INCORPORATION OF D-SERINE INTO THE CELL WALL MUCOPEPTIDE OF *MICROCOCCUS LYSODEIKTICUS*[a]

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Qualitative analysis of bacterial cell wall constituents has been proposed as an aid in classification of bacteria (Cummins and Harris, 1956). Such analyses would be of value if cell wall composition were immutable. Cummins and Harris reported that cell wall composition of several organisms was unchanged after growth on different complex media.

Tsung, *et al.*, (1962) found that hydroxylysine can substitute for lysine in the cell wall of *Streptococcus faecalis*. Incorporation of hydroxylysine caused the cells to be more resistant to lysis. Although not demonstrable by chemical procedures, Lark and Lark (1961) reported the incorporation of D-methionine into the cell wall mucopeptide of *Alcaligenes fecalis* after growth in the presence of D-[C^{14}] methionine. This incorporation was claimed to result in defective wall synthesis and subsequent spheroplasting of cells.

This communication describes an easily demonstrable modification (and partial reversals thereof) in mucopeptide composition in *Micrococcus lysodeikticus*.

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Materials and Methods

Cells were grown in a defined medium (Grula, 1962) for 40 hrs at 30C on a reciprocal shaker. They were harvested by centrifugation, washed in distilled water and the cell walls isolated by the chemical procedure of Park and Hancock (1960). Prior to final study of peptide amino acids, a portion of the cell wall preparation was hydrolyzed (18 hrs in 6 N HCl at 105C in evacuated and sealed tubes) then chromatographed in the two-dimensional system of Redfield (1953) as a check to determine if all protein had been removed during the fractionation. When pure, cell walls were hydrolyzed as above and the hydrolysate evaporated to dryness two times under a stream of warm air from a hair dryer. The residue was resuspended in distilled water and chromatographed on Whatman No. 1 filter paper using the descending technique in n-butanol-pyridine-water 6:4:3 (Whistler and Conrad, 1954) for 60 hrs at approximately 27-28C. This system was designed for separation of sugars, but excellent resolution of wall peptide amino acids can be achieved employing the longer running time. Sequence of flow (from origin) is lysine, glutamic acid, glycine, serine, and alanine; the majority of other amino acids and glucosamine run off the paper. After drying, chromatograms were sprayed with ninhydrin (0.25 % in acetone) and color developed in an oven at 105C for 5 min. For quantitative estimation, the developed papers were run through a Photovolt densitometer (570 mμ filter) fitted with a constant speed motor and attached to a recorder-integrator. Amount of each amino acid present was estimated by comparison with standard curves prepared with the same densitometer. Results given are the average of at least three individual determinations.

Results

Growth responses are given in Table I. D-Serine reduces growth to approximately one-third of control. This inhibition can be reversed by

L-serine or glycine. Addition of L-alanine to the D-serine containing medium also results in some reversal of growth inhibition; however, restoration is better with D-alanine. Pantoic acid, which partially reverses growth inhibition caused by D-serine in a species of Erwinia (Grula and Grula, 1962[a]) does not reverse growth inhibition caused by D-serine in M. lysodeikticus. D-Threonine also inhibits growth. D-Serine-grown cells do not show a greatly altered response to lysozyme (rate of clearing is slightly increased).

TABLE I

GROWTH RESPONSES OF M. LYSODEIKTICUS[a]

Medium Additions[b]	O.D.[c]
None	0.59
D-serine	0.22
D-serine + D-alanine	0.48
D-serine + L-alanine	0.39
D-serine + glycine	0.55
D-serine + pantoic acid	0.19
L-serine	0.58
D-threonine	0.25
L-serine + D-serine	0.57

a All tubes contained defined medium (Grula, 1962).
b D-Serine, D-alanine, glycine, and L-serine tested at 5×10^{-4}M, D-threonine was 0.05 M, and pantoic acid was 1.6×10^{-3}M.
c Measured at 540 mμ using tubes having an I.D. of 10 mm in a Coleman Jr. spectrophotometer.

Cells from different growth situations were fractionated to allow study of mucopeptide amino acid composition. These data are given in Table II. Cells grown in the presence of D-serine incorporated serine into the mucopeptide in an amount almost equal to lysine or glutamic acid. Incorporation of serine into the mucopeptide does not occur when cells are grown in the presence of L-serine.

