Decreased Particulate NADH Oxidase Activity in *Bacillus subtilis* Spores After Polymyxin B Treatment

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The activities of several enzymes of polymyxin B-treated dormant and germinated spores of *Bacillus subtilis* were examined. The particulate NADH oxidase of the antibiotic-treated spores showed considerably lower specific and total activities compared with those of untreated ones. The specific and total NADH oxidase activities of untreated spores increased 12- and 15-fold respectively during germination, whereas increases during germination of polymyxin B-treated spores were inhibited. The specific and total activities of particulate NADH cytochrome *c* reductase of dormant spores were decreased by polymyxin B treatment in almost the same proportion as those of the particulate NADH oxidase. The specific activity of NADH dehydrogenase of dormant spores remained unchanged after antibiotic treatment but the total activity fell considerably. The activities of other enzymes examined were similar for untreated dormant and germinated spores and antibiotic-treated spores. The respiration of polymyxin B-treated dormant spores was inhibited at the same time as the start of germination. Morphologically, polymyxin B-treated dormant spores lost a laminar structure of the cortex and details of the spore protoplast. The inhibitory mechanism of particulate NADH oxidase activity of polymyxin B-treated dormant spores is discussed.

INTRODUCTION

Cyclic polypeptide antibiotics such as polymyxin B, colistin and gramicidin S, and aminoglycoside antibiotics such as streptomycin, kanamycin and gentamicin bind to the dormant spores of *Bacillus subtilis*, inhibit outgrowth or vegetative growth after germination, and consequently act as sporocidal or sporostatic agents under certain limited conditions (Tochikubo, 1978; Tochikubo *et al.*, 1981). Morphological changes and macromolecular synthesis during germination, outgrowth, and vegetative growth of the dormant spores treated with the above antibiotics have been investigated in detail (Hayakawa *et al.*, 1981). The electrophoretic mobilities of antibiotic-treated dormant spores have confirmed that cyclic polypeptide and aminoglycoside antibiotics bind at least to the spore coat (Tochikubo, 1978; Tochikubo *et al.*, 1981). However, the inhibitory mechanism for outgrowth and vegetative growth, including the mode of penetration of antibiotics, has not yet been clarified. In order to examine this problem, the activities of some enzymes including particulate NADH oxidase of polymyxin B-treated dormant spores were examined. This report describes the correlation between low particulate NADH oxidase activity and inhibited outgrowth of polymyxin B-treated dormant spores.

METHODS

Bacterial strain, germination and other procedures. Spores of *B. subtilis* PCI 219 were used in this study. Spore preparations and treatment with polymyxin B and colistin were carried out as previously described (Tochikubo *et al.*, 1981). Antibiotic-treated dormant spores at about $5 \cdot 1 \times 10^8$ spores ml⁻¹ were incubated with shaking at 37 °C for 2 h in 20 mM-sodium/potassium phosphate buffer (pH 7.2) containing 20 mM-L-alanine, washed five times

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

with the same buffer, and used as antibiotic-treated germinated spores. Polymyxin B-treated dormant spores were incubated at 37 °C for 2 h in sterile 100 mM-CaCl₂ solution (about 3.1×10^8 spores ml⁻¹, final concentration), washed five times with sterile 20 mM-phosphate buffer (pH 7.2), and used as polymyxin B- and CaCl₂-treated dormant spores. Subsequently, these spores were also germinated in 20 mM-phosphate buffer (pH 7.2) containing 20 mM-L-alanine. Untreated dormant spores (about 5.1×10^8 spores ml⁻¹) were germinated as described above in the presence of 64 µg polymyxin B ml⁻¹ or 100 µg chloramphenicol ml⁻¹.

