ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1976, p. 361-366

Vol. 9, No. 3
Printed in U.S.A.

Modification of Bacterial Respiration by a Macromolecular Polyanionic Antibiotic Produced by a Marine *Alteromonas*

M. J. GAUTHIER

Centre d'Etudes et de Recherches de Biologie et d'Océanographie Médicale, Institut National de la Santé et de la Recherche Médicale, (U 40) 06300 Nice, France

Received for publication 2 December 1975

A macromolecular polyanionic antibiotic produced by a marine bacterium belonging to the genus *Alteromonas* causes a large modification in bacterial respiration when added to the culture of several bacterial species in their early stage of growth. This antibiotic induces an increase of oxygen uptake and the production of hydrogen peroxide. The latter fact explains the high sensitivity of bacteria with low catalase activity and the antagonistic effect of pure catalase on antibiosis. The antibiotic could act at the level of the respiratory chain by setting up a flavinic respiration.

Several previous reports (2) have presented evidence for the presence in every ocean of antibiotic-producing marine bacteria whose inhibitory activity concerns the terrestrial bacterial microflora as a whole. From those species I have selected a group of gram-negative bacilli belonging to the genus *Alteromonas*, a physiological characteristic of which is their ability to synthetize an antibacterial polysaccharide that can inhibit a number of marine and terrestrial bacteria (3). This antibiotic was extracted from cultures of one of the strains and partially purified. Some characteristics of its action led me to suspect an activity at the respiratory level: it is active only in the presence of molecular oxygen and two respiratory enzymes, catalase and peroxidase, will offset its lethal action (4).

As reported here, I have examined the physiological action of the antibiotic upon a number of terrestrial bacteria and the possible alteration of their respiratory metabolism.

MATERIALS AND METHODS

Antibiotic-producing organism. The antibiotic-producing strain used is listed in the Centre d'Etudes et de Recherches de Biologie et d'Océanographie Médicale collection (C18); it has been deposited at the National Collection of Marine Bacteria (Torry Research Station, Aberdeen, Scotland) under NCMB 1890. It was isolated in Mediterranean coastal waters off Nice, where it is common during the autumn months (5). Its taxonomic position has already been discussed; it is considered to belong to the genus *Alteromonas*, although it has not yet been given a specific name (5).

Preparation of the semipurified antibiotic. The antibiotic was partially purified from cultures of the bacterium grown in ZoBell's medium 2216 E, using a procedure described in a previous report (3). The cells were cultivated during 5 days in liquid ZoBell's medium (Peptone [Difco], 5 g; yeast extract [Difco], 1 g; $FePO_4$, 0.1 g; seawater, 800 ml; distilled water, 200 ml; pH 7.6 with 1 N NaOH) at room temperature (22 C). After being filtered on membrane filters (0.45-μm pore size; Millipore Corp., Bedford Mass.), the medium was precipitated with ethanol (respectively, 1 vol:2 vol). The precipitate was dissolved with distilled water and dialyzed against water. The residue was filtered on Sephadex G200 in 0.1 M ammonium bicarbonate buffer, pH 7. The active fractions were collected together, fixed on diethylaminoethyl-cellulose in sodium diethylbarbiturate buffer (pH 9.2), and eluted with 0.2 M NaCl. After being dialyzed against distilled water, the antibiotic was freeze dried and used directly for biological tests. It still contained a few impurities, at most 4 to 5% of the dry weight. Its electrophoretic behavior indicates a polyanionic structure, and its strong positive reaction with anthrone reagent suggests a polysaccharidic nature.

