INCORPORATION OF D-SERINE INTO THE MUCOPEPTIDE OF *ERWINIA CAROTOVORA*

By

DAVID LYNN SPESS

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Oklahoma State University

Stillwater, Oklahoma

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MUCOPEPTIDE OF ERWINIA

CAROTOVORA

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

1029492

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CHAPTER I

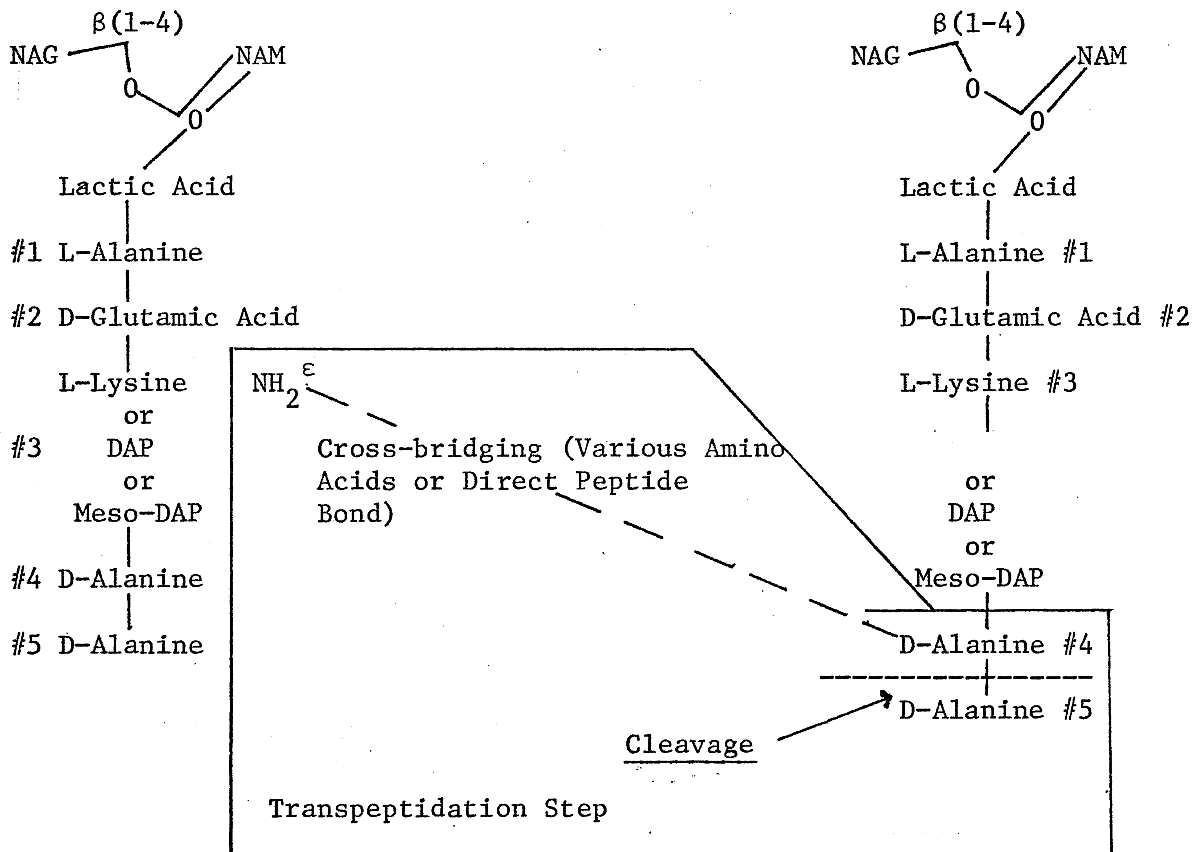
INTRODUCTION

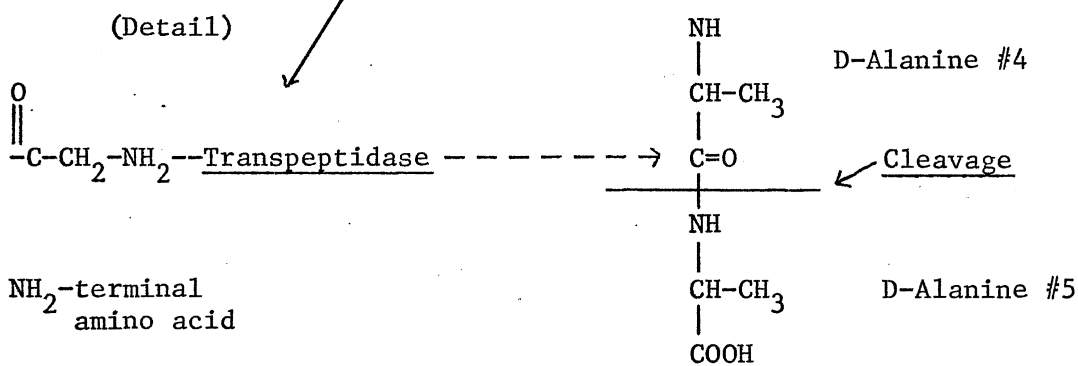
The cell wall and mucopeptide (MP) of bacteria have aroused the interest of various researchers and have been intensively studied (3) (46). The MP is characteristic in that it is responsible for shape determination, rigidity, and the resistance of cells to osmotic lysis (46). The protective MP is found in virtually all bacteria except the mycoplasms and certain halophilic species (39) (66).

The essential features (Figure 1) of the MP are: a backbone of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues having a β (1-4) linkage, a pentapeptide of the general sequence L-alanine-D-glutamic acid-a diamino acid (lysine or diaminopimelic acid) D-alanyl-D-alanine joined to the glycan chain by amide linkages between the _alpha_-amino groups of L-alanine and the lactyl carboxyl groups of the muramic acid residues. Peptide subunits are at least partially cross-linked to each other, the C-terminal #4 D-alanine residues of one being generally linked to the free amino group of the diamino acid (lysine, diaminopimelic acid (DAP) or _meso_-diaminopimelic acid) in a second tetrapeptide (43) (46) (47) (53).

Bacterial cell wall biosynthesis is one of the most complex sequences of enzymatic reactions now known and is unique because DAP and NAM are not found anywhere else in nature (7).

Figure 1. General Sequence of Mucopeptide (MP) Including Transpeptidation Step


β(1-4)
NAG
NAM
O
O
Lactic Acid
#1 L-Alanine
#2 D-Glutamic Acid
L-Lysine
or
#3 DAP
or
Meso-DAP
#4 D-Alanine
#5 D-Alanine
β(1-4)
NAG
NAM
O
O
Lactic Acid
L-Alanine #1
D-Glutamic Acid #2
L-Lysine #3
or
DAP
or
Meso-DAP
D-Alanine #4
D-Alanine #5
NH2 ε
Cross-bridging (Various Amino Acids or Direct Peptide Bond)
Cleavage
Transpeptidation Step


(Detail)
O
-C-CH2-NH2--Transpeptidase
NH2-terminal amino acid
NH
CH-CH3
C=O
NH
CH-CH3
COOH
D-Alanine #4
Cleavage
D-Alanine #5

The β (1-4) linkage between NAG-NAM in the backbone of MP is specifically hydrolyzed by the enzyme lysozyme. When done in a hypertonic medium, the resultant morphological form is called a protoplast or spheroplast. Spheroplasts and protoplasts can be produced by the action of various agents which inhibit cell wall MP synthesis. These agents produce their particular effect at least partially on the tetrapeptide amino acids through substitution or interference in the cross-linkage between tetrapeptides making up the MP (28) (30) (46) (53) (57) (61). Although many inhibitors exist we will concentrate upon the action of D-amino acids, specifically D-serine and the analogous role of glycine.

As early as 1944 and 1945, Fox et al. (13) and Fling et al. (12) demonstrated that a D-amino acid isomer of a natural amino acid, such as L-leucine or L-valine, which is required for growth by Lactobacillus arabinosus, inhibits growth of that strain. Likewise the growth of Escherichia coli is inhibited by added D-amino acids at levels at which the L-forms do not exhibit such an effect (34). Other studies have shown that D-alanine inhibits growth of L. arabinosus, L. casei and Streptococcus faecalis (52). Studies with three strains of Brucella abortus using the D-forms of the amino acids valine, leucine, histidine, methionine, and phenylalanine showed that each strain is inhibited by the D-amino acids at levels at which the L-forms do not exhibit such an effect. D-Amino acids that showed the greatest inhibition were D-phenylalanine and D-methionine (65).

Lark and Lark (37) (38) demonstrated that growth of Alcaligenes fecalis LB in the presence of certain D-amino acids results in spheroplast formation. Further studies revealed that D-methionine is incorporated into the "cell wall" of A. fecalis thereby blocking further

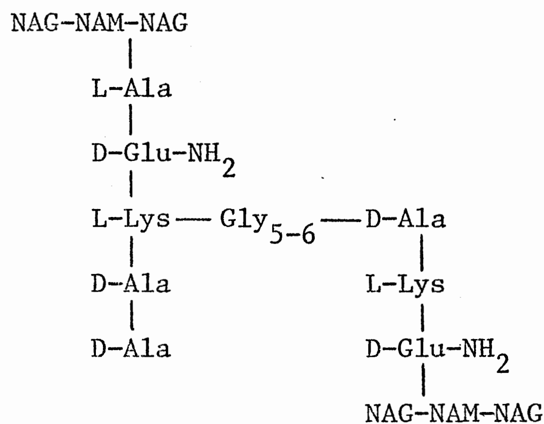
synthesis of this structure. Upon analysis it was shown that neither the amino nor the carboxyl group of methionine is free when this amino acid is incorporated into the cell wall. Electron microscopic studies revealed lesions in the wall of cells grown in the presence of D-methionine as well as liberation of fibrous material, which was lysozyme sensitive (36). Other work on D-amino acids reported by Tuttle and Gest (58) show that several D-amino acids increase the cell wall content of amino sugars in Rhodospirillum rubrum.

