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Efficient and quantitative incorporation of diaminopimelic acid into the peptidoglycan of *Salmonella typhimurium*

(*Salmonella typhimurium*; peptidoglycan; diaminopimetic acid incorporation)

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1. SUMMARY

Diaminopimelic acid is incorporated into the peptidoglycan of *Salmonella typhimurium* in an efficient and quantitative manner. The amount of DAP incorporated is similar to the number of molecules estimated to exist in the *Salmonella* cell wall. In contrast, strains of *E. coli*, including those most used for studies of cell wall synthesis, are much less efficient in the incorporation of diaminopimelic acid. The lysine-requiring strains of *E. coli* appear to excrete diaminopimelic acid related material during growth and this accounts, in part, for the inefficient incorporation of radioactive diaminopimelic acid into *Escherichia* strains. In addition, the *Escherichia* strains are much less permeable to DAP than *Salmonella* strains. Cysteine and cystine inhibit the incorporation of DAP into the cell and this result suggests that *Salmonella* uses the cystine uptake system to allow DAP into the cell.

2. INTRODUCTION

Diaminopimelic acid (DAP) is a unique constituent of the peptidoglycan of Gram-negative bacteria. It is involved in the cross-linking reaction which connects adjacent peptidoglycan chains and it is this cross-linking which produces the structural characteristics of the bacterial cell wall. Because DAP is a unique constituent of the cell wall it has been very useful in studies on the biosynthesis of cell walls. Many experiments on cell wall synthesis have used diaminopimelic acid requiring strains. Such *dap*$^-$ strains (e.g., *E. coli* W7) were used because it was felt that the labeling of cell wall in such strains would be more efficient than strains which synthesized endogenous DAP. In a detailed study of DAP incorporation in *E. coli*, Wentjes, Pas, Taschner and Woldring [1] suggest that labeling of the cell wall with DAP was more efficient if a lysine-requiring strain not auxotrophic for DAP was used. This was because the pool of nonradioactive DAP in a cell which was necessarily grown in DAP (prior to the addition of label) was much larger than the pool of DAP in cells grown on the endogenously synthesized DAP. Wentjes et al. appeared to have a relatively efficient incorporation system. When they normalized for cell growth, they observed a constant rate of incorporation of DAP for over 4 to 5 generations.

The incorporation of exogenously added DAP

by *E. coli* appears to occur by way of the cystine uptake system [2] and thus the incorporation of DAP is presumably dependent on the ability of this uptake system to allow DAP into the cell.

We wish to report that a related organism, *S. typhimurium*, is much more efficient at incorporating DAP into the peptidoglycan and that the incorporation of exogenous DAP is essentially quantitative. Incorporation in *S. typhimurium* strains is approx. 20–30 times more efficient than incorporation into *E. coli* strains. We have analyzed the differences between the uptake and incorporation of DAP into *Salmonella* and *Escherichia* and find that incorporation into *Escherichia* is lowered because of inefficient uptake as well as the excretion of compounds into the medium which inhibit the incorporation of radioactive DAP.

3. MATERIALS AND METHODS

3.1. Bacterial strains

E. coli strains W7 (*lys*$^-$, *dap*$^-$) and MC4100*lys*$^-$ [1] were obtained from Dr. Conrad Woldringh of the Department of Electron Microscopy and Molecular Cytology, University of Amsterdam, Amsterdam, the Netherlands. SCl was a lysine requiring strain derived by nitrosoguanidine mutagenesis of *E. coli* B/r. All *Salmonella* strains were obtained from Dr. Kenneth E. Sanderson of the University of Calgary, Canada. Strain 2616 (LT7, *lys*$^-$) was used most extensively in the studies reported here and had the most efficient DAP incorporation. All strains of *Salmonella* tested were very efficient at DAP incorporation and were all much more efficient than *E. coli* strains.

3.2. Radioactive materials

[^{3}H]-diaminopimelic acid (35.6 Ci/mmol) was obtained from Research Products International, Mount Prospect, IL. Lower specific activity DA was prepared when needed by adding some of the high specific activity DAP to a prepared solution of diaminopimelic acid.

