JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 7–11
0021-9193/93/010007-05$02.00/0

Vol. 175, No. 1

Turnover and Recycling of the Murein Sacculus in Oligopeptide Permease-Negative Strains of *Escherichia coli*: Indirect Evidence for an Alternative Permease System and for a Monolayered Sacculus

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Received 29 June 1992/Accepted 25 October 1992

Turnover of murein in oligopeptide permease-negative *Escherichia coli* cells appeared to be minimal or nonexistent. In one strain in which it was possible to measure turnover during the first generation of chase, it was found that the rate of turnover was constant throughout a chase of three generations. This result suggests that an "inside-to-outside" mode of growth of the sacculus does not occur in *E. coli*. Turnover, though minimal, was significantly higher from cells labeled uniformly than from cells labeled only in the lateral wall, suggesting that a significant portion of the observed turnover is related to cell separation. Actually, turnover only appeared to be minimal in *opp* mutant strains. Tripeptides were being released by turnover at a rate of about 50% per generation and then were efficiently recycled. This suggests that in addition to *opp*, a low-affinity uptake system for tripeptide derived from the sacculus may exist.

For many years, it was thought that the *Escherichia coli* cell wall consisted of a single layer of murein and that during growth there was little or no release of wall material. Both of these assumptions have recently been questioned. Since 1984, several lines of evidence have suggested that the murein sacculus of exponentially growing *E. coli* cells might be multilayered, with a thickness of up to three layers. Electron microscopic observations based on new embedding techniques and freeze substitution revealed a periplasmic space filled with an electron-dense layer of uniform thickness which was several times thicker than the peptidoglycan layer observed in previous electron micrographs (9). The authors proposed that the peptidoglycan formed a gel consisting of a cross-linked outer shell with additional less-cross-linked material attached to the inner surface. Consistent with this view was the observation that staining of thin sections of *E. coli* by the Rambourg technique (phosphotungstic acid in 10% chromic acid) revealed a similar 15-nm-thick layer in the periplasm apparently consisting primarily of peptidoglycan (13). Furthermore, beta-lactam-induced autolysis caused degradation and thinning limited to the inner side of the layer, which could indicate an uneven distribution of the polymer or of its cross-links. Leduc et al. have demonstrated that the main material stained by the Rambourg technique was indeed peptidoglycan and interpreted their measurements to indicate that in exponentially growing cells the sacculi may be two or three layers thick and in the stationary phase the sacculi may be up to five layers thick (12). In contrast, a more recent article by Labischinski et al. concludes, on the basis of neutron small-angle scattering studies, that the wall has "about 75–80% of its surface monolayered and 20–25% triple-layered" (11). Another recent article, by Wientjes and coworkers, argues for a monolayered wall on the basis of the diaminopimelic acid (DAP) content of the wall (19). Thus, recent evidence both for and against a multilayered sacculus in *E. coli* exists, although the most convincing data to me, the physical measurements of Labischinski et al., demonstrate the presence of a largely monolayered wall with some triple-layered areas (11).

Holtje and Glauner have reported the presence of trimers in muramidase digests of *E. coli* sacculi and have suggested that this is another indication that multiple layers may exist, since a trimer cannot be present in a monolayer. These authors went on to suggest that a multilayered wall, should one exist in *E. coli*, ought to be formed by the "inside-to-outside" mechanism that is known to occur in gram-positive bacilli (10).

In the case of *Bacillus subtilis*, after the murein is labeled for a short pulse and then chased, there is a lag of about one generation before any radioactivity is released into the medium. After the first generation, about 50% of the radioactivity of the cylindrical wall is released per generation (15). This is interpreted to indicate that peptidoglycan is initially laid down next to the cytoplasmic membrane and eventually reaches the external surface, where it is degraded by autolytic enzymes. Since the principal enzymes used to polymerize and cross-link murein are bound to the cytoplasmic membrane, it is self-evident that new material must be formed at the inner surface of a multilayered wall. Degradation should not occur until additional layers are added underneath. Without an attempt to explain how inside-to-outside murein synthesis and cell wall turnover are coordinated with wall elongation, synthesis of multilayered walls may be characterized by a long delay before newly synthesized murein is released from the wall, followed by an extended period during which as much as 50% of the remaining pulse-labeled wall murein is released per generation.

