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Conservation of Cell Wall Peptidoglycan by Strains of *Streptococcus mutans* and *Streptococcus sanguis*

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Turnover of the cell wall peptidoglycan fraction of six different strains of *Streptococcus mutans* and eight different strains of *Streptococcus sanguis* was examined. Cells were grown in the presence of [^{3}H]lysine and [^{14}C]leucine for at least eight generations and then chased in growth medium lacking the two labels. At intervals during the chase, samples of cultures were removed, and the amounts of the two labeled precursors remaining in the peptidoglycan and protein fractions were quantitated. Similar experiments were done in which the pulse-labeling technique was used. In addition, cells were labeled in the presence of tetracycline or penicillin, chased with growth medium containing no inhibitor, and assayed at intervals during the chase for the amount of [^{3}H]lysine present in peptidoglycan fractions. Studies of cultures of *S. mutans* strains FA-1, OMZ-61, OMZ-176, 6715, GS-5, and Ingbritt and of *S. sanguis* strains 10558, M-5, Wicky, DL-101, DL-1, 71X26, and 71X48 maintained in the exponential phase of growth in a chemically defined medium failed to show evidence of loss of insoluble peptidoglycan via turnover. Similarly, for the strains of *S. mutans*, insoluble peptidoglycan assembled during 2 h of benzylpenicillin or tetracycline treatment was also conserved during recovery from growth inhibition.

Turnover of the cell wall peptidoglycan during growth of several gram-positive bacterial species has been adequately demonstrated (2, 7, 10, 24, 25, 29, 30, 40). Loss of peptidoglycan to the supernatant medium, along with cell wall polymers that are covalently attached to the peptidoglycan, has both theoretical (e.g., physiological) and practical implications. For example, the role of cell wall turnover in surface expansion and cell division is not yet understood (11). Also, both soluble and insoluble peptidoglycan fragments have been shown to have immunological activities (8, 18, 19, 26, 28). In addition, covalently bound cell wall polysaccharides are known to carry serological (antigenic) determinants, which can elicit antibody responses (5, 6), and are important for the identification of species and strains. However, the role that released wall fragments may play in infectious diseases or resistance to infection is not understood.

In contrast to the situation with several other gram-positive species, the cell wall peptidoglycan of *Streptococcus faecalis* ATCC 9790 (*S. faecium*) was shown to be conserved during the growth of the organism under a variety of conditions (2). Indirect evidence suggests that the wall peptidoglycan of *Streptococcus pneumoniae* fails to turn over at a detectable rate (4, 39). Thus, it was of interest to examine peptidoglycan turnover in streptococci which are known to be present in the oral cavity and which have been associated with dental caries. Peptidoglycan turnover in these species is of particular biological importance because of several immunological, serological, and other properties associated with the cell wall polymers of these organisms (35).

MATERIALS AND METHODS

Organisms and growth conditions. All of the strains of *Streptococcus mutans* used were obtained from previously described sources (36). All strains of *Streptococcus sanguis* were obtained from B. Rosan, University of Pennsylvania, Philadelphia. Cultures were stored in the lyophilized state. For routine use, lyophilized cultures were rehydrated and grown to late exponential phase in Todd-Hewitt broth (BBL Microbiology Systems) supplemented with 2% glucose. These initial cultures were used to inoculate a series of slants (Todd-Hewitt agar plus 2% glucose). After growth, these slants were stored at 4°C for no longer than 4 weeks. For inocula, cells from the slants were transferred into a chemically defined medium (FMC) (36) containing 0.01 M sodium bicarbonate and grown aerobically (36) to a turbidity of 500 to 1,000 adjusted optical density (AOD) (37) units (1 AOD = 0.39 μg of cellular dry weight per ml [36]), as measured at 675 nm in a Coleman model 14 spectrophotometer. Purity of cultures was monitored by plating on mitis sali-

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varius agar (BBL) and blood agar plates incubated in candle jars, as well as by determining the ability of cultures of *S. mutans* to ferment mannitol and sorbitol and to clump in the presence of 2% sucrose.

