Genetic Studies on Microbial Cross Resistance to Toxic Agents IV. Cross Resistance of *Bacillus megaterium* to Forty-Four Antimicrobial Drugs[1]

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It has been shown in previous studies with gram negative (*Escherichia coli*), gram positive (*Micrococcus pyogenes* var. *aureus*), and acid-fast (*Mycobacterium ranae*) bacteria, that strains selected for resistance to a given toxic agent may, as a result, acquire several new properties (Szybalski, 1953b; Szybalski and Bryson, 1952, 1954). They may change their sensitivity to other agents to which they have never before been exposed. Thus, sensitivity may be increased (collateral sensitivity), decreased (cross resistance), or remain unchanged.

The following investigation employs *Bacillus megaterium*, a spore-forming bacillus representing a bacterial type hitherto not included in our analyses of cross resistance. This species is characterized by high sensitivity to many antibacterial agents, simple growth requirements, fast growth, formation of large cells suitable for cytological studies (DeLamater, 1951) and easy isolation of the resistant mutants. It was also planned to select several mutant strains having clear, independent and neutral markers. These genetic markers would be useful for testing the possibility that *B. megaterium* undergoes genetic recombination similar to *Escherichia coli* strain K-12. The search for recombination was motivated by cytological studies of DeLamater and Hunter (1953), who observed formation of "fusion tubes" between cells of *B. megaterium*, suggesting a sexual process.

This type of study affords opportunity for the selection of some mutants which are not related by cross resistance. In addition, it permits the allocation of the antibiotics of undetermined structure to established groups of biologically similar or identical substances.

MATERIALS AND METHODS

A strain of *Bacillus megaterium* obtained from Dr. E. D. DeLamater, University of Pennsylvania, (DeLamater and Hunter, 1953) was employed in these experiments. Bacterial stocks were grown in small (1-oz) screw-cap bottles filled with double strength nutrient broth[3] (16 grams per liter; Difco) and aerated by agitation with a wrist action shaker at 34 C. Heavy growth (up to 6×10^8 cells per ml) was obtained 6 to 8 hours after seeding with heavy, fresh inoculum. The comparatively large size of *B. megaterium* made it impractical to further increase the cell titers.

The following antimicrobial agents were employed: amicetin, bacitracin, carbomycin, catenulin, chloramphenicol, chlortetracycline,[4] cinnamycin, circulin, dihydrostreptomycin, erythromycin, formaldehyde, furadroxyl, hydroxystreptomycin, illudin M and S, isoniazid, licheniformin B, micrococcin, mycomycetin, neomycin, nicotinaldehyde, thiosemicarbazone, nisin, oxytetracycline,[5] PAS (sodium *p*-aminosalicylate), patulin, penicillin, pleocidin, pleuromutilin, polymyxin B, streptomycin, streptothricin, subtilin, tetracycline, thiolutin, thiolutin-like antibiotic (Sharp and Dohme), tyrocidine, thioaurin, vinactin, viomycin, xanthomycin, and four other antibiotics identified by their code names: PA-89 (Pfizer), X-206, X-464 and X-537-A (Hoffmann-LaRoche).[6]

Stock solutions of a majority of these substances were prepared at concentrations of 10 mg per ml, using demineralized water as a solvent. Low solubility or a very scarce supply of chloramphenicol, erythromycin,

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[3] Several experiments were performed to determine the best medium for the cultivation of *B. megaterium*. It was found that double strength nutrient broth supplies enough nutrient and has sufficient buffering capacity to provide for fast bacterial growth with minimal death rate. In more diluted broth, and especially when glucose was added, the highest obtainable titers were up to 10^8 cells per ml with viability decreasing rapidly after prolonged incubation, so that the majority of the cells were dead within two days. Addition of sugar also decreased the total count on agar plates. It increased, however, the rate of growth of the colonies and their mucosity.

[4] Generic name for aureomycin (Trademark of Lederle Laboratories Division).