Early experiments with *E. coli* cells in which the murein was specifically labeled with DAP had shown that little or no release of murein occurred during growth. Greenway and Perkins (7) more recently confirmed that DAP was not lost from the cell during growth but unexpectedly found that *E. coli* murein labeled with glucosamine was lost from the cell at a rate of 8% or more per generation. This result became

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or construction	Reference
CH1467	*lysA dap opp468*	Spontaneous mutant of W7	6
CH483	*thi lac pro galE* Δ(*trp-tonB-oppABCDF*)*467*		6
CBK140	W3110 *thy lysA*::Tn*5*		17
TP41	*thi lac pro galE* Δ(*trp-tonB-oppABCDF*)*467 lysA*::Tn*5*	P1(CBK140) × CH483	
JFL100	F⁻ *ilv his thyA deo ara*(Am) *lac-125*(Am) *galU42*(Am) *tyrT*[*supF*(Ts)A81] *ftsZ84*(Ts)		14
NK6732	F⁻ λ⁻ *leu-82*::Tn*10* IN(*rrnD-rrnE*)	CGSC 6488	
DRC11	F⁻ *ilv his thyA deo ara*(Am) *lac-125*(Am) *galU42*(Am) *tyrT*[*supF*(Ts)A81] *ftsZ84*(Ts) *leu-82*::Tn*10*	P1(NK6732) × JFL100	
TP42	*thi lac pro galE* Δ(*trp-tonB-oppABCDF*)*467 lysA*::Tn*5 leu-82*::Tn*10 ftsZ84*(Ts)	P1(DRC11) × TP41	
MC4100 *lysA*	F⁻ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 ptsF25 deoC opp lysA*		18
TP50	F⁻ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 ptsF25 deoC lysA leu-82*::Tn*10 ftsZ84*(Ts) *opp*	P1(DRC11) × MC4100 *lysA*	
MC1000	F⁻ *araD139* Δ(*araABC-leu*)*7679 galU galK* Δ*lacX74 rpsL thi*		3
DRC12	F⁻ *araD139 galU galK* Δ*lacX74 rpsL thi leu-82*::Tn*10 ftsZ84*(Ts)	P1(DRC11) × MC1000	
DRC13	F⁻ *araD139 galU galK* Δ*lacX74 rpsL thi ftsZ84*(Ts)	P1(C600) × DRC12	
TP44	F⁻ *araD139 galU galK* Δ*lacX74 rpsL thi ftsZ84*(Ts) *lysA*::Tn*5*	P1(CBK140) × DRC13	
PY45	F⁻ *araD139 galU galK* Δ*lacX74 rpsL thi ftsZ84*(Ts) *lysA*::Tn*5 opp*	P1(CH483) × TP44	

understandable when Goodell demonstrated that *E. coli* recycled tripeptides containing DAP (5). Thus, wall turnover in *E. coli* presumably occurs by breakdown of murein in the periplasm with some release of disaccharide units but with immediate uptake and reuse of the peptide portion of the murein. Goodell and Higgins subsequently showed that the cell wall peptides could be transported into the cell specifically via the well-known oligopeptide permease (Opp) (6).

In this study, I measured the rate of loss of DAP from various Opp-negative strains as well as the rate of recycling of DAP-containing peptides. It was initially assumed that Opp-negative cells could not recycle the peptides released during degradation of peptidoglycan (6) and hence that any turnover of murein would result in loss of DAP-containing peptides from the cells. However, as shown below, efficient recycling of DAP-containing peptides released by turnover was found to occur, and this recycling obscured the actual high rate of turnover.

