INHIBITION OF MICROBIAL GROWTH AND SEPARATION BY D-SERINE, VANCOMYCIN, AND MITOMYCIN C

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ABSTRACT

DURHAM, NORMAN N. (Oklahoma State University, Stillwater). Inhibition of microbial growth and separation by D-serine, vancomycin, and mitomycin C. J. Bacteriol. **86**:380–386. 1963. —A study was made of the growth and separation inhibition of *Pseudomonas fluorescens* by vancomycin and mitomycin C and of a *Flavobacterium* sp. sensitive to mitomycin C and D-serine. Aberrant morphological forms (primarily "boat-shaped" cells and "chain-like" filaments) were observed during growth of the sensitive organism in the presence of the inhibitors. Reversal studies indicated that Mg^{++}, and to a lesser extent Mn^{++}, partially reversed vancomycin and mitomycin C growth inhibition during the early stages of growth but did not influence D-serine inhibition. Selected metabolites also showed a tendency to partially reverse mitomycin C and vancomycin inhibition of growth during the initial growth phase, and the differences in reversal trends suggested that D-serine, vancomycin, and mitomycin C produce metabolic lesions at different sites in the cell with respect to growth. Cultivation of the sensitive organism in low concentrations of the inhibitors resulted in a structurally fragile population, indicating formation of a defective cell structure. Mg^{++} partially reversed the vancomycin inhibition of separation in these organisms. None of the other metals or metabolites, either singly or in varied combinations, was able to reverse inhibition of separation by the various inhibitors.

Interference with synthesis of microbial cell walls frequently results in unusual morphological forms. Aberrant cells are common when microorganisms are cultured in a D-amino acid-containing medium (Lark and Lark, 1959; Tuttle and Gest, 1960; Durham and Milligan, 1961). Reich, Shatkin, and Tatum (1961) reported that mitomycin C produced "filamentous" forms in *Escherichia coli*, and proposed that mitomycin C mediated breakdown of the nuclear apparatus and its constituent deoxyribonucleic acid (DNA). Kersten and Rauen (1961) also demonstrated that mitomycin C enhanced depolymerization of nuclear material. Jordan (1961) showed that vancomycin inhibited synthesis of cell-wall mucopeptide in *Staphylococcus aureus*.

Cultivation of a *Flavobacterium* sp. and *Pseudomonas fluorescens* in the presence of an appropriate inhibitor, such as D-serine, vancomycin, or mitomycin C, produces morphological variations. Durham and Milligan (1962) reported that β-alanine or pantothenic acid reversed growth inhibition of the *Flavobacterium* sp. by D-serine without reversing aberrant cell formation. This manuscript presents results which establish that all three inhibitors serve to structurally weaken the cell wall formed in their presence and to prevent separation of daughter cells formed during division.

MATERIALS AND METHODS

Growth experiments were performed at 37 C in an inorganic salts medium containing 0.2% succinic acid as the carbon and energy source, as previously described (Durham and Milligan, 1961). All additives were dissolved in 0.01 M potassium phosphate buffer (pH 7.2), sterilized by filtration, and added aseptically. The pH of the medium prior to inoculation was carefully standardized at 7.2, since the D-serine effect on the *Flavobacterium* sp. is pH-dependent.

The organisms were cultured on nutrient agar for 15 hr, washed, and suspended in buffer to an absorbancy of 0.5 at 540 mμ. In growth studies, 0.1 ml of this suspension was used as the inoculum. Growth (the increase in cell mass) was followed by measuring absorbancy in 1.9-cm tubes at 540 mμ in a Bausch & Lomb Spectronic-20 colorimeter. The tubes contained a total volume of 6 ml. Aberrant morphological forms were evaluated microscopically. Absorbancy and dry cell

mass were closely correlated during the first 10 to 12 hr in the presence of D-serine, mitomycin C, or vancomycin. However, during continued incubation, osmotically sensitive spheres appeared in some tubes, and lysis was evident.