Test bacteria and measurement of the antibiotic action. The antibiotic activity of the product was tested with bacterial strains from the Institut Pasteur Collection (IPC): *Staphylococcus epidermidis* (IPC 53124), *Staphylococcus aureus* (IPC 209P), *Streptococcus faecalis* (IPC 5432), *Lactobacillus delbrueckii* (IPC 5758), *Lactobacillus acidophilus* (IPC 6218), *Micrococcus denitrificans* (IPC 7111), *Micrococcus lysodeikticus* (IPC A270), *Diplococcus pneumoniae* (IPC 692, serotype 1), *Shigella dysenteriae* (IPC 6217, type 1), *Klebsiella pneumoniae* (IPC 53153), *Citrobacter* (IPC A 245), *Proteus morganii* (IPC 53186), *Escherichia coli* (IPC strain Monod). In addition, use was made of bacteria isolated in this laboratory from urban waste water: a strain of *Serratia marcescens* and a gram-positive bacillus with low catalase activity closely related to *Bacillus firmus* and included in our collection under no. C5.

The antibiosis tests were carried out using the standard antibiogram procedure in solid medium (Trypticase soy agar, pH 6; Difco) described by Chabbert (1). The action of cyanide was investigated on Möller agar (Peptone [Difco], 3 g; NaCl, 5 g;

KH_2PO_4, 225 mg; Na_2HPO_4, 5.64 g; agar [Difco], 10 g; and distilled water to 1,000 ml) with 75 mg of KCN added per liter of medium after sterilization. The action of riboflavin and nicotinamide, precursors of flavine adenine dinucleotide and nicotinamide adenine dinucleotide, was investigated with vitamin-free agar (peptone, 1 g; agar [Difco], 10 g; distilled water to 1,000 ml), with either vitamin added at a concentration of 20 μg/ml. Inhibition of the test bacteria was also studied at various stages of their growth in liquid medium (antibiotic medium 3 [Difco]) using a recording biophotometer (Bonet-Maury).

Production of H_2O_2. Production of H_2O_2 was measured by iodometric determination of the H_2O_2 released by the bacterial cells (6) in the culture medium (antibiotic medium 3 [Difco]), after adding the antibiotic at the beginning of exponential growth.

Variations of the oxygen uptake. The variation in oxygen uptake by bacteria in the presence of the antibiotic was determined by means of a Gilson constant-pressure differential respirometer with cell suspensions prepared in 0.1 M phosphate buffer at pH 7.3 and glucose added at a concentration of 5 g/liter. The antibiotic was added in steps until a final concentration of 500 μg/ml was reached. Some of these tests were made in the presence of catalase (bovine liver catalase, Sigma Chemical Corp., St. Louis, Mo.) present at 10 μg/ml or potassium cyanide at a level of 75 μg/ml.

RESULTS

The antibacterial activity of the investigated product, determined roughly by the antibiogram method, seems to vary according to the particular test bacterium (Table 1), the gram-positive ones being more strongly inhibited. In all cases, the activity is antagonized by catalase and strongly enhanced by KCN. Lactic bacteria are unaffected by the product.

The biophotometric study of the product in liquid medium also revealed that its acitivity is largely dependent on the growth stage reached by the bacterium when the agent is added to the medium (Fig. 1).

All bacteria, whether they have a catalase or not, become inhibited when the antibiotic is added to the medium during the lag phase of the cultures, except for *Lactobacillus, Streptococcus,* and *Diplococcus* species, which are resistant.

The addition of antibiotic at the start of the logarithmic phase of growth entails different effects depending on the test bacteria. With catalase-positive bacteria, there is a slight bacteriostasis of short duration. For the bacteria whose catalase activity is low or nil (*S. dysenteriae,* strain C5) there is a greater extent of inhibition, with the gram-positive strain appearing to be much more susceptible. In the latter case, the antibiotic effect can be offset if a small amount of catalase is added to the medium at the same time (Fig. 1B). At the end of the growth phase, the activity becomes very low, even with the most sensitive organisms.