MATERIALS AND METHODS

Bacterial strains. The strains used in these experiments are listed in Table 1. TP50 is derived from MC4100 (*lysA*), a strain used in many studies at the University of Amsterdam (18). This strain, as received, was found to be Opp negative. Thus, the tripeptide Lys-Gly-Gly would not replace Lys for growth, and the culture would grow in the presence of 400 μM triornithine, a concentration which kills normal *E. coli*. In explanation of this phenomenon, Higgins and Gibson (8) reported that *opp* mutants often are selected when strains are maintained in L broth, which sometimes contains toxic peptides. TP41 is a strain in which all five genes required for synthesis of the oligopeptide permease transport system are deleted (6).

ftsZ84(Ts) was introduced into several strains of *E. coli* so that the lateral wall could be specifically labeled. The *ftsZ* gene product appears to be required to initiate cell septation (4). When a strain containing *ftsZ84*(Ts) is shifted to 42°C, existing septa are completed but no new septa are formed, and the cells continue to form lateral wall resulting in long filamentous cells. TP50 was constructed from MC4100 (*lysA opp*) by cotransduction of *leu*::Tn*10* and *ftsZ84*(Ts) with selection for tetracycline resistance followed by screening for temperature-sensitive clones. PY45 was constructed from MC1000 by sequential transduction of *ftsZ84* and *lysA*::Tn*5*. *opp* mutants were selected on minimal glucose plates containing leucine, lysine, and 400 μM triornithine. The absence of *opp* function was confirmed by the inability of 10 μg of the tripeptide Lys-Gly-Gly per ml to provide the lysine needed for growth of PY45. TP42 is a third K-12 strain containing *ftsZ84* and *lysA*::Tn*5* in which the *opp* operon is deleted.

Determination of murein turnover from uniformly labeled cells. Conditions varied depending on the strain used. Cells of the DAP-requiring strain CH1467 (W7 *opp dap lys*) were grown in 1 ml of glucose minimal medium containing 50 μg of lysine, 5 μg of DAP, and 0.4 μCi of [^{14}C]DAP. After growth overnight at 37°C with aeration, 7 ml of glucose minimal medium containing 50 μg of lysine per ml, 2.5 μg of DAP per ml, and 0.2 μCi of [^{14}C]DAP per ml was added, and incubation was continued until the optical density as measured in a Klett-Summerson colorimeter was about 100. The cells were then harvested by centrifugation, washed once with water, and resuspended in 40 ml of minimal medium lacking DAP. After 15 min of incubation, during which most of the free DAP in the cytoplasm was utilized (2), 10 μg of DAP per ml was added to start the chase. Aliquots were taken at 5- or 10-min intervals and placed into 2 volumes of 6% sodium dodecyl sulfate (SDS) or 1 volume of 8% SDS, and the resulting mixtures were heated at 95 to 100°C for 30 min to isolate the murein sacculi. The sacculi were collected on 0.22-μm-pore-size membrane filters and washed thoroughly with 0.1% SDS and then with water. The filters were dried and immersed in toluene-based scintillation fluid, and the radioactivity in the filters was counted to 1% accuracy. For *opp* strains not requiring DAP, such as TP41, cells were uniformly labeled by growth overnight with aeration in 1 ml of L broth containing 0.2% glucose, 50 μg of lysine, and 0.1 μCi of [^{14}C]DAP. The radioactive cells were used to inoculate 5 ml of fresh radioactive medium, and incubation was continued. When the optical density reached 55 Klett units, 25 ml of fresh medium containing a total of 300 μg of DAP was added. During the chase, aliquots taken at intervals were added to an equal volume of 10% trichloroacetic acid.

