STUDIES ON BACTERIAL CELL WALL INHIBITORS*

VI. SCREENING METHOD FOR THE SPECIFIC INHIBITORS OF PEPTIDOGLYCAN SYNTHESIS

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A screening method was established for selecting new specific inhibitors of bacterial cell wall peptidoglycan synthesis. In the primary test, culture broths of soil isolates were selected based on relative microbial activity. A culture, to be retained, must be active against *Bacillus subtilis* and lack activities against *Acholeplasma laidlawii*. In the secondary test, inhibitors of bacterial cell wall synthesis were identified by their ability to prevent the incorporation of *meso*-[^{3}H]diaminopimelic acid but not to prevent the incorporation of L-[^{14}C]leucine into the acid-insoluble macromolecular fraction of growing cells of *Bacillus* sp. ATCC 21206 (Dpm$^-$). As the tertiary test, inhibitors with molecular weights under 1,000 were selected by passage through a Diaflo UM-2 membrane. By this screening procedure, six known antibiotics and one new one were picked out from ten thousand soil isolates.

A large number of antibiotics have been discovered in the past four decades, and many infectious diseases have been conquered by using antibiotics as chemotherapeutics. Additional useful antibiotics, however, are needed for the treatment of disease caused by drug-resistant microbes, of super infections, and of opportunistic infections. Unfortunately finding a new antibiotic has become more and more difficult.

Among the various types of known antibiotics, specific inhibitors of cell wall peptidoglycan synthesis are characterized in general by their lower toxicities. Consequently we attempted to establish a new screening method for such an inhibitor. The method described in this paper is based on the inactivity of cell wall inhibitors against *Mycoplasma*[1], which lack a cell wall[2,3], and on the inhibition of incorporation of *meso*-diaminopimelic acid (Dpm) into the acid-insoluble macromolecular fraction of *Bacillus*. Dpm is an amino acid present only in the peptidoglycan of Dpm-containing bacteria such as the bacilli. In the primary test, culture broths of soil isolates with activity against *Bacillus* but lacking activity against *Mycoplasma* were selected. The selected culture broths were further examined in the secondary test by determining their effect on incorporation of [^{3}H]Dpm and [^{14}C]leucine into the acid-insoluble macromolecular fraction of a Dpm-requiring strains of *Bacillus* sp. Those culture broths which inhibited the incorporation of [^{3}H]Dpm but did not influence that of [^{14}C]leucine were saved for further study.

In this paper we present the new method with evidence for its applicability.

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Materials and Methods

Mycoplasmal and bacterial strains

Mycoplasma gallisepticum KP-13 and *Acholeplasma laidlawii* PG 8 were obtained from Dr. C. KUNIYASU, National Institute of Animal Health, Japan, and Dr. M. YOSHIOKA, Tokyo Women's Medical College, Japan. *Bacillus* sp. ATCC 21206 (Dpm-requiring strain) was obtained from Tokyo Res. Labs., Kyōwa Hakkō Kōgyō Co., Ltd. *B. subtilis* PCI 219 and *Escherichia coli* NIHJ maintained in our laboratory were also used.

Preparation of seed or stock culture of *M. gallisepticum* and *A. laidlawii*

Eiken's avian PPLO medium was used for growth of *M. gallisepticum*. The PPLO medium (17.3 g) and phenol red (10 mg) were dissolved into water (1 liter) and then sterilized for 15 minutes at 120°C. To 100 ml of the medium, 20 ml of sterilized horse serum and 10,000 units of penicillin G were added and mixed. Each 8 ml of the medium was transferred into a sterilized test tube, inoculated with 2 ml of stock culture of the organism, and then incubated at 37°C for 24 hours. The culture was stored at −70°C. Phenol red was added to the medium as an indicator of growth of *Mycoplasma*, and penicillin was used to prevent contamination by bacteria. Seed and stock cultures of *A. laidlawii* were prepared with Hokken's PPLO medium by a method similar to that used for *M. gallisepticum*. Though it is known that *Acholeplasma* does not require sterols for growth[4], better growth of this strain of *Acholeplasma* was observed with serum (20% in medium) than without it.