TABLE 1. *Modifications in the activity of the antibiotic polysaccharide in a normal medium (Trypticase soy agar, pH 6) and the same medium added with catalase (10 mg/liter) or potassium cyanide (75 mg/liter)*

Test organism	Antibiotic activity (diam of inhibition ring, mm)		
	Normal medium	+ Catalase	+ KCN
Strain C5	9	0	
Staphylococcus aureus	6	0	
Staphylococcus epidermidis	7.5	0	
Micrococcus lysodeikticus	4.5	0	
Micrococcus denitrificans	1	0	
Streptococcus faecalis	0	0	
Diplococcus pneumoniae	0	0	
Lactobacillus acidophilus	0	0	
Lactobacillus delbrueckii	4.5	0	
Escherichia coli	0	0	
Klebsiella pneumoniae	0	0	5
Citrobacter	0	0	6
Shigella dysenteriae	4	0	
Proteus morganii	5	0	14
Serratia marcescens	5	0	16
Pseudomonas aeruginosa	2	0	

It should be pointed out that the lactic bacteria are only slightly affected by the antibiotic, whatever the growth phase; only *L. delbrueckii* is partially inhibited whether the antibiotic is added during the lag phase or at the beginning of exponential growth of the cultures.

In the case of bacteria with low catalase activity (strain C5), the addition of the antibiotic to the culture medium is rapidly followed by an unbalanced synthesis of H_2O_2, with the concentration in the medium rapidly increasing and reaching a peak after 3 h and then gradually decreasing to zero after 5 to 6 h (Fig. 2). All of the bacteria are killed, but cell lysis is very small and does not seem to result from the action of the antibiotic. A similar result can be obtained after the addition of pure H_2O_2 to the medium, at the concentrations observed in the presence of antibiotic (Fig. 3); thus, its lethal action is effectively due to the released peroxide.

This peroxide synthesis was found in all of the test bacteria growing under aerobic conditions, with the exception of the strains belonging to the genera *Lactobacillus, Streptococcus,* and *Diplococcus.* The release is larger and lasts