The amino acid glycine has been shown to have a somewhat similar role to that of D-amino acids and penicillin, i.e., division inhibition, cell elongation, and spheroplast or protoplast formation (9) (28) (42) (60) (61). The inhibitory effect of glycine on bacterial growth has been known for a long time (8) (9) (12) (13) (15) (16) (43) (51). A detailed study was conducted by Hishinuma et al. (30) who reported growth inhibition, using glycine, on eight different species of gram-positive bacteria of various genera representing the four most common mucopeptide types (Figure 2). The inhibited cells showed morphological aberrations including cell elongation which could be prevented by addition of L-alanine. The amount of incorporated glycine was equivalent to the decrease in the amount of alanine. With one exception glycine was also incorporated into the MP. Glycine can replace L-alanine in position 1 or D-alanine residues in positions 4 and 5 of the peptide subunit. Replacement of D-alanine residues was most common. Most of the D-alanine replaced by glycine was in the C-terminal or number 4 position.

Further studies have confirmed and added to our knowledge of D-amino acids and the effect of glycine on MP structure and synthesis.

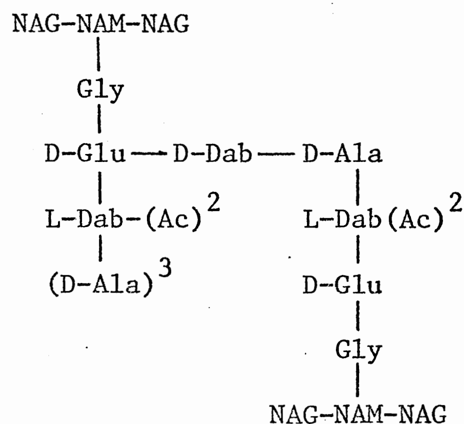
Figure 2. Fragments of the Primary Structure of Four Different Types of MP (Abbreviations of Amino Acids are: Ala = Alanine, Glu = Glutamic Acid, Lys = Lysine, Gly = Glysine, Dab = Diaminobutyric Acid, Orn = Ornithine, Asp = Aspartic Acid, Ac = Acetyl, A = *Staphylococcus aureus*, B = *Corynebacterium insidiosum*, C = *Lactobacillus plantarum*, D = *Lactobacillus cellobiosus*)

A.



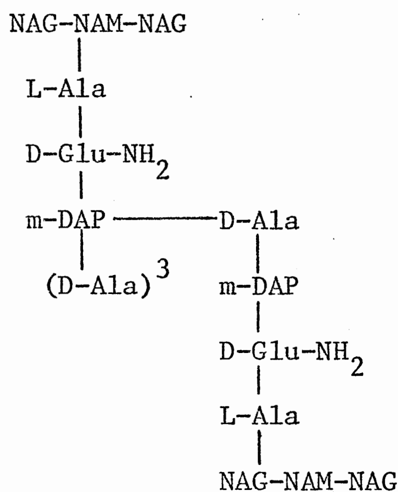
L-Lys-Gly_{5-6} type

B.



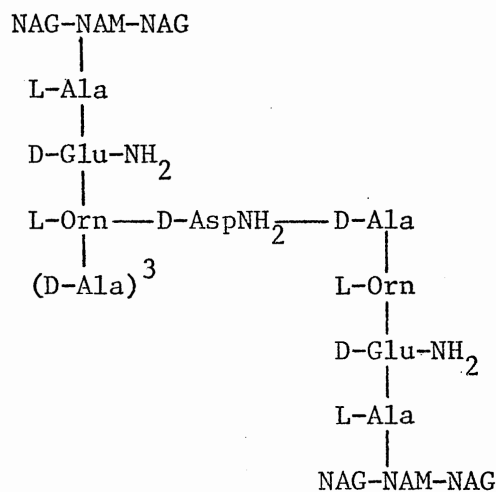
(L-Dab) D-Glu-D-Dab-type

C.



m-DAP direct type

D.



L-Orn-D-Asp type

Schleifer, Hames and Kandler (28) have shown that growth-inhibiting concentrations of glycine or D-amino acids lead to a modification of the peptide subunits and to a decrease in the extent of their cross-linkage. Glycine can replace L-alanine in position 1 and D-alanine residues in positions 4 and 5 of the peptide subunits. Incorporation of D-amino acids is restricted to positions 4 and 5. These studies were conducted upon eight different gram-positive bacteria of various genera representing the four most common MP types.

Schleifer et al. (48) have proposed that in the presence of D-amino acids or glycine at growth inhibiting concentrations modified nucleotide-activated MP precursors are formed in which D- or L-alanine residues are replaced by glycine or D-amino acids. Since they are less efficiently incorporated into the MP, a high percentage of the modified MP remains uncross-linked, showing that they are poor substrates for the transpeptidation reaction (53). This is consistent with data showing that residues 4 and 5 of the pentapeptide moiety play an important role in the donor phase of cross-linked synthesis. The transpeptidase has a higher degree of specificity in the donor phase for D-alanine in residue 4 than for D-alanine in residue 5 in the cross-linking stage of MP synthesis (4) (45).

D-Serine is one of the most commonly used D-amino acids to demonstrate growth inhibition, elongation, and incorporation into the MP. D-Serine has been shown to inhibit the synthesis of pantothenate in E. coli (41), Erwinia sp. (22) and a Flavobacterium sp. (11). The inhibition can be overcome by addition of beta-alanine or pantothenic acid (11) (22) (24). In a further study, it was demonstrated that

D-serine inhibits the alpha-decarboxylation of aspartic acid in Erwinia sp. (22). Some reports have also shown that D-serine inhibits the racemization of alanine (32) (40). In addition to these findings, there are numerous reports of its incorporation into MP (26) (48) (57) (62) (63) (64).

Dubos first reported that DL-serine is toxic for virulent and avirulent strains of Mycobacterium tuberculosis (18). It has been shown that D-serine inhibits cell wall synthesis in Bacillus subtilis and Pasteurella pestis. This inhibition can be reversed by addition of D-alanine (50) (54). Similar findings by Yabu and Heumpfner (64) have shown D-serine can induce the accumulation of uridine diphosphate-N-acetylmuramyltripeptide, a cell wall intermediate in Mycobacterium smegmatis, and this accumulation could be decreased by addition of D-alanine. D-Serine also inhibited the growth of M. smegmatis and induced morphological alterations in the organism. On the basis of their results, Yabu and Kuempfner concluded that D-serine acts at three sites of cell wall MP synthesis: (1) the racemization of alanine, (2) the incorporation of glycine, and (3) the formation of DAP from aspartic acid.

Data concerning the role of D-serine on Erwinia carotovora (24) have shown that it causes filament formation and inhibition of cell division (18) (21) (23) (27). Five other D-amino acids (histidine, tryptophan, methionine, phenylalanine, and threonine) have shown similar results (18). Analysis of the MP in growing cells of E. carotovora reveals that it consists of 2-alanine, 1-glutamic acid and one DAP residues. Smaller amounts of aspartic acid and glycine were also observed and it was suggested that these amino acids are present in the