[5] Generic name for terramycin (Trademark of Chas. Pfizer & Co., Inc.)

[6] Most of these antibiotics have been listed by Baron (1950) and Benedict (1953). The source of supply is indicated in the acknowledgments at the end of this paper.

furadroxyl, illudins, micrococcin, pleuromutilin, thiolutins, tyrocidine, thioaurin, PA-89, and X-antibiotics compelled the use of less concentrated stock solutions or nonsterilized fine suspensions. With the exception of penicillin, oxytetracycline and chlortetracycline, the Seitz-filtered solutions were adequately stable at a temperature of about 1 C. To improve stability, the pH of the solutions was adjusted to 6.5 for penicillin and 1.5 for chlortetracycline and oxytetracycline; they were kept at about −15 C until the day of actual use. All plating was done using nutrient agar of the following composition: nutrient broth, 8 grams; yeast extract, 1 gram; sodium chloride, 5 grams; NZ-amine A, 5 grams; and agar, 20 grams per 1 liter demineralized water. NZ-amine A was supplied through the courtesy of Sheffield Farms and all other components were commercial products of Difco Laboratories, Inc. Ten-ml portions of this melted medium were distributed to ½-oz prescription bottles (Armstrong-Cork Company, GX-½, black screw caps) using an automatic pipetting machine, autoclaved and kept in a water bath (about 50 C). Appropriate amounts of antibiotic solution were added to these bottles and the contents were mixed and poured into Petri dishes as required. The gradient plate technique was used both for developing resistant strains and for determining their comparative resistances. These procedures have been described in previous publications (Szybalski and Bryson, 1952).

Results

Isolation of Resistant Strains

Employing the gradient plate technique, it was always possible to obtain growth of several colonies beyond the boundary of total inhibition for the majority of the bacterial population. The proportion of these apparently resistant colonies was large for some toxic agents (for example 10^{-3} to 10^{-1} per cent of *B. megaterium* colonies were isoniazid-resistant) and very small for others (for example 10^{-8} to 10^{-6} per cent for erythromycin resistance), employing a population up to 10^{10} cells divided between several identical plates. These apparently resistant colonies, however, were in several cases just as sensitive as the parental strain when tested against the same antibiotic on another gradient plate.

The bold face figures on the diagonal of table 1 indicate the increase in resistance of the resistant strains of *B. megaterium*.

It was not possible to select strains of *B. megaterium* permanently resistant to formaldehyde, furadroxyl, nicotinaldehyde thiosemicarbazone, nisin, patulin, penicillin, subtilin, thioaurin, and xanthomycin. Strains transferred several times on gradient plates containing these agents and represented on the table by the symbols Bm/FO, Bm/FU, Bm/NTS, Bm/NI, Bm/PT, Bm/PN, Bm/SB, Bm/TA, Bm/XA, do not show significant changes in their sensitivity to any agents tested. A pronounced effect of inoculum size was observed in assaying the activity of penicillin, subtilin, and xanthomycin. This may have been one of the factors contributing to failure in the isolation of strains resistant to these antibiotics. An alternative explanation is that the resistant mutants are extremely rare or entirely absent in the tested population of *B. megaterium*, or that the wild strain shows the upper potential level of resistance.

By repeated restreaking on the gradient plates, it was not possible to obtain strains with permanent resistance increased more than twofold to the following substances: chloramphenicol, circulin, pleuromutilin, polymyxin B, thiolutin, thiolutin-like antibiotic and tyrocidine.

A high degree of resistance without impaired viability and morphology was exhibited by strains resistant to amicetin, bacitracin, cinnamycin, erythromycin, tetracyclines and streptomycins. Resistance to *p*-aminosalicylic acid and to isoniazid develops in one clear-cut step; the initial resistance of *B. megaterium*, however, is rather high and resistant mutants are very frequent. It also has been observed that several satellite colonies appear around the PAS-resistant colonies after prolonged incubation. The delay in appearance of this satellite growth depends on the concentration of PAS, indicating decomposition of this chemical. The genetic use of micrococcin, illudin M and X-537-A resistant strains was prevented by the low solubility and great scarcity of these antibiotics. Resistance to many of the antibiotics was accompanied by more or less profound changes in the morphology and viability of *B. megaterium*.

