Biosynthesis of Peptidoglycan in Staphylococcus aureus: Incorporation of the N'-Ala–Lys Moiety into the Peptide Subunit of Nascent Peptidoglycan

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UDP-MurNAc-Ala-\(d\)Glu-Lys(N'-Ala)-dAla was isolated from extracts of Staphylococcus aureus Copenhagen. This nucleotide accumulated in media deficient in glycine. To establish its role in peptidoglycan biosynthesis, the nucleotide-hexapeptide was compared with UDP-MurNAc-Ala-\(d\)Glu-Lys-dAla-dAla in the reaction catalyzed by phospho-MurNAc–pentapeptide translocase and in the membrane-catalyzed nascent peptidoglycan-synthesizing system. In the exchange reaction catalyzed by the translocase, the \(R_{max}\) and \(R_{max}/K_m\) are 1.79 \(\mu\)M/min and 4.47 \(\times\) \(10^{-2}\)/min, respectively, for UDP-MurNAc–pentapeptide and 1.81 \(\mu\)M/min and 4.46 \(\times\) \(10^{-2}\)/min, respectively, for UDP-MurNAc–hexapeptide. In the synthesis of nascent peptidoglycan, the \(V_{max}\) is 1.8 \(\mu\)M/min \(\times\) \(10^{-2}\) for both the nucleotide-hexapeptide and -pentapeptide. The \(V_{max}/K_m\) is \(5.6 \times 10^{-4}\) and \(4.3 \times 10^{-4}\)/min for the nucleotide-pentapeptide and -hexapeptide, respectively. Schleifer, Hammes, and Kandler (Adv. Microbiol. Physiol., in press) observed that growth of \(S.\) aureus Copenhagen on a glycine-poor medium results in a peptidoglycan structure in which 20% of the lysine residues are substituted at the \(\epsilon\)-amino group by \(L\)-alanine residues that do not participate in interpeptide bridge formation. In the vitro studies demonstrate that UDP-MurNAc-Ala-\(d\)Glu-Lys(N'-Ala)-dAla-dAla is a possible precursor of the N'-Ala–Lys moiety.

In Staphylococcus aureus Copenhagen, the interpeptide bridge of the peptidoglycan consists of five glycine residues (1, 19) when the organism is cultured in a glycine-rich medium. When it is grown in a glycine-poor medium, the walls show a decrease in cross-linking (K. H. Schleifer, Proc. Soc. Gen. Microbiol. 37:xiv, 1969; K. H. Schleifer, W. P. Hammes, and O. Kandler, Adv. Microb. Physiol., in press). Under these conditions a significant percentage (20%) of the lysine residues of the peptidoglycan are substituted at the \(\epsilon\)-amino group by alanine residues that do not participate in interpeptide bridge formation.

Since the presence of N'-Ala–Lys dipeptide moieties in the peptidoglycan can be correlated with the degree of cross-linking in \(S.\) aureus Copenhagen, the mechanism by which this dipeptide moiety is introduced into this polymer is of interest. The biosynthesis of peptidoglycan is catalyzed by a series of membrane-associated enzymes that utilize two nucleotide-activated precursors, UDP-\(N\)-acetylmuramic and UDP-MurNAc-Ala-\(d\)Glu-Lys-dAla-dAla. A possible precursor of the N'-Ala–Lys moiety is the UDP-MurNAc–hexapeptide: UDP-MurNAc–Ala–\(d\)Glu–Lys(N'-Ala)–dAla–dAla. Nucleotide-activated hexapeptides have been found in two different species of bacteria, Lactobacillus viridescens (11) and Streptococcus faecalis (9). Membrane preparations from \(L.\) viridescens utilize the nucleotide-activated hexapeptide for the synthesis of peptidoglycan (11). In contrast to \(S.\) aureus Copenhagen, the L-alanine residue linked to the \(\epsilon\)-amino group of lysine derived from UDP-MurNAc–hexapeptide forms an integral part of the interpeptide bridge of \(L.\) viridescens (13) and \(S.\) faecalis. In \(S.\) aureus Copenhagen it is N terminal.