Preparation of soluble and particulate fractions. A 10 ml sample of a thick spore suspension (about 10^{11} spores ml⁻¹ in 50 mM-phosphate buffer, pH 7.5), together with 10 g 0.25 to 0.45 mm diameter glass beads, were treated in a 20-kc sonic oscillator (Kubota Insonator Model 200 M) at 2–4 °C at 1.4 A and at 160 W for 30 min for dormant spores and for 10 min for germinated spores. The broken cell suspension was centrifuged at 10700 g for 30 min at 4 °C. The supernatant was centrifuged at 100000 g for 3 h at 4 °C and separated into soluble and particulate fractions; for dormant spores the dipicolinic acid was removed by freezing before ultracentrifugation (Tochikubo & Yasuda, 1983). The particulate fraction obtained by ultracentrifugation was suspended in a small volume of 50 mM-phosphate buffer (pH 7.5). Dormant spores were also sonicated in the presence of various concentrations of polymyxin B dissolved in 50 mM-phosphate buffer (pH 7.5) and soluble and particulate fractions prepared as described above.

Enzyme assays. Particulate NADH oxidase and soluble NADH oxidase (EC 1.6.99.2) were assayed at 37 °C by measuring the decrease in absorbance at 340 nm of NADH as previously described (Tochikubo, 1971; Tochikubo, 1974); 0.3 µmol flavine mononucleotide was added to the assay cuvette containing soluble NADH oxidase. The activity of particulate NADH oxidase was also measured after standing at 0 °C for 4 h in the presence of various concentrations of polymyxin B. NADH dehydrogenase (EC 1.6.99.3) activity was followed spectrophotometrically at 37 °C and at 600 nm. The test system contained 1 µmol 2,6-dichlorophenolindophenol (DCIP), 0.5 µmol KCN, 0.6 µmol NADH, 300 µmol phosphate buffer (pH 7.5), and 0.01 to 0.05 ml sample. The volume was made up to 3.0 ml in the cuvette (1 cm light path) by the addition of deionized water and the reaction was initiated by the addition of NADH. NADH cytochrome *c* reductase (EC 1.6.99.3) activity was measured at 37 °C and at 550 nm in the same manner as NADH dehydrogenase except that the assay cuvette contained 0.1 µmol cytochrome *c* in place of DCIP. Alanine dehydrogenase (EC 1.4.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were assayed at 37 °C by measuring NADH and NADPH formation at 340 nm, respectively, as previously described (Nitta *et al.*, 1974; Tanahashi *et al.*, 1976); for glucose-6-phosphate dehydrogenase, 0.1 µmol MgCl₂ was added to the assay cuvette. The activity of alkaline phosphatase (EC 3.1.3.1) was measured according to the method of Glenn & Mandelstam (1971). Enzyme solution (0.05 ml) was added to 0.6 ml 25 mM-borate/NaOH buffer (pH 10.0) containing *p*-nitrophenyl phosphate and incubated at 37 °C. After colour development, 3.0 ml 0.02 M-NaOH was added to stop the reaction and the molar absorption at 410 nm was measured. For catalase (EC 1.11.1.6) enzyme solution (0.05 ml) was added to 0.6 ml 25 mM-phosphate buffer (pH 7.2) containing 1.5% (v/v) H₂O₂ and incubated at 37 °C. After incubation, 3.0 ml 0.66 M-H₂SO₄ was added to stop the reaction and the decrease in absorbance at 250 nm of H₂O₂ was measured (Chance & Maehly, 1960).

The following molar absorption coefficients were used: 6.22 mm⁻¹ cm⁻¹ at 340 nm for NADH and NADPH; 21 mm⁻¹ cm⁻¹ at 600 nm for DCIP; and 19 mm⁻¹ cm⁻¹ at 500 nm for cytochrome *c*. Each activity was calculated from the linear portion of the time course immediately after the reaction was started.

Protein was determined by the Lowry method.

Microbiological assay of polymyxin B. The polymyxin B released from the antibiotic-treated dormant spores during germination or in CaCl₂ solution was measured quantitatively by an antibiotic assay, using the spores of *B. subtilis* as a test organism. A 0.2 ml sample of the spore suspension (about 10^7 spores ml⁻¹) was added to 19.8 ml sterile streptomycin agar (Difco), and this was poured into a sterile Petri dish. After solidification, four cups per plate were cut out with a hard steel punch (outside diameter, 8.6 mm). Polymyxin B-treated dormant spores were incubated at 37 °C for 2 h in heart infusion broth, in 20 mM-phosphate buffer (pH 7.2) containing 20 mM-L-alanine, and in 100 mM-CaCl₂ solution, and centrifuged to remove the spores. The supernatant fluid was filtered through a Millipore membrane filter and used as test solution. Polymyxin B dissolved in heart infusion broth, in 20 mM-phosphate buffer (pH 7.2) or in 100 mM-CaCl₂ solution, diluted by a twofold serial dilution method, was used as a standard solution. A 0.15 ml sample of the test or standard solution was pipetted into a cup. After standing at 4 °C for 15 h to allow diffusion of polymyxin B, the plate was incubated at 37 °C for 8 h. The diameter of an inhibitory zone on the surface of the medium was measured with a needle-point caliper.

