# GENETIC AND BIOCHEMICAL STUDIES ON CELL WALL PEPTIDOGLYCAN SYNTHESIS IN ESCHERICHIA COLI K-12

Hiroshi Matsuzawa*, Michio Matsuhashi,

The Institute of Applied Microbiology,

University of Tokyo, Tokyo, Japan

Atsuhiro Oka and Yoshinobu Sugino

Department of Biology, Faculty of Science,

University of Osaka, Osaka, Japan

Received July 11, 1969

SUMMARY - Temperature sensitive mutants of E. coli JE1011 (derived from E. coli K-12), not forming cell walls at 42° were isolated and the genes of four of those mutants were mapped. Two cell wall-ts genes were located between ara and lac and the other two close to argH on the chromosomes.

The basal structure of the bacterial cell walls consists of rigid peptidoglycans formed by strands of N-acetylmuramyl-N-acetylglucosaminyl-glycan cross-linked by peptides, the length and amino acid sequences of which differ in different strains of bacteria. In E. coli these peptides are octapeptide formed from two equal tetrapeptide strands, $N^{\alpha}$-L-Ala-D-Glu-m-Dpm-D-Ala linked together by a D-alanyl m-Dpm linkage. The amino terminal of the L-Ala residue in the tetrapeptide strand is linked to the carboxy terminal of muramyl residues in glycan strands. Although the peptidoglycan of gram-negative bacteria only represents about 10 - 20 % of the cell walls, it contributes most of the rigidity to

---

* Present address: Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo.

The following abbreviations and symbols are used: m-Dpm, meso-diaminopimelic acid; UDP-MurNAc, uridine diphosphate N-acetylmuramic acid; ts, temperature sensitive; thr, threonine; leu, leucine; trp, tryptophan; his, histidine; ilv, isoleucine-valine; met, methionine; arg, arginine; thy, thymine; thi, thiamine; ara, arabinose; lac, lactose; gal, galactose; xyl, xylose; mtl, mannitol; str, streptomycin.

the cells (1) and may, therefore, play an important role in the process of cell growth and division.

Genetic analysis of cell wall peptidoglycan synthesis was originally initiated by Sugino and Okazaki who isolated several mutants of E. coli K-12 which did not form cell walls at high temperature ($42^{o}$) (cell wall ts-mutants). They identified the product accumulated in the cells of one of the mutants at high temperature ($42^{o}$) as UDP-N-acetylglucosamine-enol pyruvate ether, which is an intermediate in the biosynthesis of UDP-N-acetylmuramic acid (Y. Sugino and R. Okazaki, manuscript in preparation).

In the present communication, further studies on the cell wall-ts mutants of E. coli K-12 are reported and some of the ts-loci on the chromosomes are mapped.

## Isolation of Cell Wall Ts-Mutant Strains

E. coli JE1011, ($F^{-}$ $thr^{-}$ $leu^{-}$ $trp^{-}$ $his^{-}$ $thy^{-}$ $thi^{-}$ $ara^{-}$ $lac^{-}$ $gal^{-}$ $xyl^{-}$ $mtl^{-}$ $str^{r}$) derived from E. coli K-12, was treated with N-methyl-N'-nitro-N-nitroso-guanidine and the mutants which grew on the nutrient-agar plate at $30^{o}$ but which did not grow on the same plate at $42^{o}$ were isolated using a replica technique. These mutants were tested in a liquid culture of nutrient-broth and nine strains which lysed on shifting the temperature from $30^{o}$ to $42^{o}$ were selected from over 600 independent ts-mutants. The growth of all but one of them on the nutrient agar plate at $42^{o}$ was restored by adding 20 % sucrose to the plate (Table 1). However, the temperature sensitivities of cell wall peptidoglycan synthesis of all nine strains were tested.

## Survey of Thermosensitive Sites of Cell Wall Peptidoglycan Synthesis

E. coli cells incorporate $^{14}$C-L-alanine exclusively into cell wall peptidoglycan from solution consisting of 0.04 M Tris HCl, pH 7.5, 0.008 M $MgCl_2$, 0.01 M glucose and 0.2 mg per ml chloramphenicol. Incubation was performed in a final volume of 5.0 ml with 2 % (wet weight of cells per volume) of freshly

TABLE I

Dependence on Sucrose and Inhibition of Peptidoglycan Synthesis on Incubation at 42°

| Strain | Growth at 42° | | Incorporation of $^{14}$C-L-Ala into peptidoglycan cpm per mg wet weight of cells | |
|---|---|---|---|---|
| | On nutrient agar plate | +20% sucrose | 30° | 42° |
| JE 1011 (parent) | + | + | 190 | 250 |
| ST 5 | - | + | 520 | 10 |
| 35 | - | + | 430 | 140 |
| 59 | - | + | 560 | 180 |
| 222 | - | + | 250 | 50 |
| 353 | - | + | 360 | 30 |
| 408 | - | + | 460 | 90 |
| 454 | - | + | 360 | 30 |
| 486 | - | -* | 290 | 10 |
| 640 | - | + | 200 | 80 |

