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Some Unique Properties of the Deoxyribonucleic Acid-Bearing Portion of the Bacterial Membrane

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By using the M-band technique we have shown that portions of the membranes of *Bacillus megaterium* and *Escherichia coli* vary in their affinity for magnesium-Sarkosyl crystals and in phospholipid composition. The portion to which deoxyribonucleic acid is attached comprises as little as 4% of the total cell membrane, has a particularly high degree of affinity for magnesium-Sarkosyl crystals, and is rich in phosphatidylethanolamine. The M-band fractionation does not depend on the use of lysozyme.

A technique for the isolation of bacterial deoxyribonucleic acid (DNA)-membrane complexes, dependent on the ability of membranes to bind to crystals of magnesium-Sarkosyl (the detergent sodium *N*-lauroylsarcosinate), has been described (13). The crystals and the DNA-membrane complex form a band (M-band) after centrifugation through layers or gradients of sucrose. Previously this technique has been used to follow the intracellular location of infecting phage DNA (5) and to distinguish DNA-bound and membrane-bound ribosomes and polyribosomes (2).

During these studies it became apparent that bacterial membranes behaved as if, during cell fractionation, they were subdivided into fragments possessing differing affinities for Sarkosyl crystals, and that DNA-bearing fragments possessed a particularly high degree of affinity. Thus, under controlled conditions, the proportion of the total cellular membrane found in the M-band can vary from 70% (or higher) to less than 10% (2; and *unpublished observations*) while the proportion of DNA is unaltered (upwards of 90%). This suggests that DNA is bound to a specific membrane fragment. Daniels (3) used a modification of the M-band technique to isolate discrete membrane fractions, one of which contained DNA.

The present study was undertaken to determine whether the DNA-bearing portion of the membrane differs recognizably from other portions or whether it derives its peculiar properties from its association with DNA.

MATERIALS AND METHODS

Strains. The bacterial strains used were *Escherichia coli* B/r, *E. coli* Ax14 (a *lon*$^-$ mutant obtained from H. Adler), and *Bacillus megaterium* KM.

Cultivation and labeling of bacteria. Organisms were usually grown at 37 C with vigorous shaking in C medium (9) containing 0.5% Casamino Acids. Labeling with glycerol-2-^3H (New England Nuclear Corp., Boston, Mass, 500 mCi/mmole), or with ^{14}C-glycerol (5.5 mCi/mmole) was carried out for two to three generations during exponential growth.

^{14}C-algal hydrolysate and ^{32}P-phosphate were also obtained from New England Nuclear Corp. (Boston, Mass.).

Enzymes. Egg white lysozyme was obtained from Worthington Biochemical Corp. *Charalopsis* enzyme, a muramidase that splits the same bonds of murein as lysozyme, was obtained from John H. Hash, Vanderbilt University.

M-band fractionation. *B. megaterium* was treated with 200 μg of lysozyme per ml at 37 C before harvesting, and the M-band procedure was used as described by Tremblay et al. (13). *E. coli* was treated with lysozyme-ethylenediaminetetraacetic acid (EDTA) and fractionated by the M-band procedure as described by Earhart et al. (5). Caution was taken not to exceed the cell concentration or the amount of EDTA indicated in the papers quoted. Failure to stay within the limits specified resulted in failure to form usable M-bands.

Preparation of cytoplasmic membranes and their components. *B. megaterium* cells were treated with 250 μg of lysozymes per ml in 0.1 M tris(hydrox-

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ymethyl)aminomethane, *p*H 7.4, for 1 hr at 25 C. The particulate material was pelleted at 10,000 rev/min for 15 min, resuspended in the same buffer with 10 μg of deoxyribonuclease per ml, and left at 4 C overnight. Membranes were washed with the same buffer by four successive centrifugations.

Preparation of structural membrane protein was done by the method of Richardson et al. (11). Phospholipids were extracted by the method of Kenfer and Kennedy (8) and prepared in micellar form according to Fleischer and Klouven (6).