It is the purpose of this paper to describe the isolation of UDP-MurNAc-Ala-\(d\)Glu-Lys(N'-Ala)–dAla–dAla from \(S.\) aureus Copenhagen and to define conditions for its optimal accumulation. Since this nucleotide represents the possible precursor of the N'-Ala–Lys dipeptide moiety observed by Schleifer et al. (in press), we present in vitro experiments to compare its activity in the reaction catalyzed by phospho-MurNAc–pentapeptide translocase and the peptidoglycan-synthesizing system from \(S.\) aureus.
MATERIALS AND METHODS

Materials. *S. aureus* Copenhagen was a gift from J. L. Strominger and was maintained as a lyophilized culture in vacuo at -25 C. *S. aureus* H (ATCC 13801) was purchased from the American Type Culture Collection. UDP-[^14]C)GlcNAc was purchased from ICN Isotope and Nuclear Division and Amersham/Searle. The plastic beads (styrene-divinylbenzene copolymer, 20 to 50 mesh, 8% cross-linked) were the kind gift of Richard Reitz of the Dow Chemical Co. Whatman DE-52 microgranular diethylaminoethyl (DEAE)-cellulose was purchased from H. Reeve Angel Co. [5-^3]H]UMP was purchased from Schwarz/Mann. Peptone and yeast extract were products of the Difco Laboratories. Levigated alumina (15 μm), manufactured by Buehler Ltd., was purchased from Fisher Scientific Co. The sources of other chemicals were described previously (3, 4).

Isolation of UDP-MurNAc-Ala-dGlu-Lys(N-AIa)-dAla-dAla. For the preparation of the nucleotide-activated hexapeptide, *S. aureus* Copenhagen was grown aerobically in the medium described by Heydanek et al. (5) at 37 C. The cells were harvested and transferred to accumulation medium (90 ml/g [wet weight] of cells) at 37 C. This medium contained per liter: L-alanine, 400 mg; d-glutamic acid, 200 mg; lysine (monohydrochloride), 200 mg; uracil, 150 mg; glucose, 10 g; and KH₂PO₄, 5 g. Penicillin G (1,600 U/ml) was added as indicated. After incubation at 37 C in the accumulation medium (2 h), the cells were harvested and extracted with cold 10% trichloroacetic acid (3 ml/g [wet weight] of cells). Partial removal of the trichloroacetic acid was accomplished by extraction with diethyl ether. After the pH was adjusted to 7.8, the UDP-MurNAc-peptide fraction was isolated from the other components of the cell extract by the method of Stickgold and Neuhaus (14).

UDP-MurNAc-hexapeptide was isolated from the UDP-MurNAc-peptide fraction by chromatography on DEAE-cellulose. This fraction was applied to a column (0.9 by 57 cm) of DEAE-cellulose, and the column was then developed with a linear gradient of ammonium bicarbonate buffer (pH 8.5; from 0.1 to 0.5 M). A typical elution pattern is shown in Fig. 1. The partially resolved contaminant in the hexapeptide fraction was not included when the fractions were pooled. The contaminant was resolved from UDP-MurNAc-hexapeptide by rechromatography, as described above, with a linear gradient of 0.15 to 0.3 M ammonium bicarbonate.