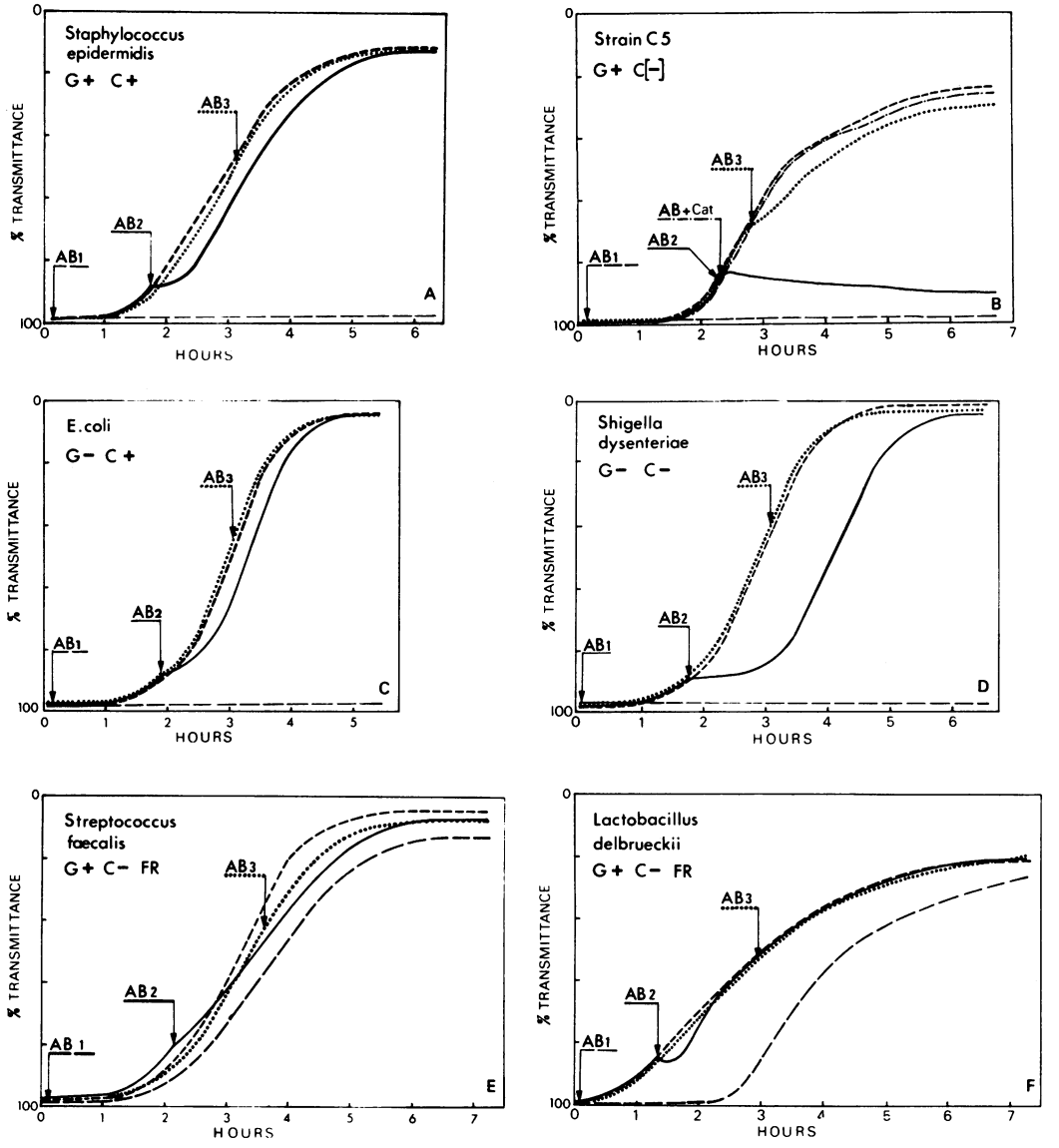

FIG. 1. *Inhibitory effect of the antibiotic on the growth of several gram-positive (G+) or gram-negative (G−) or catalase-positive (C+) or catalase-negative (C−) test bacteria studied by means of a recording biophotometer. Except for the lactic bacteria with flavinic respiration (FR), all the test bacteria are strongly inhibited when the antibiotic is added to the medium during the lag phase (AB1). At the beginning of the logarithmic phase of growth (AB2), its action decreases and mainly concerns the strains with low catalase activity. At the end of the growth (AB3), the antibiotic has a very slight low activity. Symbols: (-----) Control growth curve; (C[−]) bacterial strain with very slight and irregular catalase activity. (A) Staphylococcus epidermis (G+C+); (B) strain C5 (G+C−); (C) E. coli (G−C+); (D) Shigella dysenteriae (G−C−); (E) Streptococcus faecalis (G+C− FR); (F) Lactobacillus delbrueckii (G+C− FR).*

longer if the catalase activity of the bacterium is low. In a vitamin-deprived medium the addition of riboflavin brings about an increase in the action of the antibiotic (Fig. 4), whereas there is no change if the medium is supplemented with nicotinamide.

The metabolic changes induced by the antibiotic on susceptible bacteria result in a modification of the respiratory uptake of molecular oxygen. In the presence of the antibiotic, this uptake is substantially enhanced, the variation being more marked when the catalase activity

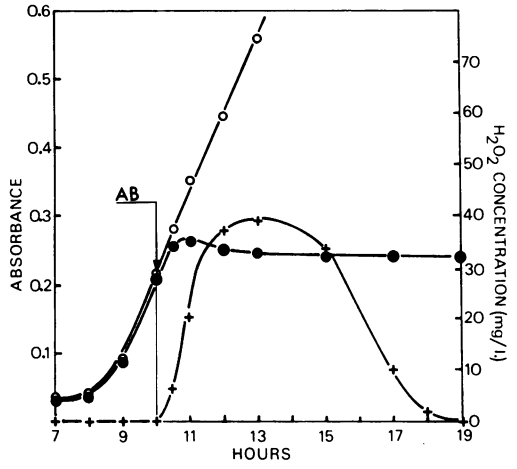

FIG. 2. *Production of hydrogen peroxide by strain C5 in the presence of antibiotic (500 μg/ml) added at the beginning of the logarithmic phase of growth. Symbols: (○) Control; (●) antibiotic (AB); (+) H_2O_2 concentration in the medium.*