Susceptibility to disruption by sonic oscillation (fragility) was tested by the following procedure. Cells, grown in the presence of the appropriate inhibitor, were washed and suspended in 0.01 M potassium phosphate buffer (pH 7.2) to an absorbancy of 1.0 at 540 mμ. Viable-cell counts were made on nutrient agar. A 7.0-ml sample was placed in a 20-ml plastic centrifuge tube cut to fit inside the cup of a Raytheon 10-kc sonic oscillator. A liquid base was prepared for the tubes by adding 40 ml of distilled water to the cup. The tubes containing the cell suspensions were then placed in the cup and treated simultaneously to insure similar exposures. At the desired time intervals, the absorbancy of the cell suspensions was measured at 540 mμ and a 1.0-ml sample was removed. The 1.0-ml sample was centrifuged to remove cells and debris, the supernatant was diluted, and the absorbancy at 260 and 280 mμ was measured in a Beckman DU spectrophotometer. A similar cell volume suspended in 0.01 M potassium phosphate buffer served as a lysis control. This suspension was not subjected to sonic oscillation. However, samples were removed at the appropriate time and the absorbancies were measured.

RESULTS

Susceptibility of organisms to inhibitors. The organisms showed pronounced differences in sensitivity to the test compounds. Inhibition of growth (approximately 70%) of the *Flavobacterium* sp. was observed with 0.91 mg/ml of D-serine and 4.0 μg/ml of mitomycin C. The organism was relatively resistant to vancomycin, since a final concentration of 1.0 mg/ml produced no inhibition. Aberrant cell types consisting of "chain-like" filaments and "boat-shaped" cells were observed with D-serine and mitomycin C but not with vancomycin. *P. fluorescens* growth was inhibited (70%) by 0.218 μg/ml of mitomycin C or 147.7 μg/ml of vancomycin but was not inhibited by 3.5 mg/ml of D-serine. Aberrant cell types, similar to those observed with the *Flavobacterium* sp., were evident when *P. fluorescens* was cultured with mitomycin C or vancomycin. Some cell enlargement was observed when *P. fluorescens* was cultured in D-serine concentrations exceeding 3.8 mg/ml. Microscopic observations of stained preparations, and phase-contrast microscopy, revealed distinct crosswalls in the "chain-like" filaments, which gave the appearance that the cells were unable to separate and thus could not complete the phenomenon known as cell division (Fig. 1). In general, similar aberrant cell types were observed in the presence of the different inhibitors, with the exception of vancomycin which did not inhibit the *Flavobacterium* sp. Increasing the concentration of an inhibitor enhanced inhibition of growth, increased the length of the "chain-like" filaments, and resulted in formation of osmotically sensitive spheres.

Oxidation of D-serine by organisms. The difference in susceptibility of *P. fluorescens* and the *Flavobacterium* sp. to D-serine could be explained in two ways: (i) the more resistant organism possesses an oxidase system for the D-amino acid, or (ii) D-serine could not readily penetrate the permeability barrier of *P. fluorescens*. The ability of washed-cell suspensions to oxidize D-serine was investigated. Both *P. fluorescens* and the *Flavobacterium* sp. rapidly oxidized D-serine with Q_{O_2} values of 365 and 384, respectively. There was no difference in the rate or extent of D-serine oxidation by these two organisms. Thus, these results do not establish the basis for the more resistant nature of *P. fluorescens*, but the ability of these organisms to oxidize D-serine does explain the extremely high levels of D-serine required to produce an inhibition. However, the biological significance of results obtained by using high levels of D-serine remains to be evaluated, since it is unlikely that these conditions exist in physiological systems.

Influence of metals on inhibition of growth by D-serine, vancomycin, or mitomycin C. Studies were conducted in which various metals were added to the medium simultaneously with the inhibitor just prior to inoculation. The medium contained trace quantities of Mg^{++}, Mn^{++}, Fe^{+++}, and Ca^{++} supplied from the mineral salts mixture prior to supplementation.