Analysis of M-band phospholipids. Phospholipids were extracted with chloroform-methanol (2:1) by the method of Kenfer and Kennedy (8) and saponified by the method of Dawson (4). Chromatography was carried out as described previously by Ballesta and Schaechter (1).

RESULTS

As shown in Tables 1 and 2, the amount of membrane material measured as cell lipids found in M-bands depends on the Mg^{2+} concentration used in the formation of crystals. This effect is largely a reflection of the amount of crystals of magnesium-Sarkosyl formed at different Mg^{2+} concentrations. Virtually all the DNA (>85%) is found in M-bands at Mg^{2+} concentrations greater than 0.005 M (13). Thus, it is possible to select conditions where M-bands, while containing the bulk of the DNA, vary greatly in membrane content, between 44% and 8% of the total under the conditions shown in Table 1. The smallest amount of membrane material in M-bands which contain over 80% of the DNA is 4% of the total. This is obtained by adding 0.5% Triton X-100 to the material layered in the standard M-band procedure. The largest proportion of membrane material in M-bands, 80 to 90%, was found when the membrane-crystals mixture was allowed to stand for at least 1 to 1.5 hr (Fig. 1).

TABLE 1. *Effect of Mg^{2+} concentration on proportion of membrane in M-band, using whole protoplasts*[a]

Mg^{2+} concn (M)	^3H in M-band (%)
0.001	8.4
0.0025	16.4
0.005	20.0
0.010	23.8
0.015	35.1
0.020	43.6

[a] *B. megaterium* KM was labeled with glycerol-2-^3H for two generations. Under the conditions employed at least 95% of the label is found in phospholipids (E. W. Green and M. Schaechter, *unpublished observations*). Protoplasts were prepared by lysozyme treatment by the procedure outlined by Schaechter (12) and suspended in TMK-15% sucrose buffer at the Mg^{2+} concentration indicated. M-bands were prepared by the standard procedure with 0.5% Sarkosyl in the layered material. The sucrose gradients contained Mg^{2+} at the concentration indicated.

TABLE 2. *Effect of Mg^{2+} concentration on proportion of purified phospholipids and membrane proteins in M-band*[a]

Mg^{2+} (M)	Percent in M-band			
	Phospholipids (^3H)	Membrane proteins (^{14}C)	Mixture of phospholipids and membrane proteins	
			Phospholipids (^3H)	Membrane proteins (^{14}C)
0.02	53	47	53	66
0.06	76	65		
0.10	89	99	99	99

[a] *B. megaterium* KM was labeled either with ^{14}C-algal hydrolysate or glycerol-*2-^3H* for two generations. Phospholipids were prepared by the method of Kenfer and Kennedy (8) and solubilized by the procedure of Fleischer and Klouven (6). Membrane proteins were prepared by the method of Richardson et al. (11). Amounts of 0.025 to 0.10 ml of the material indicated were mixed with 0.1 ml of 0.5% Sarkosyl and 0.1 ml of an Mg^{2+} solution at the concentration indicated. M-bands were prepared by the standard procedure, with sucrose gradients containing the Mg^{2+} concentration indicated.

The proportion of membrane material in the M-band was not substantially affected by the Sarkosyl concentration over a wide range (Table 3). The effect of varying the cell concentration is given in Table 4. At higher concentrations (multiply the cell number per milliliter by about 5 for *E. coli*) M-bands are not sharp, and the gradients can be fractionated into distinct portions only with great difficulty.

These results suggest that the membrane consists of portions that are heterogeneous with respect to their affinity for magnesium-Sarkosyl crystals. We have not been able to demonstrate whether this affinity resides in the lipid or in the protein components of membranes since both behave rather alike at varying Mg^{2+} concentrations (Table 2). It should be noted that under standard conditions M-bands contain only small amounts of the soluble proteins of the cell. They contain between 15 and 30% of the total cell proteins, most of which can be accounted for by the presence of membrane and ribosomes.

