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Effects of Antimicrobial Agents on the Milky Disease Bacteria *Bacillus popilliae* and *Bacillus lentimorbus*[1]

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ABSTRACT

PRIDHAM, T. G. (Northern Regional Research Laboratory, Peoria, Ill.), H. H. HALL, AND R. W. JACKSON. Effects of antimicrobial agents on the milky disease bacteria *Bacillus popilliae* and *Bacillus lentimorbus*. Appl. Microbiol. **13**:1000–1004. 1965.—The effects of antibiotics, sulfonamides, and other antimicrobial agents on vegetative cultures of five strains of milky disease bacteria were compared with those on *Bacillus subtilis* Cohn emend. Prazmowski, *Staphylococcus aureus* Rosenbach, *Sarcina lutea* Schroeter, *Escherichia coli* (Migula) Castellani and Chalmers, *Saccharomyces pastorianus* Hansen, and *Mucor ramannianus* Moel. Similar numbers of viable cells of each organism were exposed to the test materials by use of an antibiotic-sensitivity disc method adapted from techniques recommended by the Food and Drug Administration in the *Federal Register*. The results suggest that vancomycin or ristocetin, as well as a few other materials, might be useful in controlling contamination either during culture of the fastidious milky disease bacteria or in large populations of vegetative cells undergoing treatment to induce sporulation. Inhibitory concentrations of vancomycin and ristocetin in shaken-tube tests were much lower than expected in comparison with results of sensitivity-disc tests on the milky disease bacteria. Sublethal concentrations of the two antibiotics elicited some morphological change in the bacteria.

The only known report of the effects of antimicrobial agents on the milky disease bacteria *Bacillus popilliae* Dutky and *B. lentimorbus* Dutky is a short comment by Dutky (1963). He stated that growth and development of *B. popilliae* in the blood of Japanese beetle (*Popillia japonica* Newman) larvae could be intercepted by the injection of 20 μg of dihydrostreptomycin per larva. He further stated that penicillin G, chlortetracycline hydrochloride, and sodium sulfadiazine inhibited the bacteria on artificial media but not when injected into infected larvae.

The milky disease bacteria grow well only in rich media at relatively high *p*H. Because of the fastidious nature of the organisms and because of their marked susceptibility to contamination, an attempt was made to determine whether antimicrobial agents could be added to culture media to prevent the development of contaminants. Tests were run with sensitivity discs on agar and with antibiotics in liquid media. The results show that some of the antimicrobial agents probably can be employed to control contamination during growth of the milky disease bacteria.

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MATERIALS AND METHODS

Cultures. Five strains of milky disease bacteria and six strains of other microorganisms were used: *B. popilliae* NRRL B-2309 (isolated from commercial spore dust by W. C. Haynes); *B. popilliae* NRRL B-2309A (an acetate-utilizing substrain selected from NRRL B-2309 by R. N. Costilow); *B. popilliae* NRRL B-2309L (a large-celled substrain selected from NRRL B-2309S by G. A. Hrubant); *B. popilliae* NRRL B-2309S (a sporulating substrain selected from NRRL B-2309 by M. C. Shekleton and R. A. Rhodes); *B. lentimorbus* NRRL B-2522 (isolated from a dried film of Japanese beetle larval hemolymph by W. C. Haynes); *B. subtilis* Cohn emend. Prazmowski NRRL B-765 (ATCC 6633); *Staphylococcus aureus* Rosenbach NRRL B-313 (ATCC 6538P); *Sarcina lutea* Schroeter NRRL B-1018 (ATCC 9341); *Escherichia coli* (Migula) Castellani and Chalmers NRRL B-766 (ATCC 9637); *Saccharomyces pastorianus* Hansen NRRL Y-139; *Mucor ramannianus* Moel. NRRL 1839.

Inocula. For the sensitivity-disc and shaken-tube tests, inocula of the bacteria and yeast were prepared in tubes of tryptone-glucose-liver extract-yeast extract (TGLY) broth according to the procedure published by Lyons and Pridham (1965). Inoculated tubes were incubated for 16 to

72 hr at 28 to 30 C on a Gump rotary shaker operating at 200 rev/min. Although most of the control bacteria and the yeast grew well in 16 hr, a long period of incubation was necessary for the milky disease bacteria. The inoculum for tests with *M. ramannianus* was a sporangiospore suspension prepared as outlined by Lindenfelser et al. (1964). Cultures of the bacteria and yeast were diluted with sterilized TGLY broth so that there were between 10^7 and 10^8 viable cells in 10 ml of test medium. Adjustments were made by use of a Bausch & Lomb Spectronic-20 colorimeter set at 600 mμ. Counts of viable cells were made by a plate method based on that described by Pridham et al. (1964), in which a high-phosphate growth medium is used. All inocula were used at the rate of 0.1 ml per 9.9 ml of test medium.