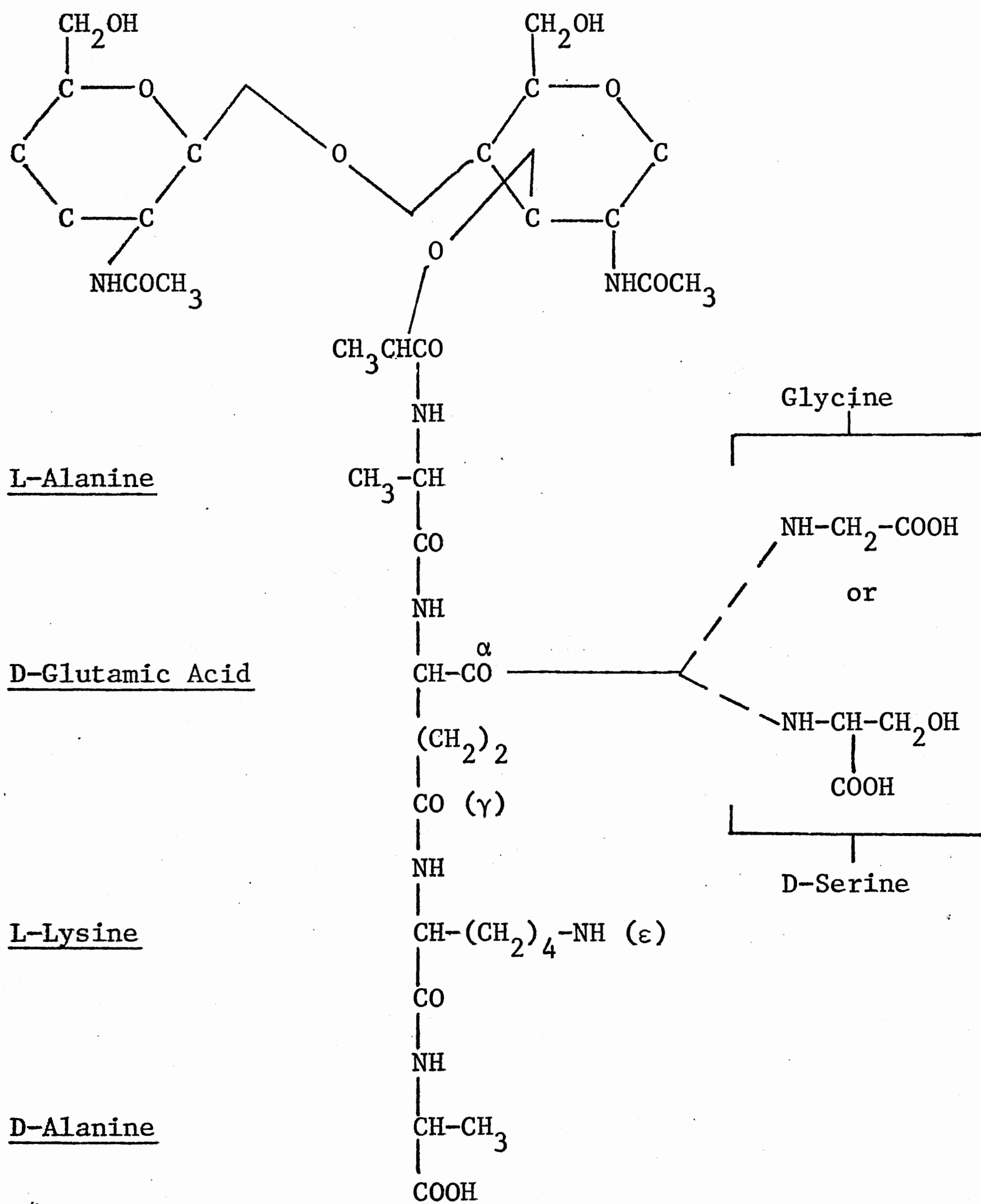
crossbridge of this organism (26). Further work has shown that D-serine incorporation into E. carotovora produces a 30 to 40% inhibition in MP synthesis during formation of filaments (23). It was also found that when glycine and D-serine are present in the growth medium, both are incorporated but glycine to a lesser extent (26).

These findings are similar to others (28) (30) (33) (48) (57) (64) which indicate that D-serine can partially replace glycine in MP; however, from these studies the site of D-serine incorporation was not determined.

Study of the effects of D-serine on Micrococcus lysodeikticus, an organism frequently used in our laboratory, has provided evidence for the site of incorporation of D-serine in the MP of this organism and, perhaps, by analogy, into E. carotovora. The tetrapeptide found in M. lysodeikticus is like that found in E. carotovora with the exception of lysine replacing DAP (14) (26). Whitney and Grula (62) reported that D-serine inhibits the growth of M. lysodeikticus to one-third that of control and is incorporated into the MP at an amount almost equal to that of lysine or glutamic acid. No morphological abnormalities such as protoplast or filament formation were observed. The greatest variation shown was the reduction of glycine to about one-half its normal value.

The cell wall MP of M. lysodeikticus grown in the presence of D-serine has been isolated and analyzed for content of C- and N-terminal amino acids. It was found that approximately 75 to 80% of the incorporated D-serine can substitute for glycine (Figure 3) and is attached via a peptide bond to the alpha-carboxyl group of glutamic acid (63). Studies using both wild type M. lysodeikticus and the dis--IIp+ mutant have shown the location of D-serine to be identical to both organisms

Figure 3. Structure of the MP in *Micrococcus lysodeikticus* Showing D-Serine Replacing Glycine on the *Alpha*-Carboxyl Group of Glutamic Acid



CH2OH
C
O
C
C
C
C
NHCOCH3
O
CH2OH
C
O
C
C
C
C
NHCOCH3
O
CH3CHCO
NH
L-Alanine
CH3-CH
CO
NH
D-Glutamic Acid
α
CH-CO
(CH2)2
CO (γ)
NH
L-Lysine
CH-(CH2)4-NH (ε)
CO
NH
D-Alanine
CH-CH3
COOH
Glycine
NH-CH2-COOH
or
NH-CH-CH2OH
COOH
D-Serine

(33). Similar findings have also been reported for M. tuberculosis where D-serine is also thought to replace glycine on the alpha-carboxyl group of glutamic acid (35) (64).

The only theory proposed on the attachment site of D-amino acids causing uncross-linked mucopeptide comes from Strominger et al. (31). This theory describes the reversibility of the terminal transpeptidation step in cell wall synthesis and indicates that it can be reversed by D-amino acids. When D-amino acids are employed, the D-alanine on the number 5 position of the pentapeptide, which remains mostly intact when the cells are subjected to penicillin (showing inhibition of the transpeptidation reaction) now becomes cleaved at a much greater rate indicating a reversal rather than an inhibition of this reaction. In addition, the added D-amino acid replaces the D-alanine that would normally be removed (Figure 4). This theory implies that D-amino acids can reverse the transpeptidation step in contrast to penicillin which always inhibits the forward reaction (53). Data already presented shows that D-amino acids can replace not only D-alanine in position number 5, as inferred by Strominger, but also in position number 4 (28) (48) (57). However, when D-serine is incorporated by being attached to the alpha-carboxyl group of glutamic acid, as in M. lysodeikticus (63), cross-linking is not involved as would be implied by Strominger's theory. There is also evidence showing that D-amino acids may not cause a reversal of the transpeptidation reaction, because a significant amount of cross-linking still remains. Grula and King (33) reported that penicillin caused a 52% inhibition of cross-linkage in MP of growing cells of M. lysodeikticus in contrast to only 14% when D-serine was

Figure 4. The Strominger Theory of D-Amino Acid Incorporation in the MP of *Escherichia coli*

Normal Cell Wall

NAG — NAM Substrate Cleavage

L-Ala-D-Glu-Meso-DAP-D-Ala-D-Ala
#1 #2 #3 #4 #5 released

NAG — NAM

L-Ala-D-Glu-Meso-DAP-D-Ala-D-Ala

direct peptide Bond

NAG — NAM (+Penicillin)

L-Ala-D-Glu-Meso-DAP-D-Ala-D-Ala
#1 #2 #3 #4 #5

NAG — NAM

L-Ala-D-Glu-Meso-DAP-D-Ala-D-Ala

Transpeptidation inhibited, no cross-linkage,
#5 D-alanine remaining intact, no cleavage.

Normal Cell Wall

NAG — NAM Substrate

L-Ala-D-Glu-Meso-DAP-D-Ala-D-Ala
#1 #2 #3 #4 #5

(+D-amino acid)

NAG — NAM

release of #5
D-Alanine replacement by

NAG — NAM

L-Ala-D-Glu-Meso-D-Ala-D-Ala

L-Ala-D-Glu-Meso-DAP-D-Ala-D-amino acid
#1 #2 $3 #4 #5

Transpeptidation reversed, no cross-
linkage, #5 D-alanine released,
complete cleavage. D-amino acid
remaining C-terminal in #5 position.

present during growth. This finding would be expected if D-serine is incorporated by being attached to the *alpha*-carboxyl group of glutamic acid as shown by Whitney and Grula (62) (63).

To check Strominger's theory we set out to determine the site of attachment of D-serine in the MP of the gram-negative organism *E. carotovora*. Other than the work of Strominger et al. (31) no one has specified a possible site for incorporation of a D-amino acid into the MP of a gram-negative organism. The results of this investigation should help to determine if Strominger's theory, relating to D-amino acid incorporation and activity, is correct and applicable to other bacteria.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism utilized in this study was a stock culture of *Erwinia carotovora* (24). Stock cultures of this organism were maintained on nutrient agar containing 0.5% sodium chloride with and without 1.0% dextrose. To ensure purity, the culture was periodically streaked on nutrient agar and observed for variation in colony morphology.

Media

The basal salts medium used in this study contained the following per 100 ml: glucose (150 mg), L-aspartic acid (280 mg), K_2HPO_4 (174 mg), KH_2PO_4 (136 mg), and $MgSO_4 \cdot 7H_2O$ (3 mg). The following components were added as trace mineral salts per 100 ml medium: H_3BO_3 (0.5 μg), $CaCO_3$ (10.0 μg), $CuSO_4 \cdot 5H_2O$ (1.0 μg), $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ (50.0 μg), $K1O_4$ (1.0 μg), $MnSO_4 \cdot H_2O$ (2.0 μg), MoO_3 (1.0 μg), and $ZnSO_4 \cdot 7H_2O$ (5.0 μg). During specific experiments either DL-serine (to 0.034 M) or D-serine (to 0.017 M) was added. All solutions were adjusted to pH 6.8 to 7.0 prior to addition to the medium. Glucose and serine were sterilized by filtration and added aseptically. All other components were sterilized together by autoclaving for 15 to 20 minutes at 121°C. All solutions were prepared using deionized water.