The uniqueness of the DNA-bearing portion

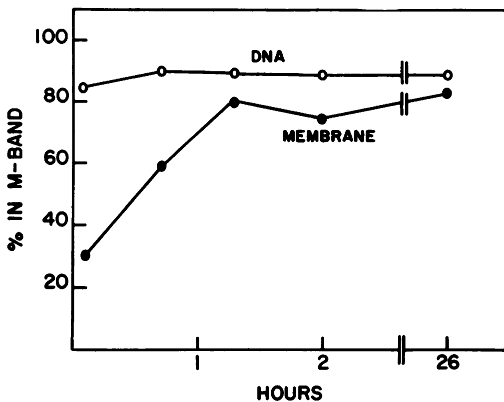

FIG. 1. *The proportion of DNA and membrane material in the M-band as a function of time of exposure to magnesium-Sarkosyl crystals. B. megaterium KM was labeled for two generations with glycerol 2-³H and ¹⁴C-thymidine and processed as in the footnote to Table 1 with buffer containing 0.01 M Mg²⁺. Individual gradients were centrifuged at different times after the addition of Sarkosyl to the top of the tube.*

TABLE 3. *Effect of Sarkosyl concentration on proportion of membrane in M-band*[a]

Sarkosyl concn in layered material (%)	Lipid ^{14}C in M-band (%)
0.05	31
0.10	32
0.25	35
0.50	39

[a] *B. megaterium* KM was labeled for two generations with ^{14}C-glycerol and processed as in the footnote to Table 1 with buffer containing 0.01 M Mg^{2+}. Sarkosyl concentrations given are in terms of the volume layered (1 ml). Lipids were estimated after extraction with chloroform-methanol by the Kenfer and Kennedy (8) procedure.

of the membrane was demonstrated by rebanding the material in M-bands and in top fractions of gradients. Since the proportion of membrane material in M-bands is influenced by several conditions (*see above*), care was taken to treat each fraction alike. As shown in Table 5, there is a marked difference in the ability of membrane material from M-bands and top fractions to reband in a second M-band. The higher rebanding ability of membranes from the first M-band material did not depend on the presence of bound DNA, since the same result was obtained after deoxyribonuclease treatment.

The content of the principal cell phospholipids in M-bands is shown in Table 6. As can be seen, M-bands contain significantly more phosphatidylethanolamine and less phosphatidylglycerol than the whole cells. The difference is more marked in *B. megaterium*, but this reflects the smaller proportion of membrane in M-bands in the experiments with this organism. A lower content of cardiolipin in M-bands was seen in the Ax14 strain of *E. coli*. Under the conditions of the experiment, it could be argued that finding less of a given constituent in M-bands may reflect its greater

TABLE 4. *Effect of cell concentration on proportion of lipids in M-band*[a]

No. of cells applied	Lipid ^{32}P in M-band (%)
4.2×10^7	12.5
8.4×10^7	10.6
2.1×10^8	11.5

[a] *B. megaterium* KM was labeled for two generations with ^{32}P. M-bands were prepared as in the footnote to Table 1 with buffer containing 0.01 M Mg^{2+}. Lipids were estimated after butanol extraction.

TABLE 5. *Rebanding ability of membrane material*[a]

Organism	Material from first gradient	^3H in second M-band (%)
E. coli B/r	Top fraction	13
	M-band	62
	Top fraction, nuclease treated	8
	M-band, nuclease treated	84
B. megaterium KM	Top fraction	2
	M-band	73

[a] *E. coli* B/r cultures in a Casamino Acids-salts medium were labeled for four generations with glycerol-2-^3H and fractionated by the procedure described by Earhart et al. (5). Deoxyribonuclease treatment was carried out on top of the first gradient by addition of 120 μg of electrophoretically purified pancreatic deoxyribonuclease per ml (Worthington Biochemical Corp.) for 10 min. *B. megaterium* KM was labeled and fractionated as in footnote to Table 1. The first *E. coli* M-bands contained 28% and 38% of the total membrane lipids for the deoxyribonuclease control and treated samples, respectively. The *B. megaterium* M-band contained 20% of the membrane. One-milliliter fractions (top fraction and M-band) were diluted 1:3 with TMK buffer and heated at 45 C for 1 min to melt the crystals. Sarkosyl was added to the same concentration as for the first M-bands, and 1-ml samples were fractionated by the standard M-band procedure.

