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Incorporation of Hydroxylysine into the Cell Wall and a Cell-Wall Precursor in *Staphylococcus aureus*

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ABSTRACT

SMITH, W. GRADY (University of Minnesota, St. Paul), DANIEL P. GILBOE, AND L. M. HENDERSON. Incorporation of hydroxylysine into the cell wall and a cell-wall precursor in *Staphylococcus aureus*. J. Bacteriol. **89**:136-140. 1965.—Recent work has shown that hydroxylysine can substitute for lysine in cell-wall synthesis of *Streptococcus faecalis*, apparently becoming incorporated into cell-wall mucopeptide. This paper extends these observations to investigate the metabolism of hydroxylysine in *Staphylococcus aureus*, an organism from which sufficiently large quantities of cell-wall precursors, uridine diphosphate-*N*-acetylmuramyl peptides, could be obtained. Hydroxylysine has been shown to be incorporated into the cell-wall precursor uridine diphosphate-*N*-acetylmuramyl L-ala·D-glu·L-lys·D-ala·D-ala from *S. aureus* (Copenhagen) apparently in lieu of lysine. Hydroxylysine was also incorporated into the cell-wall mucopeptides of *S. aureus* in resting cultures. This incorporation was inhibited by penicillin or lysine, but not by chloramphenicol. Hydroxylysine had little effect on the incorporation of lysine into *S. aureus*. Hydroxylysine acted as a growth inhibitor in this organism; the inhibition was reversed by lysine.

Recent studies on the role of hydroxylysine (2,6-diamino-5-hydroxyhexanoic acid) in bacteria have revealed that this amino acid can substitute for lysine in cell-wall synthesis in *Streptococcus faecalis*, becoming incorporated into the cell-wall mucopeptide in lieu of lysine (Leach, Smith, and Tsung, 1961; Smith, Leach, and Henderson, 1961; Tsung et al., 1962; Smith et al., 1962; Smith and Henderson, 1964). To extend these observations, it seemed desirable to investigate the metabolism of hydroxylysine in an organism from which the lysine-containing cell-wall precursors, uridine diphosphate-*N*-acetylmuramyl peptides (Strominger, Park, and Thompson, 1959), could be obtained in sufficient amounts for study. Experiments in which such studies were conducted with *Staphylococcus aureus* (Copenhagen) are reported here.

MATERIALS AND METHODS

J. T. Park, Department of Microbiology, Tufts University School of Medicine, Boston, Mass., provided us with a culture of the organism, *Staphylococcus aureus* (Copenhagen). Growth experiments were conducted with the Henderson-Snell medium (Henderson and Snell, 1948) at 0.1 its original strength. *S. aureus* (Copenhagen) cells were grown on a yeast extract-glucose-agar slant overnight, and suspended in sterile water; the cells were washed by centrifugation, and resuspended in 2 ml of sterile water. One drop of this suspension was used to inoculate the 10-ml cultures which were subsequently incubated at 37 C with vigorous shaking. Absorbance of the cultures was measured at intervals by use of a Coleman Junior spectrophotometer with the wavelength set at 660 mμ.

Cells for the isolation of uridine diphosphate (UDP)-*N*-acetylmuramyl peptides were grown as described by Park (1952). UDP-*N*-acetylmuramyl peptides and *N*-acetylmuramyl peptides containing hydroxylysine-6-C^{14} or lysine-C^{14} were prepared as described by Ito and Strominger (1962). *N*-acetyl amino sugar determinations were conducted as described by Strominger (1957). *N*-acetylmuramyl peptides were hydrolyzed in a sealed tube at 105 C for 18 hr, and the amino acids were estimated as described by Mandelstam and Rogers (1959).

Lysine and hydroxylysine were separated by paper chromatography by use of the system of Rhuland et al. (1955): methanol-water-10 N HCl-pyridine (80:17.5:2.5:10). The C^{14} on the chromatograms was located with a Nuclear-Chicago 4 π scanner (Nuclear-Chicago Corp., Des Plaines, Ill.) and by radioautography on Kodak No-Screen X-ray film. Amino acids were located by reacting them with ninhydrin.