Radiolabeling methods used to determine peptidoglycan turnover. Chases of both continuously (equilibrium-) labeled and 10-min pulse-labeled cultures were used to study turnover of peptidoglycan. In some experiments dual labels of [^{3}H]lysine and [^{14}C]leucine were used in order to monitor and correct for protein contamination of peptidoglycan fractions by their relative contents of [^{14}C]leucine (10) and to monitor for the loss of protein from cells as an indicator of cell lysis or disturbance of the cultures.

Equilibrium-labeled cells were obtained by growing cultures for at least eight generations in balanced exponential growth, as described by Roth et al. (32). Initially, cultures were inoculated to 1 to 4 AOD units into FMC containing 30 μg (1 μCi) of L-[4,5-^{3}H]lysine per ml and 30 μg (0.2 μCi) of L-[*U*-^{14}C]leucine per ml. At a turbidity of about 1,000 AOD units, cells were collected on membrane filters (0.45 μm; Millipore Corp., Bedford, Mass.) and washed three times with chase medium containing excess lysine and leucine (300 μg of each per ml). The cells were resuspended from the filter in the chase medium by vigorous blending in a Vortex mixer. These cultures were incubated at 37°C; at intervals, culture turbidity was monitored, and multiple samples (0.2 or 0.5 ml) were removed and either quickly frozen in a dry ice-ethanol bath (stored at −20°C until assayed) or immediately added to 5 ml of ice-cold 10% trichloroacetic acid.

For pulse-labeling experiments, cells were grown in the presence of L-[*U*-^{14}C]lysine (30 μg/ml; 0.1 μCi/ml) in FMC for at least eight generations. At an AOD of 900, L-[4,5-^{3}H]lysine (25 μCi/ml) was added. After 10 min, pulses were ended by rapidly collecting the cells on membrane filters and washing them with chase medium. The cells were then resuspended from the filters into chase medium, incubated at 37°C, and sampled as indicated above.

The effects of tetracycline and benzylpenicillin (PenG) on peptidoglycan turnover were also investigated. In some experiments, either tetracycline (5 μg/ml; Pfizer Inc.) or PenG (1 μg/ml; 1,584 U/mg; Wyeth) was present in the media during the chase of equilibrium-labeled cultures. In other experiments, exponentially growing cultures were treated with one of the antibiotics (5 μg of tetracycline per ml or 1 μg of PenG per ml) in the presence of L-[4,5-^{3}H]lysine (1 μCi/ml) and L-[*U*-^{14}C]leucine (0.2 μCi/ml). After these additions, the cultures were incubated for 2 h, during which the turbidity was monitored and multiple samples were removed for peptidoglycan and protein determinations.

Determination of incorporation of radiolabeled precursors. Incorporation of labeled precursors into protein and insoluble peptidoglycan was determined in an identical manner for all samples. Frozen samples were first thawed by adding 5 ml of cold 10% trichloroacetic acid; this was followed by immersing them in an ice bath for at least 30 min. For the determination of total incorporation of label, duplicate cold trichloroacetic acid precipitates were collected on glass fiber filter disks (Reeve-Angel 984-H or Whatman GF/C), washed twice with 5 ml of ice-cold 10% trichloroacetic acid, followed by two 5-ml washes of absolute ethanol, and air dried. To estimate the amount of label incorporated into protein, samples were exposed to 95°C for 15 min in a water bath and then cooled in an ice bath for 60 min and processed as described above for cold trichloroacetic acid precipitates.

The method of Boothby et al. (3) failed to yield consistent fractions of total (macromolecular) labeled lysine in peptidoglycan residues, necessitating a systematic examination of several of the factors involved in this procedure. Thus, 50 ml of an exponentially growing culture continuously labeled for eight generations with L-[4,5-^{3}H]lysine (1 μCi/ml) and L-[*U*-^{14}C]-leucine (0.2 μCi/ml) was harvested by filtration onto a 0.65-μm membrane filter and washed twice with ice-cold unlabeled medium. The cells were resuspended to an AOD of 100 and held at 0°C until sampling was completed. Multiple 0.5-ml samples were dispensed into 5 ml of ice-cold 10% trichloroacetic acid. One set of three samples was processed for total label incorporated into cold trichloroacetic acid-precipitable material, whereas the remaining samples were heated at 95°C and, at successive intervals, a set of tubes was removed to 0°C. Hot trichloroacetic acid-treated precipitates were then collected on glass fiber filters, washed with cold 10% trichloroacetic acid, 95% ethanol, and then 0.1 M sodium phosphate (pH 8), and digested at 37°C for various times with 3 ml of a 1-mg/ml solution of one of the following three proteinases: pronase (Calbiochem), trypsin (EC 3.4.4.4; Worthington Biochemicals Corp.), or subtilisin BPN' (EC 3.4.4.16; Nagarse, Enzyme Development Corp., New York, N.Y.). To one series of samples, 0.2 mg (dry weight) of unlabeled carrier cells was added before hot trichloroacetic acid hydrolysis. All other manipulations after digestion were as described previously (3). The method finally adopted is described below.