Growth of Cells

Nutrient agar slants were inoculated from stock cultures and incubated 12 to 24 hours at 25°C. The resulting growth was suspended in sterile 0.85% saline and washed two times with centrifugation (clinical model). Cells from one nutrient agar slant were then resuspended in saline to an optical density equivalent to 0.1 at 540 nm (Bausch and Lomb spectronic 20 Spectrophotometer) in 20 mm (OD) by 150 mm Kimax test tubes. One drop of this suspension was used to inoculate 5.0 ml of medium, while 1.0 ml was used to inoculate 100 ml of medium. Inoculated media (5.0 ml) were incubated in 20 mm by 150 mm test tubes at 25°C on a rotary shaker having 180 revolutions per minute whereas volumes of 100 ml were incubated in 250 ml Erlenmeyer flasks. Cells were harvested by centrifugation after 17 hours of incubation.

Procedure for Isolation of Mucopeptide (MP)

Whole cells were suspended in water and added dropwise with stirring into boiling 4% sodium dodecylsulfate (SDS) at a ratio of 1 ml cells to 6 ml SDS. The suspension was stirred for another 2 hours while it cooled and then kept overnight at room temperature (2).

The following procedure was then used for the isolation of MP from the SDS treated cells:

Wash 5X in buffer A (K_2HPO_4, 0.05 M, pH 7.8) at 30,000 rpm for 35 minutes.

|

Resuspend cells in 5 to 10 ml of the buffer A and add trypsin (0.5 mg/ml/final). Incubate 2 hours at 35°C and add same amount of buffered trypsin; continue incubation 2 additional hours then centrifuge at 30,000 rpm for 35 minutes.

|

Wash sediment 2X in buffer A.

|

Wash sediment 2X in distilled water.

|

Resuspend sediment and spin at low speed (approximately 4,000 rpm for 10 minutes); discard sediment and centrifuge supernatant for final isolation of MP (20).

All centrifugations except for the slow spin were accomplished using a number 40 head in the Beckman Spinco Model L Ultracentrifuge at 78,410 G-force (30,000 rpm) for 35 minutes. All washing procedures were done using cold (0 to 4°C) solutions. All manipulations except boiling of the cells were performed in centrifuge tubes used in the number 40 Spinco head; all volumes were adjusted to the full level of these tubes.

Acid Hydrolysis of Mucopeptide

Mucopeptide hydrolysis was performed by placing 0.3 to 2.0 ml samples of isolated MP in 10 mm (OD) by 100 mm test tubes. An equal volume of 12N hydrochloric acid was added forming a 6N solution for total hydrolysis of MP components. The incubation period for total hydrolysis was 18 to 24 hours *in vacuo* at 100°C. A 4N solution of hydrochloric acid was used for partial hydrolysis to obtain peptides. The incubation period for partial hydrolysis was 1 to 4 hours *in vacuo* at 100°C.

Amino Acid Labeling Experiments

Cells were grown in 100 ml of basal medium for 17 hours in the presence of 1 μ Ci of C^{14}-labeled D-serine added at time = 0. Following optical density readings at 540 nm on a Bausch and Lomb Spectronic 20 Spectrophotometer (for conversion of cell density to dry weight values using a previously constructed curve), the cells were washed twice in

0.85% saline solution and the MP isolated. The cell wall MP fractions were usually subjected to acid hydrolysis in 6N HCl for amino acids and analyzed for radioactive compounds by radioautography.

Formation of Dansyl Derivatives for Determination of N-Terminal Amino Acids

Determination of amino-terminal residues of proteins and peptides was carried out using 1-dimethylaminonaphyhalene-5-sulphonyl chloride (CNS-CL), which reacts with free amino and phenolic groups to form compounds having an intense yellow fluorescence. The procedure is as follows:

The MP was added to 15 µl of 0.1 M $NaHCO_3$ (pH = 9.8) and 15 µl of a solution of CNS-Cl in acetone (mg/ml), forming a one phase system. After 3 hours at room temperature the sample was further dried *in vacuo*, and 20 µl of 6N HCl were added; the tube was then sealed and heated at 105°C for 6 to 12 hours. Acid was removed *in vacuo* and the hydrolysate subjected to thin-layer chromatography. All procedures were performed in hydrolysis tubes, 10 mm (OD) by 100 mm (17). Standard R_f values for each of the three solvents used are given in Table I.

Hydrazinolysis Procedure for Determination of C-Terminal Amino Acids

One ml of anhydrous hydrazine was added to 0.2 to 0.8 ml of the peptide and heated under reflux at 125°C (oil bath) for 8 hours. An autoclave adjusted to a constant temperature of 125°C can be substituted for the oil bath. Care should be taken not to allow moisture into the

TABLE I

THIN-LAYER CHROMATOGRAPHY OF AMINO ACID DANSYL DERIVATIVES
R_f VALUES IN VARIOUS SOLVENT SYSTEMS

Source of Derivative	R_f Values		
	Solvent*		
	A	B	C
Glutamic Acid	.10	.23	.75
Aspartic Acid	.05	.14	.71
Diaminopimelic Acid	.23, .61	.83, .94	.72, .94
Alanine	.26	.92	.96
Glycine	.15	.84	.91
Serine	.07	.41	.70
Lysine	.21, .60	.79, .96	.65, .91
Tyrosine	.07	.86	.96
Phenylalanine	.20	.96	.98
Proline	.21	.94	.97
Valine	.23	.97	.98
Isoleucine	.25	.99	.99
Threonine	.01	.50	.89
Tryptophan	.07	.89	.97
Methionine	.15	.97	.99
Leucine	.22	.99	1.00

*A = Benzene:pyridine:acetic acid (80:20:2).
B = Chloroform:tert-amylalcohol:acetic acid (70:30:3)
C = Chloroform:tert-amylalcohol:formic acid (70:30:1)

tubes. This is somewhat remedied by using 20 mm (OD) by 150 mm test tubes with an overlapping lip sealed tight with a marble. After cooling the hydrazinolysate was poured into a watch glass and set inside a desiccator. Excess hydrazine was evaporated as much as possible *in vacuo* over concentrated sulfuric acid (10 ml in a petri dish). After approximately six days the hydrazinolysate slurry was dissolved in water and transferred to a 17 mm (OD) by 150 mm test tube. An excess of benzaldehyde was dropped into it under cooling (ice bath) and stirring under the hood, whereupon hydrazides condensed with benzaldehyde to form a pale yellow amorphous precipitate. The yellow color is due to the formation of benzalazine from the remaining hydrazine. After filtering off the precipitate (millipore filter) the colorless filtrate will contain the C-terminal amino acid(s) (1). All C-terminal amino acid(s) remaining using this procedure were quantitated using an amino acid analyzer. Qualitative tests were also done using thin-layer chromatography.

Chromatography and Detection of Amino Acids

Chromatograms spotted with samples of partially hydrolyzed MP were developed in the two-dimensional system of Schleifer and Kandler (49). The first solvent consisted of isopropyl alcohol, acetic acid, water (75:10:15 v/v/v/); the second of *alpha*-picoline, 25% NH_4OH, water (70:2:28 v/v/v). Chromatograms spotted with samples of totally hydrolyzed MP were developed in the two-dimensional system of Heathcote and Jones (29). The first solvent consisted of isopropanol, formic acid, water (80:4:20 v/v/v); the second of tert-butyl alcohol, methyl

ethyl ketone, concentrated NH_4OH, H_2O (50:30:10:10 v/v/v/v). Both paper and thin-layer chromatograms were used. Whatman number 1 filter paper was used for the papergrams and MN-300 cellulose (Brinkmann) was used for the thin-layer plates. Samples of 10 to 80 µl were spotted under a stream of warm air from a hair dryer. Amino acids, NAM and NAG, were detected by spraying with a solution of 0.5% ninhydrin in 100 ml of acetone. After spraying, the chromatograms were heated at 100°C for 3 minutes in an oven. Amino acids appeared as either purple, yellow, or reddish brown spots on a white background. Dansyl derivatives were spotted on silica gel G (Brinkmann) thin-layer plates and developed in three different one-dimensional solvent systems (given in Table I) and viewed for yellow fluorescence under a UV lamp (44).

Radioautography

Radioautography was performed by placing paper chromatograms or thin-layer chromatograms next to Blue Brand Medical x-ray film and allowing them to remain in contact 1 to 4 weeks in the dark. Films were developed using Edwal Hispeed-Hi Contrast x-ray developer diluted to a 1:4 ratio with tap H_2O and fixed for 15 minutes in Kodafix rapid fixer. Previous work in this laboratory has shown that this method allows detection of 100 counts per minute of a C^{14}-labeled compound after 16 days contact.