Results of these studies (Table 1) suggest that the divalent cation Mg^{++} partially reverses inhibition of *P. fluorescens* by vancomycin in the early stages of growth. Mn^{++} also showed a tendency for reversal, but Fe^{+++} and K^{+} were unable to overcome the inhibition of growth. A mixture

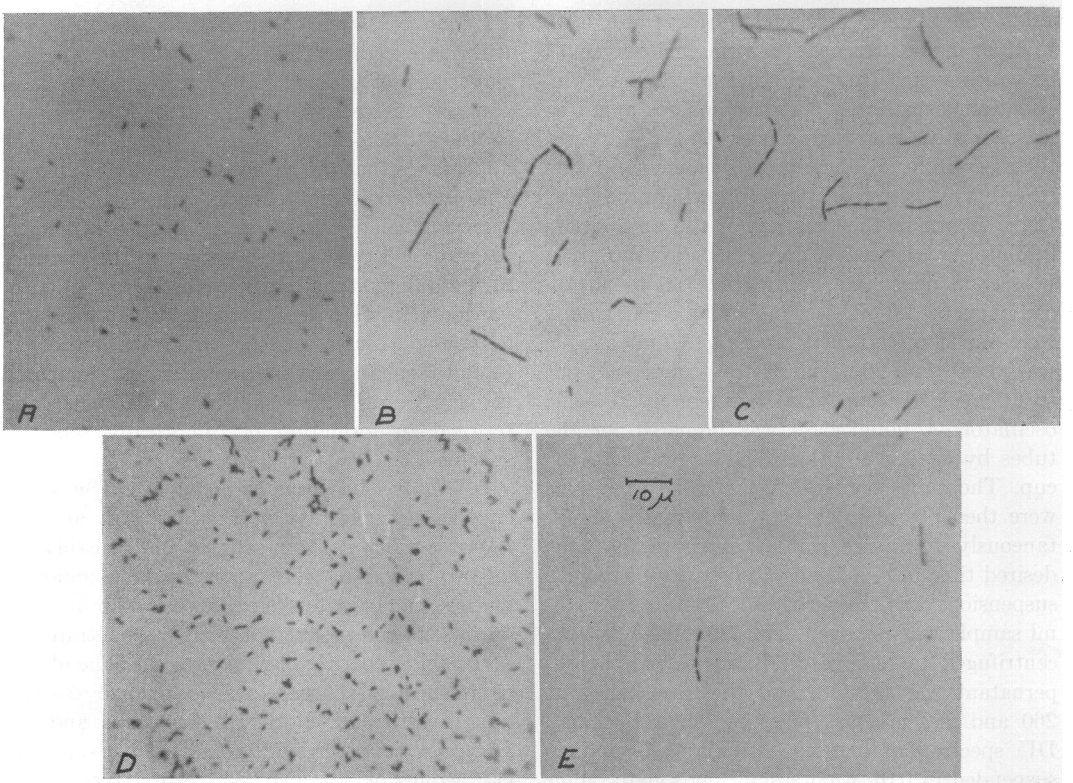

FIG. 1. *Influence of inhibitors on separation of the Flavobacterium sp. A = control; B = mitomycin C (4.5 μg/ml); C = D-serine (0.95 mg/ml); D = vancomycin (0.9 mg/ml); E = penicillin (21.8 μg/ml).*

of the ions showed growth equivalent to Mg^{++} alone. The *Flavobacterium* sp. was not inhibited by the vancomycin concentration used in this study.