* 20% sucrose also inhibited strain ST486 at 30°.

prepared cells of parent and mutant strains in the middle of the logarithmic phase of growth. After 60 min incubation either at 30° or at 42°, cells were collected by centrifugation, washed once with cold water, suspended in 3 ml of water and boiled for 3 min. Cells and insoluble materials were collected by centrifugation, washed once with 0.05 M Tris HCl, pH 7.5, containing 0.01 M $MgCl_2$, suspended in 2 ml of the same buffer and digested with trypsin. Most of the radioactivity was resistant to trypsin but was digested with lysozyme to form low molecular substances. On incubation for 60 min at 42°, the cells of the parent strain incorporated $^{14}$C-L-alanine into peptidoglycan as well as at 30°,

or even slightly better. On the contrary, in all nine ts-mutants, incorporation of $^{14}$C-L-alanine was significantly reduced (Table I).

The inhibition of incorporation of $^{14}$C-L-alanine into cell wall peptidoglycan in some mutant strains resulted in accumulation of $^{14}$C-UDP-nucleotides in which radioactivity was located in L-alanine or in L- and D-alanine. The radioactive nucleotides in the extracts obtained by boiling the mutant cells were purified by adsorption on and elution from activated charcoal and identified by paper chromatographies with the solvent systems isobutyric acid- 1 M ammonia (1 : 0.6 v/v) and ethanol- 1 M ammonium acetate (1.5 : 0.6 v/v). Appropriate reference compounds were obtained by treatment of cells with antibiotic or deprivation of amino acid (2).

Table II shows the $^{14}$C-compounds accumulated at 42°. No radioactive compounds accumulated in ST 5. However, Sugino and Okazaki found that this strain accumulates UDP-N-acetylglucosamine-enol pyruvate ether. Three other strains,

TABLE II

Accumulation of UDP-MurNAc-peptide-$^{14}$C at 42°

| Strain | $^{14}$C-Compounds accumulated |
|---|---|
| JE1011 (parent) | none |
| ST 5 | none |
| 35 | UDP-MurNAc-L-Ala-D-Glu-m-Dpm-D-Ala-D-Ala |
| 59 | none |
| 222 | unknown |
| 353 | none |
| 408 | UDP-MurNAc-L-Ala-D-Glu |
| 454 | UDP-MurNAc-L-Ala-D-Glu |
| 486 | none |
| 640 | UDP-MurNAc-L-Ala-D-Glu-m-Dpm |

ST59, ST353, and ST486 also did not accumulate $^{14}$C-compounds. Possibly these strains also accumulated UDP-N-acetylmuramic acid or its precursors. In two strains, ST408 and ST454, UDP-MurNAc-dipeptide accumulated. The m-Dpm synthesis of these strains was thermosensitive and addition of 100 μg per ml of Dpm ( a mixture of DD-, LL- and meso-compounds) completely restored the growth at 42°. Similarly D-alanine formation (probably alanine racemase-ts) is thermosensitive in mutant ST640, which accumulated UDP-MurNAc-tripeptide, and its growth at 42° was restored by addition of 1 mg per ml of D-alanine. Mutant ST35 was the only strain which accumulated UDP-MurNAc-pentapeptide, suggesting that some step(s) of peptidoglycan synthesis after this intermediate may be thermosensitive in this mutant. However, a particulate enzyme preparation actively synthesized peptidoglycan from UDP-MurNAc-pentapeptide-$^{14}$C and UDP-N-acetylglucosamine (3) at 42°.

Gene Mapping of ST5, ST35, ST222, and ST640

For gene mapping two Dpm$^{ts}$ strains of the nine mutant strains were omitted as their ts loci seemed to be related not to the cell wall loci, but to the

TABLE III

Transduction of ts genes with Plkc*, recipient AB1450 ilv-16$^-$ met-1$^-$ arg-1$^-$**

| Selection | Donor ST 5 | | ST35 | |
|---|---|---|---|---|
| | tr | ts | tr | ts |
| ilv$^+$ | 100 | 0 | 100 | 0 |
| met$^+$ | 51 | 14 | 74 | 4 |
| arg$^+$ | 15 | 35 | 22 | 28 |

* Plkc was grown on ST strains, and selections were made for ilv$^+$, met$^+$ or arg$^+$ transductants at 30°. Transductants were scored for temperature sensitivity by replica plating and incubating at 42°.
** According to the nomenclature recently proposed (4) the genetic symbols should be written as ilvD$^-$ metB$^-$ argH$^-$.

usual lysine (Dpm) loci. The genes of four strains, ST5, ST35, ST222, and ST640 were mapped. The ts locus of both ST5 and ST35 was located by mating with AB1206 F'-14, an F' carring a chromosome fragment located from arg to ilv. Then, by transduction by P1kc the loci of both strains became closely linked with argH (Table III).