Determinations of radioactivity in samples. Dried glass fiber filter disks containing radiolabeled precipitates were placed in scintillation vials, and 0.5 ml of 90% NCS (Amersham/Searle) was added to each one. Vials were capped, incubated either at 50°C for 2 h or at 37°C for 12 to 18 h, and then cooled to 10°C; 5 ml of scintillation fluid [4 g of 2,5-diphenyloxazole per liter and 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene] was added, and the vials were counted in a Nuclear Chicago Mark I liquid scintillation counter equipped with an external standard. The data were corrected for overlap of ^{14}C into the ^{3}H channel and converted to disintegrations per minute by using a suitably programmed computer.

RESULTS

Modification of the peptidoglycan determination for use with cultures of *S. mutans* and *S. sanguis*. For *S. mutans* FA-1, increased time of pronase treatment resulted in a progressive loss of [^{3}H]lysine from peptidoglycan residues on filters (Table 1), even in the presence of 0.2 mg of carrier cells. In contrast to previously reported results with *S. faecalis* and *Lactobacillus acidophilus* (3), it appears that the prep-

TABLE 1. *Effect of time of exposure to trichloroacetic acid at 95°C and of treatments with pronase, trypsin, and subtilisin on the percentage of cold trichloroacetic acid-precipitable disintegrations per minute remaining after completion of the assay procedure*[a]

Treatment	Exposure time (h)	% of [^{3}H]lysine after the following times at 95°C in 10% trichloroacetic acid								% of [^{14}C]leucine after the following times at 95°C in 10% trichloroacetic acid							
		5 min		10 min		20 min		30 min		5 min		10 min		20 min		30 min	
		Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells	Without carrier cells	With carrier cells
Pronase (1 mg/ml)[b]	0.5	14.7	20.4	14.9	20.2	11.5	20.6			4.8	5.4	3.8	4.4	3.1	3.4		
	1.0	13.8	20.1	6.3	17.3	7.3	18.9			6.8	5.2	2.6	3.1	2.5	3.1		
	2.0	2.6	11.4	2.8	11.9	6.2	15.9			2.4	2.1	3.0	2.3	3.9	2.5		
	3.0	2.1	7.0	2.8	6.4	1.6	8.9			2.3	1.5	2.5	1.3	1.4	1.4		
Trypsin (1 mg/ml)[c]	1.0			24.0	27.7	21.0	26.2	15.1	25.3			8.5	9.7	5.9	7.5	3.5	5.8
	2.0			20.7	25.2	17.1	25.5	18.3	20.9			5.8	7.3	3.6	5.9	3.4	3.5
	3.0			21.1	26.7	21.4	24.5	13.7	24.8			5.2	7.2	4.1	4.8	2.4	4.6
Subtilisin (1 mg/ml)[d]	0.5			23.1	28.4	23.0	26.0	14.9	22.3			6.6	7.2	5.2	5.7	4.2	5.0
	1.0			27.0	28.0	21.1	25.1	14.3	23.5			6.5	6.6	4.6	5.1	3.6	4.5
	2.0			24.5	25.2	21.7	25.2	11.8	20.1			5.4	5.7	4.4	4.3	2.8	3.6
	3.0			25.0	25.2	20.8	24.9	15.5	20.9			5.5	5.3	3.7	5.0	3.8	3.9

[a] Each value is the average of duplicate or triplicate determinations.
[b] Cold trichloroacetic acid-precipitable radioactivity: ^{3}H, 27,653 dpm; ^{14}C, 3,367 dpm.
[c] Cold trichloroacetic acid-precipitable radioactivity: ^{3}H, 60,172 dpm; ^{14}C, 7,374 dpm.
[d] Cold trichloroacetic acid-precipitable radioactivity: ^{3}H, 34,671 dpm; ^{14}C, 4,173 dpm.