3.3. Growth of bacteria

Bacteria were grown in Medium C [3] which contains 6 g of Na_2HPO_4, 3 g KH_2PO_4, 2 g NH_4Cl, 3 g NaCl, and 0.25 g of $MgSO_4$ per l. The medium was supplemented, when required, with 0.2% glucose and 40 μg/ml of lysine or diaminopimelic acid. Bacteria were grown at 37° with rotary shaking. Growth was monitored as the A_{450} or the cell number measured with a Coulter Model ZM particle counter with a 30 micron aperture.

3.4. Labeling with diaminopimelic acid

Radioactive DAP was added to growing cells (or in the case of DAP requiring strains, after filtration and washing of the cells on a nitrocellulose filter) and samples were removed periodically to 10% TCA at 0°C. The cells were stored in the TCA for at least 30 min and then filtered on a nitrocellulose filter. Acid hydrolysis of labeled cells and TLC analysis followed by autoradiography revealed that all of the material stayed in the form of DAP and none was converted into lysine. That all of the DAP was in cell wall was supported by the observation that all of the TCA precipitable material was also resistant to solubilization by 4% sodium dodecyl sulfate at 100°C [1]. TCA insoluble material was collected on filters and measured by liquid scintillation counting.

4. RESULTS

4.1. Incorporation of diaminopimelic acid (DAP) into the peptidoglycan of S. typhimurium and E. coli

Salmonella or *Escherichia* were labeled with DAP as described in MATERIALS AND METHODS. Typical results are shown in Fig. 1. Incorporation into the bacterial cell wall was much more efficient (approximately 25–30 times more efficient) in *Salmonella* 2616 than in any *E. coli* strain tested. With strain W7 (*dap*$^-$, *lys*$^-$) starved for DAP for different periods of time [4] there was some improvement in the incorporation of DAP but *Salmonella* was still more efficient.

4.2. Alteration of the growth medium by E. coli

We observed that the incorporation of diaminopimelic acid into *E. coli* was independent of the concentration of bacteria added to a given amount

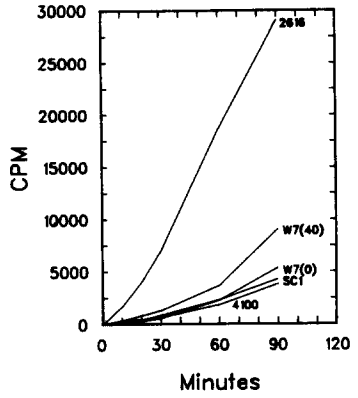

Fig. 1. Incorporation of diaminopimelic acid into *S. typhimurium* 2616 and *E. coli* strains W7, 4100*lys*$^-$ and SCI. Bacteria were grown in C medium and aliquots of the growing cultures were added to radioactive DAP (2.58 μg/ml). Samples (0.2 ml) were removed to TCA at 10, 20, 30, 60 and 90 min and processed as described in MATERIALS AND METHODS. At the start of the experiment W7 was filtered, washed and resuspended in medium with lysine and without diaminopimelic acid. At zero minutes and at 40 min of starvation the labeling of W7 was started in two separate flasks. The optical density of the W7 was 0.362 at the start of starvation and was 0.595 at 40 min of starvation. The absorbance of strains 2616, 4100*lys*$^-$, and SCI at the start of labeling were 0.680, 0.570, and 0.485 respectively. The doubling times of strains 2616, 4100*lys*$^-$, SCI and W7 were 40, 37, 38 and 63 min, respectively.

of radioactivity. That is, as the concentration of bacteria increased the radioactivity incorporated was constant. This was explained by finding that if a lysine requiring *E. coli* strain was mixed with a lysine-requiring *Salmonella* strain, the incorporation was not the sum of the incorporation of the 2 strains labeled independently. Analysis of the supernatant of medium in which *E. coli* strains have grown revealed the medium was altered so that the supernatant inhibited the incorporation of DAP into *Salmonella*. We propose that strains of *E. coli* excrete some compound or compounds which competes with added radioactive diaminopimelic acid. This compound is presumably diaminopimelic acid or a related compound. There are previous reports [5,6] of turnover and release of peptidoglycan components into the medium. We suggest that this is at least part of the reason that *Escherichia* strains are less efficient at incorporating DAP than *Salmonella*. Other experiments (not shown) indicate that *E. coli* is less permeable to DAP than *Salmonella*.