solubilization by Sarkosyl. However, free phospholipids have been found to have great affinity for magnesium-Sarkosyl crystals (Table 2, reference 13). No significant difference was found in the affinity of purified phosphatidylethanolamine, phosphatidylglycerol, or cardiolipin for the crystals (D. Bloomberg, *unpublished observations*).

Lastly, we wish to report that the presence of DNA in M-bands is not, as has been suggested by Patterson et al. (10), an artifact due to the liberal use of lysozyme. Table 7 shows that the most of the DNA of the cells is found in M-bands after a variety of treatments that involve either no lysozyme or small amounts of this enzyme. Daniels (3) reached the same conclusion using a new fractionation technique.

DISCUSSION

Several types of evidence presented here suggest that the DNA-bearing portion of the membrane is distinct with regard to phospholipid composition and affinity for magnesium-Sarkosyl crystals. Since neither the relevant properties of membranes nor the rationale of M-band fractionation are well understood, this conclusion ought to be taken with caution.

TABLE 6. *Phospholipid composition of M-bands*[a]

Organism	Fraction	Phosphatidylethanolamine	Phosphatidylglycerol	Cardiolipin	Percent total lipid in M-bands
E. coli B/r	Whole cells	56 ± 4[b]	27 ± 5	14 ± 2	
(4 determinations)	M-band	69 ± 5	19 ± 2	11 ± 1	49
E. coli A ×14	Whole cells	64 ± 5	16 ± 3	22 ± 7	
(4 determinations)	M-band	78 ± 3	10 ± 2	12 ± 1	52
B. megaterium KM	Whole cells	34 ± 2	41 ± 1	25 ± 3	
(6 determinations)	Top fraction	31 ± 2	42 ± 2	25 ± 4	
	M-band	70 ± 2	6 ± 1	22 ± 2	15

[a] *E. coli* strains were labeled with ^{32}P for three generations and fractionated as in the footnote to Table 5. *B. megaterium* KM was labeled with ^{32}P and fractionated as in the footnote to Table 1 with buffer containing 0.01 M Mg^{2+}. M-bands and whole cells were analyzed for their phospholipid content as described in Materials and Methods.

[b] Standard deviation.

TABLE 7. *Percent DNA in M-bands obtained with and without the use of lysozyme*

Organism	Treatment[a]	DNA in M-band (%)
E. coli B/r	Penicillin at 75 min	87
	Penicillin + lysozyme (200 μg/ml) at 0 min	85
	Penicillin + lysozyme (400 μg/ml) at 0 min	90
	Penicillin + lysozyme (400 μg/ml) at 75 min	85
	Penicillin + lysozyme (400 μg/ml) at 75 min	87
B. megaterium KM	Lysozyme (5 μg/ml)	85
	Lysozyme (50 μg/ml)	83
	Lysozyme (200 μg/ml)	86
	Charalopsis enzyme 45 min	84
	Charalopsis enzyme + lysozyme (20 μg/ml) at 0 min	80
	Charalopsis enzyme + lysozyme (200 μg/ml) at 0 min	72
	Charalopsis enzyme + lysozyme (20 μg/ml) at 45 min	84
	Charalopsis enzyme + lysozyme (200 μg/ml) at 45 min	84

[a] Penicillin G was added to a final concentration of 600 μg/ml. Cultures were treated for 75 min at 30 C without shaking. The culture medium was C medium plus 10% nutrient broth. *Charalopsis* enzyme (7) was added to a final concentration of 0.25 μg/ml. Cultures were treated for 45 min at 30 C without shaking.

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