aration of pronase used hydrolyzed one or more bonds in the peptidoglycan of *S. mutans*, causing its partial dissolution. For example, an activity that hydrolyzed the postulated L-alanyl-L-alanine peptide cross-bridge of *S. mutans* (1) could have been present. Either trypsin or subtilisin treatment of hot trichloroacetic acid-treated precipitates left a nearly constant (20 to 25%) residue of [^{3}H]lysine that was approximately the amount expected from the calculated amount of cell wall peptidoglycan present in exponential-phase cells of streptococci (11, 38), especially when carrier cells were added (Table 1).

Treatment with any of the three proteinases effectively reduced the presence of [^{3}H]leucine (protein) in the peptidoglycan fractions to 5% or less of the amount present in cold trichloroacetic acid precipitates (Table 1). This level of contamination can easily be corrected (10) by using dual labels (e.g., [^{3}H]lysine into peptidoglycan and protein and [^{14}C]leucine into only protein).

On the basis of these results, the procedure of Boothby et al. (3) was modified for use with *S. mutans* and *S. sanguis*. Trichloroacetic acid treatment at 95°C was reduced to 15 min, trypsin (1.0 mg/ml) treatment for 90 min at 37°C in 0.1 M sodium phosphate (pH 8.0) was substituted for the pronase treatment, and carrier cells were routinely added.

Turnover of peptidoglycan during exponential growth of *S. mutans* FA-1. When exponential-phase cultures grown in the presence of [^{3}H]lysine and [^{14}C]leucine for eight generations (fully equilibrated label) were rapidly harvested, washed, and resuspended in the chase medium, an immediate resumption of turbidity increase at nearly the same rate was observed (Fig. 1A), with complete recovery of cellular turbidity and radioactivity. The chase was efficient, since an increase in neither ^{14}C (data not shown) nor ^{3}H was observed. Losses of [^{14}C]leucine from the cold or hot trichloroacetic acid-precipitable fractions (protein) (data not shown) or of [^{3}H]lysine from either cold trichloroacetic acid precipitates (protein plus peptidoglycan) or peptidoglycan fractions (Fig. 1B) did not occur, either during 5 h of exponential growth or after an overnight chase. As expected (11, 38), the amount of [^{3}H]lysine in the peptidoglycan fractions was 20 to 27% of that in cold trichloroacetic acid precipitates.

Although evidence for peptidoglycan turnover was not obtained in the experiment described above, it seemed possible that small amounts of newly assembled peptidoglycan could be lost from cells via turnover. Therefore, we examined peptidoglycan turnover in cells grown in the presence of [^{14}C]lysine for eight generations and [^{3}H]lysine for the final 10 min before resuspension in chase medium (Fig. 1C). Such experiments showed that turnover of the [^{3}H]lysine-pulse-labeled peptidoglycan was also below a detectable level.