The UDP-MurNAc-hexapeptide was characterized as follows. With paper chromatography the hexapeptide has R₀ values of 0.26 and 0.39 in solvents A and B (see below), respectively. For comparison, the pentapeptide has R₀ values of 0.22 and 0.46 and the tripeptide has R₀ values of 0.17 and 0.60 in solvents A and B, respectively. Hydrolysis of the nucleotide with 0.1 M HCl at 100 C for 15 min results in the formation of UMP and UDP. The amino acid composition of the hexapeptide is: glutamic acid-alanine-lysine-uridine (1.00:3.95:0.98:1.10). Small amounts of glycine are consistently detected in this fraction, UDP-MurNAc-pentapeptide has the ratio 1:3:1:1. Thus, the nucleotide-hexapeptide differs from UDP-MurNAc-pentapeptide by one additional residue of alanine. Since the additional alanine is not sensitive to the action, of d-amino acid oxidase, it is concluded that the extra residue is L-alanine. Thus, the nucleotide has two residues of d-alanine and two of L-alanine. Reaction of the nucleotide with 1-dimethylaminonaphthalene-5-sulfonyl-chloride (dansyl-chloride) followed by hydrolysis results in the release of dansyl-alanine, indicating that this residue is N terminal. To determine the location of the N-terminal alanine, the sequence analysis procedure of Gray and Smith

![Fig. 1. Separation of UDP-MurNAc-hexapeptide and UDP-MurNAc-pentapeptide by ion-exchange chromatography. The UDP-MurNAc-peptide fraction, isolated by the method of Stickgold and Neuhaus (14), was applied to a column of DEAE-cellulose, and the nucleotides were eluted with the linear gradient described in the text. The absorbance at 280 nm (O), the exchange activity (R) (Δ) observed with a 30-μl sample, and the molarity of the NH₄HCO₃ (−) are presented.](http://jb.asm.org/)

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peptide translocase. For measuring the reaction catalyzed by phospho-MurNAc-pentapeptide translocase, the exchange assay described by Hammes and Neuhaus (3) was used. This assay measures the rate of exchange of [3H]UMP with the UMP moiety of UDP-MurNAc-pentapeptide. Exchange activity was calculated by the method of Struve et al. (18). The rate of exchange, R, is reported as moles exchanged per liter per minute. The reaction mixture contained: 0.05 M Tris-hydrochloride (pH 7.8); 0.21 M KCl; 0.042 M MgCl₂; membrane fragments (41.3 μg of protein); and [3H]UMP and UDP-MurNAc-pentapeptide as indicated, in a total volume of 60 μl. The incubation was carried out at 25°C for 10 min. After the reaction was terminated by placing the mixture in boiling water for 2 min, the amount of exchange was determined as described previously (3).

Peptidoglycan synthesis assay. Peptidoglycan synthesis was assayed by determining the incorporation of N-acetyl-[14C]glucosamine from UDP-[14C]-GlcNAc into a lysozyme-sensitive, chromatographically immobile fraction. That this labeled fraction is peptidoglycan was concluded from its sensitivity to lysozyme and its dependence for synthesis on the second substrate UDP-MurNAc-peptide. The reaction mixture contained: 0.10 M Tris-hydrochloride, pH 8.5; 3 mM magnesium acetate; 10 mM NH₄Cl; 1 mM ATP; 1 mM 2-mercaptoethanol; 98 μM UDP-[14C]GlcNAc (25 to 35 counts/min per pmol); 20 to 200 μg of membrane protein; and the indicated concentration of UDP-MurNAc-peptide, in a total volume of 25 μl. Duplicate incubations were carried out at 20°C for 1 h. The reactions were terminated by placing the incubation mixtures into a boiling-water bath for 2 min. One of the samples was incubated for an additional 30 min at 37°C with 150 μg of lysozyme. The reaction mixtures were then quantitatively applied to Whatman 3 MM paper, and the paper chromatograms were developed by descending chromatography in solvent A (see below) for about 20 to 22 h. The origins were assayed for chromatographically immobile [14C]-labeled material.