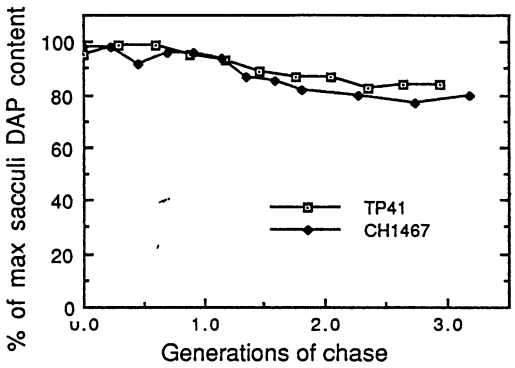

FIG. 1. Loss of murein from uniformly labeled *E. coli* during a chase. All data are averages of four experiments.

Cells were collected on 0.22-μm-pore-size filters prewashed with 0.1% DAP. The filtered cells were washed two times with 5% trichloroacetic acid and three times with water, and the radioactivity was counted after several hours of equilibration with a scintillation fluid suitable for aqueous samples. The chase was for three generations except when noted otherwise.

Determination of murein turnover from the lateral wall of strains carrying the *ftsZ84*(Ts) mutation. To selectively label the lateral wall of these strains, cultures growing exponentially at 28°C in M9 glucose medium containing 100 μg each of methionine, threonine, and needed amino acids per ml were shifted to 42°C. Microscopic examination showed that the percentage of cells that were septating dropped from 20% to about 2% within 20 min. Radioactive DAP was then added (0.1 μCi of [^{14}C]DAP per ml) to label the lateral wall for about 0.2 generation and then chased by diluting the culture fivefold with prewarmed medium to yield a final concentration of 100 μg of DAP per ml. Aliquots taken at intervals thereafter were added to an equal volume of 10% trichloroacetic acid and filtered, and the radioactivity was counted as described above. The chase was for three generations except when noted otherwise.

RESULTS

Rate of loss of peptidoglycan from cells uniformly labeled with DAP. In four experiments with strain CH1467, the average loss of [^{14}C]DAP per generation over three generations was 8%, with a variation of about 25% (Fig. 1). With the *E. coli* K-12 strains, i.e., TP41 and three strains carrying the *ftsZ84*(Ts) mutation, the average loss was 5% per generation (Fig. 1; Table 2). This result contrasts with the reduced rate of loss observed from the same strains labeled only in their lateral walls (see below).

Loss of peptidoglycan from the lateral wall of strains carrying the *ftsZ84*(Ts) mutation. Table 2 summarizes the rate of loss of murein observed with several strains that were either uniformly labeled or labeled in their lateral walls. The lateral wall was specifically labeled either at 42°C as described above or at 28°C in cells treated with 1 μg of furazlocillin, which prevents septation by binding specifically to penicillin-binding protein 3 (1), per ml. Table 2 shows there was no apparent turnover of lateral wall from PY45 labeled by either of these methods. Figure 2 shows the results of a typical experiment in which no loss of DAP from the lateral wall of PY45 was observed. This is the only strain for which no loss from the lateral wall could be demonstrated. I have no explanation for this result other than the possibility that PY45, which is derived from MC1000, is more efficient than other strains at conserving its wall. Table 2 shows that TP42 and TP50 each lost from 2 to 4% of their lateral-wall DAP per generation whether they were labeled at 42°C or labeled in the presence of furazlocillin. Table 2 also shows that all three strains lost label at a greater rate from uniformly labeled cells than from lateral wall-labeled cells. Since *ftsZ84* caused these strains to form elongated cells even at 28°C, MC4100 (*fts*$^+$) was tested, and as shown in Table 2, its turnover rate was comparable to those of the other strains.

TABLE 2. Turnover of murein in various *E. coli* strains uniformly labeled or labeled in the lateral wall only

Genotype and strain	Temp (°C) during labeling	Presence of furazlocillin during labeling	Temp (°C) during chase	Label	Murein loss (%)/ generation[a]
ftsZ84 opp					
TP42	42	–	28	Lateral wall	5
	28	+	28	Lateral wall	2–3.5
	28	–	28	Uniform	8
PY45	42	–	28	Lateral wall	0
	28	+	28	Lateral wall	0
	28	–	28	Uniform	5
TP50	42	–	28	Lateral wall	3.5
	28	+	28	Lateral wall	2
	28	–	28	Uniform	5
ftsZ$^+$ *opp*					
TP41	37	–	37	Uniform	5
CH1467	37	–	37	Uniform	8
MC4100 *lysA*	28	–	28	Uniform	6

[a] All results are averages of two to four experiments.

