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Production of L Phase Variants of Bacteria with Cycloserine. * (27733)

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Available evidence indicates that cycloserine acts by interfering with cell wall biosynthesis. Biochemical studies have shown that this antibiotic causes accumulation of N-acetylaminosugar(1), inhibits incorporation of lysine into cell wall fractions(2), and inhibits alanine racemase and alanyl-alanine synthetase in *Staphylococcus aureus*(3,4,5). Nonetheless, cycloserine may affect mammalian cells as demonstrated by its clinical toxicity(6) in contrast to penicillin which clearly acts by specific inhibition of cell wall mucopeptide formation in growing bacteria. Michel and Hijmans were able to obtain L growth from a single strain of streptococcus by exposing the bacterium to cycloserine but stated that the L form was inhibited by low concentrations of this antibiotic(7). Since L forms of bacteria represent the organisms which are capable of growth and reproduction yet lack a rigid cell wall(8,9,10), the L forms should be resistant to cycloserine action if indeed this antibiotic exerts a primary and specific action on cell wall formation. Therefore, we have studied the ability of cycloserine to induce L phase growth of bacteria. The cycloserine susceptibilities of L phase variants derived by exposing bacteria to penicillin have been compared with L phase variants obtained with cycloserine. Also the penicillin sensitivities of L forms derived with cycloserine were compared with those obtained with penicillin. The susceptibilities of the bacteria from which the L forms were isolated were determined and compared with the sensitivities of the L forms. The sensitivities of selected strains of *Mycoplasma* to penicillin and to cycloserine also have been compared.

Materials and methods. Ninety-six bacterial strains isolated locally were studied (Table I). They were identified by the usual cultural and serologic technics. Nine strains of *Mycoplasma* were examined.† The L phase variants were isolated on agar plates (Oxoid sensitivity test medium)‡ containing 3% NaCl and 20% heated human ascitic fluid. Penicillin (1,000 U/ml) or cycloserine (1,000 μg/ml) was placed in wells on plates streaked with the test organism. The plates were incubated at 37°C semianaerobically by the Fortner method(11). L phase growth was isolated from the zone of inhibited bacterial growth after 4-7 days incubation and was maintained by serial agar block transfer on agar plates containing penicillin (500 U/ml) or cycloserine (500 μg/ml). The *Mycoplasma* were maintained by serial transfer on PPLO agar (Difco) containing 20% heated human ascitic fluid.

Antibiotic susceptibilities were determined

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† The Preston, 4430, Washington, "L" and C 15 strains were obtained from Dr. L. Dienes, Boston, Mass. Goat and bovine strains were obtained from Dr. H. R. Adler, Davis, Calif., and the L4 strain from Dr. Kuzell, San Francisco, Calif. The "Cont." strain was isolated from rats and showed cultural and virulence differences from the L 4 and Preston strains.

‡ Obtained from Consolidated Laboratories, Inc., Chicago Heights, Ill.

TABLE I. Number of Strains of Bacteria Producing L Phase Growth on Exposure to Penicillin or Cycloserine Is Shown According to Species.

Bacterial species	No. strains tested	No. producing L phase growth	
		Penicillin	Cycloserine
Group A β-hemolytic streptococcus	25	2	2
Hemolytic *Staphylococcus aureus**	50	4	4
Proteus sp.†	5	5	5
Sarcina lutea	1	1	0
Pseudomonas aeruginosa	1	1	1
Salmonella sp.‡	9	3	3
Hemophilus influenzae	1	0	0
Micrococcus lysodeikticus	1	0	0
Bacillus megaterium	2	0	0
Corynebacterium diphtheriae	1	0	0

* All staphylococci were pathogenic and reduced tellurite.

† Three *Proteus* species were *P. rettgeri* and 2 were *P. vulgaris* (Table II).