of the test bacterium is lower (Fig. 5 and 6). The rate of increase (ratio of the amount of oxygen absorbed per hour in the presence of antibiotic to that absorbed in the same time without the antibiotic) is on the order of 1.2 for catalase-positive bacteria and 1.7 for strain C5. It is remarkable that the increase in oxygen uptake remains comparable in a medium with catalase added, the only difference being an overall increase of the respiratory activity of the test bacteria. The same phenomenon occurs in a medium with added KCN for those bacteria capable of growing in the presence of the cyanide. In that case, the oxygen uptake, while markedly reduced, is decidedly increased after the addition of the antibiotic (Fig. 6) but never reaches the level observed in a normal medium.

DISCUSSION

The bacterial activity of the polysaccharide is actually indirectly related to a modification of the respiratory process in susceptible bacteria. Such a modification entails an increase in molecular oxygen consumption and a concomitant release of H_2O_2. This fact accounts for both the antagonistic activity of catalase, which decomposes the peroxide and thus suppresses its lethal activity, and the higher susceptibility of catalase-negative bacteria to the antibiotic. As a matter of fact, all the bacteria that consume molecular oxygen under aerobic conditions are susceptible, to various degrees, to this antibiotic. Against catalase-positive bacteria, the antibiotic is effective only during the lag phase of the culture growth, where the synthesis of proteins is probably not sufficient to supply the cells with the requisite respiratory enzymes.

From the overall results of this study, it is possible to define an outline of the action of this antibiotic. The increase in its activity in a synthetic medium enriched with a flavin adenine dinucleotide precursor possibly suggests the participation of flavinic systems or flavoproteins in the test bacteria. At the membrane level the antibiotic seems to modify the respiration and induce a direct reoxidation of the flavinic coenzymes by molecular oxygen, resulting in the formation of peroxide. If this is true, its action would consist in the setting up, in the bacteria, of a flavinic-type respiration similar to that of the lactic bacteria. The latter are resistant because they have a coupled enzymatic system in which, first, a molecule of reduced nicotinamide adenine dinucleotide is oxidized and H_2O_2 is released, and, second, an-

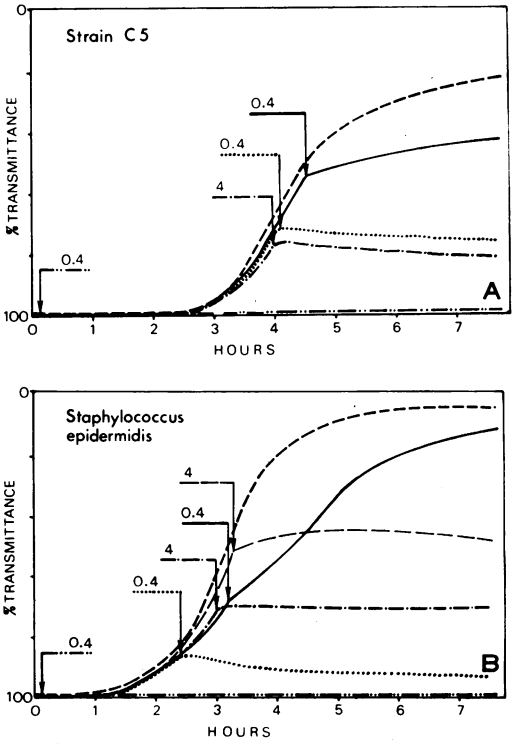

FIG. 3. *Inhibitory effect of pure hydrogen peroxide on the growth of two test bacteria. The peroxide, added to the medium at the rates of 0.4 and 4 mg/liter, mimics the killing action of the antibiotic both in the lag and growth stages. Furthermore, such a test indicates that the threshold of lethality is reached for low concentrations of H_2O_2, readily occurring in the test media added with antibiotic. Symbol: (-----) Control growth curve. (A) Strain C5; (B) Staphylococcus epidermidis.*