The amount of polymyxin B in the soluble fractions of polymyxin B-treated dormant and germinated spores, polymyxin B- and CaCl₂-treated dormant spores, and dormant spores sonicated in the presence of various concentrations of polymyxin B was measured as described above. In this case antibiotic dissolved in 20 mM-phosphate buffer (pH 7.2) was used as the standard solution.

Respiration measurement. Untreated, polymyxin B-treated or polymyxin B- and CaCl₂-treated dormant spores were suspended in 50 mM-phosphate buffer (pH 7.2) to give about 5.6×10^8 spores ml⁻¹. A 3.6 ml sample of the spore suspension was saturated with O₂ by gently bubbling air through the solution. Monitoring of O₂

consumption was started at the same time as the initiation of germination by the addition of 3 μmol L-alanine. O_2 consumption was determined at 37 °C in a stirred cell suspension with a Fieldlab oxygen analyser (Beckman Instruments) in the presence and absence of 0.4 μmol NADH. The rate of O_2 consumption was continually monitored up to 120 min and plotted as $\mu\text{mol O}_2 (10^9 \text{ spores})^{-1} \text{ ml}^{-1}$ consumed at the indicated time in Fig. 2(b).

Electron microscopy. Dormant spores were fixed by the triple-fixation method with a sequential application of 5% (v/v) glutaraldehyde, 1% (w/v) osmium tetroxide (OsO_4), and 2% (w/v) potassium permanganate as previously described (Kozuka & Tochikubo, 1983) and suspended in molten 2% (w/v) agar. After solidification, the agar was cut into 1 mm cubes. The agar cubes were dehydrated by passage through an ethanol series, substituted by *n*-butyl glycidyl ether, and embedded in Quetol 653 (Kushida, 1980). Germinated spore samples were prefixed in 2.5% (v/v) glutaraldehyde for 3 h at 4 °C and centrifuged at 1500 *g*. The pellet was suspended in 1 ml 1% (w/v) OsO_4 and 0.1 ml tryptone medium (Difco) (Kellenberger *et al.*, 1958) and was fixed overnight at room temperature. After washing with Kellenberger buffer (Kellenberger *et al.*, 1958), the cell pellet was fixed in 0.5% (w/v) uranyl acetate and suspended in molten 2% (w/v) agar. The agar was cut into 1 mm cubes and treated as described above. Sections were cut on a Porter-Blum MT ultramicrotome, stained with 6% (w/v) uranyl acetate and Reynolds lead citrate (Reynolds, 1963), and examined in a JEOL JEM-200CX electron microscope at an accelerating voltage of 200 kV.

Chemicals. NAD, NADP, NADH and glucose 6-phosphate were purchased from Boehringer-Mannheim. Crystalline cytochrome *c*, prepared from *Candida krusei*, was purchased from Sankyo, polymyxin B sulphate from Sigma, DCIP and OsO_4 from Merck, and Quetol 653 from Nisshin EM. All other chemicals were of the finest grade.

RESULTS

Enzyme activities of antibiotic-treated dormant and germinated spores

The activities of several enzymes were examined to elucidate the effect of polymyxin B and colistin treatment on dormant and germinated spores. The particulate NADH oxidase of untreated dormant spores showed high specific and total activities, which increased 12- and 15-fold respectively during germination (Table 1). Polymyxin B treatment decreased the specific and total activities of particulate NADH oxidase of dormant spores by 70 and 80%, respectively. The specific and total NADH oxidase activities of polymyxin B-treated germinated spores increased to about three and six times those of the corresponding dormant spores and their values were similar to those of untreated dormant spores (Table 1). Treatment with colistin also resulted in a decrease in the specific and total NADH oxidase activities of dormant and germinated spores, but the extent of inhibition was less than that due to polymyxin B treatment