‡ All *Salmonella* strains were pathogenic.

on agar plates (*vide supra*). Serial 2-fold dilutions of penicillin§ and cycloserine‖ were preserved in the frozen state until added to the medium before pouring the plates. Final concentrations ranged from 0.001 to 13.6 μmoles per ml (0.5 to 8,000 U/ml) for penicillin and 0.005 to 81.2 μmoles per ml (0.5 to 8,000 μg/ml) for cycloserine. The test plates were inoculated with L forms and *Mycoplasma* by agar blocks cut from 48- to 72-hour cultures as this procedure gave more uniform growth than transfer from broth. L phase variants derived by bacterial exposure to cycloserine were used for determining cycloserine sensitivity except for *Sarcina lutea* and *Proteus* 18. No L growth was isolated from *S. lutea* with cycloserine and the L phase growth from *Proteus* 18 obtained with cycloserine could not be maintained in serial subculture on cycloserine plates. For penicillin sensitivities, L forms derived by exposure to penicillin were used. For assay of bacterial sensitivities, approximately 1×10^4 bacteria from 18-hour broth cultures were transferred to the test plates. Examination for bacterial growth was made at 24 hours. Growth of L forms and *Mycoplasma* was examined at 48 and 96 hours with a hand lens and checked by stained agar block preparations(12). Minimal inhibitory sensitivity of an organism was expressed as the lowest concentration of the antibiotic in which no growth was observed. Lethal effect was determined by transfer of agar from inoculated plates to agar plates containing no antibiotic. Lethal concentration of the antibiotics was expressed as the lowest concentration in which no growth was observed on transplant. At least 2 tests were made with each antibiotic and every organism at different times using different batches of media. Results were comparable.

All L forms were reverted to bacteria by serial transfer on media containing no antibiotics. The sensitivities of these bacteria were then determined as described above.

Results. Of the 96 bacterial strains examined, 16 produced L growth which could be maintained in serial transfer (Table I). The organisms were tested in a systematic fashion for their L production except for *Proteus, S. lutea* and *Pseudomonas*. These strains were organisms which had been selected in prior experiments as being able to produce L phase growth. In all strains examined, exposure of the bacteria to cycloserine resulted in L phase growth only in strains which also produced L growth on exposure to penicillin. In general L growth was more luxuriant with penicillin, and cycloserine failed to induce L forms in the one strain of *S. lutea*.

When the sensitivities of the parent bacteria were contrasted with the sensitivities of the L phase variants, a significant difference was apparent (Table II). In every instance the L phase variants were resistant to these antibiotics. With penicillin the resistance of L phase variants ranged from 1.7 to 13.6 μmoles per ml as contrasted with bacterial sensitivities of 0.007 to 3.4 μmoles per ml. The concentrations of penicillin which inhibited L growth ranged from 2 to 1,000 times the concentrations required for inhibition of bacterial growth. Similar but less striking differences were noted for cycloserine. Concentrations of cycloserine inhibitory for the

§ A commercial preparation of crystalline penicillin G Potassium was used.

‖ Crystalline D-cycloserine was supplied as Seromycin (R) by Dr. S. N. Gellis, Lilly Research Labs., Indianapolis, Ind.

TABLE II. *In vitro* Sensitivities of Bacteria and Their L Forms to Cycloserine and Penicillin Are Expressed as Inhibitory and Lethal Concentrations.

Organism		Cycloserine (μmoles/ml)		Penicillin (μmoles/ml)	
		Inhib.	Lethal	Inhib.	Lethal
Proteus rettgeri 10347	Bacteria	2.5		.21	
	L	>81.6	>81.6	>13.6	>13.6
52	Bacteria	2.5		.05	
	L	>81.6	>81.6	>13.6	>13.6
Clay	Bacteria	5.1		.11	
	L	20.3	>81.6	>13.6	>13.6
Proteus vulgaris 18*	Bacteria	5.1		.05	
	L	>81.6	>81.6	>13.6	>13.6
Wells	Bacteria	5.1		.21	
	L	>81.6	>81.6	>13.6	>13.6
*Sarcina lutea**	Bacteria	2.5		.05	
	L	>81.6	>81.6	>13.6	>13.6
Pseudomonas aeruginosa	Bacteria	1.3		.03	
	L	>81.6	>81.6	>13.6	>13.6
Salmonella typhimurium (TM)	Bacteria	2.5		.03	
	L	>81.6	>81.6	>13.6	>13.6
typhosa 10565	Bacteria	2.5		.05	
	L	>81.6	>81.6	>13.6	>13.6
unclassified 10715	Bacteria	2.5		.01	
	L	>81.6	>81.6	>13.6	>13.6
Streptococcus 11011	Bacteria	1.3		.007	
	L	40.6	40.6	6.8	6.8
10790	Bacteria	2.5		.03	.05
	L	20.3	20.3	13.6	13.6
Staphylococcus 2212	Bacteria	2.5		.007	1.7
	L	10.1	10.1	1.7	13.6
VA_4	Bacteria	5.1		.21	3.4
	L	40.6	40.6	6.8	13.6
236	Bacteria	5.1		.21	3.4
	L	20.3	20.3	13.6	13.6
13	Bacteria	2.5	40.6	3.4	6.8
	L	40.6	80.6	6.8	13.6