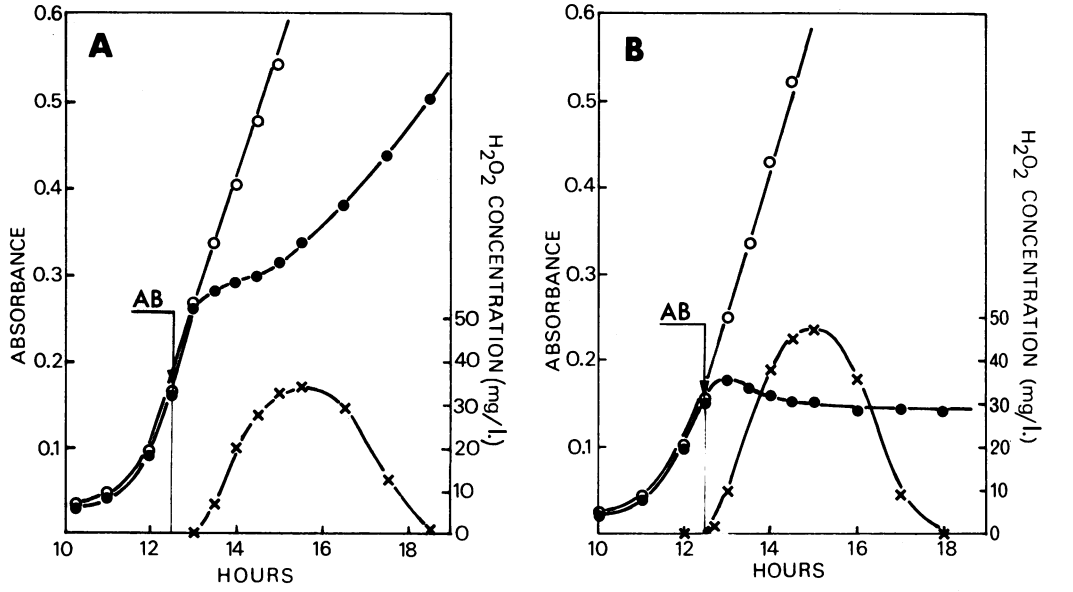

FIG. 4. *Comparative effect of the antibiotic on growth of strain C5 and hydrogen peroxide production in a vitamin-free medium (A) and in the presence of 20 mg of riboflavin per liter (B). Symbols: ○, control; ●, antibiotic (AB) added at the beginning of the logarithmic phase of growth; X, H_2O_2 concentration in the medium.*

