TURNOVER OF THE CELL WALL OF BACILLUS SUBTILIS W-23 DURING LOGARITHMIC GROWTH*

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Summary: Extensive turnover of the mucopeptide and teichoic acid of B. subtilis W-23 occurs during logarithmic growth. The half life of the mucopeptide is of the order of 0.6 generations, while that of the teichoic acid is of the order of 2 generations, and is independent of the generation time over a four fold change in growth rate.

It is generally assumed that the bacterial cell wall is a stable structure. Although a large number of bacterial cells, including B. subtilis, contain autolytic enzymes (1, 2, 3), these are assumed to cause only a limited breakdown of the cell wall, to allow insertion of new material for growth, and cell separation after septum formation. In the course of examining the effects of phosphate limitation on teichoic acid synthesis in B. subtilis, a phenomenon first described by Ellwood and Tempest (4), we noted extensive turnover of the cell wall mucopeptide. In this communication we present evidence that in B. subtilis W-23, there is extensive turnover of the cell wall during logarithmic growth.

MATERIALS AND METHODS: All radioactive isotopes were obtained from New England Nuclear. B. subtilis W-23 was grown in minimal medium (5) containing 5mM P_i. Carbon source was present at 0.6 gm/l. In cultures containing trypticase and yeast extract, these were present at a level of 10 gm/l. A typical experiment to measure mucopeptide turnover was carried out as follows. To 60 ml of a B. subtilis W-23 culture $OD_{650m\mu}$ = 0.1 in minimal medium with glucose as carbon source, were

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added 0.5 mC of D,L-glutamic acid-3-^{3}H (2.6 C/mM). After the culture reached an $OD_{650m\mu}$ of 0.200, 200 μmoles of non-radioactive D,L-glutamic acid was added. Ten ml samples were removed when the culture had reached an $OD_{650m\mu}$ = 0.26, 0.5, 0.7 and 1.28 for the isolation of cell walls.

The cells from 10 ml of radioactive culture were collected by centrifugation, at 10,000 x g for 10 min, the cells suspended in 5 ml of 5% trichloroacetic acid (TCA) at 0° and again collected by centrifugation. To the cells were added 5 ml of carrier cells (ca. 360 mg dry weight)(see below), and the cells were broken in a Nossal shaker at 0° with glass beads, the supernatant removed and the beads washed with 10 ml of 1% sodium dodecyl sulfate (SDS). Unbroken cells and glass beads were removed from the combined supernatant fluid by centrifugation at 2,000 x g for 10 min, and the cell walls collected by centrifugation at 15,000 x g for 20 min. The walls were suspended in water and heated at 100° for 5 min. They were collected by centrifugation, suspended in 1% SDS and incubated at 37° for 3 hrs. The walls were washed 4 times with distilled water and incubated in 4 ml of 0.10M Tris-Cl (pH-8.0) containing 1 mg/ml DNase and RNase at 37° for 6 hrs. The walls were then collected by centrifugation and digested with 1 mg/ml of trypsin for 12 hrs. at 37°. The trypsin treatment was repeated and the walls finally washed 4 times with distilled water. Purity of the cell walls was confirmed by the fact that in experiments where ^{14}C-leucine or ^{14}C-proline were used, in addition to ^{3}H-glutamic acid, the final cell walls were essentially free of ^{14}C. Acid hydrolysis of the ^{3}H walls showed that the major portion of the radioactivity chromatographed with glutamic acid in 2 solvents; a minor fraction chromatographed with diaminopimelic acid. The recovery of cell walls was 10 - 15 mg dry weight per sample.

Carrier cells were prepared by harvesting 3.6 liters of *B. subtilis* W-23 in minimal medium with glucose, washing successively with 0.1 M Tris-Cl

(pH-8.0), 0.01 M $MgCl_2$, then with 50 ml of 5% TCA, and H_2O and finally suspending the cells in 25 ml of 0.1 M Tris-Cl (pH-8.0). In some experiments carrier cells which had not been treated with TCA (to inactivate possible autolytic enzymes) were used with identical results.

For ^{32}P experiments, the labelling was similar using 5 mC of $^{32}P_i$ per culture, the phosphate was diluted 30 fold with P_i at the end of the labelling period. The data have been corrected for a small incorporation of $^{32}P_i$ during the chase period.