Mg^{++} and Mn^{++} also showed some tendency to reverse mitomycin C inhibition of both *P. fluorescens* and the *Flavobacterium* sp. during the initial growth period. However, during continued incubation, cells growing in the presence of vancomycin and mitomycin C showed an inclination to lyse. None of the ions tested was effective in reversing inhibition of growth of the *Flavobacterium* sp. by D-serine, and *P. fluorescens* was not unusually sensitive to this antimetabolite and was not investigated. Additional studies showed that Fe^{++}, Ca^{++}, and Zn^{++} all failed to influence growth inhibition in these systems. Microscopy revealed that Mg^{++} partially reversed formation of aberrant cell types, when vancomycin was used as the inhibitor, with both the *Flavobacterium* sp. and *P. fluorescens*. However, none of the other metals showed significant reversal of aberrant morphological forms.

Influence of inhibitors on structural fragility of the cells. Interference with cell-wall synthesis by an inhibitor generally alters the structural strength of the cells. *Flavobacterium* cells were cultured 9 hr in the succinate-salts medium in the presence and absence of D-serine (0.28 mg/ml), mitomycin C (1.18 μg/ml), or vancomycin (0.80 mg/ml). These concentrations approached "non-inhibitory" levels, since absorbancy readings indicated growth was not inhibited and microscopy revealed only an occasional aberrant cell in the D-serine and mitomycin C environments. The latter observation was made to eliminate the possibility that filamentous forms are more fragile than the normal small individual cells. Results from this study are presented in Table 2. Cell counts showed that each cell suspension contained approximately the same number of viable cells. The difference in absorbancy of the cell suspensions during treatment in the sonic oscillator demonstrated that cells grown in the presence of D-serine and mitomycin C were more fragile than the control or cells grown with vanco-

TABLE 1. *Influence of metals on growth inhibition of Pseudomonas fluorescens and Flavobacterium sp. by* D-*serine, vancomycin, and mitomycin C**

Supplement	Inhibitor	Absorbancy (540 mμ)			
		P. fluorescens		*Flavobacterium* sp.	
		6 hr	12 hr	6 hr	12 hr
None	—	0.41	0.86	0.28	0.62
Mg^{++}	—	0.45	0.92	0.24	0.69
Fe^{+++}	—	0.38	0.82	0.31	0.59
Mn^{++}	—	0.43	0.89	0.29	0.65
K^{+}	—	0.40	0.93	0.34	0.64
Mg^{++}, Mn^{++}, Fe^{+++}, K^{+}	—	0.44	0.81	0.27	0.71
None	D-Serine	0.44	0.81	0.08	0.21
Mg^{++}	D-Serine			0.10	0.23
Fe^{+++}	D-Serine			0.07	0.21
Mn^{++}	D-Serine			0.12	0.17
K^{+}	D-Serine			0.13	0.22
Mg^{++}, Mn^{++}, Fe^{+++}, K^{+}	D-Serine			0.09	0.19
None	Mitomycin C	0.06	0.28	0.10	0.15
Mg^{++}	Mitomycin C	0.11	0.41	0.09	0.27
Fe^{+++}	Mitomycin C	0.10	0.31	0.04	0.10
Mn^{++}	Mitomycin C	0.09	0.38	0.11	0.20
K^{+}	Mitomycin C	0.05	0.22	0.10	0.14
Mg^{++}, Mn^{++}, Fe^{+++}, K^{+}	Mitomycin C	0.14	0.43	0.12	0.30
None	Vancomycin	0.09	0.31	0.25	0.58
Mg^{++}	Vancomycin	0.16	0.52		
Fe^{+++}	Vancomycin	0.10	0.29		
Mn^{++}	Vancomycin	0.12	0.44		
K^{+}	Vancomycin	0.08	0.34		
Mg^{++}, Mn^{++}, Fe^{+++}, K^{+}	Vancomycin	0.18	0.54		

* Growth studies were conducted in the succinate-salts medium supplemented as indicated. Concentrations used with *P. fluorescens* were D-serine, 3.0 mg/ml; mitomycin C, 0.25 μg/ml; and vancomycin, 160 μg/ml; with *Flavobacterium* sp. were D-serine, 0.95 mg/ml; mitomycin C, 5 μg/ml; and vancomycin, 1.0 mg/ml. Metal concentrations were $MgCl_2$, $MnCl_2$, and KCl, 5.4 μmoles/ml; and $FeCl_3$, 0.54 μmoles/ml.