4.3. Quantitation of DAP incorporation into S. typhimurium

From the measured specific activity of the radioactive DAP we have determined the number of molecules incorporated per cell. With high specific activity DAP we find that there are 6.4×10^6 molecules incorporated per cell during one doubling period. This is consistent with previous determinations of the number of molecules of DAP present in the cell wall determined chemically or by calculation from the cell dimensions and the dimensions of a unit cell of peptidoglycan [7,8]. Measurements of the number of DAP molecules incorporated into cells were done either by completely labeling cells (infinite labeling period), or by labeling for a short period of time and determining in that experiment the cell increase during the labeling period. The specific activity (CPM/molecule) of the DAP was determined on the original material or on DAP solutions prepared by accurately weighing DAP and adding radioactivity to the freshly prepared solution. With both labeling procedures and with both types of label the results were equivalent. Diaminopimelic acid is incorporated into the peptidoglycan of *Salmonella* in an essentially quantitative manner.

4.4. Effect of cystine and cysteine on incorporation of DAP by Salmonella

Lieve and Davis [2] reported that *E. coli* was taken into the cell by way of a cystine uptake

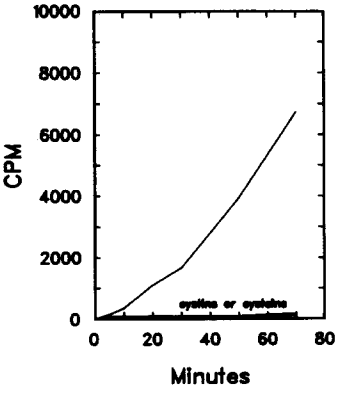

Fig. 2. Effect of cystine and cysteine on the incorporation of DAP into *S. typhimurium* strain 2616. Conditions were similar to those described in Fig. 1. The competing amino acids were added at a concentration of 100 μg/ml.

system. Meadow, Hoare and Work [9] reported that cystine inhibited the growth of DAP requiring strains. Presumably the inhibition of incorporation of DAP by cystine occurred due to competition of the two compounds for the cystine uptake system described by Lieve and Davis [2]. Both cystine and cysteine inhibit the incorporation of DAP by *Salmonella* (Fig. 2) and we suggest that a similar mode of uptake of DAP exists in *Salmonella* and in *E. coli*.

5. DISCUSSION

Diaminopimelic acid is the preferred label for measuring the synthesis of bacterial peptidoglycan as it is a unique constituent of the peptidoglycan. In lysine-requiring strains there is no further metabolism of the DAP and therefore the incorporation of the DAP is specifically into the peptidoglycan. Most studies of peptidoglycan synthesis have used diaminopimelic acid-requiring strains as these strains were presumed to be more efficient at incorporating DAP than strains unimpaired in their endogenous DAP synthetic system. Wientjes, Pas, Taschner and Woldringh [1] suggested that lysine-requiring strains were actually more efficient at incorporation of DAP. They suggested that the large pool of DAP in the cell due to the requirement that DAP auxotrophs have for growth in DAP was the cause of the higher efficiency of DAP prototrophs.

The experiments presented in this paper indicate that *S. typhimurium* is much more efficient at incorporating DAP than any *E. coli* strain we have tested. No starvation of the *Salmonella* strains is required for an almost quantitative incorporation of DAP into the peptidoglycan. The slight curvatures noted in the DAP incorporation curves are possibly due to either the existence of a very small pool of DAP in the cell or the exponential growth of the cells. We did not correct for this exponential growth [1]. Estimates of the number of molecules incorporated into the cell compare favorably with the number of DAP moieties estimated to be present in the cell wall. The incorporation of DAP into *Salmonella* appears to be due to the cystine-cysteine uptake system as was reported previously for *E. coli*. We suggest that *Salmonella* is an excellent organism to use for studies on the biosynthesis of bacterial peptidoglycan.

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