Peptidoglycan synthesis was found to continue linearly for at least 110 min under the conditions described. A slight drop from linearity was observed as the concentration of membrane fragments increased. When UDP-[14C]GlcNAc was used to label the immobile product, significant amounts of origin material were found to be lysozyme insensitive. This lysozyme-insensitive product also was formed in the absence of UDP-MurNAc-peptide. All measurements of peptidoglycan synthesis with [14C]GlcNAc have been corrected for lysozyme-sensitive material. When incorporation of MurNAc–Ala–dGlu–Lys(Nᵈ-Ala)–dAla–dAla was measured, the quantity of the peptidoglycan produced was the same as the amount of lysozyme-sensitive product when [14C]-GlcNAc was the label.

Analytical methods. For the determination of radioactivity in aqueous samples, the scintillation fluid described by Patterson and Greene (10) was used; for paper chromatograms the fluid contained 0.3% 2,5-diphenyloxazole and 0.025% 1,4-bis-(2)-(5-phenyloxazolyl)benzene in toluene. Descending
paper chromatography was performed with Whatman 3 MM paper. Solvent A contains isobutyric acid-concentrated NH₄OH-water (66:2:33, vol/vol/vol). Solvent B contains 0.1 M phosphate buffer (pH 6.8)-(NH₄)₂SO₄-n-propanol (100:60:2, vol/wt/vol). Amino acids were analyzed on a Durrum amino acid analyzer (model D-500) after hydrolysis of the samples with 5.7 N HCl for 12 h at 100 C. Protein was determined by the method of Lowry et al. (7), with bovine serum albumin as the standard.

RESULTS

Accumulation of UDP-MurNAc-Ala-dGlucose (N'-Ala)-dAla-dAla. In preparing UDP-MurNAc-Ala-dGlucose-Lys-dAla-dAla and UDP - MurNAc - Ala - dGlucose - Lys(N'-Ala) - dAla-dAla from S. aureus Copenhagen, it was observed that the quantities of these nucleotides varied with the growth phase of the bacteria at the time of harvest for transfer to the accumulation medium. To determine the influence of the growth phase on the accumulation of these nucleotides, samples of S. aureus from the growth medium were harvested at different times and transferred to the accumulation medium for 2 h. In Fig. 3A the amount of each nucleotide per 10¹² cells is presented as a function of the time of harvest before transfer to the accumulation medium. The incubation in this medium (2 h) does not allow growth of the cells but does result in maximal accumulation of nucleotides (8, 15). Therefore, this plot represents the potential (or capacity) of the cells to synthesize these nucleotides at the time of their harvest from the growing culture. During the late log phase of growth, there is a large increase in the capacity for synthesis of UDP-MurNAc-pentapeptide followed by a smaller increase in UDP-MurNAc-pentapeptide(N'-Ala). The ratio of UDP-MurNAc-Ala-dGlucose-Lys(N'-Ala)-dAla-dAla to UDP-MurNAc-Ala-dGlucose-Lys-dAla-dAla increases from 0.06 at mid-log to approximately 0.15 at the transition from the late log to the stationary phase.

The effect of penicillin in the accumulation medium was tested to optimize conditions for the production of UDP-MurNAc-Ala-dGlucose-Lys(N'-Ala)-dAla-dAla. Cells were harvested from the growing culture at the time of maximal accumulation of the two nucleotides (Fig. 3A, 3.5 h). They were transferred to accumulation medium modified in the amount of penicillin present. As shown in Fig. 3B, the concentration of penicillin in the accumulation medium has no effect on the accumulation of UDP-MurNAc-hexapeptide. The presence of penicillin results in a small decrease in the accumulation of UDP-MurNAc-pentapeptide.

Strominger and Threnn (17) reported a significant accumulation of N-acetylamino sugar nucleotides in S. aureus in the absence of penicillin (6.2 μmol/liter of culture at half-maximal growth) in a medium similar to the accumulation medium used in the present work. When penicillin (100 μg/ml) was added to the accumulation medium, only a 2.5-fold increase (15.5 μmol) in the amount of these nucleotides was observed. Thus, the lack of effect of penicillin in the accumulation medium in the present work can be compared with the low increase (2.5-fold) observed by Strominger and Threnn.

