

Effect of Penicillin on the In Vivo Formation of the D-Alanyl-L-Alanine Peptide Cross-Linkage in Cell Walls of *Micrococcus luteus*

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Penicillin G was found to inhibit the formation of the D-alanyl-L-alanine cross-linkage in intact cells of *Micrococcus luteus*. This reaction was approximately 50-fold less susceptible to penicillin than the formation of the D-alanyl-L-lysine cross-linkage in the same organism. The presence of two penicillin-susceptible transpeptidation reactions that function in the incorporation of peptidoglycan precursors into the cell wall is proposed.

The cell wall peptidoglycan of *Micrococcus luteus* (*lysodeikticus*) contains two types of peptide cross-linkages (4, 9), of which the predominant one is between a terminal D-alanine from one peptide side chain and the α -amino group of an L-alanine from another chain (D-Ala \rightarrow L-Ala). The other type of cross-linkage is between a terminal D-alanine from one peptide side chain and the ϵ -amino group of L-lysine of another chain (D-Ala \rightarrow L-Lys).

We have recently shown that penicillin inhibits the formation of the D-Ala \rightarrow L-Lys cross-linkage both in vitro and in vivo and that the limited incorporation of newly synthesized peptidoglycan strands, which occurs in the presence of high concentrations of the antibiotic, is the consequence of linear elongation of existing chains by transglycosylation (6, 7; D. Mirelman, R. Bracha, and N. Sharon, Ann. N. Y. Acad. Sci., in press). These results led us to propose that the penicillin-susceptible transpeptidase, which catalyzes the formation of the D-Ala \rightarrow L-Lys bond, functions not only in the cross-linking of peptide side chains but also in the incorporation proper of linear peptidoglycan strands into the pre-existing cell wall.

In the present communication we wish to report further experiments with intact cells of *M. luteus* which show for the first time that penicillin also inhibits the formation of the D-Ala \rightarrow L-Ala peptide cross-linkage. The formation of this linkage is approximately 50-fold less susceptible to penicillin than that of the D-Ala \rightarrow L-Lys linkage. Moreover, the inhibitory effect of increasing concentrations of penicillin G on the in vivo formation of the D-Ala \rightarrow L-Ala cross-linkage was similar to the inhibitory effect of increasing concentrations of the antibiotic on

the incorporation of L-alanine and of L-lysine into the cell-wall peptidoglycan.

MATERIALS AND METHODS

The in vivo incorporation of radioactive L-lysine or L-alanine into cells of *M. luteus* NCTC 2655 was studied in a minimal medium in which cell-wall synthesis can take place (2). For this purpose, early logarithmic-phase cells grown as described before (7) were suspended in 5 or 10 ml of a minimal medium (2×10^8 cells/ml) containing glycine (1 mM), L-glutamic acid (1 mM), and glucose (0.0285 M), together with salts and cofactors as described (7) and penicillin G when indicated.

For the study of the incorporation of radioactive L-lysine, unlabeled L-alanine (0.5 mM) was added to the minimal medium and, after incubation for 5 min with shaking at 32 C, uniformly labeled L-[14 C]lysine (Radiochemical Centre, Amersham; >99.9% pure; specific activity, 342 mCi/mM, diluted with unlabeled L-lysine to a specific activity of 110 mCi/mM) was added to the medium to a final concentration of 3 nmol/ml. To investigate the incorporation of radioactive L-alanine, unlabeled L-lysine (1 mM) was added to the minimal medium and, after incubation for 5 min with shaking at 32 C, uniformly labeled L-[14 C]alanine (Radiochemical Centre, Amersham; >99.9% pure; specific activity, 102 mCi/mM) was added to the medium to a final concentration of 3 nmol/ml.

Incubation with shaking was continued for 10 min. The cells were then harvested, thoroughly washed with phosphate buffer (3×20 ml, 0.05 M, pH 7.2), and disintegrated in water (10 ml) with glass beads (10 g) in a Braun homogenizer. The cell walls were isolated by differential centrifugation and extensively purified by heating with 0.1% sodium dodecylsulfate in water and then washing in water (7). Amino acid analysis (8) of the purified walls showed that more than 97% of the amino acids detected belong to the cell-wall peptidoglycan.