CHAPTER III

RESULTS

Concentration of D-Serine

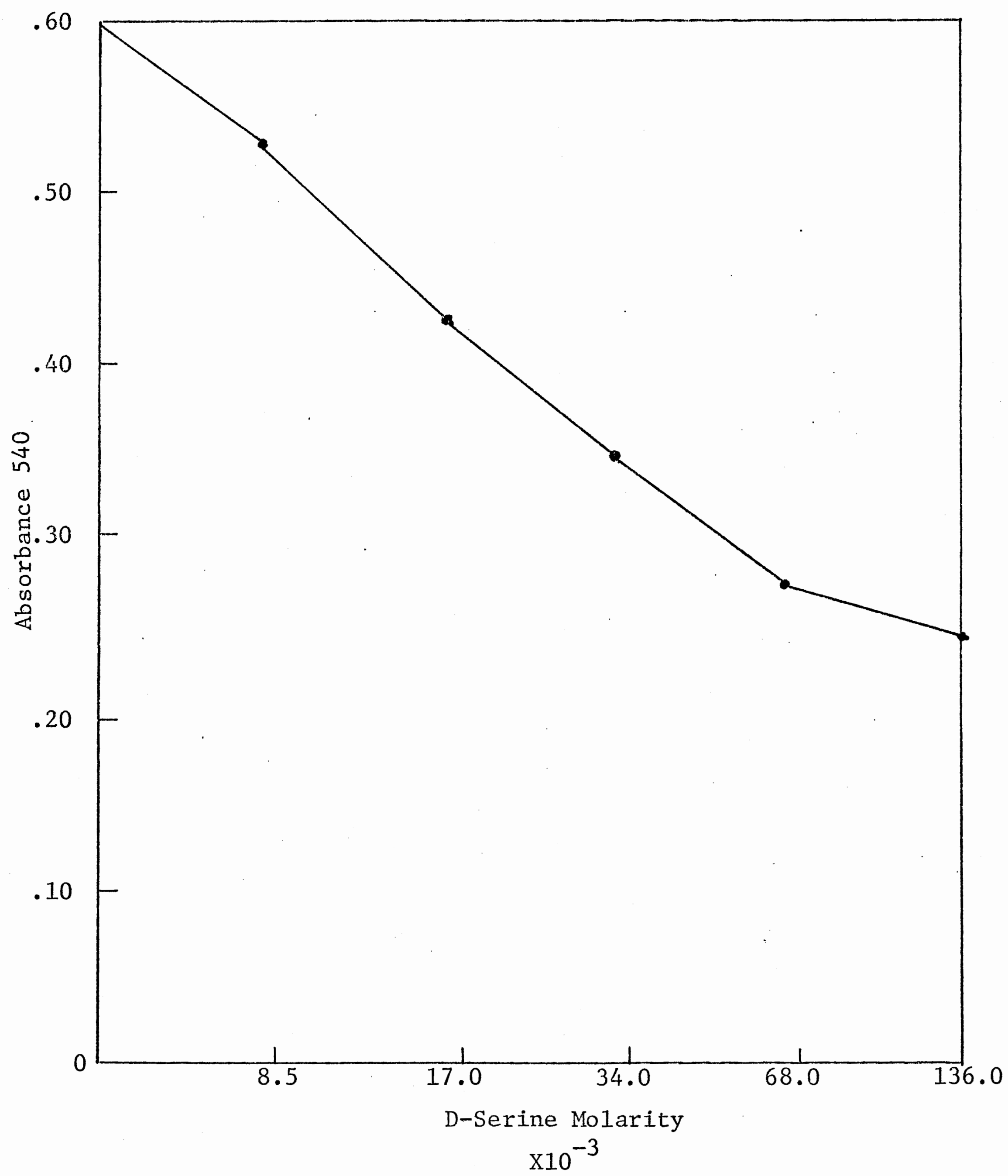
At the beginning of this study it was necessary to determine at what concentration added D-serine would produce the longest filaments while still permitting good growth of cells. A standard titration was conducted and is shown in Figure 5.

It was observed (at 17 hours) that a concentration of D-serine of 17.0×10^{-3} M permitted acceptable growth yields (0.42) and satisfactory cell length (average about 59.0 μ), as shown in Figure 6. Such conditions were necessary for incorporation of D-serine and to obtain a sufficient cell mass for isolation of MP.

Determination of Mucopeptide Composition

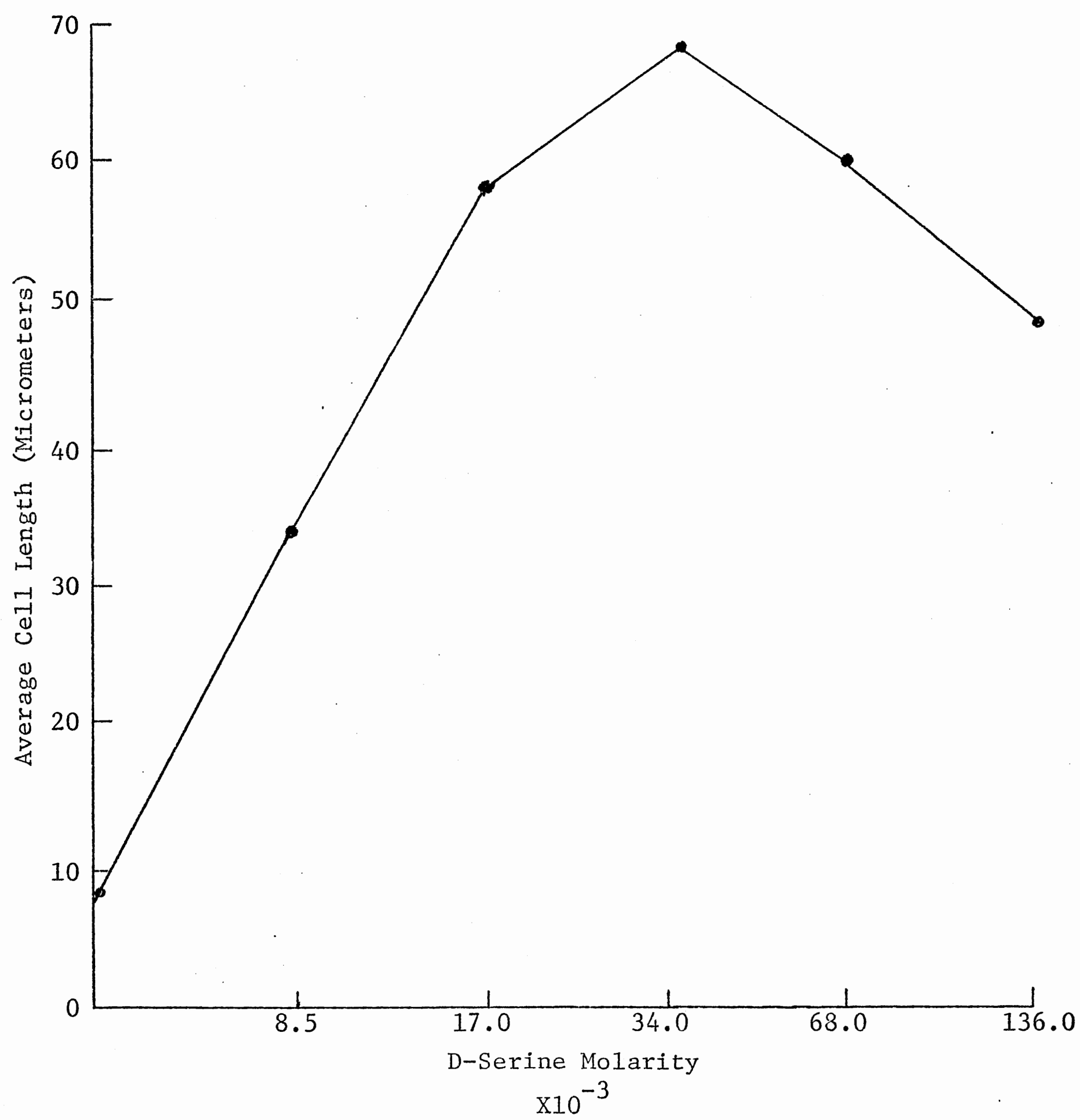
The amino acid composition of normal and serine-containing MP of *E. carotovora* is given in Table II. Molar ratios of each amino acid comprising the MP were determined by taking DAP as 1.0. Control (normal) MP of *E. carotovora* showed an amino acid composition comparable to that reported previously. An alanine to glutamic acid to DAP ratio of 1.5:1:1 was present. Lysine was most probably present as the N-terminal amino acid of Braun's protein (3). Although it has not been specifically shown that Braun's protein exists in *E. carotovora*,

Figure 5. Growth of *E. carotovora* as a Function of the D-Serine Concentration After 17 Hours Incubation



.60
.50
.40
.30
.20
.10
0
Absorbance 540
8.5
17.0
34.0
68.0
136.0
D-Serine Molarity
X10-3

Figure 6. Cell Length in *E. carotovora* as a Function of the D-Serine Concentration After 17 Hours Incubation


70
60
50
40
30
20
10
0
Average Cell Length (Micrometers)
8.5
17.0
34.0
68.0
136.0
D-Serine Molarity
X10-3

a protein was present which must be removed by continued washings and trypsinization during isolation of MP. It was suspected, therefore, that E. carotovora is similar to E. coli and contains a Braun's protein or a similar type which links the cell wall to the lipopolysaccaride layer. Mucopeptide from cells grown in the presence of D-serine possesses an alanine to glutamic acid to DAP ratio of 1.2:1:1. An increased amount of serine was also present showing that the compound was incorporated into the MP when present in the growth medium. Small amounts of aspartic acid and lysine were also present as in control cell walls. The decreased glycine content along with the concomitant rise in D-serine indicated that serine was most likely replacing glycine as reported previously (26).