Since the accumulation of UDP-MurNAc-peptides in S. aureus Copenhagen is not greatly affected by the addition of penicillin to the accumulation medium, the presence or absence
of another component in the medium might be responsible for the accumulation that is observed. Glycine is a major component of the interpeptide bridge of *S. aureus*. Its deficiency in the accumulation medium might result in an inhibition of cross-linked peptidoglycan synthesis and accumulation of nucleotide precursors. Thus, addition of glycine to the accumulation medium might reduce the accumulation of UDP-MurNAc-pentapeptide and -hexapeptide. As shown in Table 1, the addition of 0.7 g of glycine (9.3 mM) per liter results in a significant decrease in the accumulation of UDP-MurNAc-pentapeptide and UDP-MurNAc-Ala-\(\beta\)Glu-Lys(\(N'\)-Ala)-dAla-dAla is not observed. When penicillin G (250 mg/liter) was added to the accumulation medium with glycine, the quantity of UDP-MurNAc-pentapeptide was similar to that observed in the accumulation medium without penicillin. UDP-MurNAc-hexapeptide, on the other hand, was not detected in the accumulation medium containing glycine and penicillin. From the above results it is concluded that the presence of glycine in the accumulation medium is a significant factor in determining the amount of UDP-MurNAc-hexapeptide.

**Effect of UDP-MurNAc-Ala-\(\beta\)Glu-Lys(N'-Ala)-dAla-dAla on phospho-MurNAc-pentapeptide translocase.** The initial membrane reaction in the biosynthesis of peptidoglycan is catalyzed by phospho-MurNAc-pentapeptide translocase (UDP-MurNAc-Ala-\(\beta\)Glu-Lys-dAla-dAla:undeacryyl-phosphate phospho-MurNAc-pentapeptide transerase). In addition to the transfer of phospho-MurNAc-pentapeptide from UMP to undeacryyl-phosphate, this enzyme catalyzes the exchange of \([3H]\)UMP with the UMP moiety of UDP-MurNAc-pentapeptide according to the reaction:

\[
[3H]\text{UMP} + \text{UDP-MurNAc-peptide} = \left[\frac{1}{3H}\right]\text{UDP-MurNAc-peptide} + \text{UMP}
\]

This exchange reaction has been employed extensively as a routine assay because of its sensitivity. In addition, a single labeled substrate \([3H]\)UMP can be used in the presence of unlabeled analogues of UDP-MurNAc-pentapeptide. As shown recently, there was good correlation between the results obtained from the exchange and transfer reactions (3). It was concluded that results from the exchange reaction provide an index of specificity for comparing UDP-MurNAc-Ala-\(\beta\)Glu-Lys-dAla-dAla with other analogues.

In Fig. 4, the activity of UDP-MurNAc-Ala-\(\beta\)Glu-Lys(N'-Ala)-dAla-dAla is compared with that of UDP-MurNAc-pentapeptide. From the Lineweaver-Burk plots (Fig. 4A, B), values for \(R_{\text{max}}\) are determined at different concentrations of UDP-MurNAc-peptide. From the intercept replots (Fig. 4C, D), values for \(K_m\) of the UDP-MurNAc-peptides are established. Within experimental error, the Michaelis-Menten constants for the two UDP-MurNAc-peptides are identical. A useful index for comparing nucleotides in this assay is the ratio \(R_{\text{max}}/K_m\) (3). This ratio represents the apparent first-order rate constant for the reaction of substrate at low substrate concentrations. Under these conditions, \(R_{\text{max}}/K_m\) reflects the effectiveness of an analogue in the exchange assay. The values of \(R_{\text{max}}\) are used to compare the effectiveness of the substrates at high concentration. As summarized in Table 2, the ratios are almost identical for UDP-MurNAc-pentapeptide and -hexapeptide.