Loss of murein during the first generation of chase. In *B. subtilis* W23, a gram-positive organism with a multilayered wall, there is no loss of murein during the first generation of chase (15). Hence, it was of interest to measure murein loss during this period. However, with several of the strains, it was not possible to measure the loss of murein during the first generation of chase because [^{14}C]DAP incorporation from the cytoplasmic pool continued throughout this period.

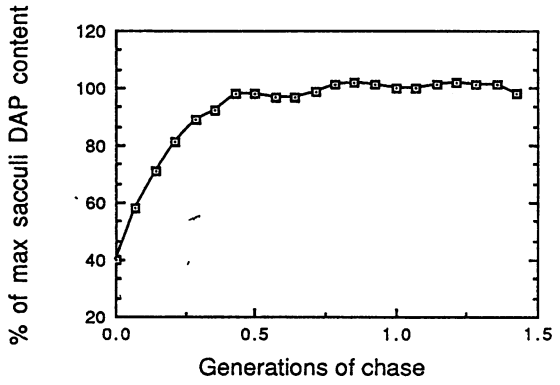

FIG. 2. No loss of murein from the lateral wall of *E. coli* PY45 during 1.5 generations of chase.

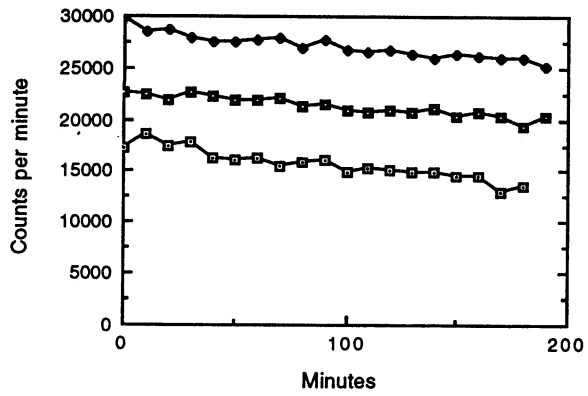

FIG. 3. The rate of loss of DAP from the murein sacculus is constant during the first and subsequent generations of chase. *E. coli* TP50 was uniformly labeled with [^{14}C]DAP and chased as described in Materials and Methods. Data are for three separate experiments. Generation time = 70 min.

On the other hand, TP50, a derivative of MC4100 (*lysA*), had a small pool of DAP when grown in minimal glucose medium containing threonine, methionine, and lysine (18), so that during the chase the radioactivity of the murein increased for only 10 or 15 min and then dropped at a slow, constant rate. Since the generation time was about 70 min during the chase, loss of murein in this strain during the first generation could be measured. In three such experiments, losses of about 5% during the first generation and each subsequent generation were consistently observed (Fig. 3).

Recycling of murein in *opp* strains. A set of experiments using *opp* strains for another purpose led me to suspect that recycling may occur in the *opp* strains. In summary, these experiments showed that the relative amounts of radioactivity in the acceptor and donor halves of muropeptide dimers derived from the lateral walls of sacculi after a long chase did not differ significantly whether the cells were *opp* or *opp*$^+$ (unpublished data). This suggested that recycling may occur in both strains. Hence, I investigated recycling in *opp* cells by measuring the rate of loss of radioactivity from the intracellular pool of the precursor, UDP-MurNAc-L-Ala-D-Glu-[^{14}C]DAP-D-Ala-D-Ala. Since the pool of this precursor is very small relative to the amount of peptidoglycan synthesized in one generation (16), one would expect a rapid depletion of radioactivity from the precursor during a chase. In contrast to this expectation, the radioactivity in the nucleotide precursor pool of an *opp* strain dropped from 55,000 to about 20,000 cpm during the first generation of chase and thereafter dropped slowly during the next two generations of chase. This indicates that the cell wall peptides are released from the wall and recycled at a rate of about 40% per generation or that if only the lateral wall turns over, as seems likely, then the rate of recycling from the lateral wall is more than 50% per generation. The radioactive precursor present after two generations of chase behaved as an authentic precursor as determined by paper chromatography in two solvent systems, by ion-exchange chromatography on Dowex-1, by size fractionation on Fractogel TSK HW-40-S (5), and by its ability to be utilized for murein synthesis by freeze-thawed cells.