Studies of Cross Resistance

The degree of cross resistance was determined by assaying the sensitivity of each resistant strain against the entire series of antibiotics through the use of the gradient plate technique. The "factor of resistance" is the quantitative value used for describing the degree of cross resistance. It is the ratio of the sensitivities of the resistant and parental strains. The figures in table 1 represent the factor of resistance. Increased sensitivity is shown as a fraction. The table is organized as in previous publications on cross-resistance patterns of *Escherichia coli*, *Micrococcus pyogenes* var. *aureus* and *Mycobacterium ranae* (Szybalski and Bryson, 1952, 1954; Szybalski, 1953b). The top row indicates the concentration of toxic agents required to inhibit the parental strain of *B. megaterium*. For instance, it is seen from the table that a strain Bm/CB with 20 times increased resistance to carbomycin (the diagonal of the table) is 3 times more resistant to erythromycin and 4 times more sensitive (¼) to neomycin. The inhibitory concentrations of carbomycin, erythromycin and

TABLE 1. *Cross-resistance relationship of certain anti-bacterial agents as tested with* Bacillus megaterium

STRAIN	ANTIBIOTIC																																										
	Micrococcin	Bacitracin	Nisin	Subtilin	Tyrocidine	Polymyxin B	Circulin	Licheniformin	Vinactin	Viomycin	Streptothricin	Pleocidin	Catenulin	Neomycin	Streptomycin	Chlortetracycline	Tetracycline	Oxytetracycline	Erythromycin	Carbomycin	Pa-89	Thiolutin	Thiolutin-like	Thioaurin	Cinnamycin	Amicetin	Mycomycetin	X-206	X-464	X-537-A	Illudin M	Illudin S	Pleuromutilin	Chloramphenicol	Xanthomycin	Penicillin	Patulin	Isoniazid	Sodium *p*-amino-salicylate	Nicotinald thio-semicarbazone	Furadroxyl	Formaldehyde
Bm/MI	>6†	10	4	1	1	1	1.1	1	1	1	1	1	1	1	1	1.1	1	1	1	2	1	1	1	1	1	1	1	1	1	1.5	1	1	1	1	1	1	1	1.1	1	1
Bm/BC	1	**100**	4	1.5	1	1.1	1	1	1.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1/1	1	1	1	1	1	1.1	1	1	1	1	1	1	1	1
Bm/NI	1	1	**1**	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/SB	1	1	1	**1**	1.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1	2	1	1	1.1	1	1	1	1	1	1	1	1	1	1
Bm/TR	1	1.1	1.1	6	**2**	1	1	1	1	1.1	1	1	1.1	1	1.1	1	1	1	1	1	1	1	1	1	1	1	3	2	5	1.5	2	1	½	1	1	½	1	1	1	1	1
Bm/PB	1.1	1	1	1.5	2	**2**	1.1	1	1*	1*	2*	3	1.1	1.5	3	1	1	1	½	1	1	1	1	1	1	1.1	1.1	1.1	1.1	1.1	1	1	1	1	1	1	1	1	1	1	1
Bm/CR	1	1	1	1	2	1.1	**2**	1.1	1	2	5	3	3	4	1	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1.1	1	1	1	1	1	1
Bm/LI	1.5	½	1.1	2	4	1	2	**5**	4	5	6	5	3	2	3	½	1	1	½	1	1	1	1	1	1	1	1	3	2	1	1.1	1	1	1	1	1	1	1	1	1	1
Bm/VI	1	½	1	2	1	1.1	2	8	**3**	5	6	5	3	4	6	½	1	1	½	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1
Bm/VM	1	½	1	1	1	1	2	4	3	**3**	3	4	2	2	4	½	½	1	½	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	½	1	1	1	1	½	1
Bm/ST	½	1	½	1	1	1	½	10	10	4	**4**	3	5	8	4	1.1	1	1	½	½	1	1	½	½	1	1	⅓	1	1	1	⅓	1	1	1	1	1	1	½	1	1	1
Bm/PC	1	1	1	1	1	1.1	1.1	>30	12	10	8	**10**	10	15	4	1.1	1	1	1	½	1	1	1	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1