Table 1. Specific and total activities of NADH oxidase and alkaline phosphatase in the particulate fraction of polymyxin B-treated and colistin-treated dormant spores and germinated spores

Enzyme	Treatment of spores	Dormant spores		Germinated spores	
		Specific activity*	Total activity†	Specific activity*	Total activity†
NADH oxidase	None‡	262 $\pm$ 59	1417 $\pm$ 207	3085 $\pm$ 482	21837 $\pm$ 1845
	Polymyxin B§	74	287	225	1747
	Polymyxin B, incubated with CaCl_2	70	229	3094	16885
	Colistin§	97	677	672	5078
Alkaline phosphatase	None‡	14 $\pm$ 4	75 $\pm$ 24	16 $\pm$ 3	103 $\pm$ 14
	Polymyxin B§	4	14	14	108
	Colistin§	5	32	15	115

* Expressed as nmol NADH oxidized or $\mu\text{mol p}$ -nitrophenylphosphate hydrolysed $\text{min}^{-1} (\text{mg protein})^{-1}$.

† Expressed as nmol NADH oxidized or $\mu\text{mol p}$ -nitrophenylphosphate hydrolysed min^{-1} per 10^{12} spores sonicated.

‡ Each value represents the mean of ten determinations $\pm$ SD.

§ Each value represents the mean of five determinations.

|| Each value represents the mean of two determinations.

Table 2. *Specific and total activities of particulate NADH oxidase in spores germinated by L-alanine in the presence of polymyxin B and chloramphenicol*

Antibiotic	Concn ($\mu\text{g ml}^{-1}$)	Specific activity*	Total activity†
None	-	3331	20217
Polymyxin B	64	183	1281
Chloramphenicol	100	2840	16798

* Expressed as $\text{nmol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$.† Expressed as $\text{nmol NADH oxidized min}^{-1}$ per 10^{12} spores sonicated.

(Table 1). Calcium treatment after exposure to polymyxin B did not restore either specific or total enzyme activities of dormant spores whereas those of germinated spores were almost completely restored (Table 1).

Spores germinated in the presence of polymyxin B had almost the same specific and total activities of particulate NADH oxidase as those of polymyxin B-treated germinated spores (Tables 1 and 2). The specific and total activities of the spores germinated in the presence of chloramphenicol were about 84 and 85% of those of untreated germinated spores, respectively (Table 2).

When the particulate fractions from untreated spores were incubated at 0°C for 4 h in the presence of polymyxin B, the activity of particulate NADH oxidase fell to a similar value for both dormant and germinated spores as the concentration of antibiotic increased (Fig. 1).

The specific and total activities of alkaline phosphatase in the particulate fraction of polymyxin B- and colistin-treated dormant spores were lower than those of untreated ones, but in germinated spores the enzyme showed almost similar specific and total activities regardless of polymyxin B or colistin treatment (Table 1).

The specific and total activities of catalase, NADH oxidase, alanine dehydrogenase and glucose-6-phosphate dehydrogenase in the soluble fractions of dormant and germinated spores exhibited little or no differences after polymyxin B or colistin treatment except that the soluble fraction from polymyxin B- and colistin-treated germinated spores had about one-half the specific and total activities of alanine dehydrogenase compared with that of untreated germinated spores (Table 3).

Comparison of particulate NADH oxidase, NADH dehydrogenase and NADH cytochrome c reductase activities

The extent of the decrease in specific and total activities of NADH oxidase in the particulate fraction of polymyxin B-treated dormant spores corresponded more to that of the specific and total activities of NADH cytochrome c reductase than to that of NADH dehydrogenase (Table 4). The same result was obtained also with polymyxin B- and CaCl_2 -treated dormant spores (Table 4), with dormant spores sonicated in the presence of 7 or 10 mg polymyxin B (Table 4), and with polymyxin B-treated germinated spores (data not shown). The specific activity of NADH dehydrogenase of dormant spores was not significantly affected by polymyxin B treatment (Table 4).

As can be calculated from Table 4, the protein content in the particulate fraction of polymyxin B-treated dormant spores was considerably lower than that of untreated spores and the protein content of the particulate fraction of dormant spores sonicated in the presence of polymyxin B decreased as the concentration of the antibiotic was increased.