The experiments involving the effect of chloramphenicol (Parke, Davis & Co., Detroit, Mich.),

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penicillin (New England Nuclear Corp., Boston, Mass.), and the competition between lysine and hydroxylysine (hydroxylysine was a mixture of the four stereo-isomers) were conducted by suspending a sufficient quantity of cells in the exponential-growth phase to give an absorbance of 0.50 in a total volume of 4 ml of the appropriate medium. The "wall medium" refers to the medium described by Strominger (1957) in which there was accumulation of UDP-*N*-acetylmuramyl peptides. It contains 0.5% K_2HPO_4, 0.3% glucose, 0.012% $MgSO_4 \cdot 7H_2O$, 0.015% L-glutamic acid, 0.01% DL-alanine, and lysine-C^{14} or hydroxylysine-C^{14} as indicated. The complete medium was the same as the wall medium, except that 250 mg per 100 ml of a lysine-free amino acid mixture (Henderson and Snell, 1948) replaced the glutamic acid and alanine.

These cultures were incubated at 37 C with shaking for 90 min. The cells were then harvested by centrifugation, washed with water, and fractionated by the method of Park and Hancock (1960). Samples of each fraction were assayed for C^{14} with a liquid scintillation spectrometer as previously described (Tsung et al., 1962). The mucopeptide fractions were hydrolyzed in 6 N HCl in a sealed tube at 105 C for 18 hr, and the lysine and hydroxylysine were separated by paper chromatography as described above.

RESULTS

The results of the growth experiment (Fig. 1A) showed that hydroxylysine was inhibitory to *S. aureus*, and that this inhibition was relieved by lysine (Fig. 1B). Hydroxylysine had similar inhibitory properties in a lysine-independent strain of *Streptococcus faecalis* (Smith and Henderson, 1964). In general, hydroxylysine inhibition can be most readily demonstrated in lysine-independent strains. In the lysine-dependent cell, the growth inhibition appears to be competitive, because by increasing the amount of lysine present the hydroxylysine inhibition may be overcome. However, in lysine-independent organisms, it appears that hydroxylysine interferes with the biosynthesis of lysine. The exact nature of this interference is not known and is presently under investigation.

Hydroxylysine was incorporated into the cell wall in the same manner as was lysine (Table 1). The incorporation of both of these compounds into protein was inhibited by chloramphenicol, an inhibitor of protein synthesis, and their incorporation into the cell-wall mucopeptide was inhibited by penicillin, which interferes with cell-wall synthesis. These data are in agreement with those obtained in similar experiments using *S. faecalis* and *Leuconostoc mesenteroides* (Smith and Henderson, 1964). As expected, incorporation into protein was greatly enhanced when all 18 amino acids were present.

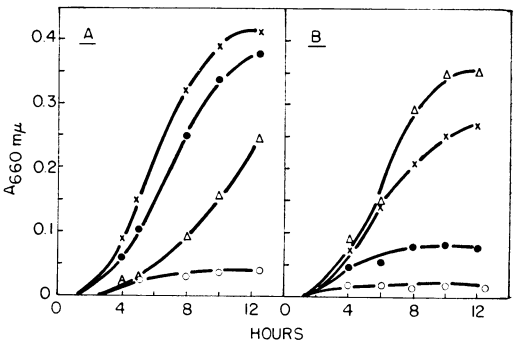

FIG. 1. *Effect of hydroxylysine and lysine on growth of Staphylococcus aureus (Copenhagen). (A) Growth inhibition by hydroxylysine;* ● = *no lysine, no hydroxylysine;* × = *lysine, 200 μg/ml, no hydroxylysine;* △ = *hydroxylysine, 20 μg/ml, no lysine;* ○ = *hydroxylysine, 40 μg/ml, no lysine. (B) Reversal of hydroxylysine inhibition by lysine;* ○ = *hydroxylysine, 200 μg/ml, no lysine;* ● = *hydroxylysine, 200 μg/ml, lysine, 1 μg/ml;* × = *hydroxylysine, 200 μg/ml, lysine, 5 μg/ml;* △ = *hydroxylysine, 200 μg/ml, lysine, 10 μg/ml. Organisms were grown in 10-ml cultures in matched tubes (18 × 150 mm) at 37 C with shaking in a water bath. L-Lysine·HCl and the mixed racemates of hydroxylysine monohydrochloride were used.*