Bm/CT	1.1	1	1.5	1	1	1.1	1.1	8	10	5	5	8	**10**	10	10	1	1	½	1	½	½	1.1	1.1	1	1	1	1	½	½	½	1	1	1	½	1	1	½	3	1	1	½	1
Bm/NM	1	10	1	1	1	1.5	2	10	10	5	4	3	6	**10**	4	1	1	1	½	1	1	½	1	1	1	1	1	1	1.5	1	½	1	1	1	1	1	1	1	1	½	½
Bm/SM	1	8	½	1	1	5	4	10	20	12	8	10	10	10	>900	1	1	1	½	½	1	1	½	1	1	1	½	1	1	½	½	1	1	1	1	½	1	1	1	1	½
Bm/AU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**10**	10	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/TC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	10	**15**	10	4*	1*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/TM	1	10	1	1	1	1	1	1	1	1	1	1	1	1	1	20	10	**10**	3*	1.1	1	1	1	½	1	1	1	1	1	1	½	1	1.1	1	1	1	1	1	1	1	1
Bm/ER	1	1.5	1	1	1	½	½	1	½	1½	1	1	1	1	1	10	10	10	**150**	1	1	1	½	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/CB	1	1	⅓	1	1	½	½	⅓	¼	¼	¼	¼	¼	¼	½	1	1	1	3	**20**	5	1	1	1	1	1	1	1	1	1	½	½	1	1	1	¼	1	1	1	½	1½
Bm/P8	2	1	¼	1	1	⅓	⅓	⅙	⅛	⅓	¼	¼	⅓	⅙	1.1	1.1	10	10	3*	5	>20†	2	2	1	1	4	3	2	1.5	2	½	½	1	1	1	1	5	1	1.1	½	1
Bm/TL	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1.1	1	1	1	1	1	**2**	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/TLL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	**2**	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/TA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/CI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	>200	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bm/AM	1	1	1.1	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	>20	1	½	1	1	1.1	1	1	1	1	½	1	½	1	1	1
Bm/MM	1	20	1	1.5	1	1	1	2	1	1	1	1	1	1	1.5	1	1	1	1	1	2	1	1	1	1	1	**20**	2	2	1	1	½	1	1.1	1	1	1	1	½	1	1
Bm/X2	1	1.1	1.1	8	2	1	1	1	1	1.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	**3**	10	6	1	1	1.1	1	1	1	1	1	1	1	1
Bm/X4	1	1.1	1.1	8	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	4	**12**	8	1	1	1	1	1	1	1	1	1	1	1
Bm/X5	1	1	1	1	2	1	1	1.1	1	1	1	1	1	1.5	1.5	1	1	1	1	1	1	1	1	½	½	1	2	>4†	>20†	>10†	½	½	1	1	1	1	1	1	1	1	1
Bm/IM	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**3**	>20	1	1	1	1	1	1	1	1	1
Bm/IS	1	2	1.1	1	1	1	⅓	½	½	½	½	½	½	1	½	1	1	1	1	1	1	1	½	1	1	1	½	1	1	1	5	**>20**	1	1	1	1	1.5	5	1	1	1
Bm/PL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.1	1	1	1	1	1	1	1	1	1	1	**2**	1	1	1	1	1.5	1	1	1
Bm/CM	1	1	1	2	1	1	½	1	½	⅓	1	1	1	½	1	1	1	1	1	1	1	2	½	½	1	1	1	1	1	1	½	½	3	**2**	1	1	1	1	1	1	½
Bm/XA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**	1	1	1	1	1	1
Bm/PN	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**	1	1	1	1	1
Bm/PT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**	1	1	1	1
Bm/INH	1	1	1.5	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1.5	1	1.1	1	1	1	1	**30**	1	1	1
Bm/PAS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**20**	1	1
Bm/NTS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**	1
Bm/FU	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
Bm/FO	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**1**
Sensitivity (μg/ml)	.5	.05	.2	.2	.5	1.5	6	.3	10	10	8	.8	.3	.04	.7	.3	1.5	.8	.2	.8	4	.4	.2	2	.2	5	3	.3	.4	.6	10	.1	10	1.5	.1	20	5	400	400	800	6	20