Estimation of the extent of cross-linking of the

L-[¹⁴C]lysine incorporated into the walls was by the nitrous acid deamination procedure previously described (7). To estimate the formation of radioactive alanine dipeptides, cell walls labeled with [¹⁴C]alanine were partially hydrolyzed according to the method of Schleifer and Kandler (9) (4 N HCl, 100 C, 60 min), and the hydrolysate was analyzed on the medium column (20 cm) of the amino acid analyzer (Beckman 120C), using citrate buffer (0.2 M, pH 3.25) as eluant. The effluent of the column was passed directly through a flow scintillation spectrometer (Packard Tri-Carb 3200). Under these conditions, alanine emerged after 39 min, L-Ala-D-Glu after 54 min, D-Ala-L-Ala after 64 min, and D-Ala-D-Ala after 72 min. The partial acid hydrolysates were also examined on paper electrophoresis (50 V/cm, 120 min, pH 3.5), where their rate of migration was compared with that of authentic synthetic peptide markers. Radioactive spots were detected by autoradiography (Kodak Royal Blue X-ray film). The peptides were eluted from the paper (3), and rechromatographed on the medium column of the amino acid analyzer. Radioactive L-Ala-D-Glu, D-Ala-L-Ala, and D-Ala-D-Ala were further identified by amino acid analysis after acid hydrolysis (6 N HCl, 105 C, 20 h), and the configuration of the radioactive alanine released was established by the use of D-amino acid oxidase (5). The N-terminal radioactive amino acid of the dipeptides was identified by dinitrophenylation (5).

RESULTS

Effect of penicillin on the incorporation and cross-linking of L-alanine. The inhibitory effect of increasing concentrations of penicillin G on the incorporation of radioactive alanine into the cell-wall peptidoglycan was the same as its effect on the incorporation of L-lysine into the cell wall (Fig. 1A, 2A). The inhibition

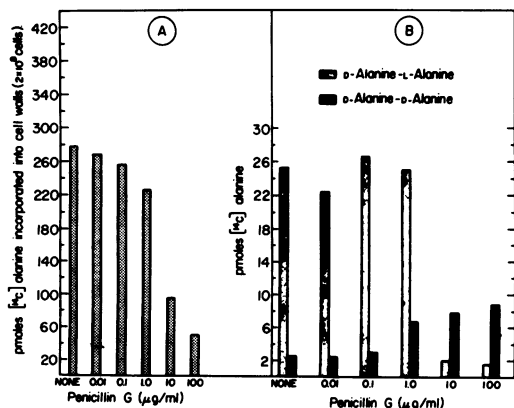


FIG. 1. Effect of penicillin on (A) the incorporation of L-[¹⁴C]alanine into cell walls of intact cells of *M. luteus*; (B) the amount of the radioactive dipeptides, D-Ala-L-Ala and D-Ala-D-Ala, obtained from partial acid hydrolysates of cell walls prepared from cells (2×10^8) labeled for 10 min with L-[¹⁴C]alanine in the presence or absence of penicillin. Radioactive compounds were estimated as described in text.

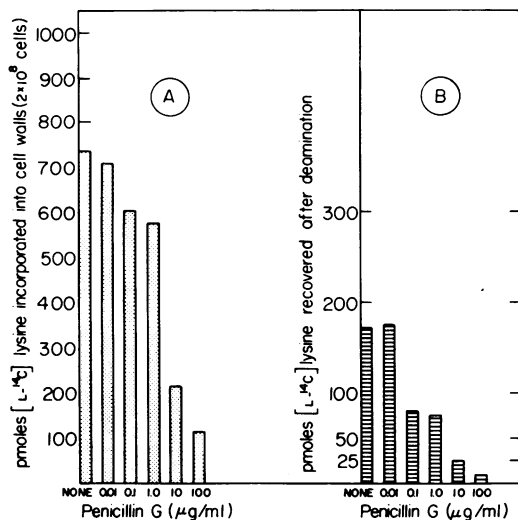


FIG. 2. Effect of penicillin on (A) the incorporation of L-[¹⁴C]lysine into the cell walls of *M. luteus*; (B) the amount of [¹⁴C]lysine found in walls after deamination and hydrolysis. Cell walls were prepared from (2×10^8) intact cells which incorporated L-[¹⁴C]lysine for 10 min in the presence or absence of penicillin. For further details, see text.

observed at concentrations of 0.1 µg/ml was approximately 15%, whereas at 10 µg/ml the inhibition was 68%.

