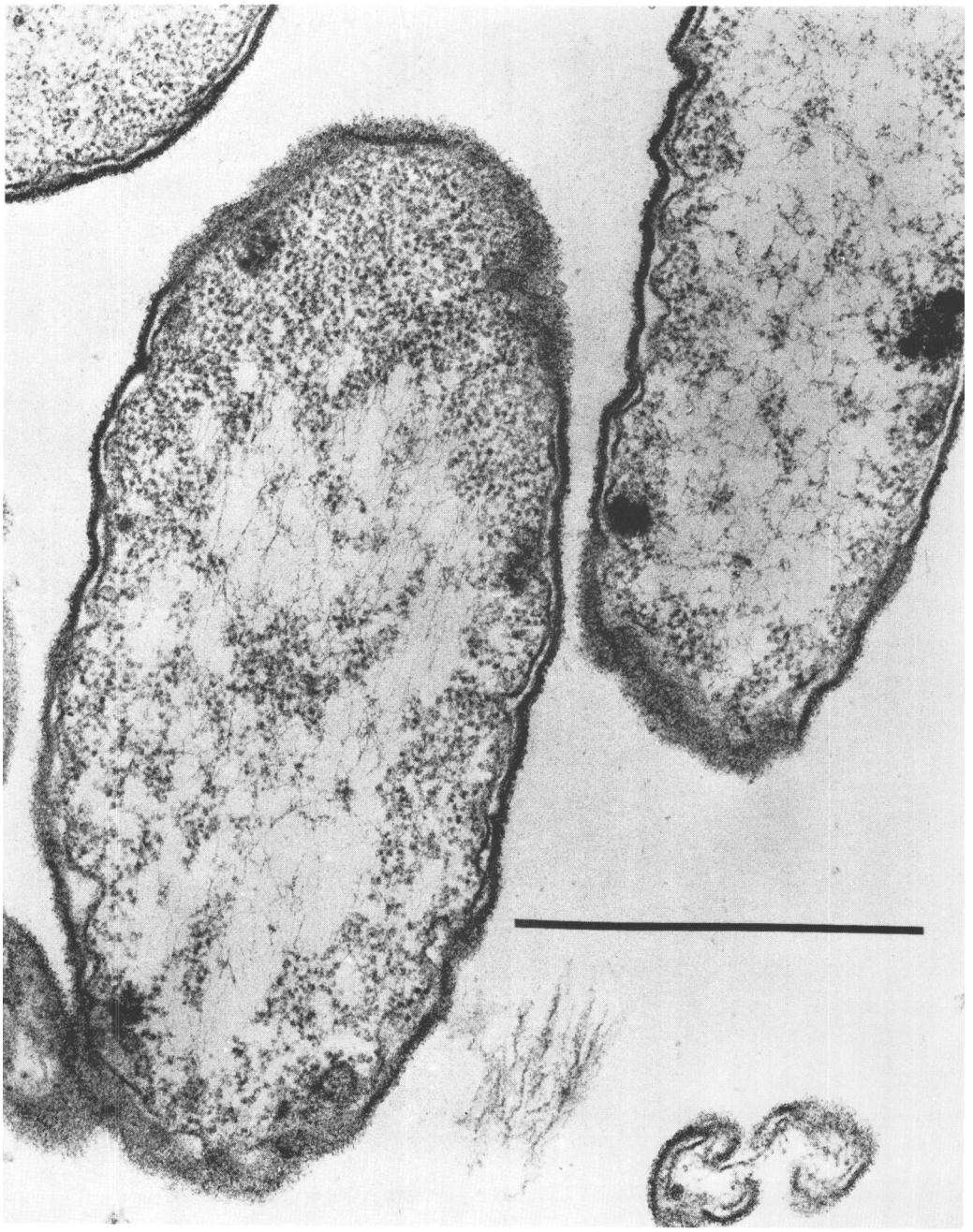

FIG. 1. A. *Proteus mirabilis* 6, exposed to 0.78 μg/ml ampicillin, 1:2 MIC, for 3 hr before fixation and sectioning. Note reduction in number of ribosomes compared to Fig. 1b. Line indicates 1 μm (×82,000).