TABLE II

AMINO ACID COMPOSITION OF THE CELL WALL MUCOPEPTIDE FROM M. LYSODEIKTICUS CULTURED IN THE PRESENCE OF DIFFERENT AMINO ACIDS[a]

Medium Additions	Amino acid molar ratios				
	alanine	glycine	serine	glutamic acid	lysine
None	2.1	1.1	0.0	1.0	1.0
D-serine	2.1	0.4	0.9	1.0	1.0
L-serine	2.3	1.2	0.0	1.0	1.0
D-serine + L-alanine	2.0	0.3	0.8	1.0	1.0
D-serine + D-alanine	2.0	1.0	0.4	1.0	1.0
D-serine + glycine	2.0	1.1	0.3	1.0	1.0
D-serine + L-Serine	1.9	0.5	0.6	1.0	1.0

[a] Growth conditions, measurements, and molar concentrations as given in Table I and Materials and Methods.

The incorporated serine appears to be the D-isomer since isolated serine from the wall mucopeptide, when treated with D-amino acid oxidase, (50 ml of a 1 per cent solution of Worthington hog kidney D-amino acid oxidase solution dialyzed for 6 hr against 3 changes of 1 liter of sodium pyrophosphate buffer, 0.05 M pH 8.5) results in the formation of keto acid whereas treatment with L-amino acid oxidase results in no keto acid formation (Haidle & Knight, 1960).

There is no evidence that serine is incorporated through an O-ester linkage since treatment with ammonium hydroxide (Critchley, et al., 1962) causes no release of serine.

No morphological abnormalities such as protoplast or filament formation have been noted with cells grown in the presence of D-serine. The greatest variation in mucopeptide composition caused by D-serine incorporation involves glycine. The amount of glycine is decreased to about one-half of normal, but the amount of serine incorporated exceeds the amount that would be required only to replace the missing glycine. Therefore, the effect of serine incorporation appears to be replacement of approximately 50 per cent of the glycine and, also, extension of the length of the wall peptide.

Incorporation of D-serine is decreased in the presence of D-alanine, L-alanine, L-serine or glycine. Where D-serine incorporation is lowered by D-alanine or glycine, glycine levels increase to what may be considered normal. L-alanine is not as effective as D-alanine or glycine. In no case is incorporation of D-serine completely inhibited.

Although data are not given, D-threonine is not incorporated into the peptide and pantoic acid does not inhibit D-serine incorporation. No morphological variations have yet been noted when cell walls isolated from D-serine-grown cells are examined using the electron microscope.

Discussion

Addition of D-serine to the growth medium causes a qualitative and quantitative alteration in composition of the cell wall mucopeptide of M. lysodeikticus.

Tanaka (1963) and Morrison (1963) reported that D-alanine reverses growth inhibition caused either by O-carbamyl-D-serine or D-serine. The mechanism of D-alanine reversal could be profitably studied at the cell wall amino acid level since we have shown that although D-alanine reverses to a great extent both growth inhibition and cell wall modification caused by D-serine, glycine, rather than D-alanine, is the target compound.

Neuhaus (1962) has reported that in the presence of D-serine, D-alanyl-D-alanine synthetase causes the formation of D-alanyl-D-serine with a resultant inhibition in activity of the synthetase. Synthetase activity has not been reported for this organism; however, incorporation of D-serine into the cell wall peptide of M. lysodeikticus does not involve alanine deletion.

Apparently, the wall modification caused by D-serine is partially responsible for inhibition in growth caused by this amino acid. However, although growth is restored to very near normal by glycine, incorporation of D-serine is still approximately 30 per cent that found in the absence of exogenous glycine. This amount of incorporation should have a greater effect on growth than is apparent if D-serine incorporation were to be accepted as the only reason for growth inhibition. It has been shown (Grula and Grula, 1962[b]; 1963) that D-serine inhibits synthesis of pantothenic acid and Coenzyme A in a species of Erwinia. Our nutritional data (Table I) and unpublished data using Erwinia sp. (Grula and Grula) suggest that D-serine also causes an inhibition in synthesis of glycine.

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