Partial acid hydrolysates of cell walls of *M. luteus* were shown to afford a very complex mixture of compounds (9). Similar hydrolysates of cell walls labeled for a short period (10 min) with [¹⁴C]alanine afforded consistently a small number of radioactive peptides together with free alanine (Fig. 3). The relative distribution of radioactivity in the different compounds was highly reproducible. Total acid hydrolysis of the compound, which migrated on the amino acid analyzer column and on paper electrophoresis as D-Ala-L-Ala, followed by digestion with D-amino oxidase, revealed that approximately 53% of the radioactivity present as [¹⁴C]alanine was converted into [¹⁴C]pyruvate. Furthermore, upon dinitrophenylation of the dipeptide and acid hydrolysis, 45% of the radioactivity was converted into 2,4-dinitrophenyl-alanine. The remaining free alanine released by the hydrolysis was not affected by D-amino acid oxidase.

The pattern of elution of the radioactive peptides obtained from cell walls which incorporated L-[¹⁴C]alanine, in the absence of penicillin, was significantly different from that found in partial hydrolysates from cells incubated in the presence of high concentrations of penicillin G (10 µg/ml) (Fig. 3B, C). In the absence of penicillin, the amount of radioactive

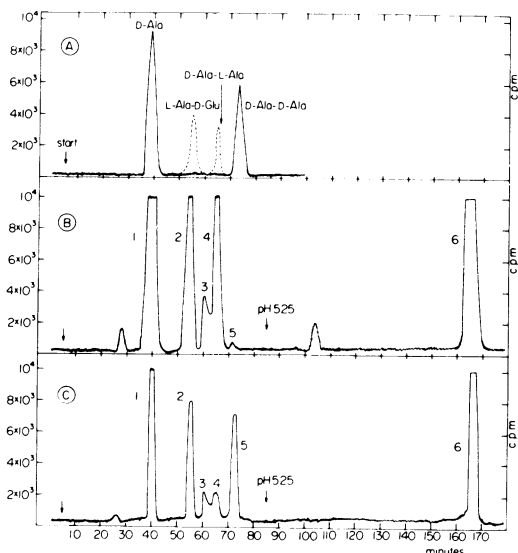


FIG. 3. Flow scintillation charts of the effluent from the amino acid analyzer. (A) Standards of authentic compounds; (B) partial acid hydrolysates of cell walls labeled *in vivo* for 10 min with [^{14}C]alanine (4×10^8 cells); (C) same as (B) but labeling was done in the presence of penicillin G (10 $\mu\text{g/ml}$). For experimental conditions, see text. (1) Alanine; (2) L-Ala-D-Glu; (4) D-Ala-L-Ala; (5) D-Ala-D-Ala. Some compounds in the partial acid hydrolysates have not yet been identified (No. (3), (6)).

D-Ala-L-Ala obtained was 25 pmol/ 2×10^8 cells or approximately 10% of the total radioactivity in the walls, whereas only traces of D-Ala-D-Ala were detected (2.6 pmol or 1.0% of total radioactivity) (Fig. 1B). In the presence of 10 μg of penicillin G per ml, the amount of D-Ala-L-Ala was only 2.0 pmol/ 2×10^8 cells, whereas that of D-Ala-D-Ala was considerably higher (8.0 pmol or approximately 10% of total radioactivity incorporated into the wall). It is of interest to note, however, that an increase in the relative proportion of the D-Ala-D-Ala in the partial hydrolysates was already noted at 0.1 μg of penicillin G per ml (Fig. 1B).

When incorporation of alanine into the cells was in the absence of penicillin, 50% of the [^{14}C]alanine in the wall was of the D-configuration, whereas, when incubation was in the presence of high amounts of penicillin (100 $\mu\text{g/ml}$), more than 65% of the radioactive alanine found in the cell walls was of the D-configuration (Table 1).

Effect of penicillin on the incorporation and cross-linking of L-lysine. Fifty-percent inhibition of incorporation of L-lysine into the cell walls was observed at concentrations of about 5 μg of penicillin G per ml (Fig. 2A).

However, formation of the peptide cross-linkage involving the ϵ -amino moiety of the radioactive lysine incorporated into the cell walls was much more sensitive to penicillin G (50% inhibition at about 0.1 μg of penicillin G per ml (Fig. 2B).