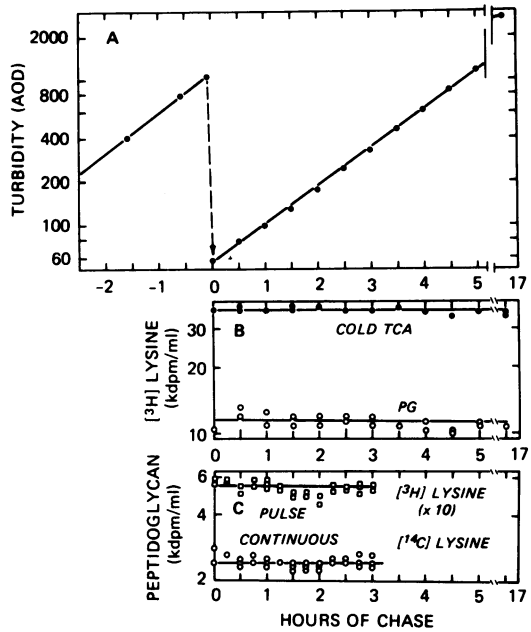

FIG. 1. *Turnover of the cell wall peptidoglycan of S. mutans FA-1 (serotype b) during balanced, exponential growth. Cultures that had grown exponentially for eight generations at doubling times of about 75 min in the presence of [^{3}H]lysine and [^{14}C]leucine (A and B) or (in a separate experiment) in the presence of [^{14}C]lysine for eight generations and [^{3}H]-lysine for the last 10 min before harvesting of cells (C) were harvested, washed, and resuspended in chase medium containing excess unlabeled lysine and leucine as described in the text. (A) Turbidity of the culture is shown for 2 h before transfer to the chase medium (left) and during the chase (right). (B) Levels of [^{3}H]lysine found in cold trichloroacetic acid (TCA) precipitates (●) and in peptidoglycan (PG) fractions (○) at various intervals during the chase. In all experiments the levels of [^{14}C]leucine in the cold and hot trichloroacetic acid precipitates followed the same pattern as that observed for [^{3}H]-lysine in cold trichloroacetic acid and are not shown in this or the other figures. (C) Levels of [^{14}C]lysine (□) and [^{3}H]lysine (○) present in the peptidoglycan fractions of a similar culture in which [^{14}C]lysine was present for eight generations before the chase and [^{3}H]lysine was present for 10 min preceding the chase.*

Effects of unbalanced growth on peptidoglycan turnover in *S. mutans* FA-1. Although no evidence of peptidoglycan turnover was obtained in the experiments described above, it seemed possible that turnover could occur during unbalanced growth conditions, such as during cell wall thickening induced by

inhibition of protein synthesis (22, 23), during inhibition of peptidoglycan synthesis, or during recovery from such inhibitions. In view of electron microscopic observations of the apparent shedding of large pieces of cell wall (34), it seemed particularly appropriate to examine the effects of physiological stresses on peptidoglycan turnover.

Cultures grown in the presence of [^{3}H]lysine and [^{14}C]leucine and then chased in the presence of tetracycline or PenG did not show evidence of loss of the radiolabels during the chases (data not shown). Similarly, cultures exposed to the two labeled precursors only during 2 h of either tetracycline treatment (Fig. 2) or PenG treatment (Fig. 3) also did not show losses of [^{14}C]-leucine (data not shown) or [^{3}H]lysine from either cold trichloroacetic acid precipitates or peptidoglycan fractions. Although Fig. 3 indicates incorporation of lysine into peptidoglycan in the presence of concentrations of PenG that are 25 to 50 times the minimal inhibitory concentration (0.02 to 0.04 μg/ml), after 2 h in PenG the level of [^{3}H]lysine in the peptidoglycan fractions was only 5% of the [^{3}H]lysine level in cold trichloroacetic acid precipitates. In view of the reduced synthesis of protein in the presence of PenG by this organism (22; Mychajlonka et al., Antimicrob. Agents Chemother., in press) and the non-equilibrated labeling conditions used, this level of [^{3}H]lysine represents an extremely low level of synthesis of peptidoglycan. However, even this extremely low level of apparent peptidoglycan was conserved during the chase.

In all of the experiments described above, samples of entire cultures were treated with trichloroacetic acid. Thus, the loss of only trichloroacetic acid-soluble peptidoglycan fragments that did not coprecipitate with the cells would be detected. For this reason, cultures of *S. mutans* FA-1 were grown for eight generations in the presence of [^{3}H]lysine and [^{14}C]leucine and chased as described above. At intervals during the chase, samples were either precipitated with cold trichloroacetic acid or centrifuged at 12,000 × *g* for 30 min, and the amounts of trichloroacetic acid-precipitable radioactivity in the supernatant fractions were determined. During the chase, both ^{3}H and ^{14}C in the trichloroacetic acid precipitates of the culture supernatant increased with time, so that after 5 h about 9% of the total trichloroacetic acid-precipitable disintegrations per minute was in the supernatant medium. However, treatment of parallel trichloroacetic acid precipitates with