The ts-loci of ST222 and ST640 were mapped by mating them each with two Hfr strains, Hfr C W2252 ($met^-$ $str^s$) and Hfr H JE1031 ($met^-$ $thi^-$ $str^s$). By timing and marker linkage experiments both the ts-loci were shown to be between ara and lac. Table IV shows examples of linkage marker frequencies of recombinants obtained by mating Hfr H x ST222 and ST640 as a function of the time of crosses. Furthermore, by switching experiments between ara and ts and between ts and lac by mating with Hfr C and Hfr H in xylose and tryptophan selections,

TABLE IV

Linkage Marker Frequencies from the crosses K-12 Hfr $ara^+$ $lac^+$ $str^s$ tr (JE1031) x $F^-$'s $ara^-$ $lac^-$ $str^r$ ts (ST222 and ST640) as a Function of Time After Mixing

| Primary Selection | | tr $str^r$ | | | $ara^+$ $str^r$ | | |
|---|---|---|---|---|---|---|---|
| $F^-$ | Time (minutes) | Number tested | Percent of unselected markers positive ara+ | lac+ | Number tested | Percent of unselected markers positive tr | lac+ |
| ST222 | 10 | 0 | - | - | 1 | 0 | 0 |
| | 20 and 30 | 5 | 100 | 0 | 7 | 43 | 0 |
| | 40 and 50 | 15 | 80 | 0 | 26 | 77 | 0 |
| | 60 | 33 | 85 | 0 | 14 | 72 | 7 |
| ST640 | 10 | 0 | - | - | 2 | 0 | 0 |
| | 20 and 30 | 6 | 83 | 0 | 11 | 73 | 0 |
| | 40 and 50 | 25 | 92 | 0 | 28 | 68 | 4 |
| | 60 | 44 | 82 | 5 | 32 | 72 | 9 |

TABLE V

Frequency of Switches in $xyl^+$ and $trp^+$ Selections from Hfr's $trp^+$ $ara^+$ $lac^+$ $xyl^+$ tr $str^s$ (HfrC W2252 and HfrH JE1031) x $F^-$'s $trp^-$ $ara^-$ $lac^-$ $xyl^-$ ts $str^r$ (ST222 and ST640)

| Crosses | ST222 x Hfr C | ST222 x Hfr H | ST640 x Hfr C | ST640 x Hfr H |
|---|---|---|---|---|
| Primary Selection | $xyl^+str^r$ | $trp^+str^r$ | $xyl^+str^r$ | $trp^+str^r$ |
| Number tested | 100 | 202 | 166 | 73 |
| A $ara^+lac^-$ | 25 | 14 | 64 | 19 |
| A $ara^-lac^+$ | 4 ) 29 | 23 ) 37 | 17 ) 81 | 5 ) 24 |
| B $ara^+ts$ | 7 | 1 | 4 | 3 |
| B $ara^-tr$ | 1 ) 8 | 6 ) 7 | 16 ) 20 | 2 ) 5 |
| C tr $lac^-$ | 21 | 15 | 69 | 16 |
| C ts $lac^+$ | 6 ) 27 | 18 ) 33 | 10 ) 79 | 3 ) 19 |
| $\frac{B}{B+C}$ | 0.23 | 0.18 | 0.20 | 0.21 |

the distance from ara to ts was roughly calculated as 0.21 of the distance between ara and lac in both ts-mutant strains (Table V).

In the present study we selected from many ts-mutant strains only those which were lysed in a liquid culture of nutrient broth on shifting the temperature from 30° to 42° and studied their cell wall synthesis. At 42°, in liquid-cultures containing 20 % sucrose these mutants did not always form spheroplasts, which were readily obtained by treatment of the parent cells with penicillin, D-cycloserine or lysozyme in the presence of the same concentration of sucrose. In medium containing sucrose most mutant cells grew rather slowly and irregular shaped cells were obtained on incubation for a few hours at 42°. The effect of sucrose is a little complex. Cell wall ts-mutants may also include mutants which just stop growing at 42° but do not lyse. A more extensive survey of these mutants is planned and results will be reported subsequently.

REFERENCES

1. M. R. J. Salton, The Bacterial Cell Wall, Elsevier Publishing Co., Amsterdam, London, and New York, 1964, p. 100.
2. E. Ito, S. G. Nathenson, D. N. Dietzler, J. S. Anderson and J. L. Strominger, Methods in Enzymology, Vol. 8 (Editors: E. F. Neufeld and V. Ginsburg), Academic Press, New York and London, 1966, p. 324.
3. K. Izaki, M. Matsuhashi and J. L. Strominger, J. Biol. Chem. 243, 3180 (1968)
4. A. L. Taylor and C. D. Trotter., Bacteriol. Reviews 31, 332 (1967).