**Biosynthesis of peptidoglycan(N'-Ala) with UDP-MurNAc-Ala-\(\beta\)Glu-Lys(N'-Ala)-dAla-dAla.** Since isolated membranes from *S. aureus* Copenhagen prepared by several different procedures do not catalyze a high rate

| Table 1. Accumulation of UDP-MurNAc-pentapeptide and UDP-MurNAc-hexapeptide in the presence of glycine |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Additions to media** | Nucleotide accumulated* (\(\mu\)mol) | UDP-MurNAc-pentapeptide | UDP-MurNAc-hexapeptide |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Series A | | | | | | |
| Complete | 13.6 | 1.5 |
| Complete + 9.3 mM glycine | 7.5 | 0 |
| Complete + 9.3 mM glycine and 0.25 g of penicillin G per liter | 13.1 | 0 |
| Series B | | | | | | |
| Complete | 10.0 | 1.8 |
| Complete + 9.3 mM glycine | 4.3 | 0 |
| Complete + 9.3 mM glycine and 0.25 g of penicillin G per liter | 11.9 | 0 |

*The accumulation medium used in series A is the standard accumulation medium as described in Materials and Methods. The medium used for series B is the same medium to which 40 g of sucrose has been added per liter.

**Cells for nucleotide accumulation were grown to an optical density of 3 and transferred to the appropriate accumulation medium. Nucleotides were isolated and quantitated.**

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of peptidoglycan synthesis, we used the system from *S. aureus* H, prepared by the method of Strominger et al. (16) (Fig. 2). The requirements for the synthesis of nascent peptidoglycan are summarized in Table 3. The incorporation of $[^{14}C]$GlcNAc from UDP-$[^{14}C]$GlcNAc into peptidoglycan requires the addition of UDP-MurNAc-pentapeptide and is stimulated by the addition of NH$_4^+$ in this preparation. ATP also stimulates peptidoglycan synthesis. Lysozyme renders approximately 90% of the product from UDP-MurNAc-$[^{14}C]$pentapeptide soluble, whereas about 40% of the labeled material from UDP-$[^{14}C]$GlcNAc is lysozyme sensitive. When incorporation of $[^{14}C]$GlcNAc is corrected for the lysozyme-insensitive incorporation, the rates of GlcNAc and MurNAc-pentapeptide incorporation into peptidoglycan are equal.

To compare the effectiveness of each nucleotide in the nascent peptidoglycan-synthesizing system, the values of $V_{\text{max}}$ and $K_m$ were established from Lineweaver-Burk plots (Fig. 5). The values obtained from these plots are compiled in Table 2. Comparison of values for $V_{\text{max}}$ indicates that UDP-MurNAc-pentapeptide and -hexapeptide are used equally well at high substrate concentrations. The values of $V_{\text{max}}/K_m$ are also similar; the $V_{\text{max}}/K_m$ for UDP-MurNAc-pentapeptide is $5.6 \times 10^{-4}$/min, and the $V_{\text{max}}/K_m$ for UDP-MurNAc-hexapeptide is $4.3 \times 10^{-4}$/min. The difference between the two nucleotides as determined in these kinetic studies is the presence of a second line segment (Fig. 5A, dotted line) in the Lineweaver-Burk plot of UDP-MurNAc-pentapeptide at low substrate concentrations. In the substrate range from $K_m/2$ to 5 $K_m$, UDP-MurNAc-pentapeptide and UDP-MurNAc-hexapeptide are equally effective for the synthesis of peptidoglycan by the membrane system from *S. aureus* H.