DISCUSSION

Several conclusions can be drawn from the experiments described. My observation that recycling proceeds as efficiently in *opp* cells as it was previously shown to proceed in wild-type cells (5) indicates that turnover occurs at a high rate, although direct measurement of turnover seems to indicate that *E. coli* can elongate without detectable loss of peptides from the sacculus (Fig. 2) and that three other strains lost only 2 to 4% of their lateral wall muropeptides per generation.

A critical finding which suggests that inside-to-outside growth of the sacculus may not occur in *E. coli* is that TP50 turns over its murein during the first generation of chase as rapidly as during subsequent generations (Fig. 3). Synthesis of lateral wall via the inside-to-outside pathway as observed in gram-positive bacteria is characterized by no loss of newly incorporated wall material during the first generation of chase followed by rapid loss thereafter.

I was surprised to find that the rate of loss of murein from uniformly labeled cells was consistently more rapid (5 to 8% per generation) than the rate of loss from cells which had been labeled in their side walls only (0 to 4% per generation) (Table 2). Since the poles of cells are believed to be relatively stable, one might have expected that uniformly labeled cells in which about 30% of the murein is in the poles (assuming a uniform thickness for poles and side walls) would have lost less murein per generation. Instead, the uniformly labeled TP42, PY45, and TP50 cells all lost murein at a more rapid rate than when they were labeled solely in the lateral wall, indicating that a significant loss must occur during cell separation or thereafter. As noted above, Labischinski et al. (11) have reported evidence that the *E. coli* sacculus may be 75 to 80% monolayered and 20 to 25% triple layered. If the interpretation of the data from the present study, namely, that the lateral wall is monolayered, is correct, then the data of Labischinski et al. imply that the poles are triple layered. To explain the greater turnover from uniformly labeled cells, one might postulate that polar murein turns over slowly but cannot be recycled. Alternatively, loss during cell separation might occur by removal of one or more layers that may initially fuse the two nascent poles to each other during septation.

If the poles, which occupy about 30% of the surface area, were in fact the only triple-layered regions of the wall, then the poles would contain almost 50% of the total murein of the sacculus. This might explain the well-known fact that mecillinam, an amidinopenicillin, which binds selectively to penicillin-binding protein 2, inhibits murein synthesis by roughly 50%.

In conclusion, the experiments demonstrating turnover of murein during the first generation of chase suggest that the lateral walls of *E. coli* consist essentially of a monolayer. This is in agreement with the results of Labischinski et al. (11) and Wientjes et al. (19). Thus, the following dilemma remains: how can the apparent thickness of the murein layer as measured by electron microscopy (9, 13) be reconciled with our evidence against the inside-to-outside mode of growth that is indicative of a multilayered wall and the other cited evidence in favor of a monolayered wall (11, 19)?

The observation that recycling of wall DAP (presumably as a tripeptide) occurs at a rate approaching 50% per generation in *opp* mutant cells, as was previously observed in normal *E. coli* W7 (5), indicates that another uptake system, perhaps a low-affinity transport system, exists in *E. coli* in addition to the high-affinity *opp* system.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 05090 from the National Institutes of Health.

I thank Sarah Shreter and Polly Yin for excellent technical assistance and J. Lutkenhaus, C. L. Woldringh, and the late E. W. Goodell for strains.

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