Test media and procedures. There are a number of standardized media recommended in the *Federal Register* (Food and Drug Administration, 1963) for sensitivity-disc tests with particular antibiotics or other antimicrobial agents. Because of the fastidious nature of the milky disease bacteria, they grow poorly or not at all when incorporated into the recommended media. In a preliminary experiment, two strains of the milky disease bacteria (*B. popilliae* NRRL B-2309 and *B. lentimorbus* NRRL B-2522) were tested with 11 different standard antibiotic assay media as seed layers; dishes were held for as long as 7 days at 28 to 30 C. The media were: Antibiotic Mediums 1, 2, 5, 7, 8, 9, 10, 11, and 12, as well as Mueller-Hinton (M-H) Medium (all Difco products). The two strains of milky disease bacteria either failed to grow, or grew only sparsely, on all but the M-H medium and Antibiotic Medium 11. Growth on the M-H medium was especially good and appeared equivalent to that obtained with the high-phosphate medium developed especially for growth of these bacteria. Consequently, the sensitivity-disc tests were run with a seed layer of high-phosphate medium solidified with 2% agar, and the shaken-tube tests were run with the M-H medium supplemented with 0.05 ml of a sterilized 20% glucose solution.

For the sensitivity-disc tests, the general procedures outlined in Part 147 of the *Federal Register* (Food and Drug Administration, 1963) were used. A 42-ml amount of sterilized 2% water agar was used as a base layer for each petri dish. After the base layer had solidified, the dishes were left at room temperature overnight. To each base layer were added 10 ml of inoculated seed layer. The seed layer was composed of 9.8 ml of high-phosphate medium, 0.1 ml of sterilized 20% glucose (Difco), and 0.1 ml of adjusted inoculum (added after the based medium was cooled to 50 C). The high-phosphate medium was composed of: yeast extract (Difco), 15 g; tryptone (Difco), 5 g; anhydrous K_2HPO_4, 6 g; distilled water to 980 ml after *p*H adjustment to 7.5; agar (Difco), 20 g; sterilized 20 min at 121.5 C. The milky disease bacteria, the other organisms, and the yeast grew well in the high-phosphate medium seed layer. When the seed layer had solidified, sensitivity discs were laid on the surface. Duplicate dishes were used for each test. The dishes were held for 16 hr at 28 to 30 C, and the zones of inhibition around the discs were noted. With the milky disease bacteria it was necessary to incubate for periods up to 1 week before inhibition zone readings could be made. Sensitivity-disc tests with *M. ramaniannus* were made with Mucor synthetic agar (MSA) according to the procedure outlined by Lindenfelser et al. (1964).

Four kinds of sensitivity discs were used: Unidisks (Difco), sensitivity discs (Difco), Multidisks (Colab Laboratories, Inc., Chicago Heights, Ill.), and Sensi-Disks (BBL). Forty-six different antimicrobial agents were used, generally at low, medium, and high concentrations. In some cases only one concentration was available. The materials tested are listed in Table 1.

For the shaken-tube tests a basal medium based on the M-H medium was used. The M-H broth consisted of: laboratory-prepared beef infusion, 300 ml; amino acids (Difco), 17.5 g; corn starch (Argo), 1.5 g (made into a paste with 100 ml of distilled water); distilled water to 980 ml after *p*H adjustment to 7.5 with 1 N NaOH. The broth was dispensed with stirring into 25 by 150 mm tubes (9.8 ml per tube); the tubes were stoppered with cotton and then sterilized for 15 min at 121.5 C.

Four antibiotics—cycloheximide (Acti-Dione, containing 85 to 100% active ingredient; The Upjohn Co., Kalamazoo, Mich.); vancomycin hydrochloride (95% purity; Eli Lilly & Co., Indianapolis, Ind.); ristocetin A sulfate, 80.5%, and ristocetin B sulfate, 9.5% (Spontin; Abbott Laboratories, North Chicago, Ill.); and actinomycin D (Merck Sharp & Dohme, Rahway, N.J.)—were added individually in appropriate concentration in 0.1-ml amounts to tubes of the M-H broth. Except for actinomycin D, the antibiotics were dissolved in distilled water so that the final range of concentrations tested was from 0.1 through 100 μg/ml. Actinomycin D was dissolved in acetone and water. All antibiotic solutions were sterilized by passage through sintered-glass filters before addition to the sterilized broth.

The shaken-tube sensitivity tests were carried out with *B. popilliae* NRRL B-2309 and *B. lentimorbus* NRRL B-2522, and the other bacteria and the yeast. After inoculation, the tubes were placed on a Gump rotary shaker operating at 200 rev/min and incubated for 16 hr. At that time, 0.5 ml of formalin was added to each tube, and the per cent transmittance of light at 600 mμ was determined with a Spectronic-20 colorimeter. Duplicate or triplicate tubes were used for each test.