* L phase growth derived from penicillin was used for these two organisms (see text).

bacteria ranged from 1.3 to 5.1 μmoles per ml and for L phase variants from 10.2 to 81.6 μmoles per ml. The concentrations of cycloserine required for inhibition of L phase growth were 4 to 60 times greater than those required for inhibition of bacterial growth. The cycloserine sensitivities of L forms were the same for L phase growth derived from penicillin or cycloserine; similarly, the penicillin sensitivities of L forms were identical for the L phase growth obtained with either cycloserine or penicillin. We were unable to produce L growth in defined media. Because D-alanine is known to inhibit cycloserine action, the susceptibilities may not represent the accurate concentrations of cycloserine required to inhibit growth(13,14). The concentrations of cycloserine used were limited by the availability of crystalline material and it is possible that at least in some instances more striking resistance of L growth could have been observed. Bactericidal concentration was difficult to determine as the bacteria would produce L forms on exposure to the antibiotic in the media containing 3% salt and these would, in turn, revert to bacterial growth when the antibiotic was not present. The sensitivities of the bacteria obtained from the L phase variants were identical with those for the parent bacteria used in the initial isolation of L forms. Thus mutation in the bacteria or L phase variants could be excluded.

When selected strains of *Mycoplasma* were tested for susceptibility to penicillin and cy-

TABLE III. *In vitro* Sensitivities of Mycoplasma to Penicillin and Cycloserine Are Illustrated.

Organism	Cycloserine (μmoles/ml) Inhib.	Cycloserine (μmoles/ml) Lethal	Penicillin (μmoles/ml) Inhib.	Penicillin (μmoles/ml) Lethal
Preston	>81.6	>81.6	>13.6	>13.6
L 4	40.6	40.6	>13.6	>13.6
Cont.	40.6	40.6	>13.6	>13.6
Goat	81.6	81.6	>13.6	>13.6
Bovine	20.3	20.3	>13.6	>13.6
4330	40.6	40.6	>13.6	>13.6
Washington	81.6	81.6	>13.6	>13.6
"L"	>81.6	>81.6	>13.6	>13.6
C 15	20.3	20.3	13.6	13.6

closerine all strains exhibited marked resistance to both agents (Table III). Of significance was the observed identity of inhibitory and lethal concentrations of both antibiotics for the *Mycoplasma* and L phase variants.

Discussion. The rigid cell wall is a unique structure which distinguishes bacterial from mammalian cells and therefore provides a target for selective antibiotic action. Penicillin has been shown to impair cell wall formation as measured by accumulation of N-acetyl-aminosugar precursors of cell walls in *S. aureus*(2,15) and inhibition of amino acid incorporation into cell wall mucopeptides(16). Additional evidence for the primary action of penicillin on cell wall biosynthesis is the production of protoplasts and L forms by exposing growing bacteria to this antibiotic. L phase variants of bacteria have the same morphology, mechanical fragility, and osmotic fragility as protoplasts prepared by enzymatic damage to the rigid cell wall(10). Sharp *et al.*(8) have shown by chemical and serologic methods that penicillin-isolated L forms of group A streptococci lack cell wall components and Weibull(9) found that stable L forms of *Proteus* contained much less diaminopimelic acid and hexosamine than the parent bacteria. Thus, L phase variants of bacteria lack the rigid cell wall, yet are capable of growth and reproduction.