* Relatively high proportion of cells with a resistance factor higher than indicated on the table.

† Growth in saturated solution.

Figures represent the factor of resistance (ratio of the sensitivities of the resistant and parental strains = fold increase) compared with that of parental strain set as a unit. Values larger than 1 indicate an increase of resistance; fractions indicate a decrease of resistance (collateral sensitivity). The symbol 1.1 represents slightly (1.1 to 1.5 times) increased resistance; and the symbol 1/1, slightly increased sensitivity; other figures are also rough estimates. The values in the bottom row are the concentrations of the agents, heading each vertical column, required to inhibit the parental strain. Resistant derivatives of *B. megaterium* are listed in the first vertical column in the same sequence as the antibacterial agents heading the table; resistance is conventionally represented by a bar. Figures on the diagonal of the table show the resistance factor of each of these experimental strains to the agent used in its selection.

neomycin for Bm/CB strain are, in this case, 16 (20 × 0.8), 0.6 (3 × 0.2) and 0.01 (¼ × 0.04) μg per ml, respectively.

The data have been tabulated so as to place the agents related by cross resistance in proximity to each other and thereby reveal their relationships. The relationships will be discussed in the next section of this paper and compared with the results obtained with similar or different organisms both in our previous experiments and in the investigations of other workers.

Discussion

Several conclusions may be derived from the present work, generally confirming those previously obtained with gram negative rods, gram positive cocci, and acid-fast bacteria, although differing in certain details. Also, a considerably wider range of antibacterial agents has been used.

The various studies employing several bacterial species have been discussed in previous papers (Szybalski, 1953b; Szybalski and Bryson, 1952, 1954). No references have been found describing cross-resistance studies employing *B. megaterium*, and only Kaipainen (1951) has used a spore former (*Bacillus subtilis*) in studies of the relationship between chlortetracycline, oxytetracycline, chloramphenicol, streptomycin and penicillin. Rather irregular results were obtained, with pronounced cross resistance noted only between chlortetracycline and oxytetracycline.