Assay method of antimicrobial activities

Antimycoplasmal and antibacterial activities were assayed by the paper disc method. Eiken's avian PPLO medium (17.3 g), phenol red (10 mg) and agar (12 g) were dissolved into water (1 liter) and sterilized at 120°C for 15 minutes. The medium (6 ml), sterilized horse serum (2 ml) containing penicillin G (10,000 units), and the seed culture of *M. gallisepticum* (2 ml) were mixed and poured into a sterilized Petri dish (9 cm in diameter) to prepare an agar plate. Inhibitory zones could be observed by 24 hours' incubation. Agar plates of *A. laidlawii* in Hokken's PPLO medium were prepared by a similar procedure. Agar plates of bacteria were prepared by the conventional method using a medium (pH 7) containing peptone (0.5%), meat extract (0.5%) and agar (1.0%).

Incorporation of [^{3}H]Dpm and [^{14}C]leucine into acid-insoluble macromolecular fraction by growing cells of bacteria

Cells of a fresh slant culture of *B. subtilis* PCI 219 or *Bacillus* sp. ATCC 21206 (Dpm$^-$) incubated overnight at 37°C were suspended in 5 ml of a medium (0.5% peptone, 0.5% meat extract, 200 μg/ml L-lysine, 10 μg/ml Dpm, pH 7.0). The cell suspension was transferred into 10 ml of the medium in a Monod tube to give an absorbance of 0.05 at 660 nm, and then incubated at 37°C until the absorbance reached 0.2. A solution (0.1 ml) containing *meso*-[^{3}H]Dpm (0.1 μCi) and L-[^{14}C]leucine (0.5 μCi) and 0.1 ml of the sample solution were added to 0.8 ml of the culture and incubated for 10 minutes at 37°C with aeration. Growth was stopped by adding 5% trichloroacetic acid (TCA) into the culture. The acid-insoluble fraction was collected on a membrane filter (pore size, 0.22 μ) and washed twice with 5% TCA. The radioactivity of the acid-insoluble fraction on the filter was counted with a liquid scintillation counter using toluene scintillation fluid (4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene).

Measurement of passing ratio through Diaflo UM-2 membrane

After discarding about 0.5 ml of the initial filtrate through a Diaflo UM-2 membrane, the next fraction (1~2 ml) was collected for assay by paper disc method using *B. subtilis* as test organism.

Antibiotics

Antibiotics were generous gifts from the following: actinomycin, novobiocin and streptonigrin from Chas Pfizer & Co., Inc., N. Y., A-16886 A and vancomycin from Lilly Research Laboratories, Indiana, hedamycin from Bristol Laboratories, N. Y., lividomycin from Kōwa Co., Ltd., Tokyo, macarbomycin from Institute of Microbial Chemistry, Tokyo, polyoxin from Kaken Chemical Co., Ltd., Tokyo, and viomycin from Sankyo Co. Ltd., Tokyo. Bacillin, cerulenin, iyomycin, kinamycin,

leucomycin and mitomycin were isolated in our laboratory. Others were stock materials of our laboratory or obtained commercially.

Radiochemicals

L-[U-^{14}C]Leucine (330 mCi/mmol) and 2,6-[G-^{3}H]diaminopimelic acid (300 mCi/mmol) were obtained from the Radiochemical Center, Amersham, U. K.

Results and Discussion

Antimycoplasmal Activity of Antibiotics

Antimycoplasmal and antibacterial activities of various antibiotics were compared to determine whether comparative activity can be useful as the primary test for screening for specific inhibitors of the synthesis of bacterial cell wall peptidoglycan. As shown in Table 1, inhibitors of protein biosynthesis or nucleic acid synthesis, such as tetracyclines, macrolides, actinomycin D, iyomycin, dauno-

Table 1. Antimicrobial activities of various antibiotics against *M. gallisepticum*, *A. laidlawii*, *B. subtilis* and *E. coli*.