^{3}H and ^{14}C were counted in Bray's solution in a Packard scintillation

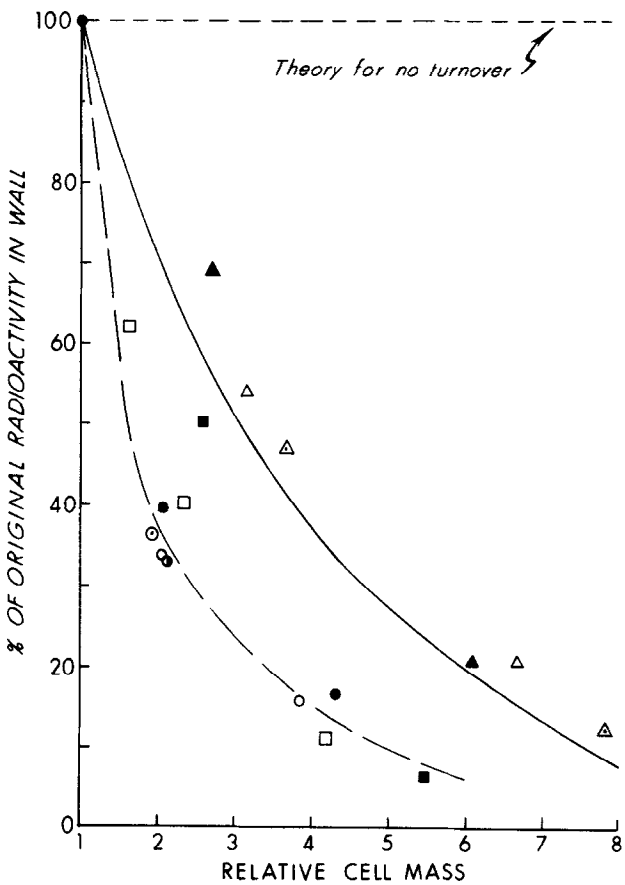

Fig. 1: Turnover of cell wall components of B. subtilis W-23. Experiments were carried out as described under methods. Different symbols indicate different experiments. In $^{32}P_i$ experiments, the following media were used: ■ △ minimal glucose, ▲ minimal maltose, ▲ minimal trypticase. All other experiments are ^{3}H-glutamic acid labelling in minimal glucose medium. The cell mass from the first sample is always taken as unity.

counter with absolute activity analyzer. ^{32}P was counted in gas flow counter. Specific activity of the isolated walls is expressed as cpm/mg dry weight or as dpm/μmole amino sugar. Amino sugar was determined after hydrolysis with 6N HCl at 100° for 4 hrs. (6)

RESULTS AND DISCUSSION: In Fig. 1 are summarized a number of experiments which show that when B. subtilis W-23 is labelled with either ^{3}H-glutamic acid or $^{32}P_i$, followed by growth in non-radioactive medium, there is a net loss of radioactivity from the cell wall. It should be noted that in these experiments cell walls were isolated from a fixed volume of radioactive culture with large excess of carrier cells, and therefore in the absence of turnover the specific activity of the isolated cell walls would remain constant. This is clearly not observed and therefore these data suggest extensive turnover of the cell wall. In addition, it should be noted that the turnover of teichoic acid is the same in cells grown on glucose, maltose or trypticase, and in the case of mucopeptide they are the same whether glucose of maltose (not shown) is used as the carbon source. The generation times of B. subtilis in these cultures is respectively 1 hr, 2 hrs and 0.5 hrs.

There are three possible sources of error in these experiments: 1) the walls are variably contaminated with ^{32}P-labelled lipid or nucleic acid, or ^{3}H protein; 2) the cell walls isolated at various times with carrier cells are not a representative sample; 3) the turnover represents cell death, rather than specific turnover of the wall. The following experiments were carried out to exclude these possibilities.

1) To exclude contamination by other polymers in P^{32} experiments, ribitol diphosphate was isolated from the cell wall (7). The apparent turnover calculated from the whole cell wall, and from the ribitol diphosphate were identical (Table 1).

TABLE I.

TURNOVER OF P^{32} LABELLED TEICHOIC ACID

The experiment was carried out as described under experimental methods. Sample 1 was taken immediately after the addition of carrier P_i to the culture

Exp.	Sample	OD_{650} of Culture	Cell Wall CPM/mg dry weight	%	Ribitol di-P CPM/μmole	%
	1	0.131	3,260	100	5,530	100
A	2	0.484	1,700	52	2,520	47
	3	1.026	700	21	640	12
	1	0.170	11,500	100	9,850	100
B	2	0.530	5,700	49.5	5,375	54
	3	1.110	2,700	23.5	2,190	22

To show that the cell walls were not contaminated with protein, cultures were simultaneously labelled with ^3H-glutamic acid, and with a "non-wall" amino acid either ^{14}C-L-leucine or ^{14}C-L-proline. These experiments indicated that the contamination by protein was negligible. Thus, in a culture in which total TCA precipitable material per ml of culture contained 2.75×10^6dpm-^3H (from glutamic acid) and 1.47×10^6dpm^{14}C (from proline) i.e., $^3H/^{14}C$ - 1.87. The isolated walls contained per μmole of amino sugar 1.91×10^5dpm-^3H and 3×10^3dpm^{14}C i.e., $^3H/^{14}C$ = 50. Similarly, in a leucine ^{14}C experiment the ratio $^3H/^{14}C$ for whole cells was 4 and for isolated cell walls, 190.