Quantity of polymyxin B in the soluble fraction

A microbiological assay was carried out to investigate the total amount of polymyxin B released during sonication into the soluble fractions of polymyxin B- and polymyxin B- and CaCl_2 -treated dormant spores and polymyxin B-treated germinated spores. Whereas about $860 \mu\text{g}$ polymyxin B (which was similar to that of the dormant spores sonicated in the presence of 10 mg polymyxin B) were released into the soluble fraction of polymyxin B-treated dormant

Fig. 1. Effect of polymyxin B on the activity of particulate NADH oxidase from untreated dormant (●) and germinated (○) spores of *B. subtilis*. The enzyme activity was measured after incubation at 0 °C for 4 h in the presence of various concentrations of polymyxin B.

Table 3. *Specific and total activities of catalase, NADH oxidase, alanine dehydrogenase, and glucose-6-phosphate dehydrogenase in the soluble fraction of polymyxin B-treated and colistin-treated dormant spores and germinated spores*

Enzyme	Treatment of spores	Dormant spores		Germinated spores	
		Specific activity*	Total activity†	Specific activity*	Total activity†
Catalase	None‡	21 ± 4	351 ± 62	46 ± 7	881 ± 82
	Polymyxin B§	19	430	30	422
	Colistin§	24	554	51	740
NADH oxidase	None‡	200 ± 56	3461 ± 541	156 ± 20	2726 ± 280
	Polymyxin B§	139	2807	187	2842
	Colistin§	135	3150	222	3276
Alanine dehydrogenase	None‡	118 ± 37	2112 ± 427	521 ± 160	8908 ± 1171
	Polymyxin B§	68	1514	232	3570
	Colistin§	63	1457	235	3430
Glucose-6-phosphate dehydrogenase	None‡	297 ± 75	5288 ± 726	363 ± 114	6357 ± 724
	Polymyxin B§	215	4859	474	7331
	Colistin§	220	5115	514	7588

* Expressed as mmol H₂O₂ decomposed, nmol NADH oxidized, nmol NAD reduced or nmol NADP reduced min⁻¹ (mg protein)⁻¹.

† Expressed as mmol H₂O₂ decomposed, nmol NADH oxidized, nmol NAD reduced or nmol NADP reduced min⁻¹ per 10¹² spores sonicated.

‡ Each value represents the mean of ten determinations ± SD.

§ Each value represents the mean of five determinations.

spores, no detectable concentration of the antibiotic was released from polymyxin B- and CaCl₂-treated ones. The soluble fraction of polymyxin B-treated germinated spores contained about 290 µg polymyxin B. When dormant spores were sonicated in the presence of various concentrations of the antibiotic, no detectable concentration of antibiotic was present in the soluble fractions from dormant spores sonicated at ≤ 5 mg polymyxin B.

Quantity of polymyxin B released during germination and by treatment with CaCl₂

The amount of antibiotic removed from polymyxin B-treated dormant spores during germination and by treatment with CaCl₂ was determined. Approximately 2.1 × 10⁸ spores

Table 4. *Specific and total activities of NADH oxidase, NADH dehydrogenase and NADH cytochrome c reductase in the particulate fractions of polymyxin B-treated and polymyxin B- and CaCl₂-treated dormant spores and dormant spores sonicated in the presence of various concentrations of polymyxin B*

Specific activity is expressed as nmol NADH oxidized, nmol 2,6-dichlorophenolindophenol reduced or nmol cytochrome *c* reduced min⁻¹ (mg protein)⁻¹. Total activity is expressed as nmol NADH oxidized, nmol 2,6-dichlorophenolindophenol reduced or nmol cytochrome *c* reduced min⁻¹ per 10¹² spores sonicated. Each value represents the mean of two determinations. Numbers in parentheses show percentage of activity expressed as activity of polymyxin B-treated and polymyxin B- and CaCl₂-treated dormant spores and dormant spores sonicated in the presence of polymyxin B divided by activity of untreated spores.