mycin. These data were augmented by absorbancy readings of the supernatants at 260 and 280 mμ. Cells suspended in phosphate buffer, which served as fragility controls, showed no tendency to lyse. Similar studies were conducted with *P. fluorescens*, and results indicated that cells grown in the presence of vancomycin or mitomycin C were more sensitive to sonic oscillation than were control cells. *P. fluorescens* is not sensitive to D-serine, and cells grown in an inhibitor concentration of 1.5 mg/ml did not show increased fragility.

These findings establish that sensitive cells grown in the presence of D-serine, vancomycin, or mitomycin C show an increased fragility to sonic oscillation. Thus, one of the end results of growth in the presence of these inhibitors is a structurally weakened cell wall.

Influence of selected metabolites on inhibition. Durham and Milligan (1962) established that pantothenic acid, β-alanine, L-alanine, or L-aspartic acid reversed D-serine inhibition of growth of the *Flavobacterium* sp. but were unable to overcome formation of aberrant cell types. These findings were augmented by the report that β-alanine also reversed D-serine inhibition in an *Erwinia* sp. (Grula and Grula, 1962*b*). Studies were conducted to determine whether metabolites influenced inhibition of the *Flavobacterium* sp. and *P. fluorescens* by vancomycin or mitomycin C (Table 3).

L-Aspartic acid, L-glutamic acid, or pyruvic

TABLE 2. *Structural fragility of Flavobacterium sp. cells grown in presence of* D-*serine, mitomycin C, and vancomycin*

Additive to succinate medium	Initial absorbancy	Viable cells per ml × 10^6	Absorbancy change/15 min in sonically treated cells*		
			Decrease in cell mass	Increase in	
				260 mμ	280 mμ
None	0.94	189	−0.26	0.164	0.107
D-Serine	0.85	174	−0.58	0.319	0.200
Mitomycin C	0.88	194	−0.60	0.377	0.224
Vancomycin	0.92	178	−0.32	0.192	0.131

* The change in absorbancy was determined from the linear portion of the absorbancy of the cell mass versus time curve, or from the absorbancy at 260 or 280 mμ versus time curves. Values have been corrected for the buffer control.

TABLE 3. *Influence of metabolites on growth inhibition of Flavobacterium sp. and Pseudomonas fluorescens by vancomycin and mitomycin C**

Additions to basal medium	*Flavobacterium* sp.			*P. fluorescens*		
	C	+MC	+VAN	C	+MC	+VAN
None	0.38	0.15	0.39	0.72	0.28	0.35
β-Alanine	0.41	0.20	0.41	0.79	0.34	0.31
L-Alanine	0.48	0.27	0.36	0.75	0.26	0.40
L-Aspartic acid	0.45	0.35	0.38	0.81	0.61	0.65
L-Glutamic acid	0.46	0.39	0.43	0.69	0.58	0.54
Pyruvic acid	0.41	0.37	0.45	0.69	0.34	0.51

* Absorbancy readings were made at 540 mμ after 12 hr of incubation. Basal medium consisted of 0.2% succinate-salts. Inhibitor concentration for *Flavobacterium* sp. was mitomycin C (MC), 5 μg/ml; and vancomycin (VAN), 1.0 mg/ml; for *P. fluorescens* was mitomycin C, 0.25 μg/ml; and vancomycin, 160 μg/ml. Column C shows readings without inhibitor. Metabolite concentrations were: β-alanine, 1.0 μg/ml; L-alanine, L-aspartic, L-glutamic, and pyruvic acids, 10.67 μg/ml.

acid partially reversed mitomycin C growth inhibition of the *Flavobacterium* sp. during the early stages of growth. L-Alanine showed slight reversal after approximately 10 to 12 hr. However, none of these compounds was able to reverse "filament" formation, and some lysis was observed in the mitomycin C environment when incubation exceeded 12 to 14 hr. Pantothenic acid, β-alanine, D-alanine, D-aspartic acid, D-glutamic acid, glycine, L-lysine, and *p*-aminobenzoic acid did not reverse mitomycin C inhibition of growth or filament formation. The organism was not sensitive to vancomycin in the concentration used in this experiment.