Because cycloserine has been shown to inhibit cell wall formation, Michel and Hijmans (7) attempted the production of L phase variants of streptococci with this antibiotic. They obtained L growth with cycloserine in one strain of group A streptococcus which was greatly enhanced when high concentrations of glycine or DL-serine were added to the media. While the amino acid content of our media was not determined, cycloserine induced L forms which were moderately resistant to this antibiotic as compared with the bacteria from which they were derived. It is known that alanine will inhibit the anti-bacterial action of cycloserine, while other amino acids do not have this propensity. It is possible that sufficient alanine was present to give falsely high concentrations of cycloserine needed for inhibition of bacterial growth. As neither serine nor glycine was added to our media, it can be assumed that cycloserine exerted a primary action on cell wall synthesis.

Because Park(2) has shown accumulation of cell wall precursors in *S. aureus* by bacitracin, chlortetracycline and glycine and Mandelstam and Rogers(16) have reported that bacitracin and chlortetracycline will inhibit cell wall mucopeptide formation in *S. aureus*, these substances might be expected to induce protoplast formation and permit L phase growth. However, Park(2) observed that low concentrations of chlortetracycline inhibited protein synthesis and Ward *et al.*(17) were unable to induce L growth in a variety of bacteria exposed to bacitracin or chlortetracycline. L forms derived from penicillin were found to be as sensitive or more sensitive to these antibiotics than their parent bacteria. As the primary defect of L phase variants seems to be the inability to synthesize cell wall mucopeptide, demonstration that continued growth and reproduction of L phase variants in the presence of penicillin or cycloserine, in concentrations which inhibit the bacteria, provides convincing evidence to support the chemical observations that these antibiotics act by blocking complete biosynthesis of the rigid cell wall. In contrast, the inability to induce L phase growth with other agents suggests that the sites of action of those antibiotics are different.

Mycoplasma are similar in many ways to L phase variants of bacteria. Plackett(18) demonstrated that 2 strains of *Mycoplasma mycoides* contained only small quantities of hexosamine and α-ε-diaminopimelic acid. These studies indicate that *Mycoplasma* lack

the "mucocomplex" characteristic of bacterial cell walls. The marked resistance of *Mycoplasma* to cycloserine and penicillin therefore adds evidence to the concept that these antibiotics act by affecting bacterial pathways which synthesize the cell wall.

Summary. L phase growth of bacteria was produced by exposing growing organisms to high concentrations of penicillin and D-cycloserine. Of 96 bacterial strains examined, 16 produced L growth which could be maintained in serial subculture. Exposure of bacteria to cycloserine gave L phase growth only in strains which also produced L growth on exposure to penicillin. The penicillin and cycloserine sensitivities of the bacteria from which the L forms were isolated were compared with the sensitivities of the L forms. The concentrations of penicillin which inhibited L growth ranged from 2 to 1000 times the concentrations required for inhibition of bacterial growth. The concentrations of cycloserine required for inhibition of L phase growth were 4 to 60 times greater than those required for inhibition of bacterial growth. *Mycoplasma* exhibited marked resistance to both agents. The resistance of the L phase growth and *Mycoplasma* to penicillin and cycloserine is evidence to substantiate the role of these antibiotics in affecting bacterial pathways which synthesize the cell wall.

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Evidence for a Common Hapten Associated with Endotoxin Fractions of *E. coli* and other *Enterobacteriaceae.** (27734)

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E. coli may be serologically characterized by 3 major groups of antigens; the 0, or heat stable, antigens located in the endotoxin moiety; the H or flagellar antigens; and the K or envelope antigens. Kauffmann(1) and Ewing *et al.*(2) have characterized the antigenic relationships among the 145 known *E. coli* 0 antigen groups by means of the bacterial agglutination test, and have demonstrated a number of cross reactions between various groups. Serologic cross reactions have also

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