Antibiotic (100 μg/ml)	Mode of action*	Antimicrobial activity*			
		M. gal.	*A. lai.*	*B. sub.*	*E. coli*
Tetracycline	P	+	+++	+	+
Oxytetracycline	P	++	++	+	−
Leucomycin	P	++	++	+	±
Amaromycin	P	++	+	+	−
Streptomycin	P	−	++	+	++
Dihydrostreptomycin	P	−	++	++	+
Kanamycin	P	−	+	+	++
Kanamycin B	P	−	+	+	++
Lividomycin	P	−	−	+	+
Bluensomycin	P	−	+	+	+
Viomycin	P	−	−	+	+
Colistin	M	−	−	−	+
Actinomycin D	N	+++	+++	++	−
Iyomycin	N	+	+	+	−
Daunomycin	N	+	+	+	−
Streptonigrin	N	+	++	+	+++
Mitomycin C	N	++	+++	++	+++
Hedamycin	N	++	+	+	+
Kinamycin D		+	+	+	+
Cerulenin	L	+	−	++	++
Azalomycin F		−	+	+	−
Xanthomycin		+	+	+	−
Penicillin G	W	−	−	+	−
D-Cycloserine	W	−	−	+	−
Vancomycin	W	−	−	+	−
Ristocetin	W	−	−	+	−
Cephaloridine	W	−	−	++	+

* P: Inhibition of protein synthesis
N: Inhibition of nucleic acid synthesis
M: Interference with cytoplasmic membrane
L: Inhibition of lipid synthesis
W: Inhibition of cell wall synthesis

** −: negative, +: 10~20 mm, ++: 20~30 mm, +++: 30~40 mm of diameter of inhibitory zone.

mycin, streptonigrin, mitomycin C and hedamycin, were active against both mycoplasmas and bacteria. Penicillin G, D-cycloserine, vancomycin, ristocetin and cephaloridine, all inhibitors of bacterial cell wall synthesis, were active against *B. subtilis* but did not affect the growth of both *Mycoplasma* and *Acholeplasma*. *M. gallisepticum* KP-13 was not sensitive to aminoglycoside antibiotics (100 μg/ml) such as streptomycin, kanamycin and so on, but these antibiotics that are inhibitors of protein synthesis were active against *A. laidlawii* PG 8. Some antibiotics such as lividomycin, viomycin and colistin were found to be inactive against the mycoplasmas at the concentrations tested.

From these results, it was ascertained that cell wall inhibitors could be selected in the primary screen by picking out antibacterial substances that were inactive against mycoplasmas. Antibiotics other than the cell wall inhibitors, which were inactive against mycoplasmas, could be eliminated by the following procedure.

Influence of Antibiotics on Incorporation of [^{3}H]Dpm and [^{14}C]Leucine into Acid-insoluble Fraction of Bacterial Cells

In order to eliminate antibiotics that are not inhibitors of cell wall synthesis, effects of these antibiotics on incorporation of precursors of macromolecules biosynthesis were examined. *B. subtilis* PCI 219 and *Bacillus* sp. ATCC 21206 (Dpm$^-$), containing *meso*-Dpm in peptidoglycan of cell wall, were used as test organisms and *meso*-Dpm and L-leucine were used as precursors of macromolecules. As shown in Table 2, incorporation of [^{3}H]Dpm into the acid-insoluble fraction of *B. subtilis* PCI 219 was about one-tenth of that in *Bacillus* sp. ATCC 21206 (Dpm$^-$). This difference can be explained by the fact that Dpm normally permeates through bacterial cell membrane with difficulty. So, the latter strain with the better incorporation was selected as the test organism. Next the medium and cultural conditions for *Bacillus* sp. ATCC 21206 (Dpm$^-$) were standardized, as described in "Materials and Methods", so that adequate incorporation of the isotopes might be observed. L-Lysine was added to the medium to prevent the formation of labeled L-lysine from [^{3}H]Dpm. In the selected experimental method, incorporation of both Dpm and leucine into *Bacillus* sp. ATCC 21206 (Dpm$^-$) was observed to be adequate, as shown in Table 2. It was also observed that penicillin G prevented only the incorporation of [^{3}H]Dpm while chloramphenicol only prevented that of [^{14}C]leucine.