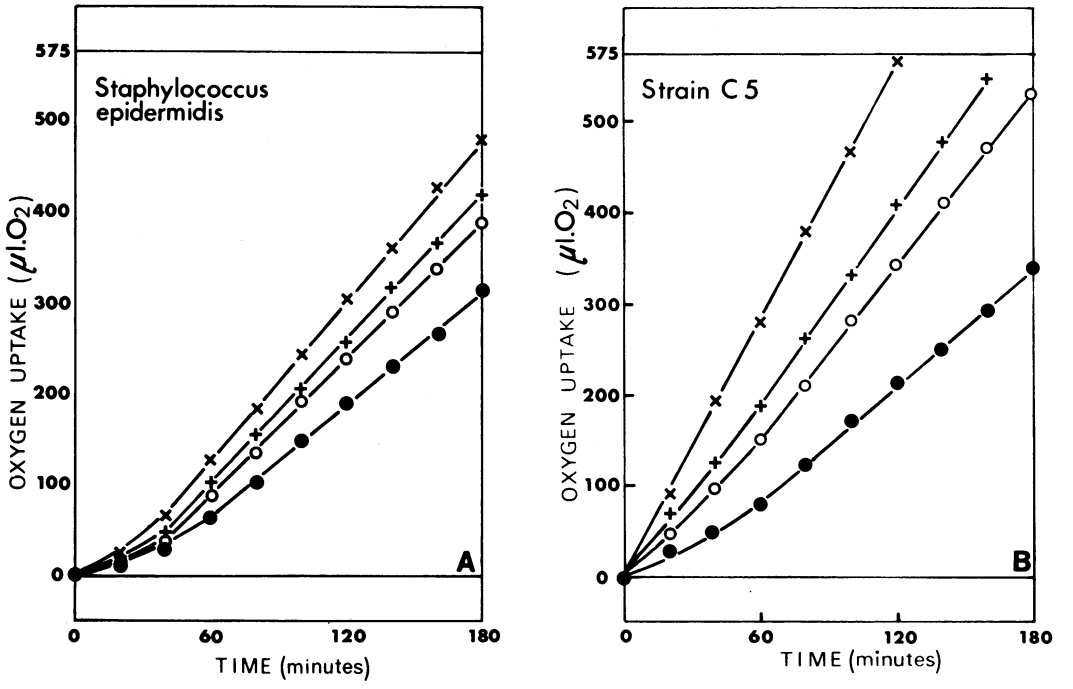

FIG. 5. *Oxygen uptake by (A) Staphylococcus epidermidis (gram-positive, catalase-positive) and (B) strain C5 (gram-positive, catalase-negative) in the presene of antibiotic (+), catalase (○), and antibiotic + catalase (×); (●) control without enzyme and antibiotic. The cells were rinsed three times and suspended in 0.1 M phosphate buffer with added glucose (5‰ wt/vol); 2 ml of each suspension was used for respirometric measurements (750 μg of dry cells per 2 ml for S. epidermidis, 1,340 μg of dry cells per 2 ml for strain C5). Concentrations: antibiotic, 500 μg/ml; catalase, 10 μg/ml.*

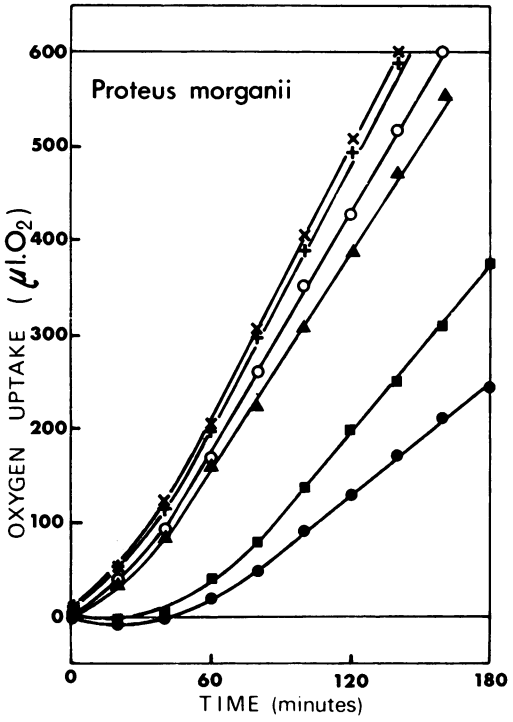

FIG. 6. *Oxygen uptake by Proteus morganii in the presence of KCN (●), KCN + antibiotic (■), catalase (○), antibiotic (+), and catalase + antibiotic (X); (▲) control without enzyme, antibiotic, or KCN. The cells were prepared in 0.1 M phosphate buffer with added glucose (5‰ wt/vol). Two milliliters of the cell suspension was used for respirometric measurement in each condition (280 μg of dry cells per 2 ml). Concentrations: KCN, 75 μg/ml; catalase, 10 μg/ml; antibiotic, 500 μg/ml.*