TABLE II

AMINO ACID COMPOSITION OF NORMAL AND SERINE-CONTAINING MUCOPEPTIDE OF E. CAROTOVORA

Amino Acid	Amino Acid Molar Ratios**	
	Control	D-Serine
Alanine	1.5	1.2
Glutamic Acid	1.0	1.0
*Diaminopimelic Acid	1.0	1.0
Serine	0.08	0.40
Glycine	0.24	0.17
Aspartic Acid	0.10	0.10
Lysine	0.10	0.10

*DAP taken as 1.0.
**DL-Serine added to the medium to a concentration of 3.4×10^{-2} M.

Previous work using this organism showed the ratio of alanine to DAP to be 1.8:1 (26). Data reported in Table II indicates a lower alanine to DAP ratio. This type of result may be consistent with other findings (59) which show that during simple repeated centrifugations, portions of the terminal D-alanine can be removed from MP.

Amino Acid Dansyl Derivatives

Amino terminal analyses were performed using MP from normal as well as D-serine grown cells. These data are presented in Table III.

TABLE III

N-TERMINAL AMINO ACIDS IN NORMAL AND D-SERINE-CONTAINING MUCOPEPTIDE

	R_f Values*		
	Solvent**		
Source of Mucopeptide	A	B	C
D-Serine Grown Cells	.20, .61	.80, .95	.66, .93
Control Cells	.20, .61	.80, .95	.66, .93

*See Table I for R_f values of amino acids.
**A = Benzene:pyridine:acetic acid (80:20:2).
B = Chloroform:tert-amylalcohol:acetic acid (70:30:3).
C = Chloroform:tert-amylalcohol:formic acid (70:30:1).

In solvent A, MP from control cells showed two spots giving R_f values of .20 and .61. Mucopeptide from cells grown in the presence of D-serine also showed two spots having the same R_f values. These values

correspond to the *beta*-amino group of lysine and the *epsilon*-amino group of either lysine or DAP. In solvents B and C, normal and D-serine containing MP both showed two spots with the same R_f values again corresponding to the *beta*-amino group of lysine and the *epsilon*-amino group of DAP or lysine. It was also observed that the slower running spot (*beta*-amino group of lysine) was always present in greater proportion to the faster running spots (*epsilon*-amino groups of lysine or DAP) in all cases. From the N-terminal data we were unable to distinguish between the *epsilon*-amino groups of DAP and lysine. No other N-terminal amino acids were detected, either from control or D-serine containing MP.

C-Terminal Amino Acids in Normal and Serine-Containing Mucopeptide

Results obtained in carboxyl-terminal analyses are presented in Table IV. The first two columns show the averages of four separate C-terminal experiments. Since methionine comes off the column in the amino acid analyzer together with DAP, it was necessary to correct the DAP figures by subtracting 10% from the total DAP content. Corrected DAP is shown in the second two columns.

Previously it was shown that a ratio of 1:10 exists between lysine and DAP in the MP of *E. carotovora* of normal and D-serine grown cells. We assume that DAP in *E. carotovora* is 90% free C-terminal while 10% is occupied by lysine as has been shown in *E. coli* where Braun's protein is attached via lysine to *meso*-DAP 12 to 14% of the time (3).

Total DAP, as shown in Table IV, was determined by the following equation, where X represents the total DAP content:

TABLE IV

HYDRAZINOLYSIS: C-TERMINAL AMINO ACIDS IN NORMAL AND SERINE-CONTAINING MUCOPEPTIDE OF _E. CAROTOVORA_

Amino Acid	C-Terminal*		Corrected DAP**		Total DAP***		C-Terminal Ratios****	
	Control MP	D-Serine MP	Control MP	D-Serine MP	Control MP	D-Serine MP	Control MP	D-Serine MP
Alanine	43.32	58.96					1.27	1.22
Glutamic Acid	4.72	4.21					.14	.10
Diaminopimelic Acid	34.02	48.14	30.62	43.33	34.02	48.14	1.00	1.00
Serine	9.23	38.80					.27	.81
Glycine	16.61	13.74					.50	.28
Aspartic Acid	5.14	4.73					.15	.09
Lysine	4.58	3.57					.13	.07

*Averages of four separate C-terminal determinations, expressed as nM/0.1 ml.

**DAP corrected for methionine (C-terminal DAP--10% as methionine), expressed as nM/0.1 ml.

***Total DAP based upon: $\frac{\text{Amount of C-terminal DAP}}{X} = .90$, X = total DAP content expressed as nM/0.1 ml.

****Ratios based upon: $\frac{\text{C-terminal amount of each amino acid}}{\text{total amount of DAP nM/0.1 ml}}$.

$$\frac{\text{Amount of C-terminal DAP}}{X} = 90.$$

Since DAP is in a ratio of one to all other amino acids in the MP it can be used to determine the C-terminal ratios of each amino acid. Carboxyl terminal ratios for both control and D-serine grown cells are based upon the corrected total DAP divided into the C-terminal of each amino acid as also shown in Table IV. The C-terminal ratios show that alanine is the major free carboxyl component in control cells. Alanine compared to DAP shows that possibly one alanine residue has its carboxyl group completely free. In addition, glycine contained a relatively high amount of free carboxyl groups. Moderate amounts of aspartic acid and some serine free carboxyl groups were also observed. Lesser amounts of lysine free carboxyl groups were also found. Interestingly, glutamic acid, which is normally in a molecular ratio of 1:1 to DAP, and which should contain a free *alpha*-carboxyl group (no N-terminal group; therefore, this group is not aminated as it would be in glutamine), contained a very low amount of free C-terminal carboxyl groups. D-Serine MP also has alanine as the major free carboxyl terminal component. Serine was seen to more than triple as compared to control values indicating it exists largely as a C-terminal component. Values in both lysine and aspartic acid were relatively the same as control values with a slight decrease in both. Carboxyl terminal glycine decreased to one-half that of control MP showing that it is largely replaced by D-serine. Amounts of carboxyl terminal glutamic acid were again very low.

Structure of Mucopeptide in Normal Cells

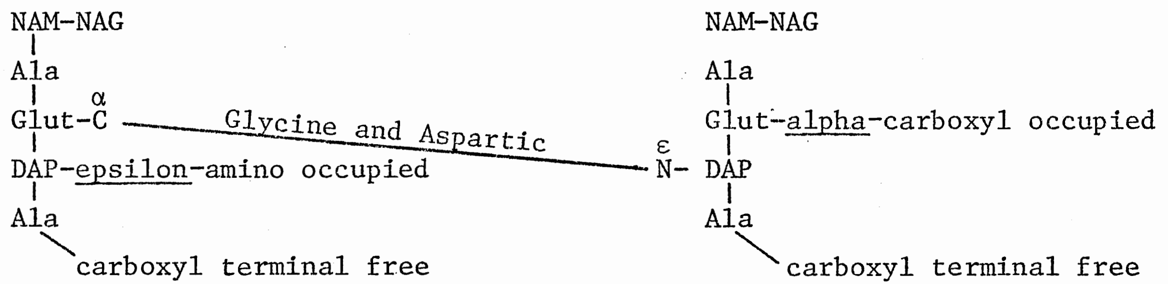
Concerning the structure of control cell wall MP of *E. carotovora*,

five possible models are shown in Figure 7. All five models are based on the assumption that the order of the tetrapeptide amino acids are: L-alanine-D-glutamic acid-DAP-D-alanine. Model I is based primarily on the C-terminal data. As observed, D-alanine is C-terminal in control cell MP. Very little of the alpha-carboxyl group of glutamic acid exists as a free C-terminal group; therefore, it appears that it is occupied either by a single amino acid or it is involved in cross-bridging. Amino-terminal data indicate that the beta-amino groups of lysine and the epsilon-amino group of DAP or lysine are at least partially free. However, as noted earlier, the epsilon-amino groups of lysine or DAP are present in small amounts compared to the beta-amino group of lysine. It is possible that not all the tetrapeptides are cross-linked, therefore leaving some epsilon-amino DAP free. Since glycine and aspartic acid are found in moderate amounts in comparison to the tetrapeptide amino acids, we assume that they could be involved in a cross-bridge as shown in Model I. Also, we observed relatively large amounts of C-terminal glycine and aspartic acid indicating that they are bonded via their N-terminal groups.

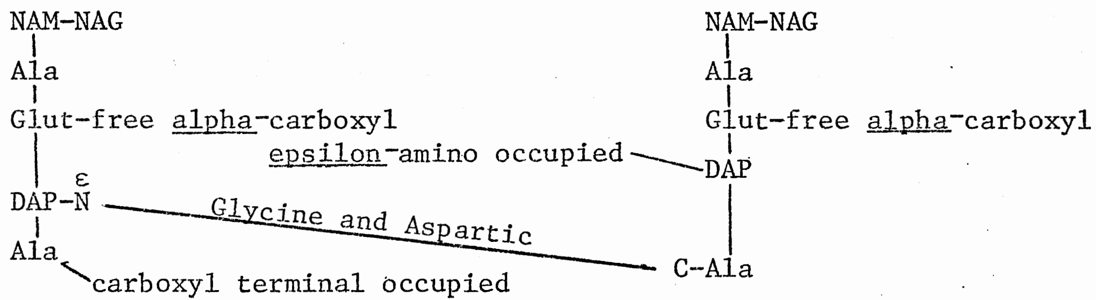
Model II shows a different type of cross-bridging and is observed to exist in most bacteria. Model II is in disagreement with our C-terminal data in two major instances. First, the alpha-carboxyl group of glutamic acid would have to be completely free, which it is not. Second, since the glycine aspartic acid cross-bridge is shown to be between the epsilon-amino group of DAP and the carboxyl group of alanine, we would expect little or no C-terminal alanine. This is not in agreement with any experimental data obtained which relates to alanine.