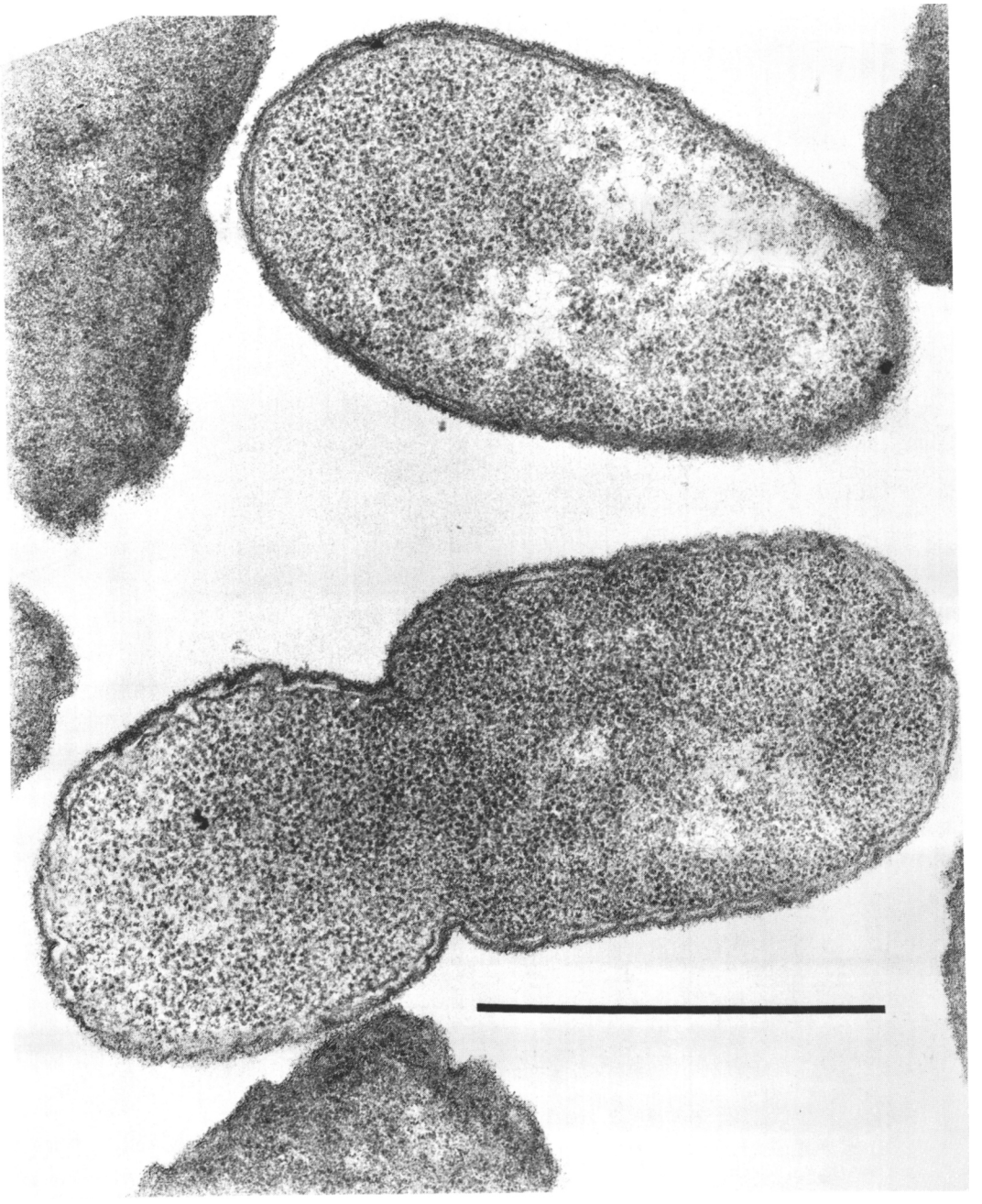

FIG. 1. B. *Proteus mirabilis* 6, control cell grown 3 hr on Millipore membrane on drug-free TSA before fixation and sectioning; line indicates 1 μm ($\times$82,000).

Chairman, Division of Microbiology and Epidemiology, The Bronx-Lebanon Hospital Center, 1276 Fulton Avenue, Bronx, NY 10456.

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