Enzyme	Activity	Treatment of dormant spores			Dormant spores (about 10 ¹²) sonicated in the presence of polymyxin B				
		None	Polymyxin B	Polymyxin B and CaCl ₂	1	Polymyxin (mg):			
NADH oxidase	Specific	263 (100)	98 (37)	86 (33)	291 (111)	270 (103)	265 (101)	114 (43)	91 (35)
	Total	1688 (100)	244 (14)	269 (16)	1647 (98)	1744 (103)	1304 (77)	267 (16)	244 (14)
NADH dehydrogenase	Specific	2184 (100)	2042 (93)	1595 (73)	2456 (112)	2270 (104)	2227 (102)	1934 (89)	1681 (77)
	Total	14021 (100)	5085 (36)	4984 (36)	13901 (99)	14664 (105)	10597 (76)	4522 (32)	4505 (32)
NADH cytochrome <i>c</i> reductase	Specific	88 (100)	38 (43)	27 (31)	87 (99)	76 (86)	71 (81)	45 (51)	28 (32)
	Total	565 (100)	95 (17)	84 (15)	492 (87)	491 (87)	349 (62)	105 (19)	75 (13)

ml⁻¹ in heart infusion broth or in 100 mM-phosphate buffer (pH 7.2) containing 20 mM-L-alanine (the physiological germinant for *B. subtilis* spores) released no detectable concentration of polymyxin B, but in 100 mM-Tris/HCl buffer (pH 7.5) containing 100 mM-CaCl₂ about 3.3 µg ml⁻¹ was released. However, this concentration was about one-quarter of the minimum inhibitory concentration (MIC; 12.5 µg ml⁻¹; Tochikubo *et al.*, 1981) for the same number of dormant spores. When 2.4 times the number of polymyxin B-treated dormant spores (5.1 × 10⁸ spores ml⁻¹) were incubated in heart infusion broth or in 20 mM-L-alanine, 5.3 and 6.1 µg polymyxin B ml⁻¹ were released. These values represent one-quarter to one-fifth of the MIC (25 µg ml⁻¹) for the same number of dormant spores. In 100 mM-CaCl₂ solution they released 10 µg polymyxin B ml⁻¹, which was less than one-half of the MIC.

Respiration during germination of polymyxin B-treated dormant spores

Although the germination rate was slightly stimulated compared with untreated dormant spores (Fig. 2*a*), the respiration of polymyxin B-treated dormant spores was inhibited at the same time as the start of germination and the inhibition of respiration after 120 min was more than 90% (Fig. 2*b*). Subsequent treatment with CaCl₂ before germination restored the respiration of polymyxin B-treated dormant spores to that of untreated spores (Fig. 2*b*). Similar results were also obtained in the absence of NADH (data not shown).

Morphological changes of polymyxin B-treated dormant and germinated spores

The majority of untreated dormant spores demonstrated a laminar cortex and a spore protoplast with a clear roundish germ cell wall and granules (Fig. 3*a*), whereas a considerable number of polymyxin B-treated dormant spores lost structural details of the cortex and the spore protoplast, and moreover, the latter was irregular in form (Fig. 3*b*). Insufficient infiltration of resins was also observed.

The untreated germinated spores 2 h after incubation in 20 mM-L-alanine solution showed a thinness of the spore coat, a diminution in size of the cortex, a swollen core, a cell wall, some

Fig. 2. Time course of L-alanine-initiated germination (a) and respiration during germination (b) of polymyxin B-treated dormant spores. Germination experiments were done by incubation of dormant spores (about 2×10^8 cells ml^{-1}) at 37°C in 3 ml 50 mM-phosphate buffer (pH 7.2) containing 3 μmol L-alanine. Germination was measured by loss of optical density of the spore suspension and is expressed as a percentage of the reduction in OD_{650} determined with a Bausch & Lomb Spectronic 20A spectrophotometer by the method of Hachisuka *et al.* (1955). See text for details of respiration measurement. ●, Untreated dormant spores; ○, polymyxin B-treated spores; ▲, polymyxin B- and CaCl_2 -treated spores.

mesosomes and DNA fibrils (Fig. 4a). Fig. 4(b) shows a thin section of the germinated spores derived from polymyxin B-treated dormant spores after incubation for 2 h in 20 mM-L-alanine solution. The spore core appears irregular in form and the thin cell wall was detached from the cytoplasmic membrane in some places. Moreover, the cytoplasm was densely homogeneous and not granular, and the nuclear material was not visible. These features are in agreement with those of the germinated spores derived from polymyxin B-treated dormant spores after incubation for 3 and 24 h in heart infusion broth (Hayakawa *et al.*, 1981).