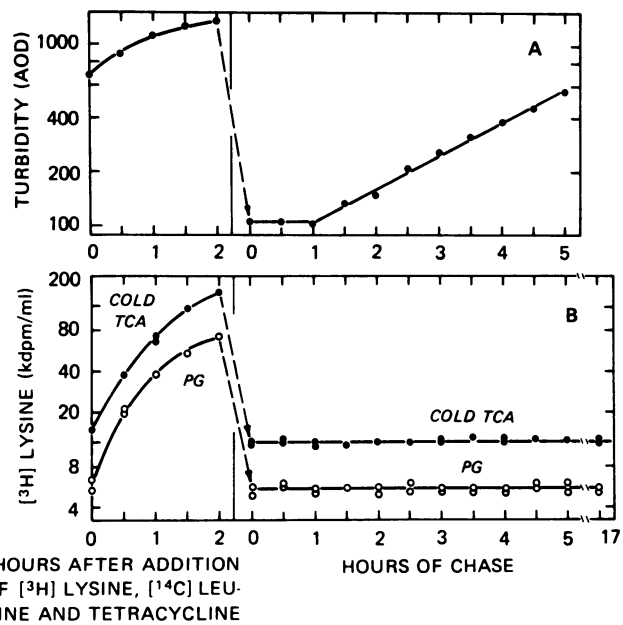

FIG. 2. *Turnover of peptidoglycan of S. mutans FA-1 during regrowth after tetracycline inhibition. At zero time, tetracycline (5 μg/ml), [^{3}H]lysine, and [^{14}C]leucine were added to an exponentially growing culture. Cells were permitted to incorporate the two labeled precursors for 2 h; they were then washed, transferred to chase medium, and allowed to regrow. (A) Turbidity of the culture during the 2 h of tetracycline treatment (left) and in the chase medium during recovery from tetracycline inhibition (right). (B) Incorporation of [^{3}H]lysine during tetracycline treatment (left) and the fate of incorporated [^{3}H]lysine during the chase (right) in cold trichloroacetic acid (TCA) precipitates* (●) *and in peptidoglycan (PG) fractions* (○).

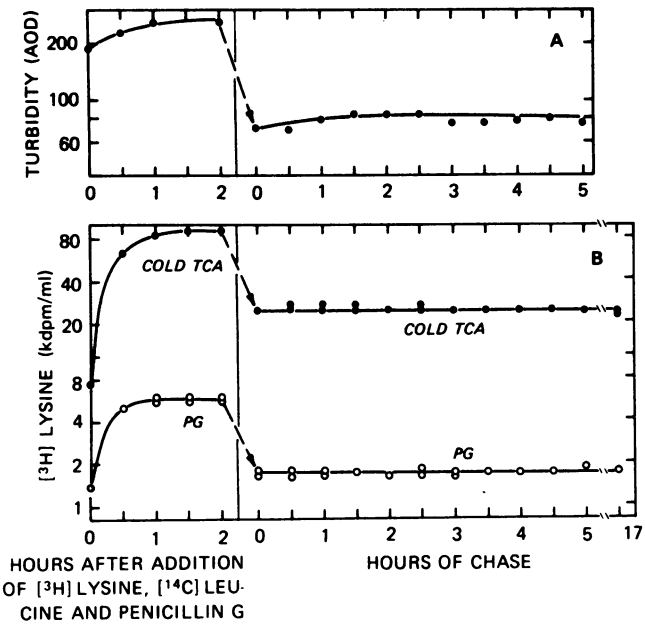

FIG. 3. *Turnover of peptidoglycan of S. mutans FA-1 during regrowth after PenG inhibition. PenG (1.0 μg/ml), [^{3}H]lysine, and [^{14}C]leucine were added to exponentially growing cells at zero time. Cells were allowed to incorporate the two labeled precursors for 2 h; they were then washed, transferred to chase medium, and allowed to regrow. (A) Turbidity of the culture during 2 h of PenG inhibition (left) and in the chase medium after PenG treatment (right). (B) Incorporation of [^{3}H]lysine during PenG treatment (left) and the fate of incorporated [^{3}H]lysine during the chase (right) in cold trichloroacetic acid (TCA) precipitates (●) and in peptidoglycan (PG) fractions (○). The kinetics of incorporation and chase of [^{14}C]leucine in cold trichloroacetic acid precipitates closely followed the pattern observed for [^{3}H]lysine in cold trichloroacetic acid precipitates (data not shown).*

hot trichloroacetic acid and trypsin for the isolation of peptidoglycan fractions resulted in the loss of more than 97% of both labels. Thus, the material containing the two radioactive amino acids was apparently extracellular protein and not peptidoglycan. Therefore, in contrast to results reported for *Bacillus megaterium* (12), no evidence was obtained for the presence of trichloroacetic acid-insoluble peptidoglycan turnover products released from the cells.