2) To determine whether the cell walls were a representative sample, the cells were grown on ^{14}C glucose, and the walls labelled with ^3H-glutamic acid. Under these conditions glucose uniformly labels the cell wall, and can be used to measure the recovery of cell wall material. In these experiments a decrease in the $^3H/^{14}C$ ratio greater than predicted from cell growth, is an indication of cell wall turnover. Such experiments are shown in Table II. As can be seen the recovery ^{14}C parallels the increase in labelled cell mass added to the carrier cells.

T A B L E II.

TURNOVER OF CELL WALL MUCOPEPTIDE

The experiment was carried out as described in methods but the culture contained at all times ^{14}C-glucose. In this experiment the ^{14}C represents the mass of cell walls from the radioactive culture, and the amino sugar is derived from carrier walls.

Exp.	$OD_{650m\mu}$ culture	Cell Wall ^{3}H dpm/μmole of amino sugar	Cell Wall ^{14}C dpm/μmole of amino sugar	^{3}H/^{14}C Theory no turnover	^{3}H/^{14}C found
	0.311	228,000	4,200	54.5	
A	0.598	162,000	7,050	32.5	23.0
	1.280	36,425	18,235	12.5	2.0
	0.653	21,200	4,970	4.25	
B	1.280	5,850	11,400	2.12	0.51

3) The fact that the cell wall is specifically turning over is shown by the fact that the total acid precipitable radioactivity remained stable in the culture after dilution of the radioactive amino acid with unlabelled carrier (Table III). The data in Table III also shows that if cell walls are labelled for at least 5 generations with ^{14}C-glutamic acid and approximately 1 generation with ^{3}H-glutamic acid, that during the chase period both radioactivities in the wall decay together, showing that the apparent turnover is not due to a small segment of the wall, which is preferentially labelled during a short labelling period.

It is clear from the preceding observations that the cell wall of B. subtilis W-23 turns over extensively during logarithmic growth, and we have made similar observations with B. subtilis ATCC 6051. It will be extremely important to establish whether similar phenomenon can be demonstrated in other bacterial species, and to try to isolate mutants with impaired turnover to determine whether this turnover is obligatory for cell growth.

It appears at first hand that these data are contradictory to the

TABLE III.

TURNOVER OF CELL WALL MUCOPEPTIDE LABELLED FOR DIFFERENT TIME PERIODS

Cells were grown as described under methods. The culture medium contained 2 μmoles (0.125mC) of ^{14}C-glutamic acid, cells were innoculated at OD_{650} = 0.01 and allowed to grow to OD_{650}= 0.11 when ^{3}H-D,L-glutamic acid was added. After culture reached OD_{650} = 0.240, cold glutamic acid was added and samples taken at the cell densities indicated for cell wall isolation. 0.50 ml of culture was precipitated with 0.50 ml of 10% TCA and 0.10 ml of this filtered on millipore filters, washed with 5% TCA and counted in a gas flow counter.

OD_{650} of culture	Total TCA Precipitable Counts ^{14}C CPM/ml x 10^{-5}	Cell Wall dpm/μmole of amino sugar x 10^{-3} ^{3}H	^{14}C	^{3}H/^{14}C
0.300	3.4	111	6.12	18.1
0.501	3.1	72.5	3.3	21.7
0.706	3.3	44.4	2.2	20.2

observations with fluorescent antibody which show that there is a specific growth zone in most Gram positive cells (8) . It would appear however that in these experiments, the area of septum formation, which is exclusively new wall, becomes extremely prominent, compared to the relatively slow turnover of teichoic acids observed in our experiments. It should be pointed out that no fluorescent antibody studies have been done with B. subtilis W-23, or with antibodies to the mucopeptide part of the wall.

There are other experiments in the literature which suggest that the cell wall synthesis can take place over the whole cell surface; these include the observation that teichoic acid can be replaced by teichuronic acid in approximately 1/10 of a generation (4, 11); the fact that during inhibition of protein synthesis uniform cell wall thickening is observed (2, 9); and that during reversion of B. subtilis protoplast to bacillary form cell wall appears to be deposited simultaneously over the whole cell surface (10).

The significance of the differences in turnover of the teichoic acid

and mucopeptide portions of the wall remains to be explored. It would appear that since under conditions of phosphate limitation (4, 11) teichoic acid loss is more rapid than mucopeptide turnover, the degradation of these two polymers is under independent control.

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