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# NOTES

## Mucopeptide Biosynthesis by Minicells of *Escherichia coli*

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Minicells produced by *Escherichia coli* χ925 incorporated amino acids and *N*-acetyl-D-glucosamine into mucopeptide.

It has been consistently observed that, although minicells are anucleate, they are capable of converting amino acids into trichloroacetic acid-precipitable material (e.g., references 4, 6, 10). An explanation for this phenomenon in *Escherichia coli* minicells was provided by the identification of stable messenger ribonucleic acids that segregate into minicells and that permit the synthesis of cell membrane proteins (3, 7). We recently investigated the phenomenon with minicells produced by a strain of *Bacillus subtilis* (8) and found that these minicells synthesized teichoic acid and mucopeptide from precursor amino acids. The polymerization of amino acids by minicells of *B. subtilis* was inhibited by vancomycin, D-cycloserine (CYC), and penicillin G (PEN). The effect of PEN on amino acid incorporation by *E. coli* minicells has been disputed (6, 10) and, indeed, a technique employing PEN to purify minicells has been proposed (5). We therefore undertook an investigation of the ability of minicells produced by the strain *E. coli* χ925 (10) to synthesize mucopeptide and the sensitivity of this synthesis to antibiotics.

One-liter cultures of *E. coli* χ925 were grown at 37°C in TY medium (1) to an absorbance at 660 nm of 0.5. Minicells were purified by using sucrose gradients as previously described (8), with the slight modification that BSG (2) was employed in place of minimal salts. Minicells were washed and resuspended in M9 minimal salts (9) medium supplemented with 0.4% glucose, 10 μg of leucine per ml, 10 μg of threonine per ml, and 1 μg of thiamine per ml. Purified minicells contained approximately 1 colony-forming unit per $10^6$ minicells as assayed on nutrient agar plates (Difco).

In Fig. 1 is shown the incorporation of the $^{14}$C-labeled amino acid mixture, D-[U-$^{14}$C]alanine, and *N*-acetyl-D-[1-$^{14}$C]glucosamine. Minicells incorporate these precursors into hot trichloroacetic acid-precipitable material, and the incorporation is inhibited to varying extents by CYC. Incorporation of the mixture of amino acids is inhibited 20% by CYC, whereas incorporation of the direct precursors of mucopeptide, i.e., D-alanine and *N*-acetyl-D-glucosamine, is inhibited by over 80%. The composition of the amino acid mixture has been previously described (8), and the possibility that the inhibition observed in Fig. 1A was due to inhibition of incorporation of the known mucopeptide precursor L-alanine was further tested. In Table 1 is presented the inhibition of incorporation of the mixture of L-amino acids, L-alanine, and L-methionine by several antibiotics. It can be seen that, whereas the incorporation of L-alanine is strongly inhibited by CYC, the incorporation of L-methionine is unaffected by the presence of CYC. PEN, at the concentration employed (33 U/ml), inhibits the incorporation of L-alanine approximately 50% and inhibits the incorporation of the mixture of amino

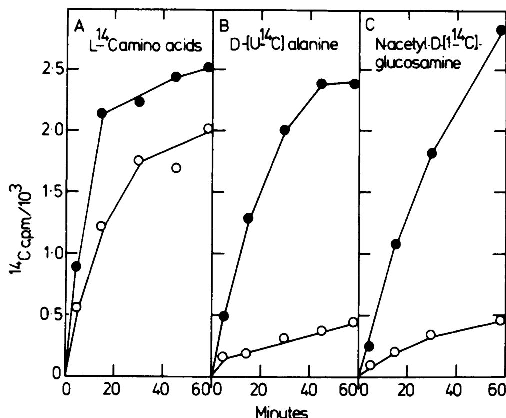


FIG. 1. *Incorporation of* L-$^{14}$*C-labeled amino acids, [*D-$^{14}$*C]alanine, and N-acetyl-*D-*[1-*$^{14}$*C]glucosamine by minicells. Minicells (absorbance at 660 nm = 0.2) were incubated at 37°C in supplemented M9 medium containing: (A) 2 μCi of* L-$^{14}$*C-labeled amino acid mixture (8) per ml; (B) 1 μCi of* D-*[U-*$^{14}$*C]alanine (37 mCi/mmol) per ml; (C) 0.55 μCi of N-acetyl-*D-*[1-*$^{14}$*C]glucosamine (52 mCi/mmol) per ml. Samples, 100 μl, were removed, and the radioactivity in hot trichloroacetic acid-precipitable material was measured in the absence (●) or in the presence (○) of 20 μg of CYC per ml.*