Electron micrographs of the spores germinated in the presence of chloramphenicol and polymyxin B resembled those of untreated germinated spores and polymyxin B-treated ones, respectively (data not shown).

DISCUSSION

The basic polypeptide antibiotic polymyxin B kills bacteria by interaction with the cytoplasmic membrane (Few, 1955; Newton, 1956; Storm *et al.*, 1977). The mode of action may be an irreversible breakdown of the permeability barrier of the membrane (Imai *et al.*, 1975).

The activity of particulate NADH oxidase of polymyxin B-treated dormant spores was considerably lower when compared with that of untreated ones, and the antibiotic treatment inhibited the remarkable increase in particulate NADH oxidase activity during germination (Table 1) as well as growth after germination (Tochikubo *et al.*, 1981; Hayakawa *et al.*, 1981). Although the enzyme activity of polymyxin B- and CaCl_2 -treated dormant spores was inhibited to the same extent as that of polymyxin B-treated spores (Tables 1 and 4), they recovered the growth ability after germination (Tochikubo *et al.*, 1981). It is therefore necessary to distinguish between the effect of polymyxin B on enzyme activity and that of the antibiotic on growth after germination. Polymyxin B treatment clearly has direct or indirect effects upon the membranous structure of dormant spores which is the site of the electron transport chain and oxidative phosphorylation. At least two possibilities can be considered with regard to the inhibitory mechanism of particulate NADH oxidase activity of polymyxin B-treated dormant spores. The

Fig. 3. Electron micrographs of thin sections of untreated (a) and polymyxin B-treated (b) dormant spores of *B. subtilis*. OSC, outer spore coat; ISC, inner spore coat; CX, cortex; GCW, germ cell wall; CR, core. Bars represent 0.5 μ m.

first possibility is that polymyxin B not only binds to the spore coat (Tochikubo *et al.*, 1981) but can also penetrate to the inner cytoplasmic membrane, leading to the inhibition of respiration. However, since treatment of polymyxin B-treated dormant spores with CaCl_2 reversed the inhibitory effect of the antibiotic on growth (Tochikubo *et al.*, 1981) and the activity of particulate NADH oxidase from their germinated spores was almost the same as that from untreated spores (Table 1), it is considered that an irreversible breakdown of the cytoplasmic

Fig. 4. Electron micrographs of thin sections of untreated (a) and polymyxin B-treated (b) germinated spores of *B. subtilis*. CW, cell wall; CM, cytoplasmic membrane; M, mesosome; N, fibrillar nucleoid. Bars represent 0.5 μ m.

membrane did not occur by treatment with the antibiotic. Therefore the antibiotic is not able to reach the cytoplasmic membrane of dormant spores. The second possibility is that interaction of polymyxin B with the outer pericortex membrane of dormant spores can affect the cytoplasmic membrane in some way to inhibit respiration without binding directly to the membrane. In *Escherichia coli* and *Pseudomonas aeruginosa* perturbation of the outer membrane structure by immobilized polymyxin can indirectly affect bacterial growth and respiration without direct

interaction of the antibiotic with the cytoplasmic membrane (LaPorte *et al.*, 1977). The existence of an outer membrane in the dormant spores of *Bacillus megaterium* has been demonstrated biochemically since the isolated outer integument contains cytochromes, electron transport enzymes and polypeptides (Cra'ts-Lighty & Ellar, 1980) and by the morphological observation of variant spores lacking exosporium (Koshikawa *et al.*, 1984). Accordingly, the second possibility seems most likely. The unclear structure and irregular form of the protoplast of polymyxin B-treated dormant spores (Fig. 3*b*) may be due to insufficient infiltration of fixatives caused by perturbation of the outer membrane. In this case, although the disorder of the outer membrane by polymyxin B does not seem to be recoverable, the cytoplasmic membrane appears to bear the antibiotic damage because of complete reversion of both particulate NADH oxidase activity (Table 1) and O₂ consumption (Fig. 2*b*) during germination of polymyxin B- and CaCl₂-treated dormant spores.