Examinations under a microscope at 1,800 × magnification were made of samples from some of the cultures to determine whether the antibiotics had elicited any morphological changes in the milky disease bacteria.

TABLE 1. *Effects of antimicrobial agents on milky disease bacteria and other microorganisms via sensitivity-disc testing**

Antimicrobial agent† (added as sensitivity disc)	*Bacillus popilliae* NRRL B-2309	*B. popilliae* NRRL B-2309A	*B. popilliae* NRRL B-2309L	*B. popilliae* NRRL B-2309S	*B. lentimorbus* NRRL B-2522	*B. subtilis* NRRL B-765	*Micrococcus pyogenes* var. *aureus* NRRL B-313	*Sarcina lutea* NRRL B-1018	*Escherichia coli* NRRL B-766	*Mucor ramannianus* NRRL 1839	Maximal concn tested per disc
Methicillin	S	S	S	S	S	S	S	S	R	R	5 μg
Oxacillin	S	R	±	S	S	S	S	S	R	–	1 μg
Penicillin	S	S	S	S	S	S	S	S	±	–	10 units
Phenethicillin	S	S	S	S	S	S	S	S	R	–	3 units
Dihydrostreptomycin	S	S	S	S	S	S	S	S	S	–	10 μg
Streptomycin	S	S	S	S	S	S	S	S	S	–	10 μg
Chlortetracycline	S	S	S	S	S	S	S	S	S	–	30 μg
Demethylchlortetracycline	S	S	S	S	S	S	S	S	S	–	30 μg
Tetracycline	S	S	S	S	S	S	S	S	S	–	30 μg
Erythromycin	S	S	S	S	S	S	S	S	±	–	15 μg
Oleandomycin	S	S	S	S	S	S	S	S	R	R	2 μg
Spiramycins	S	S	S	S	S	S	S	S	R	–	30 μg
Triacetyloleandomycin	S	S	S	S	±	±	S	S	R	R	2 μg
Bacitracin	S	S	S	S	S	±	S	S	R	–	10 units
Colistin	R	R	R	±	R	±	R	R	±	R	2 μg
Polymyxin B	R	R	R	R	S	±	R	R	±	R	50 units
Viomycin	±	±	±	±	±	±	R	R	±	–	10 μg
Kanamycin	S	S	S	S	S	S	S	S	S	S	30 μg
Neomycin	S	S	S	S	S	S	S	S	S	±	30 μg
Paromomycin	S	S	S	S	S	S	S	S	S	±	30 μg
Ristocetin	±	±	±	±	S	S	S	S	±	–	30 μg
Vancomycin	R	R	±	R	S	S	S	S	±	–	30 μg
Chloramphenicol	S	S	S	S	S	S	S	S	±	–	30 μg
Cycloserine	R	R	R	R	R	R	R	R	R	R	2 μg
Novobiocin	S	S	S	S	S	S	S	S	±	–	30 μg
Amphotericin B	R	R	R	R	R	R	R	R	R	S	30 μg
Nystatin	R	R	R	R	R	R	R	R	R	S	25 units
Sulfadiazine	R	±	R	R	R	R	R	±	±	±	300 μg
Sulfadiazine / Sulfamerazine / Sulfamethazine	R	±	R	R	R	R	R	±	±	±	300 μg
Sulfadimethoxine	R	R	R	R	±	±	±	±	±	±	300 μg
Sulfamerazine	R	±	R	R	R	R	R	±	±	±	300 μg
Sulfamethoxypyridazine	R	±	R	R	R	±	±	±	±	±	300 μg
Sulfamethylthiadiazole	R	±	R	R	R	±	R	±	±	±	300 μg
Sulfathiazole	R	±	R	R	R	±	±	±	±	–	300 μg
Sulfisomidine	R	±	R	R	R	±	±	±	±	–	300 μg
Sulfisoxazole	R	±	R	R	R	±	±	±	±	±	300 μg
Furaltadone	S	S	S	S	S	S	S	±	S	–	300 μg
Furazolidone	S	S	S	S	S	S	S	R	S	R	50 μg
Nitrofurantoin	S	S	S	S	S	S	S	R	S	R	50 μg
Nitrofurazone	S	S	S	S	S	S	S	R	S	R	50 μg
Methenamine mandelate	R	S	S	S	±	±	±	±	±	–	3,000 μg
Triclobisonium chloride	S	S	S	S	S	S	S	S	S	S	1,000 μg
p-Aminosalicylic acid	R	R	R	R	R	R	R	R	R	–	100 μg
Isonicotinic acid hydrazide	R	R	R	R	R	R	R	R	R	–	25 μg

* S = inhibition of test organism at all concentrations tested; R = organism resistant at all concentrations tested; ± = organism inhibited by higher concentrations but not by lower ones; – = not tested.