The effects of lysine on the incorporation of hydroxylysine-C^{14}, and of hydroxylysine on the incorporation of lysine-C^{14} are presented in Table 2. Small amounts of lysine were markedly inhibitory toward the incorporation of hydroxylysine into the cell-wall mucopeptide. This was in agreement with previous observations made with *S. faecalis*. However, hydroxylysine was not incorporated into the protein fraction of *S. aureus* nearly to the extent that it was in *S. faecalis*. In this respect, *S. aureus* resembles *L. mesenteroides* which also incorporates hydroxylysine into protein to a much lesser degree than it does into mucopeptide. Hydroxylysine, on the other hand, did not inhibit the incorporation of lysine into protein or mucopeptide, an observation similar to those made with *S. faecalis* and *L. mesenteroides* (Smith and Henderson, 1964). Thus, it appeared that even though hydroxylysine may be available in the bacterial environment, it will not be utilized unless the exogenous lysine supply is limited.

When the mucopeptides from the experiments (Table 1) were hydrolyzed and chromatographed as described, the results presented in Fig. 2A were obtained. The presence of lysine-C^{14} in the mucopeptide from cells exposed to lysine-C^{14} is shown in Fig. 2A (no. 1). Hydroxylysine was not detected on the chromatogram. The presence of hydroxylysine-C^{14} and unlabeled lysine in the

TABLE 1. *Effect of penicillin and chloramphenicol on the incorporation of lysine-C^{14} and hydroxylysine-C^{14} into protein and mucopeptide of Staphylococcus aureus (Copenhagen)**

Medium	Labeled compound	Inhibitor	Count/min × 10^{-1} in	
			Protein	Mucopeptide
Wall	Hydroxylysine-C^{14}	None	477	5,031†‡
Wall		Penicillin	322	401
Wall		Chloramphenicol	264	3,555
Complete		None	1,389	11,633
Complete		Penicillin	1,385	2,191
Complete		Chloramphenicol	166	12,673
Wall	Lysine-C^{14}	None	1,472	5,139†
Wall		Penicillin	1,347	1,220
Wall		Chloramphenicol	1,054	6,574
Complete		None	21,424	19,067
Complete		Penicillin	22,473	6,525
Complete		Chloramphenicol	1,789	18,877

* Exponential cells were incubated in the experimental medium for 90 min, harvested by centrifugation, and washed and fractionated by the method of Park and Hancock (1960). The trypsin-soluble fraction is represented by the protein, and the remaining residue is represented by the mucopeptide. Concentrations were as follows: penicillin, 10 μg/ml; chloramphenicol, 100 μg/ml; hydroxylysine HCl-C^{14}, 25 μg/ml (specific activity, 41.6 μc/μmole); lysine HCl-C^{14}, 25 μg/ml (specific activity, 37 μc/μmole). Wall medium and complete medium refer to media containing three amino acids (glutamic, lysine or hydroxylysine, and alanine), or 18 amino acids, respectively.

† These mucopeptide fractions were hydrolyzed for the data obtained in Fig. 2.

‡ These figures are the total number of counts obtained from each of the two fractions listed, protein and mucopeptide, and represent the total amount of radioactivity in these fractions isolated from all the cells of the 4 ml of incubation mixture (absorbance = 0.50).

mucopeptide from cells exposed to hydroxylysine-C^{14} is shown in Fig. 2A (no. 2).

These results established that hydroxylysine was incorporated into the mucopeptide without being transformed chemically, and suggested that hydroxylysine can substitute for lysine in cell-wall synthesis in *S. aureus* as it can in *S. faecalis*. Hydroxylysine was incorporated into the cell wall; thus, it should also be incorporated into the presumed cell-wall precursor UDP-*N*-acetylmuramyl·L-ala·D-glu·L-lys·D-ala·D-ala (Strominger et al., 1959), probably in lieu of lysine. When this nucleotide peptide was isolated by paper chromatography from cells exposed to penicillin in the presence of hydroxylysine-C^{14} or lysine-C^{14}, and the *N*-acetylmuramyl peptide was prepared from it as described by Ito and Strominger (1962), the expected labeled amino acid was present. When the *N*-acetylmuramyl peptide was hydrolyzed and chromatographed as before, the results presented in Fig.