**DISCUSSION**

The composition and degree of cross-linking of peptidoglycan from *S. aureus* Copenhagen is partially determined by the amount of glycine in the growth medium (Schleifer et al., in press). For example, when this organism is
grown in a glycine-poor medium, 20% of the lysine residues are substituted with N-terminal alanyl residues and 20% of the lysine residues are unsubstituted. At most, 60% of the ε-amino groups can be substituted with pentaglycine. In glycine-sufficient media almost all of the ε-amino groups of lysine are linked to pentaglycine. Thus, the degree of L-alanine substitution reflects the amount of glycine available to the organism and may play an important role in determining the degree of cross-linking. In this paper we describe a possible precursor of the N'-alanyl-lysine dipeptide moiety, i.e., UDP-MurNAc-Ala-Ala-εGlu-Lys-(N'-Ala)-dAla-dAla. The utilization of this nucleotide by phospho-MurNAc-pentapeptide translocase and its incorporation into peptidoglycan by systems from S. aureus is established.

Several reports describing the accumulation of nucleotide-activated MurNAc-peptides in various strains of S. aureus have been published (6, 12, 15, 17). None of these reports suggested the presence of UDP-MurNAc-Ala-εGlu-Lys-(N'-Ala)-dAla-dAla in this organism. In the present experiments, the accumulation of UDP-MurNAc-hexapeptide by resting cells was not affected by the presence of penicillin in the accumulation medium. The addition of glycine to the accumulation medium eliminates the accumulation of UDP-MurNAc-hexapeptide. Since glycine is required for the formation of the interpeptide bridge, it might be expected that a glycine deficiency could lead to a reduced rate of peptidoglycan synthesis with an accompanying accumulation of UDP-MurNAc-peptides. This requirement for glycine is reflected by the accumulation of UDP-MurNAc-pentapeptide in a glycine-deficient medium and the reduction of accumulation when glycine

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**Table 2.** Comparison of kinetic data for UDP-MurNAc-pentapeptide and UDP-MurNAc-hexapeptide

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$R_{max}$ (μM/min)</th>
<th>$R_{max}/K_m$ (min⁻¹ × 10⁻⁴)</th>
<th>$V_{max}$ (μM/min)</th>
<th>$V_{max}/K_m$ (min⁻¹ × 10⁻⁴)</th>
</tr>
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<tbody>
<tr>
<td>A. Exchange activity catalyzed by phospho-MurNAc-pentapeptide translocase*</td>
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</tr>
<tr>
<td>UDP-MurNAc-pentapeptide</td>
<td>40.0</td>
<td>1.79</td>
<td>4.47</td>
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<td></td>
</tr>
<tr>
<td>UDP-MurNAc-hexapeptide</td>
<td>40.6</td>
<td>1.81</td>
<td>4.46</td>
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<tr>
<td>B. Peptidoglycan synthesis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>UDP-MurNAc-pentapeptide</td>
<td>32*</td>
<td>1.8*</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-hexapeptide</td>
<td>42</td>
<td>1.8</td>
<td>4.3</td>
<td></td>
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</tr>
</tbody>
</table>

*Data in A and B were established from the Lineweaver-Burk plots presented in Fig. 4 and 5, respectively.

Values for the indicated substrate are extrapolated to infinite UMP concentration.

These values were determined from data in the concentration range 13 to 160 μM (solid line, Fig. 5A inset).

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**Table 3.** Requirements for the synthesis of peptidoglycan

<table>
<thead>
<tr>
<th>Additions</th>
<th>Peptidoglycan synthesis (pmol/h)</th>
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</thead>
<tbody>
<tr>
<td>UDP-[¹⁴C]GlcNAc</td>
<td></td>
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<tr>
<td>UDP-MurNAc-Ala-εGlu-Lys-(N'-Ala)-dAla-dAla</td>
<td></td>
</tr>
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</table>

Expt I*:

A. Complete | 24.5 |
Complete – UDP-MurNAc-pentapeptide | 0 |
B. Complete | 24.0 |
Complete – UDP-GlcNAc | 0 |

Expt II:

Complete | 46.7 |
Complete – ATP | 27.3 |
Complete – NH₄⁺ | 31.8 |
Complete – UDP-MurNAc-pentapeptide | 6.6 |