other molecule of reduced nicotinamide adenine dinucleotide is reoxidized by H_2O_2, leading to the synthesis of water. The lack of such a system in the bacteria that normally consume molecular oxygen brings about an increase in the H_2O_2 concentration, which is lethal unless neutralized by catalase. The comparative susceptibility of *L. delbrueckii* to this antibiotic might be accounted for by the fact that this bacterium, which can grow aerobically as well as under anaerobic conditions and has no cytochromes, shows some respiratory activity when it is allowed to grow in the presence of molecular oxygen. It then produces H_2O_2 by direct transfer of electrons from substrates to molecular oxygen through flavine adenine dinucleotide and flavoproteins. Here again, the antibiotic activity is neutralized by catalase, which is naturally absent in that bacterium.

In contrast, *Micrococcus denitrificans*, a strictly aerobic bacterium like all the aerobic species in the genus, has a catalase and a cytochrome chain and is readily capable of consuming oxygen through the flavinic pathway. It therefore has some resistance to the lethal activity of the antibiotic, which is not the case for *M. lysodeikticus*.

Cyanide inhibits heme enzymes, such as catalase, and stops the transfer of electrons and protons at the last stage of the reduction of molecular oxygen by the cytochromes. Thus, its synergistic action with the antibiotic may be partly accounted for by its blocking catalase, thereby preventing the peroxide formed from being neutralized. It should be emphasized, however, that where this inhibitor is present the bacteria that can develop show the same increase in oxygen uptake in the presence of the antibiotic, which confirms that the respiratory shunt induced by the latter takes place earlier in the respiratory chain, at the flavoprotein or nicotinamide adenine dinucleotide level.

Such respiratory modifications are suggestive of a possible primary site for the antibiotic on the bacterial membrane. Therefore, the nature of the envelopes, in particular the cell wall, is critical as regards the possibility for this high-molecular-weight antibiotic to be incorporated. One thus could explain the higher susceptibility of gram-positive bacteria, whose wall appears to be a simple structure.

ACKNOWLEDGMENTS

I thank J. P. Belaïch (Laboratoire de Chimie Bactérienne-C.N.R.S., Marseille) for helpful discussion and criticism during the course of this work and G. J. Bonde (Hygiejnisk Institut, Aarhus University, Denmark) for the identification of the test bacterium included in our collection under no. C5.

LITERATURE CITED

1. **Chabbert, Y. A.** 1963. L'antibiogramme. Coll. techniques de base. Ed. La Tourelle, St. Mandé, France.
2. **Gauthier, M. J.** 1969. Substances antibactériennes produites par les bactéries marines. I. Etude systématique de l'activité antagoniste de souches marines vis-à-vis de germes telluriques aérobies. Rev. Int. Oceanogr. Med. 15-16:41-59.
3. **Gauthier, M. J.** 1970. Substances antibactériennes produites par les bactéries marines. II. Lipopolysaccharides antibiotiques produits par certains germes marins appartenant aux genres *Pseudomonas* et *Chromobacterium*. Rev. Int. Oceanogr. Med. 17:23-45.
4. **Gauthier, M. J.** 1972. Antagonismes et synergies dans l'antibiose chez certains bactéries marines. Rôle de certains enzymes respiratoires dans ces phénomènes. Rev. Int. Oceanogr. Med. 26:65-84.
5. **Gauthier, M. J., J. M. Shewan, D. M. Gibson, and J. V. Lee.** 1975. Taxonomic position and seasonal variations in marine neritic environment of some gram-negative antibiotic-producing bacteria. J. Gen. Microbiol. 87:211-218.
6. **Halbwachs-Strich, M.** 1969. Travaux Pratiques d'analyse quantitative chimique. Ed. Masson, Paris.