TABLE 1. *Incorporation of L-amino acids by minicells in the presence and absence of inhibitors*

| Label incorporated[a] | Amt incorporated | Synthesis (%) in the presence of[b] | | | |
|---|---|---|---|---|---|
| | | CAM | RIF | CYC | PEN |
| L-$^{14}$C-amino acid mixture | 3,622 cpm | 36 | 87 | 76 | 79 |
| L-[U-$^{14}$C]alanine | 3.53 ng[c] | 75 | 102 | 23 | 49 |
| L-[U-$^{14}$C]methionine | 0.33 ng | 59 | 96 | 106 | 93 |

[a] Minicells (0.1 ml; absorbance of 660 nm = 0.4) were incubated for 45 min at 37°C in supplemented M9 medium plus 0.5 μCi of L-$^{14}$C-labeled amino acid mixture (8) or 0.1 μCi of L-[U-$^{14}$C]alanine plus 0.0025% (wt/vol) pancreatic casein hydrolysate (Merck, Germany) (final concentration of L-alanine = 1.80 μg/ml) or 0.09 μCi of L-[U-$^{14}$C]methionine (250 mCi/mmol) plus 25% (vol/vol) methionine assay medium (Difco) (6). Two milliliters of 5% (wt/vol) trichloroacetic acid containing 0.1% (wt/vol) casein hydrolysate was added to each sample, and the hot trichloroacetic acid-precipitable material was measured as previously described (8).

[b] Final concentrations of antibiotics: CAM, 150 μg/ml; RIF, 100 μg/ml; CYC, 20 μg/ml; PEN, 20 μg/ml (equivalent to 33.3 U/ml). The amount of material synthesized in the presence of the antibiotics is expressed as a percentage of the amount synthesized in the absence of antibiotics.

[c] Synthesis of 1 ng of material from L-alanine or L-methionine was equivalent to the incorporation of 791 and 2,420 cpm, respectively.

TABLE 2. *Incorporation of amino acids by minicells and lysozyme sensitivity of the product*

| Label incorporated[a] | Antibiotic added[b] | Hot trichloroacetic acid-precipitable material (ng/ml)[c] | | | Digested by added lysozyme (%) |
|---|---|---|---|---|---|
| | | 0 min | After 15-min incubation in buffer | After 15-min incubation in buffer plus lysozyme | |
| L-$^{14}$C-amino acids | None | 13,640[d] | 9,704[d] | 6,056[d] | 27 |
| L-[U-$^{14}$C]alanine | None | 16.5[e] | 14.0 | 2.9 | 67 |
| | CYC | 3.9 | 3.3 | 3.5 | 0 |
| | CAM | 15.4 | 12.5 | 2.1 | 67 |
| | RIF | 16.7 | 10.9 | 3.5 | 45 |
| D-[U-$^{14}$C]alanine | None | 22.0 | 18.6 | 5.8 | 57 |
| L-[U-$^{14}$C]methionine | None | 1.46 | 1.01 | 1.04 | 0 |

[a] Minicells (absorbance at 660 nm = 0.4) were incubated in supplemented M9 medium for 45 min at 37°C in the presence of 4 μCi of L-$^{14}$C-labeled amino acid mixture per ml (8) or 1 μCi of L-[U-$^{14}$C]alanine plus 0.0025% (wt/vol) pancreatic casein hydrolysate (final concentration of L-alanine = 1.80 μg/ml) or 1 μCi of D-[U-$^{14}$C]alanine (37 mCi/mmol) per ml. For incorporation of methionine, the minicells were suspended in M9 medium containing one-quarter strength methionine assay medium (6) plus 1 μCi of L-[U-$^{14}$C]methionine (250 mCi/mmol) per ml.