DISCUSSION

Considerable evidence is available which shows that the D-Ala \rightarrow L-Lys cross-linkage is formed by transpeptidation (6, 7, 9; D. Mirelman, R. Bracha, and N. Sharon, Ann. N.Y. Acad. Sci., in press). However, no direct evidence on the formation of the D-Ala \rightarrow L-Ala cross-linkage has been presented. In this communication, we show that the formation of the D-Ala \rightarrow L-Ala cross-linkage is susceptible to penicillin, though this susceptibility is some 50-fold less than that of formation of the D-Ala \rightarrow L-Lys linkage. In this connection it is pertinent to note that preliminary data obtained by us (manuscript in preparation) shows that the cleavage of the amide bond between *N*-acetyl-muramic acid and the L-alanine moiety of the peptide chain by an endogenous amidase is not inhibited by penicillin G (at 10 $\mu\text{g/ml}$). This finding clearly indicates that the reason for the inhibition of the formation of the D-Ala \rightarrow L-Ala cross-linkage by penicillin (10 $\mu\text{g/ml}$) is not due to the absence of the specific acceptor group (α -amino group of L-alanine).

These results may be taken as evidence for the presence in *M. luteus* of two penicillin-susceptible transpeptidases, one of which catalyzes the formation of the D-Ala \rightarrow L-Lys linkage, the other forming the D-Ala \rightarrow L-Ala linkage. Alternatively, it is possible that only one enzyme is responsible for both cross-linking reactions. In this case the differences in susceptibility to penicillin may be ascribed to differences in the nature of the enzyme-donor-acceptor complex

TABLE 1. Effect of penicillin on the relative amount of D-[^{14}C]alanine in cell wall hydrolysates^{a, b}

Penicillin G ($\mu\text{g/ml}$)	D-[^{14}C]alanine (%)
0	49.8
0.01	50.4
0.1	50.9
1.0	51.5
10.0	62.0
100.0	65.5

^a [^{14}C]alanine-labeled cell walls were isolated from cells incubated in the absence or presence of penicillin as described in Materials and Methods.

^b D-[^{14}C]alanine was enzymatically determined in acid hydrolysates of labeled cell walls (6 N HCl, 105 C, 20 h) (5).

in the two transpeptidations or to differences in the location of the two reactions in the cell.

The present results also lend further support to our earlier suggestion that elongation of cell-wall peptidoglycan occurs both by penicillin-susceptible transpeptidation and by penicillin-unsusceptible transglycosylation (6, 7; D. Mirelman, R. Bracha, and N. Sharon, Ann. N.Y. Acad. Sci., in press). Thus, the increase in the relative proportion of radioactive D-alanine found in walls synthesized in the presence of high concentrations of penicillin is ascribed by us to the incorporation of peptidoglycan strands with terminal-D-Ala-D-Ala units into the cell wall by transglycosylation. In the absence of penicillin, the terminal D-alanine of those newly synthesized strands is removed by transpeptidation (4, 6, 7, 9); D. Mirelman, R. Bracha, and N. Sharon, Ann. N.Y. Acad. Sci., in press). The discrepancy found in the amounts of radioactive alanine and lysine which is incorporated into the cell wall during 10 min under identical conditions is not yet understood (Fig. 1A, 2A). A possible reason for this may be the difference in the intracellular pools of these two amino acids.

Another interesting finding is that the concentration of penicillin (5 $\mu\text{g}/\text{ml}$) required to inhibit by 50% the formation of the D-Ala \rightarrow L-Ala cross-linkage is very similar to that which inhibits to the same extent the in vivo incorporation of L-alanine and L-lysine into the cell wall (Fig. 1, 2). On the other hand, the concentration of penicillin (0.1 $\mu\text{g}/\text{ml}$) required for 50% inhibition of the formation of the D-Ala-L-Lys linkage causes only 15% inhibition of incorporation of amino acids into the cell wall. This concentration is, however, very close to that required to inhibit growth of *M. luteus* cells by 50% (1, 10). It would appear, therefore, that part of the lethal effect of low concentrations of penicillin G on *M. luteus* cells may be ascribed to the inhibition of the formation of the D-Ala \rightarrow L-Lys linkage.

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