Slightly different results were observed with *P. fluorescens*. L-Aspartic acid, L-glutamic acid, and D-glutamic acid showed partial reversal of vancomycin inhibition of growth. L-Alanine showed a slight reversal trend at later time intervals (12 to 14 hr), whereas β-alanine, D-alanine, glycine, and *p*-aminobenzoic acid were unable to reverse vancomycin inhibition of growth. L-Aspartic acid and L-glutamic acid showed tendencies to overcome mitomycin C inhibition of growth. Pyruvic acid and β-alanine showed some reversal during continued incubation (12 to 24 hr), but L-alanine, D-alanine, D-aspartic acid, D-glutamic acid, and glycine showed no reversal of mitomycin C inhibition of growth.

The results suggest that L-aspartic acid and L-glutamic acid are fairly effective reversing agents for all three inhibitors during the early stages of growth. The similarity between reversal of inhibition of growth by vancomycin and mitomycin C in the two organisms is very good. An earlier observation established that D-aspartic acid and D-glutamic acid were unable to reverse mitomycin C and D-serine inhibition (Durham and Milligan, 1961), but the present findings show that these metabolites can reverse vancomycin inhibition, suggesting that the inhibitors produce metabolic lesions at different sites in the microbial cell with respect to growth. None of the compounds studied was able to reverse formation of aberrant morphological forms.

Pantoyl lactone (Mann Research Laboratories) was studied as a reversing agent. The additive was dissolved in 0.01 M potassium phosphate buffer, adjusted to pH 7.2, and sterilized by filtration just prior to use. A similar experiment was conducted in which the additive was adjusted to pH 6.7. The maximal concentration of pantoyl lactone used in this study was 3.2 mg/ml (0.0246 M). Higher concentrations were inhibitory to both the *Flavobacterium* sp. and *P. fluorescens*. Reversal studies were conducted in which (i) pantoyl lactone was added simultaneously with the inhibitor (D-serine, vancomycin, or mitomycin C); and (ii) the cells were incubated in

the presence of the inhibitor for 6 hr (absorbancy readings and microscopy revealed growth inhibition and aberrant-cell formation), at which time pantoyl lactone was added to the system.

Pantoyl lactone was unable to reverse either growth or aberrant-cell formation owing to D-serine or mitomycin C in the *Flavobacterium* sp. or the inhibitions resulting from vancomycin or mitomycin C in *P. fluorescens*, regardless of the time at which pantoyl lactone was added to the inhibited system.

Similar studies were conducted in a medium supplemented with Mg^{++} in the case of vancomycin and mitomycin C, or supplemented with β-alanine (0.03 mg/ml) when D-serine was the inhibitor since β-alanine will reverse D-serine inhibition of growth but not formation of aberrant cell types (Durham and Milligan, 1962). However, pantoyl lactone was unable to prevent or reverse aberrant-cell formation in the supplemented environments.

Discussion

D-Serine, vancomycin, or mitomycin C inhibit microbial growth and prevent the separation of cells, which results in formation of "chain-like" filaments in certain microorganisms. High concentrations of the inhibitors resulted in formation of osmotically sensitive structures. Alteration of the cell wall is generally associated with conversion of osmotically stable bacilli to osmotically sensitive spheres.