[b] Final concentrations of antibiotics: CYC, 20 μg/ml; CAM, 150 μg/ml; RIF, 100 μg/ml.

[c] Minicell suspensions were diluted 20 times with 5% (wt/vol) trichloroacetic acid containing 0.1% (wt/vol) pancreatic casein hydrolysate and 1 mg of D-alanine per ml. The suspensions were held at 90°C for 30 min, and cooled to 0°C, and the precipitates were removed from suspensions by centrifugation (24,000 × g; 10 min, 0°C). The precipitates were washed twice with ice-cold buffer [0.05 M tris(hydroxymethyl)aminomethane, 0.1 M NaCl, 0.05 M ethylenediaminetetraacetic acid, pH 7.6] and resuspended in a volume of this buffer equal to the original volume of the minicell suspensions. Portions of these suspensions were placed on 20 volumes of 5% trichloroacetic acid either directly (0 min) or after incubation in buffer with or without lysozyme (2 mg of lysozyme per ml, final concentration) for 15 min at 37°C. The hot trichloroacetic acid-precipitable material in each sample was determined as previously described (8).

[d] In counts per minute per milliliter.

[e] Synthesis of 1 ng of material from L-alanine, D-alanine, or L-methionine was equivalent to the incorporation of 791, 521, or 2,420 cpm, respectively.

acids approximately 20%. PEN at this concentration inhibits the incorporation of L-methionine by less than 10%. However, at much higher concentrations, as suggested in the purification procedure (5), the incorporation of all amino acids tested (L- and D-alanine, L-proline, L-methionine) is inhibited at least 50%, as has also been described for the incorporation of L-leucine (10). Table 1 also includes the inhibition effects of chloramphenicol (CAM) and rifampin (RIF) on amino acid incorporation. The results are consistent with the existence of stable messenger ribonucleic acid (7) in that CAM inhibits the incorporation of L-amino acids, whereas RIF produces far less inhibition. The choice of precursor amino acid is very important: whereas methionine incorporation would indicate mostly protein synthesis,

the use of L-alanine would indicate mostly mucopeptide biosynthesis. This has been verified by checking the lysozyme solubility of the product produced in minicells allowed to incorporate a variety of precursor amino acids (Table 2). L- and D-alanine are polymerized into material that is over 50% digested by exposure to lysozyme. This may be an underestimate of mucopeptide biosynthesis, as it has been consistently found that some hot trichloroacetic acid-precipitable material becomes hot trichloroacetic acid soluble during incubation in buffer without lysozyme. This amount has been substracted from the amount of solubilization occurring in the presence of lysozyme to obtain the percentage of material that is directly converted to trichloroacetic acid-soluble material by lysozyme digestion. In the presence of CYC, L-alanine is not converted to a product that can be digested by lysozyme, whereas synthesis in the presence of CAM or RIF does produce a lysozyme-digestible product (Table 2). The product from the mixture of amino acids is 27% solubilized by lysozyme, which is in good agreement with the extent of inhibition of the incorporation of the amino acid mixture by CYC and PEN (Table 1). L-Methionine is, however, converted to a product that is not solubilized by lysozyme and is therefore most likely converted to protein, as previously described (6, 7).

It is clear from the results presented that minicells produced by *E. coli* $\chi$925 do synthesize mucopeptide and, indeed, on a mass basis (absorbance of 660 nm of 0.5 is equivalent to $4 \times 10^9$ *B. subtilis* minicells and $5 \times 10^9$ *E. coli* minicells), the amount synthesized is very similar to that synthesized by minicells produced by *B. subtilis* (8). The results indicate that careful choice of precursor and inhibitors must be made in order to interpret incorporation curves depicting synthesis in minicells of macromolecules from amino acid precursors.

## ADDENDUM IN PROOF

Goodell and Schwartz (personal communication, 1976; submitted to Eur. J. Biochem., 1977) have demonstrated the presence of both murein-synthesizing and -hydrolyzing enzymes in the cell envelope fraction isolated from *E. coli* minicells and assayed in vitro. Their in vitro results agree very well with the in vivo results presented here.

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