B. megaterium is rather sensitive to many toxic agents to which other bacteria previously tested were either insensitive, or only selectively sensitive. There remains, however, a considerable number of substances to which resistance either fails to develop or develops to an insignificant extent. This fact is a severe handicap in the identification of some antibiotics by means of cross-resistance studies. The difficulty could perhaps be overcome in some cases by the use of other bacterial variants or species exhibiting higher sensitivity to a given toxic substance and producing clear-cut resistant mutants. *E. coli*, *M. pyogenes* var. *aureus* and *B. megaterium*, for instance, are not properly suited for differentiating between viomycin and other antibiotics of the streptomycin-streptothricin-neomycin group. These species are comparatively insensitive to viomycin; strains resistant to this antibiotic have very markedly decreased viability and rate of growth, and show increased resistance to all other drugs of the viomycin-streptomycin-streptothricin-neomycin category (Szybalski, 1953b; Szybalski and Bryson, 1952). Acid-fast *Mycobacterium ranae* is much more satisfactory for comparing the members of this group. Its sensitivity to viomycin, streptomycin, neomycin and catenulin is of a similar order; it produces mutants highly resistant to these drugs with unimpaired growth properties and shows selectively different patterns of cross resistance which easily differentiate viomycin-vinactin on one hand and neomycin, catenulin, streptomycin and streptothricin on the other (Szybalski and Bryson, 1954). The same *M. ranae*, however, would be very poorly suited for comparing differences and similarities between tetracyclines, erythromycin, carbomycin and chloramphenicol, primarily because of low sensitivity and the lack of highly resistant mutants. *E. coli* or *B. megaterium* would be much better suited for this purpose. It may be concluded that a bacterial mutant resistant to one agent is generally resistant to all other chemically and biologically related toxic substances. However, on occasion it is possible to isolate specific resistant mutants which show small but definite differences in response to such similar substances as streptomycin and N′-α-hydroxypropylstreptomycylamine (Treffers *et al.*, 1953) or tetracyclines (Szybalski and Bryson, 1952).

The studies with *B. megaterium* reveal several groups of antibiotics related by cross resistance. As in previous investigations of this series, the largest group is composed of streptomycins, neomycin, catenulin, pleocidin, streptothricin, viomycin, vinactin, licheniformin, polymyxin B and circulin. As was found in previous studies, streptomycin, dihydrostreptomycin and hydroxystreptomycin show complete cross resistance and cross dependence and are therefore collectively designated as streptomycins. Pleocidin, which we have not studied before, undoubtedly belongs to this group. Charney *et al.* (1952) who discovered pleocidin classify it as closely related to streptothricin. Dutcher (1953), on the other hand, believes that it is a form of neomycin. Resistant strains of *B. megaterium* do not display enough selectivity to be used for distinguishing between these two possibilities. *M. ranae* may, in this case, be a more appropriate species. Dutcher (1953) subdivides the above-mentioned antibiotics into two classes: a basic, glycosidic group containing streptomycins, neomycins, catenulin and pleocidins; and a basic, polypeptide group consisting of streptothricin, streptolin, viomycin, vinactin, cinnamycin, nisin and amicetin. According to our studies, the last three antibiotics do not show any biological similarity to each other or to the rest of the class. As a group, the basic polypeptides should be augmented to include the biologically similar bacterial polypeptides: licheniformin, circulin and polymyxin B. When studied with *B. megaterium* and *M. pyogenes*, tyrocidine and subtilin, two other bacterial polypeptides, show, in a few cases, a low order cross-resistance relationship to the other basic polypeptides which have been discussed. Bacitracin, micrococcin and nisin do not show any appreciable relationship to other bacterial polypeptides. *B. megaterium* is very sensitive to these three antibiotics. It produces mutants highly resistant to bacitracin and micrococcin, but otherwise apparently unchanged; it is not possible

to isolate any strains resistant to nisin. Similarly *M. pyogenes* and *M. ranae* hardly develop any resistance to nisin, being originally very resistant to this antibiotic. It is interesting to note in the table that although we were unable to isolate nisin- and subtilin-resistant strains directly, an indirect selection was possible. Thus, bacitracin- and micrococcin-resistant strains of *B. megaterium* are four times more resistant to nisin. Similarly, tyrocidine, X-206 and X-464 selected strains are six to eight times more resistant to subtilin than is the parental strain. Using *B. megaterium*, the studies with subtilin were obstructed by a pronounced effect of the inoculum size in the determination of sensitivity (inoculum effect). Resistance to cinnamycin, on the other hand, develops easily and to a very high degree. No biological relationship was noted between cinnamycin and subtilin which are both polypeptides containing a new $C_7H_{14}N_2O_4S$ amino acid (Benedict *et al.*, 1952). Netropsin was not included in these studies because of relative inactivity of this substance against *B. megaterium*. The studies with *M. pyogenes* have related this antibiotic to the group of actinomyces polypeptides.