Figure 7. Possible Structure of MP in Normal Cells of *E. carotovora*

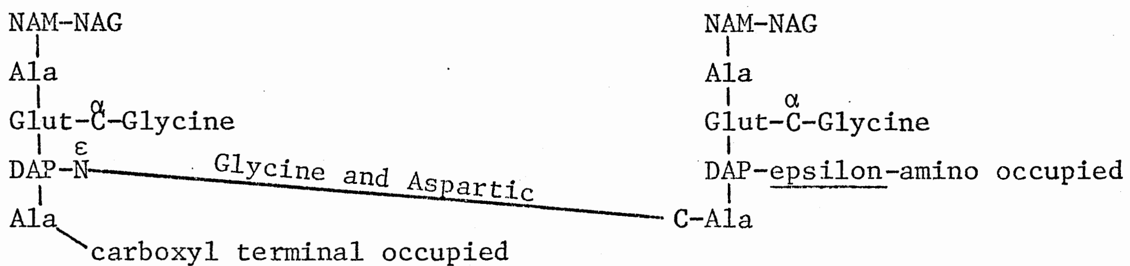
Model I



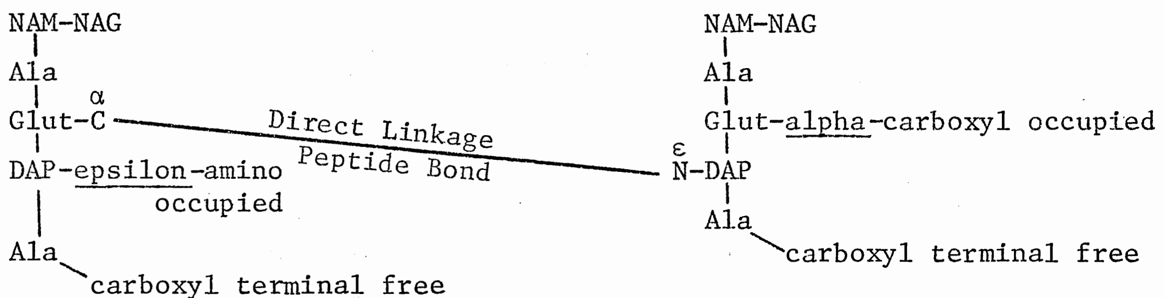
Model II



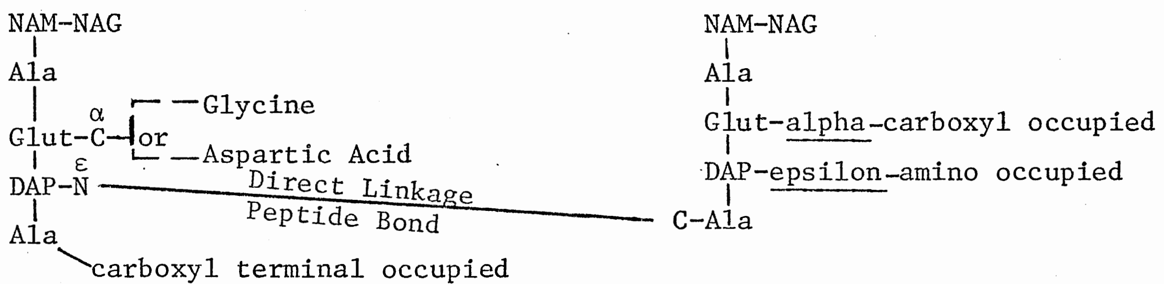
Model III



Model IV



Model V



Model III is in agreement with the data in regard to the _alpha_-carboxyl group of glutamic acid. In this model, glycine is attached to glutamic acid. This is a good assumption since moderate amounts of C-terminal glycine have been observed. A major disagreement with Model III is the glycine-aspartic acid cross-bridge extending from the _epsilon_-amino group of DAP to the carboxyl group of D-alanine. If this were the case, we would observe little or no C-terminal alanine.

Model IV shows a novel type of cross-bridge which consists of a direct peptide bond between the _alpha_-carboxyl group of glutamic acid and the _epsilon_-amino group of DAP. This model is in agreement with the C-terminal data in regard to the _alpha_-carboxyl group of glutamic acid being occupied and the carboxyl group of alanine being free. This model does not consider the possible locations of glycine and aspartic acid in the mucopeptide structure.

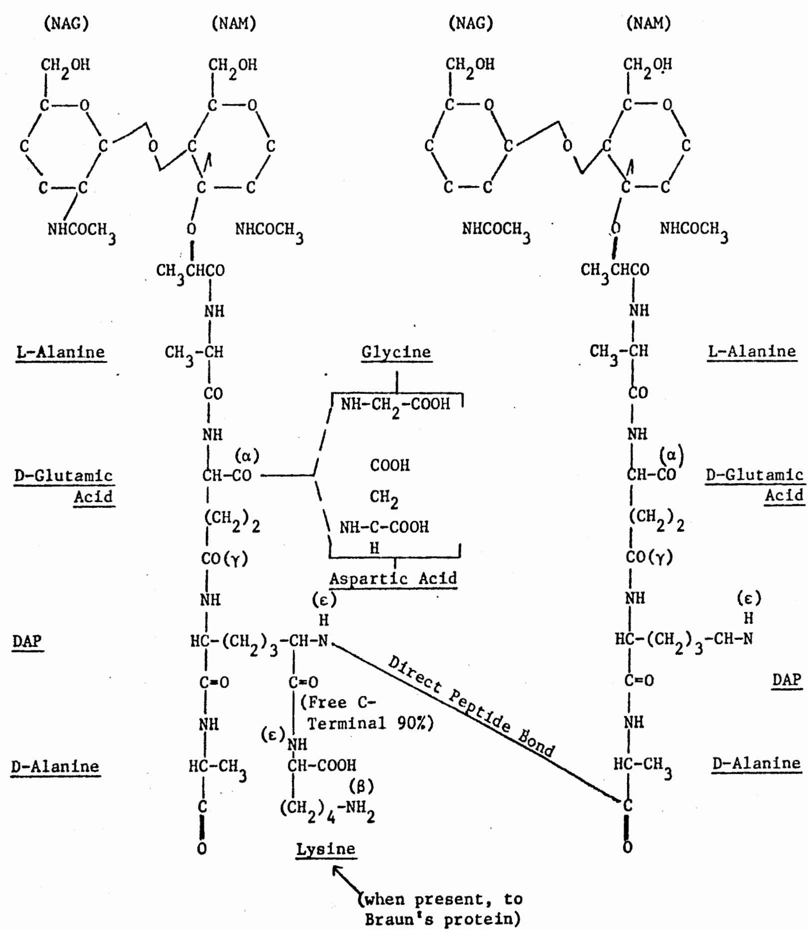
Model V is in agreement with the C-terminal data except in regard to free carboxyl terminal alanine. This model shows a direct peptide cross-bridge from the _epsilon_-amino group of DAP to the carboxyl group of alanine. Model V most closely agrees with the C-terminal data concerning the additive effect of carboxyl terminal groups of normal cell MP. The additive effect of glutamic acid (.14), serine (.27), glycine (.50), and aspartic acid (.15) (equal to 1.06), seems to indicate that these amino acids are completely C-terminal, and their amino groups attached to the _alpha_-carboxyl of glutamic acid and, therefore, not involved in a cross-bridge. Model V agrees with all the data we have obtained on the MP as regards N- and C-terminal data, with the exception of the carboxyl group of alanine. The carboxyl group of alanine is shown to be mostly free according to our C-terminal data.

After evaluating the experimental data in light of all models, it would appear that Model V most closely fits our findings, with the exception of data relating to C-terminal alanine. Therefore, this researcher proposes the structure shown in Figure 8 for the normal cell wall MP of E. carotovora. This model most clearly correlates to the C-terminal data obtained in regard to glycine, aspartic acid, and serine as described in Model V. This structure shows that most of these amino acids are C-terminal and, therefore, most probably attached via their amino groups to the alpha-carboxyl group of glutamic acid. This is in agreement with the findings which show the alpha-carboxyl group of glutamic acid is nearly completely occupied. Although there is no proof of the exact cross-linkage, the N-terminal data indicate the epsilon-amino group of DAP is also nearly completely occupied. This would indicate its possible involvement in a cross-bridging.