Table 2. Effects of penicillin G and chloramphenicol on incorporation of [^{3}H]Dpm and [^{14}C]leucine into the acid-insoluble fraction in *B. subtilis* PCI 219 or *Bacillus* sp. ATCC 21206 (Dpm$^-$)

	Antibiotic	Concentration μg/ml	Incorporation, cpm (%)	
			[^{3}H]Dpm	[^{14}C]Leucine
Bacillus subtilis PCI 219	—	—	212 (100)	2,684 (100)
	Penicillin G	10	222 (105)	2,930 (109)
	〃	100	173 (82)	2,851 (106)
	Chloramphenicol	10	257 (122)	744 (28)
	〃	100	174 (82)	199 (7)
Bacillus sp. ATCC 21206 (Dpm$^-$)	—	—	1,873 (100)	2,074 (100)
	Penicillin G	10	678 (36)	2,133 (103)
	〃	100	605 (32)	2,153 (104)
	Chloramphenicol	10	1,706 (91)	596 (29)
	〃	100	1,717 (92)	264 (13)

In order to examine whether the method is useful for differentiating inhibitors of cell wall synthesis from inhibitors of other metabolic function, known antibiotics of various modes of action were tested at various concentrations. As shown in Table 3, incorporation of [^{3}H]Dpm was inhibited while that of [^{14}C]leucine was not prevented by inhibitors of cell wall synthesis. The inhibition of incorporation of [^{3}H]Dpm by moenomycin or macarbomycin was limited, suggesting low sensitivity of the organism to an antibiotic of moenomycin group. The inhibition of incorporation of [^{3}H]Dpm by a β-lactam antibiotic, such as penicillin G, cephaloridine or A-16886 A, seems also to be limited. The incorporation ratios did not decrease under 30% even by 100 μg/ml of these antibiotics. This may be explained by the site of action of these antibiotics; they inhibit cross-linking reaction of nascent peptidoglycan chain, but do not inhibit formation of single chain of peptidoglycan. As shown in Table 4, protein synthesis inhibitors such as kanamycin and gentamicin did not affect the incorporation of [^{3}H]Dpm, while streptomycin, leucomycin and tetracycline prevented it somewhat. In contrast, incorporation of [^{14}C]leucine was inhibited more strongly than that of [^{3}H]Dpm. Colistin and polymyxin B, which interfere with the cytoplasmic membrane of bacterial cells, inhibited the incorporation of both [^{3}H]Dpm and [^{14}C]leucine possibly resulting from disruption of the bacterial cells. Inhibitors of DNA

Table 3. Influence of cell wall synthesis inhibitors on the incorporation of [^{3}H]Dpm and [^{14}C]leucine into acid-insoluble fraction in *Bacillus* sp. ATCC 21206 (Dpm$^-$).

Antibiotic	Concentration (μg/ml)	Incorporation (%)	
		[^{3}H]Dpm	[^{14}C] Leucine
Bacillin (Tetaine)	5	45	103
	50	10	119
	500	9	99
D-Cycloserine	2	97	100
	20	32	103
	200	9	102
Enduracidin	0.1	35	99
	1	9	96
	10	9	93
	100	0	9
Ristocetin	10	8	108
	100	8	90
Vancomycin	10	9	95
	100	4	98
Moenomycin	100	98	105
	1000	59	103
Macarbomycin	10	85	97
	100	52	98
Bacitracin	10	21	92
	100	7	95
Penicillin G	10	36	103
	100	32	104
Cephaloridine	10	44	101
	100	41	100
A-16886 A	10	74	103
	100	51	99

Table 4. Influence of antibiotics other than cell wall synthesis inhibitors on the incorporation of [^{3}H]Dpm and [^{14}C]leucine into the acid-insoluble fraction in *Bacillus* sp. ATCC 21206 (Dpm$^-$)

Antibiotic	Concentration (μg/ml)	Incorporation (%)	
		[^{3}H]Dpm	[^{14}C] Leucine
Streptomycin	1	109	93
	10	70	25
Kanamycin	10	100	18
	100	99	7
Gentamicin	10	94	14
	100	83	5
Leucomycin	10	40	10
	100	37	4
Tetracycline	10	72	9
	100	2	7
Colistin	1	93	95
	10	4	20
	100	1	3
Polymyxin B	1	69	127
	10	5	31
Mitomycin C	1	110	105
	10	100	94
Novobiocin	1	102	105
	10	100	81
	100	91	70
Bleomycin	100	109	111
Rifampicin	100	91	45
Cerulenin	100	71	49

synthesis such as mitomycin C, novobiocin and bleomycin did not affect the incorporation of both [^{3}H]Dpm and [^{14}C]leucine, but rifampicin, an inhibitor of RNA synthesis, prevented that of [^{14}C] leucine. This inhibition of protein synthesis may be a secondary effect caused by inhibition of RNA synthesis. Finally, cerulenin, an inhibitor of fatty acid synthesis, prevented incorporation of both [^{3}H]Dpm and [^{14}C]leucine, with the latter more strongly affected than the former.