Tetracycline, oxytetracycline and chlortetracycline are again very closely related, with more pronounced similarity between the spectra of tetracycline and its oxy-derivative. Erythromycin-resistant strains are also resistant to these three antibiotics (see table). Resistance to erythromycin develops easily to a high degree in a single step, but the mutation rate is rather low. Erythromycin-resistant strains, like those resistant to tetracyclines, show full viability and a normal rate of growth. Resistance to carbomycin, on the other hand, results in poorly growing strains. Cross resistance between carbomycin- and erythromycin-resistant *B. megaterium* is unidirectional and of very low order, seemingly contrary to the findings of Finland *et al.* (1952) who reported a complete cross resistance for carbomycin- and erythromycin-resistant staphylococci. Our additional experiments have confirmed that *M. pyogenes* var. *aureus* shows similar patterns of resistance development both to erythromycin and carbomycin. It develops a high degree of resistance in only a few steps with almost complete cross resistance of fully viable resistant strains. *B. megaterium*, however, distinguishes clearly between the action of both these drugs.

Some cross resistance was noted between carbomycin and antibiotic PA-89. Strains resistant to both antibiotics grow very slowly which may be a common reason for their increased resistance to these drugs.

Difficulties in isolating strains resistant to thioaurin, thiolutin and a thiolutin-like preparation prevented a comparison of the biological similarities of these sulphur-containing substances.

Resistance to amicetin develops easily and to a very high degree. This antibiotic, however, is not related to any other antibiotic tested. On the basis of cross-resistance patterns, it was fairly routine to tentatively classify as amicetin several previously unidentified antibiotics supplied by independent laboratories.

In previous studies with *M. pyogenes* (Szybalski, 1953b) it was not possible to isolate strains resistant to antibiotic X-537-A, although isolation of strains resistant to X-206 and X-464 was achieved without difficulty. The last two antibiotics showed pronounced cross resistance, with no evident relationship to X-537-A. *B. megaterium* easily produces strains resistant to all three of the "X" antibiotics, which here prove to be closely related by cross resistance to each other, and in a much lower degree to the unrelated antibiotic, mycomycetin. This is another example of the finding that different bacterial species show different selectivity of their resistant mutants in distinguishing between apparently similar toxic substances.

Strains of *B. megaterium* resistant to illudin M and S show complete cross resistance, as do resistant strains of *M. ranae*. Illudin S is 100 times more active against *B. megaterium* and 5 times less active against *M. ranae* than is illudin M.

B. megaterium develops little resistance against chloramphenicol and pleuromutilin. A strain with two-fold resistance to chloramphenicol is three times more resistant to pleuromutilin, similar to the chloramphenicol resistant or dependent *M. pyogenes*, which showed increased resistance to pleuromutilin or even partial dependence. The inverse relationship does not obtain with either organism.

One-step resistance develops to isoniazid and sodium *p*-aminosalicylate. Strains resistant to these drugs do not show a significant increase in resistance to other toxic agents. The mutation rate to isoniazid resistance is rather high (6×10^{-5} per bacterium per generation) (Szybalski and Bryson, 1953). This may explain why a few strains resistant to other antibiotics showed increased resistance to isoniazid, which was also the case with *M. ranae*. As stated previously, it was not possible to select strains resistant to several other agents as penicillin, xanthomycin, patulin, furadroxyl, formaldehyde and nicotinaldehyde thiosemicarbazone. Neither was it possible to isolate strains of other microorganisms tested that are resistant to some of these substances, but in the case of penicillin and furadroxyl it was easy to obtain strains of *E. coli* and *M. pyogenes* highly resistant to these drugs. For instance, a first step mutant of *E. coli* strain B is 30 times more resistant to furadroxyl and identical with the radiation-resistant strain B/r (Szybalski and Nelson, 1953). The failure in isolating strains resistant to penicillin and furadroxyl within the *B. megaterium* population may be due to its initial high resistance and penicillinase production

in the case of penicillin, or in great rarity or inherent lack of resistant mutants.