† Generic names of agents available under a number of trade names.

TABLE 2. *Effects of selected antibiotics on growth of milky disease bacteria and other microorganisms in shaken-tube liquid culture**

Antibiotic	Amt added	*Bacillus popilliae* NRRL B-2309	*B. lentimorbus* NRRL B-2522	*B. subtilis* NRRL B-765	*Sarcina lutea* NRRL B-1018	*Escherichia coli* NRRL B-766	*Saccharomyces pastorianus* NRRL Y-139
	μg/ml						
Vancomycin hydrochloride	0	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
	1	+	±	−	−	+	+
	5	±	−	−	−	+	+
	10	±	−	−	−	+	+
	50	±	−	−	−	+	+
	100	±	−	−	−	+	+
Ristocetin A and B sulfates	0	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
	1	+	±	−	−	+	+
	5	+	−	−	−	+	+
	10	±	−	−	−	+	+
	50	±	−	−	−	+	+
	100	−	−	−	−	+	+
Actinomycin D	0	+	+	+	+	+	+
	0.1	−	±	−	−	+	+
	1	−	−	−	−	+	+
	5	−	−	−	−	+	+
	10	−	−	−	−	+	+
	50	−	−	−	−	NT	+
	100	−	−	−	−	NT	+
Cycloheximide	0	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
	1	+	+	+	+	+	−
	5	+	+	+	+	+	−
	10	+	+	+	+	+	−
	50	+	+	+	+	+	−
	100	+	+	+	+	+	−

* + = growth (per cent transmittance at 600 mμ) equivalent to control with no antibiotic; ± = partial inhibition of growth; − = complete inhibition of growth; NT = not tested.

RESULTS AND DISCUSSION

Sensitivity-disc tests. Results obtained with the sensitivity-disc tests (Table 1) suggest that it might be possible to include selected agents in growth media for the milky disease bacteria at concentrations which did not inhibit milky disease bacteria but did inhibit some other organisms. These agents were vancomycin, ristocetin, amphotericin B, nystatin, and possibly some of the sulfa compounds. The effects of the two antifungal agents amphotericin B and nystatin, of course, were not unexpected and suggest that cycloheximide also could be used for control of fungal contaminants. In previous experiments in which Japanese beetle larval hemolymph was plated for recovery of injected milky disease bacteria, contamination with fungi had been a problem. It appears that any of the antifungal antibiotics could be added to cultivation or plating media for control of fungal contaminants.

It was not possible to repeat the sensitivity-disc tests with M-H medium rather than the high-phosphate medium used. The relatively high concentration of yeast extract in the latter might have affected the results of some of these tests. Possibly some of the sulfa compounds might also exert activity against the milky disease bacteria when grown in M-H medium recommended for testing these compounds.

Because of the unusual resistance of the milky disease bacteria to vancomycin and ristocetin, shaken-tube tests were run with these antibiotics and with cycloheximide and actinomycin D. The results of the shaken-tube tests are given in Table 2.

Inhibitory levels of vancomycin hydrochloride and ristocetin A and B sulfates were lower than expected from the results of the sensitivity-disc tests. Nevertheless, it appears that these two antibiotics could be added to culture media at relatively low levels without causing undue inhibition of cell populations of milky disease bacteria and, at the same time, could exert some control over contaminants. Cycloheximide, and

probably the two antifungal antibiotics used in the sensitivity-disc tests, could be added at rather high levels to either liquid or plating media to prevent development of fungi.

Examination of some of the liquid cultures of *B. popilliae* NRRL B-2309 which contained sublethal concentrations (0.1 to 1 μg/ml) of vancomycin hydrochloride and ristocetin A and B sulfates showed that morphological changes had occurred in some of the cells. These changes appeared to correspond to certain of the gross changes that occur in sporulating cells of this organism in larval hemolymph, i.e., central swelling of the cell, rounding-off of one tip and narrowing of the other tip of the cell. The central area in some cells contained partially refractile material. No parasporal bodies, characteristic of *B. popilliae*, were noted. Marked elongation of cells (filament formation) was seen with higher concentrations of the antibiotics; at intervals along the filaments there were swollen cells. It has been reported that the primary site of action of vancomycin is the cell wall (Russel, 1964). The partial resistance of the milky disease bacteria to this antibiotic and to the ristocetins suggests that the walls of these bacteria are different from those of other sporeforming bacilli. Because these antibiotics in low concentration elicited gross morphological changes, some effect on the cell wall or cell membrane possibly occurs.

In general, the milky disease bacteria exhibit the same sensitivities to antibiotics as do other gram-positive bacteria. The results with the sensitivity-disc and shaken-tube tests further suggest that species and strains of these bacteria differ quantitatively in their response to these antibiotics.

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