Generalizing from these results, incorporation of [^{3}H]Dpm was prevented by inhibitors of cell wall synthesis, cytoplasmic membrane, protein synthesis and fatty acid synthesis. The cell wall synthesis inhibitors were the only antibiotics that did not inhibit [^{14}C]leucine incorporation even when the antibiotics were used in relatively high concentrations. Therefore, it seemed reasonable to eliminate antibiotics having a mode of action other than by inhibition of cell wall synthesis by this procedure.

Molecular Weights and Passing Ratios of Antibiotics through Diaflo UM-2 Membrane Filter

Elimination of antibiotics of high molecular weight was attempted at an early stage of the screening system, because of their general lack of medical utility. Identification of antibiotics of molecular weight under 1,000 was tried with a membrane filter. As shown in Table 5, the passing ratio of colistin, molecular weight 967, through a Diaflo UM-2 membrane filter was 7.0%. Vancomycin and ristocetin, with molecular weights of over 1,000, also passed through the membrane by several per cent. Therefore, the criterion for eliminating an antibiotic having a molecular weight of greater than 1,000 was set about 7~8%.

From the results mentioned above, the following procedure was established for screening specific inhibitors of cell wall peptidoglycan synthesis. In the primary test, culture broths were selected that showed activity against *B. subtilis* but lacked activity against *A. laidlawii*. In the secondary test, the broths were further selected by retaining those which inhibited only the incorporation of [^{3}H]Dpm into the acid-insoluble fraction of *Bacillus* sp. ATCC 21206 (Dpm$^-$). Finally antibiotics having molecular weights under 1,000 were retained.

Table 5. Passing ratios of various antibiotics through Diaflo UM-2 membrane filter.

Antibiotic	Molecular weight	Passing ratio (%)
D-Cycloserine	90	72.5
Penicillin G	356	22.0
Cephalosporin C	415	17.5
Streptomycin	581	32.0
Colistin	967	7.0
Vancomycin	1,600	5.4
Ristocetin B	>1,000	6.0

Results of this Screening Program

Broth filtrates of about ten thousand strains including fungal, bacterial and actinomycete soil isolates were submitted to this screening program. One new antibiotic, azureomycin, and six known antibiotics, 3-amino-3-deoxy-D-glucose, amphomycin, D-cycloserine, penicillin G, ristocetin A and ristocetin B, were identified in this program. Three antibiotics, amphomycin and ristocetins A and B, had molecular weights over 1,000. Among the six antibiotics, the mechanisms of action of four have been reported[5], while those of amphomycin and 3-amino-3-deoxy-D-glucose have not been known.

Amphomycin was found by HEINEMANN *et al.*[6], and the structure was clarified by BODANSZKY *et al.*[7] It is active against Gram-positive bacteria and consists of a fatty acid combined with eleven amino acids. The mechanism of action of amphomycin was studied by the authors as an extension of this screening program, and the primary site of action was found to be phospho-MurNAc-penta-

peptide translocase in the lipid cycle in peptidoglycan synthesis[8~10].

3-Amino-3-deoxy-D-glucose, one of the constituents of kanamycin, was isolated in this screening program and was found to be an inhibitor of cell wall synthesis. In contrast kanamycin is a known inhibitor of protein synthesis. 3-Amino-3-deoxy-D-glucose was produced by *Bacillus cereus* var. SA-1127 in our laboratory, but it had previously been isolated from *Bacillus aminoglucosidicus*[11]. The primary site of action of 3-amino-3-deoxy-D-glucose was found to be the formation of glucosamine-6-phosphate from fructose-6-phosphate, a reaction catalyzed by glucosamine synthetase[12,13].

Azureomycin, the new antibiotic discovered by this screening procedure, is a new cell wall inhibitor with a relatively low molecular weight. The taxonomy, isolation, properties and mode of action will be reported in the following papers[14,15].

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