Only a few cases of collateral sensitivity (Szybalski and Bryson, 1952) of a higher order were observed. For example, a strain resistant to antibiotic PA-89 was more sensitive to several actinomyces and bacterial polypeptides and to neomycins. The highest increase in sensitivity was ninefold in the case of licheniformin. As previously mentioned, resistant strains often showed a decreased rate of growth which may simulate increased sensitivity in turbidometric assays.

All these studies indicate that controlling the development of bacterial resistance to antibiotics is not such a hopeless problem as has sometimes been stated. Extensive investigations indicate that certain bacteria do not produce resistant mutants to certain antibiotics, or that the production of such mutants is highly improbable. The other encouraging fact is that some resistant strains lose their vigor, viability and virulence (for example: isoniazid-resistant tubercle bacilli—Middlebrook and Cohn, 1953; "artificial" penicillin-resistant staphylococci—Szybalski, 1953a). This loss may become even more evident in the case of multiple-resistant mutants. *In vivo*, however, a sufficiently long selection process might produce new resistant mutants of improved growth characteristics and restored virulence. In the absence of the toxic substance, or in the presence of an unrelated drug, resistance is often lost after several subcultures due to selective overgrowth of better suited mutants which, in this case, may be less resistant and sometimes even more sensitive (collateral sensitivity) to the agent previously employed. All the facts, together with the use of simultaneous multiple chemotherapy, indicate that bacteria may be controlled effectively when a sufficient number of unrelated antibiotics becomes available. This poses two additional problems: search for new effective antibiotics and proper use of the variety of substances now at our disposal.

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Summary

An attempt has been made to isolate strains of *Bacillus megaterium* resistant to 44 toxic agents, to screen out any strains that might conveniently be utilized for genetic studies, and to determine patterns of cross resistance.

First-step mutants were isolated, with considerably increased resistance to amicetin, bacitracin, cinnamycin, erythromycin, illudin S, isoniazid, micrococcin, PAS, streptomycins, tetracyclines and X-537-A. Strains of *B. megaterium* resistant to several other toxic substances were less suitable for genetic studies either as a consequence of poor viability or because of an insufficient development of resistance. On the basis of

cross-resistance patterns the substances have been divided into the following classes:

The first group of basic glycosides and polypeptides includes streptomycins, neomycin, catenulin, pleocidin, streptothricin, viomycin, vinactin, licheniformin, polymyxin B and circulin. The other bacterial and actinomyces polypeptides or polypeptide-like antibiotics (micrococcin, bacitracin, nisin, subtilin, tyrocidine, cinnamycin, amicetin and mycomycetin) do not show any significant cross-resistance relationship to this first group.

The second group consists of tetracycline, chlortetracycline, oxytetracycline, and the less closely related substances, erythromycin, carbomycin and antibiotic PA-89.

Close relationships are exhibited within the third group composed of illudin M and S and within the fourth group, which includes the three X-antibiotics (X-206, X-464, X-537-A) and the more distantly related mycomycetin.

Chloramphenicol and pleuromutilin are related by a low degree, unidirectional cross resistance. Inadequate resistance development makes the comparison of thiolutins and thioaurin difficult. No increase in resistance was observed in *B. megaterium* exposed to xanthomycin, penicillin, nicotinaldehyde thiosemicarbazone, furadroxyl and formaldehyde, and no other groups of pronounced cross resistance were observed.

This paper summarizes the results of previous studies on cross resistance which employed representative species of gram negative, gram positive and acid-fast bacteria (Szybalski and Bryson, 1952, 1954; Szybalski, 1953b) and integrates them with the present work on the spore-forming *B. megaterium*.

The implications of these studies on the identification of unknown antibiotics and on the planning of suitable measures to prevent development of bacterial resistance are discussed.

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