TABLE 2. *Effect of lysine on the incorporation of hydroxylysine-C^{14} and of hydroxylysine on the incorporation of lysine-C^{14} into protein and mucopeptide of Staphylococcus aureus (Copenhagen)**

Medium	Labeled compound	Unlabeled compound†	Count/min × 10^{-1} in	
			Protein	Mucopeptide
Wall	Hydroxylysine-C^{14}	None	477	5,021‡
Wall		Lysine (10)	31	1,129
Wall		Lysine (50)	15	728
Complete		None	1,389	11,633
Complete		Lysine (10)	734	7,272
Complete		Lysine (50)	41	191
Wall	Lysine-C^{14}	None	1,472	5,139
Wall		Hydroxylysine (25)	1,550	5,424
Wall		Hydroxylysine (125)	1,880	8,271
Complete		None	21,424	19,067
Complete		Hydroxylysine (25)	22,330	18,871
Complete		Hydroxylysine (125)	21,847	19,976

* Experimental conditions and the concentrations of radioactive compounds were the same as given in Table 1.

† Numbers in parentheses give the concentration in micrograms per milliliter.

‡ These figures are the total number of counts obtained from each of the two fractions listed, protein and mucopeptide, and represent the total amount of radioactivity in these fractions isolated from all the cells of the 4 ml of incubation mixture (absorbance = 0.50).

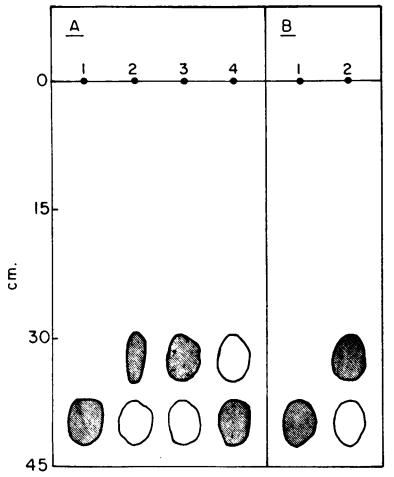

FIG. 2. *Paper chromatography of the hydrolysates of mucopeptides from Table 1, and of nucleotide peptides. (A) Mucopeptide from cells incubated with: (1) lysine-C^{14}, (2) hydroxylysine-C^{14}, (3) standard mixture of lysine-C^{14} and hydroxylysine-C^{14}, and (4) standard mixture of lysine-C^{14} and hydroxylysine. (B) Paper chromatography of the hydrolysate of N-acetylmuramyl peptide prepared from penicillin-inhibited cells exposed to: (1) lysine-C^{14}, or (2) hydroxylysine-C^{14}. Chromatograms were run in descending direction on Whatman no. 1 paper in methanol-water-10 N HCl-pyridine, 80: 17.5:2.5:10. The other amino acids and amino sugars moved ahead of lysine and hydroxylysine and were allowed to run off the paper. Shaded areas represent ninhydrin-reactive material containing C^{14}. Unshaded areas represent ninhydrin-reactive material only.*

2B were obtained. Figure 2B (no. 1) shows only the presence of lysine-C^{14} in the *N*-acetylmuramyl peptide obtained from cells exposed to lysine-C^{14}. Figure 2B (no. 2) shows the presence of both lysine (unlabeled) and hydroxylysine-C^{14} in the *N*-acetylmuramyl peptide obtained from cells exposed to hydroxylysine-C^{14}. This probably represents a mixture of two compounds, one containing lysine and the other hydroxylysine. This was supported by the amino acid molar ratios. The hydroxylysine-C^{14} muramyl peptide had a glutamic acid–lysine + hydroxylysine–alanine ratio of 1.00:1.02:2.8. The lysine-C^{14} muramyl peptide had a glutamic acid-lysine-alanine ratio of 1.00:1.03:3.3.

DISCUSSION

Recent studies of hydroxylysine have somewhat clairfied its role in bacteria. Hydroxylysine may act in a stimulatory or inhibitory manner depending on the organism and cultural conditions. It confers a stability on the cell wall when incorporated (Smith et al., 1962; Smith and Henderson, 1964). These results along with those of others (Snell, Radin, and Ikawa, 1955; Whitney and Grula, 1964) establish that the structure of the bacterial cell wall is not invariant, but depends at least to some degree on nutritional factors. The present study demonstrates the incorporation of hydroxylysine into the cell wall of *S. aureus* under conditions of a limited lysine supply, and indicates that hydroxylysine is incorporated via the usual pathway for wall synthesis, that is, through the UDP-*N*-acetylmuramyl peptides. Recent work by Chatterjee and Park (1964) and Meadow, Anderson, and Strominger (1964) on cell-free wall biosynthesis confirms that these compounds are indeed wall precursors.

ACKNOWLEDGMENTS

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