*In experiment I the complete reaction mixture contained 98 μM UDP-MurNAc-Ala-εGlu-Lys-dAla-dAla and 98 μM UDP-[¹⁴C]GlcNAc (28.8 counts/min per pmol) in A or 98 μM UDP-GlcNAc and 98 μM UDP-MurNAc-Ala-εGlu-Lys-dAla-dAla (23.6 counts/min per pmol) in B, with 117 μg of membrane protein (preparation A) in addition to the standard reaction components. The reaction was carried out, and peptidoglycan synthesis was determined as a chromatographically immobile, lysozyme-sensitive product. The amount of labeled material incorporated into lysozyme-insensitive material was 38.8 pmol from UDP-[¹⁴C]GlcNAc and 4.2 pmol from UDP-MurNAc-[¹⁴C]-pentapeptide.

*In experiment II all conditions and concentrations are the same as in experiment I, except that the reaction mixture contained 100 μg of membrane protein (preparation B). The amount of [¹⁴C]GlcNAc incorporated into lysozyme-insensitive material was 34.9 pmol.
is added. The relationship between glycine deficiency and UDP-MurNAc-hexapeptide production is not well understood. Since N-terminal alanine in the peptide moiety of the peptidoglycan is not further substituted in this organism, we might view the formation of the hexapeptide as a mechanism for conserving the glycine pool of the growing bacterium.

Acylation of the ε-amino group of the lysine residue with L-alanine does not affect the activity catalyzed by phospho-MurNAc-pentapeptide translocase. From the results with the exchange reaction, it was concluded that the rate of undecaprenyl-diphosphate-MurNAc-hexapeptide formation is similar at both high and low substrate concentrations when compared with UDP-MurNAc-pentapeptide. With membrane preparations from *L. viridescens*, the rate of undecaprenyl-diphosphate-MurNAc-hexapeptide formation from UDP-MurNAc-Ala-DGlu-Lys(N'-Ala)–dAla–dAla is similar to that described for undecaprenyl-diphosphate-MurNAc-pentapeptide formation (11). Thus, acylation of residue 3 with L-alanine does not result in a discrimination against this nucleotide in the reaction catalyzed by the initial membrane-associated enzyme in peptidoglycan synthesis.

Since isolated membranes from *S. aureus* Copenhagen do not catalyze a high rate of nascent peptidoglycan synthesis, the membrane preparation from *S. aureus* H prepared by alumina grinding (16) has been used for comparing UDP-MurNAc-pentapeptide and -hexapeptide. In the concentration range of $K_m/2$ to $5 K_m$, UDP-MurNAc-hexapeptide is as effective as UDP-MurNAc-pentapeptide in the biosynthesis of peptidoglycan. The parameters $V_{\text{max}}$ and $V_{\text{max}}/K_m$ are similar for both nucleotides. With UDP-MurNAc-hexapeptide the incorporation is characterized by a second line segment in the Lineweaver-Burk plot at low substrate concentrations. The second line segment is not observed with the hexapeptide. The results presented in this paper and those described by Schleifer et al. (in press) suggest that in *S. aureus* UDP-MurNAc-hexapeptide is utilized effectively for synthesis of nascent peptidoglycan.

In *L. viridescens*, the N-terminal alanine of the hexapeptide serves as an acceptor at the lipid intermediate stage for an additional serine residue required for the formation of the interpeptide bridge. In contrast, on the basis of the in vivo results described by Schleifer et al., the N-terminal alanine of the hexapeptide of *S. aureus* Copenhagen does not function as an acceptor for the glycine residues required for interpeptide bridge formation. The presence of the N-terminal alanine decreases the number of potential interpeptide bridges for cross-linking the glycans strands. Thus, growth in a glycine-poor medium indirectly affects the degree of cross-linking of the peptidoglycan.

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**LITERATURE CITED**