Growth inhibition resulting from vancomycin and mitomycin C can be partially reversed during the early stages of growth by the divalent cation Mg^{++}, to a lesser extent by Mn^{++}, but not by Ca^{++}, Zn^{++}, K^{+}, Fe^{++}, or Fe^{+++}. With the exception of Mg^{++}, none of these ions was able to overcome formation of aberrant morphological forms resulting from an inhibition of the separation phase of cell division. The mechanism of action of the ions in the inhibited system remains somewhat obscure. Mitomycin C reportedly acts by enhancing depolymerization of DNA, and Mg^{++} is essential for optimal depolymerization (Reich et al., 1961). Desreux, Hacha, and Fredericq (1962) proposed that hydrolysis of DNA by deoxyribonuclease occurred in several steps and that Mg^{++} was a specific activator for hydrolysis of double-stranded molecules. Results obtained in reversal studies indicate that Mg^{++} may also act by another mechanism(s) in the cell. Mg^{++} may function by preserving the structural integrity of the ribonucleoprotein particles (Hershko, Amoz, and Mager, 1961). Weibull (1956) pointed out that membranes are stabilized by Mg^{++}, and Brown (1962) gave a probable explanation of di- and multivalent cations as preventing membrane swelling by influencing configuration of membrane proteins.

Cells cultured in low concentrations of the inhibitors (D-serine, vancomycin, mitomycin C) showed a loss of structural strength, suggesting an interference with the cell wall formed in the presence of the inhibitors. In one respect, these inhibitors are similar to penicillin, which inhibits synthesis of the "basal structure" of the cell wall, and the abnormal structure lacks the mechanical strength of the normal cell wall (Wylie and Johnson, 1962). Neuhaus (1962) proposed that a mixed dipeptide (for example, D-alanyl-D-serine) may replace that D-alanyl-D-alanine moiety in the cell-wall mucopeptide, resulting in an altered or defective structure, or the mixed dipeptide may inhibit the dipeptide synthetase enzyme. Fragility studies and the lysis observed in some growth experiments indicate formation of a defective structure. Thus, it is suggested that environmental conditions (D-serine, vancomycin, mitomycin C, ultraviolet light, penicillin) which result in formation of "chain-like" filaments may do so by interfering with reactions regulating wall or membrane synthesis. However, this does not necessarily imply that the site of action of each inhibitor is the same. It is difficult to evaluate objectively the D-serine inhibition, since an extremely high concentration is required to inhibit the cell and it is doubtful that this condition exists in a biological system.

Durham and Milligan (1962) established that β-alanine, L-alanine, or L-aspartic acid reversed D-serine inhibition of growth of the *Flavobacterium* sp. and proposed that D-serine inhibited formation of β-alanine in this organism. Amino acids also showed a tendency to partially reverse vancomycin and mitomycin C inhibition of growth in the early stages. L-Aspartic acid and L-glutamic acid gave some reversal of mitomycin C inhibition in both *P. fluorescens* and the *Flavobacterium* sp. In addition to these two metabolites, the D isomers of aspartic acid and glutamic acid gave some indication of reversing inhibition of growth due to vancomycin. Reversal studies with amino acids show some similarities

for the three inhibitors, but over-all the results suggest that the inhibitors produce metabolic lesions at different sites in the cell even though the same end result was obtained.

None of the metabolites tested in this study was able to reverse significantly the formation of aberrant cell types characteristic of these inhibitors. Grula and Grula (1962*a*) reported that substrate levels (0.046 M) of pantoyl lactone triggered cell division in an *Erwinia* sp. regardless of whether the division process had been inhibited by D-serine, ultraviolet light, or penicillin. Pantoyl lactone did not reverse growth inhibition or filament formation due to D-serine, vancomycin, or mitomycin C in either the *Flavobacterium* sp. or *P. fluorescens*, establishing that reversal of "division inhibition" by pantoyl lactone is not a general biological phenomenon. Thus, at this time, it is not possible to define completely the nutritional aspects of aberrant-cell formation in these two microorganisms.

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