Addition of CaCl₂ to polymyxin B-treated dormant spores reverses the electrophoretic mobility within the normal range by releasing the antibiotic bound to the spore coat surface (Tochikubo *et al.*, 1981). Since 10 µg polymyxin B is released from about 5.1×10^8 spores, about 20 mg polymyxin B will be released from 10^{12} polymyxin B-treated dormant spores by treatment with 100 mM-CaCl₂. No detectable concentration of the antibiotic was found in the soluble fraction of polymyxin B- and CaCl₂-treated dormant spores and therefore the total amount of the antibiotic binding to 10^{12} spores after CaCl₂ treatment is ≤ 5 mg. It seems probable that about 20 mg polymyxin B binds to the spore coat and can be easily dissociated by treatment with CaCl₂, whereas a small quantity (5 mg and less) of the antibiotic binds to the outer pericortex membrane and cannot be dissociated by CaCl₂ treatment. Thus the low activity of particulate NADH oxidase of polymyxin B- and CaCl₂-treated dormant spores may be due to the inhibitory effect of a small amount of the antibiotic binding to the outer pericortex membrane.

The low activity of particulate NADH oxidase (Table 1) and the inhibited outgrowth of polymyxin B-treated germinated spores (Tochikubo *et al.*, 1981) could be due to antibiotic released from the spore coat during germination. It seems that germinating spores directly incorporate the released antibiotic into the inside, since about 89% of the polymyxin B-treated dormant spores are not able to form colonies on heart infusion agar plates (Tochikubo *et al.*, 1981) and the respiration of polymyxin B-treated dormant spores was inhibited at the same time as the start of germination (Fig. 2*b*). The recovery of growth ability of polymyxin B- and CaCl₂-treated dormant spores may be due to the removal of bound antibiotic.

The inhibitory action of polymyxin B on the activity of particulate NADH oxidase may occur during spore fractionation. However, the following two facts support our opinion that inhibition of the enzyme activity by polymyxin B occurs before spore fractionation: (i) in spite of very low specific and total enzyme activities, the soluble fraction of polymyxin B- and CaCl₂-treated dormant spores contained no detectable concentration of the antibiotic; (ii) inhibition of respiration of polymyxin B-treated dormant spores occurred at the same time as the initiation of germination (Fig. 2*b*).

The decrease in the specific and total activities of particulate NADH oxidase by polymyxin B treatment agreed well with those for NADH cytochrome *c* reductase activities but not with those for NADH dehydrogenase activities (Table 4). The specific activity of NADH dehydrogenase after polymyxin B treatment was almost in agreement with that of untreated dormant spores, but about 64% of the total enzyme activity was lost (Table 4). This loss originated in the decrease in the protein content from the particulate fraction of polymyxin B-treated dormant spores. If the protein content of the latter is corrected to the same protein content as that of untreated spores in Table 4, 93% of the total enzyme activity was recovered. This suggests that there is little or no decrease in the total activity of NADH dehydrogenase by a specific action of polymyxin B on the enzyme. The site inhibited by the antibiotic in the electron transport chain seems to be between coenzyme Q and cytochrome *c*.

The protein content of the particulate fraction of polymyxin B-treated dormant spores decreased by approximately one-half compared with that of untreated spores, and sonication of dormant spores in the presence of polymyxin B also decreased the protein content in particulate fractions depending on the antibiotic concentration (Tables 1 and 4). A high concentration of

polymyxin B may therefore damage membranous structures and induce solubilization of membrane-bound proteins during fractionation even at low temperature. The decrease in total activities of particulate NADH oxidase and NADH cytochrome *c* reductase by polymyxin B treatment is therefore caused both by the decrease in the specific activities of the enzymes and by that in the particulate protein content.

Wilkinson *et al.* (1977) have reported that a rapid chloramphenicol-resistant activation of certain membrane-bound respiratory chain enzymes occurs in the first 10 min of germination of *B. megaterium* KM spores. The same phenomenon was observed also with the particulate NADH oxidase of *B. subtilis* spores (Table 2).

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