Structure of Mucopeptide in D-Serine-Grown Cells

In comparing the amino terminal data in control and D-serine-grown cells, no differences were observed. Upon comparing the carboxyl terminal data, approximately identical figures for each amino acid (except serine and glycine) were obtained. As shown in Table IV, there is about three times more C-terminal serine present in D-serine-grown cell wall MP. At the same time, it was observed that the number of glycine C-terminal residues decreased to about one-half that of control cell wall MP. This suggests that D-serine is replacing glycine. Again, it was shown that very little glutamic acid is C-terminal. These data suggest that the alpha-carboxyl group of glutamic acid is occupied (most

Figure 8. Proposed Cell Wall MP Structure in *E. carotovora*, Showing a Possible Direct Peptide Bond Between the *Epsilon*-Amino Group of DAP and the Carboxyl Group of Alanine



(NAG)
(NAM)
(NAG)
(NAM)
NHCOCH3
NHCOCH3
NHCOCH3
NHCOCH3
CH3CHCO
CH3CHCO
L-Alanine
L-Alanine
Glycine
NH-CH2-COOH
D-Glutamic Acid
D-Glutamic Acid
COOH
CH2
NH-C-COOH
H
Aspartic Acid
DAP
DAP
Direct Peptide Bond
(Free C-Terminal 90%)
D-Alanine
D-Alanine
Lysine
(when present, to Braun's protein)

likely by glycine, serine and aspartic acid), and the same possible cross-bridge exists as in control cells, i.e., between the carboxyl group of alanine and the _epsilon_-amino group of DAP. Keeping these observations in mind, we propose the cell wall MP structure shown in Figure 9 for D-serine-grown cells.

Peptide Containing D-Serine

Once the structures of control and D-serine-grown cell wall had been partially deduced, the next step was to establish the exact location of D-serine in the MP in order to obtain direct proof for the structure. To accomplish this, cells were grown with ^{14}C-D-serine added at time zero. These cells were harvested and MP isolated. To find the peptide in which D-serine was present, the researcher performed partial hydrolysis of D-serine MP samples (4N-HCl for 1 hour and 4N-HCl for 1.5 hours at 100°C). These samples were spotted on thin-layer cellulose plates and subjected to the appropriate solvent system (49) and thereafter placed next to x-ray film for 6 weeks. Several radioactive spots were observed in both the 1 hour and 1.5 hour hydrolyzed samples. These spots were scraped and totally hydrolyzed (6N-HCl 18 to 24 hours _in vacuo_ at 100°C), re-spotted on thin-layer cellulose plates, again developed in the appropriate solvent (29), and then exposed to x-ray film. In no instance could this researcher positively identify any D-serine-containing peptide. Regardless of the hydrolytic procedure used, serine always appeared on the plates in the free form rather than in any di- or tri-peptide.

Figure 9. Proposed Cell Wall MP Structure for D-Serine-Grown _E. carotovora_, Showing a Possible Direct Peptide Bond Between the _Epsilon_-Amino Group of DAP and the Carboxyl Group of Alanine

(NAG) (NAM) (NAG) (NAM)

CH_2OH CH_2OH CH_2OH CH_2OH

C—O C—O C—O C—O

C C O C C C C O C C

C—C C—C C—C C—C

$NHCOCH_3$ O $NHCOCH_3$ $NHCOCH_3$ O $NHCOCH_3$

Glycine

CH_3CHCO CH_3CHCO

$NH-CH_2-COOH$

NH OH NH

L-Alanine CH_3-CH or CH_2 CH_3-CH L-Alanine

CO NH-C-COOH CO

NH H NH

D-Glutamic Acid (α) CH-CO D-Serine (α) CH-CO D-Glutamic Acid

Aspartic Acid

$(CH_2)_2$ $(CH_2)_2$

H

CO(γ) NH-C-COOH CO(γ)

NH (ε) H CH_2 NH (ε) H

DAP $HC-(CH_2)_3-CH-N$ COOH $HC-CH_2)_3-CH-N$ DAP

C=O C=O C=O

(Free C-Terminal 90%)

Direct Peptide Bond

NH (ε) NH NH

D-Alanine $HC-CH_3$ CH-COOH $HC-CH_3$ D-Alanine

(β)

C $(CH_2)_e-NH_2$ C

O O

Lysine

(when present, to Braun's protein)

CHAPTER IV

DISCUSSION AND SUMMARY

The amino acid composition of normal cell MP in *E. carotovora* has been obtained by amino acid analysis. A ratio of 1.5 alanine residues to 1 each of glutamic acid and DAP have been reported. These amounts are consistent with previous observations (26) which show a ratio of 2 alanine residues to 1 of glutamic acid and DAP in this organism. Lowered amounts of alanine in the preparation could be accounted for because of losses through centrifugation during MP isolation (59). Glycine is present in the next highest concentration (ratio of .24 residues per residue of both glutamic acid and DAP). Aspartic acid and lysine are present in less than half the concentration of glycine. Since lysine is present in both control and D-serine MP, the researcher assumes that it is connected by its *epsilon*-amino group to the free carboxyl terminal group of DAP and then to Braun's protein as shown in *E. coli* (3). No evidence was obtained to indicate that D-serine incorporation interfers with attachment to or presence of Braun's protein in *E. carotovora*.

With regard to D-serine-grown cell wall MP, it was observed that approximately the same proportion of amino acids are present with the exception of D-serine, which more than triples in amount compared to control values. In addition, levels of glycine are decreased. The data

suggests that some glycine normally present is replaced by D-serine as reported previously (26).

Identical results were obtained for both control MP and D-serine containing MP in regard to N-terminal amino acids. Experimental data reveal that some of the epsilon-amino groups of DAP are free. This type of finding can be expected in D-serine-grown cells if DAP is involved in cross-bridging and D-serine interfers in such linkage. However, the finding that the epsilon-amino group of DAP is partially free in control MP would indicate that not all of the tetrapeptides are cross-linked. Unfortunately, the chromatographic procedures that were employed will not distinguish between the epsilon-amino groups of DAP and lysine.

Carboxyl terminal analysis using normal *E. carotovora* MP revealed that one alanine C-terminal residue remains completely free thereby eliminating it from involvement in a possible cross-bridge. This was also true in MP containing D-serine. The observation that only very small amounts of free alpha-carboxyl groups of glutamic acid are present in both control and D-serine-grown cells indicates this group is probably involved in cross-bridging or occupied by serine, glycine, or both, as pointed out previously.

The major difference observed in the C-terminal data obtained, using control versus D-serine-grown cell wall MP, is the increased levels of D-serine in MP and an accompanying decrease in glycine C-terminal groups. This obviously means that D-serine is replacing glycine as previously observed (26). These findings are similar to others and indicate that D-serine can partially replace glycine in MP (28) (30) (33) (48) (57) (64). This replacement is at least partially on the alpha-carboxyl of glutamic acid as inferred from the low C-terminal content

of the glutamic acid. Usually D-serine incorporates into positions number 4 or number 5 of the tetrapeptide or partially replaces glycine wherever it might be located in the mucopeptide (30) (48) (57) (64). As suggested by Schleifer et al. (48), D-serine added at growth inhibiting concentrations would possibly form modified nucleotide-activated MP precursors by which glycine would be replaced by D-serine. Since D-serine is less efficiently incorporated into MP, a certain percentage of the modified MP would remain uncross-linked, showing that D-serine is a poor substrate for the transpeptidation reaction.

After reviewing the data in comparison to the theory proposed by Strominger et al. (31) on the attachment site of D-amino acids in gram-negative bacteria, it can be concluded that their main thesis on the attachment site of the D-amino acid and the nature of its function (reversal of the transpeptidation step) does not seem to hold true for the organism under investigation. When D-serine is added to the growth medium it is incorporated into the MP but not in position number 4 or number 5 of the tetrapeptide as indicated by our C-terminal data for alanine (no change in C-terminal alanine was measured).

Also, as stated previously, there is little or no difference in the free C-terminal residues of alanine, glutamic acid or DAP existing in normal versus D-serine-grown cell wall MP. When glycine or D-serine are considered and molar proportions of these amino acids obtained (see final column in Table IV), some differences seem to exist (glycine is decreased by about 30% and D-serine increased about 30%). These differences could indicate increased cross-linking exists in D-serine-grown cell wall MP or, alternatively, that serine and glycine are bonded to the <u>alpha</u>-carboxyl group of glutamic acid as a mixed (seryl-glycine

or glycyl-serine) dipeptide. Until the amino acids existing with or on either side of D-serine are identified, these possibilities cannot be distinguished.

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VITA[2]

David Lynn Spess

Candidate for the Degree of

Master of Science

Thesis: INCORPORATION OF D-SERINE INTO THE MUCOPEPTIDE OF ERWINIA CAROTOVORA

Major Field: Microbiology

Biographical:

Personal Data: Born in Tulsa, Oklahoma, December 12, 1951, the son of Frank E. and Rozella M. Spess.

Education: Graduated from Mannford High School, Mannford, Oklahoma, in May, 1970; received the Bachelor of Science degree in Microbiology at Oklahoma State University in May, 1974; completed the requirements for the Master of Science degree at Oklahoma State University in May, 1979.

Professional Experience: Graduate Teaching Assistant, Microbiology Department, Oklahoma State University, 1975-1977; Research Assistant, Microbiology Department, Oklahoma State University, Spring, 1978; presently employed as Microbiologist, Food and Drug Administration, Washington, D. C.