Examination of other strains of oral streptococci for peptidoglycan turnover. Five additional strains of *S. mutans*, representative of serotypes a, c, d, and g (Table 2), and eight strains of *S. sanguis* (Table 3) were examined for peptidoglycan turnover by using the methods described above. During exponential growth, peptidoglycan turnover was below detectable levels in all 13 strains (Tables 2 and 3). Similarly, no evidence of peptidoglycan turnover was obtained during recovery from tetracycline or PenG inhibition for the five strains of *S. mutans* examined (Table 2). It is of some interest to note that, particularly during the experiments dealing with recovery from tetracycline inhibition, in some strains (such as *S. mutans* GS-5, Ingbritt, and 6715) the harvesting, washing, and resuspension procedure was not as effective as it was for the other strains, as witnessed by some increase in [^{3}H]lysine in the cold trichloroacetic acid-precipitable materials, but not in the peptidoglycan or protein ([^{14}C]leucine-containing) fractions (not shown). These results could be due to the presence of a larger fraction of soluble peptidoglycan (27) in these strains when they are grown under these conditions.

DISCUSSION

Turnover of cell wall peptidoglycan has been shown to occur in a variety of bacterial species, including *B. megaterium* (7, 24), *Bacillus subtilis* (24, 25), *L. acidophilus* (2, 10), *Neisseria gonorrhoeae* (13), and *Staphylococcus aureus* (40). In contrast, the wall peptidoglycan of *Escherichia coli* (33) or *S. faecalis* (2) does not seem to turn over.

The data presented here, which indicate the absence of peptidoglycan turnover in six strains of *S. mutans* and eight strains of *S. sanguis*, along with the indirect evidence for the lack of wall turnover in *S. pneumoniae* (4, 39) and our previous data with *S. faecalis*, suggest that the

TABLE 2. *Peptidoglycan and protein turnover in S. mutans strains*

Chase time (h)	Turnover (×10³ dpm) in the following strains									
	OMZ-61(a)[a]		Ingbritt(c)		GS-5(c)		OMZ-176(d)		6715(g)	
	PG[b]	Protein[c]	PG[b]	Protein[c]	PG[b]	Protein[c]	PG[b]	Protein[c]	PG[b]	Protein[c]
During exponential growth										
0	29.5	13.5	20.3	10.4	28.0	18.2	8.8	5.2	13.4	9.0
1	28.3	13.4	21.7	10.0	22.9	13.9	9.4	4.9	15.5	9.3
5	27.3	13.5	22.6	9.5	23.6	16.6	9.3	4.9	14.5	9.0
17	26.1	11.8	20.5	9.1	28.3	13.3	8.6	3.9	10.1	8.6
During recovery from 2 h of treatment with 5 μg of tetracycline per ml										
0	15.4	2.2	11.4	1.8	8.3	1.5	5.3[d]	0.2	6.8	2.2
1	14.1	2.2	15.5	1.7	9.9	1.3	5.6[d]	0.5	9.6	2.1
5	15.2	2.1	12.7	1.6	10.6	1.1	5.4[d]	0.4	10.5	2.0
17	15.1	2.0	10.5	1.4	8.8	1.0	5.5[d]	0.5	10.1	2.0
During recovery from 2 h of treatment with 0.5 μg of PenG per ml										
0	2.8	4.7	1.4	3.5	2.2	5.8	1.0	1.7	3.5	9.2
1	2.4	4.7	1.7	3.4	2.4	6.0	0.9	1.6	4.1	8.7
5	2.5	4.3	2.2	3.1	2.8	5.6	1.0	1.4	7.3	8.8
17	3.4	3.7	2.0	2.8	2.6	4.8	0.7	1.2	7.4	8.5

[a] Letters in parentheses are serotype designations.
[b] Amount of [³H]lysine incorporated into hot trichloroacetic acid-insoluble, trypsin-resistant fractions (peptidoglycan [PG]), corrected for residual contamination by protein (10).
[c] Amount of [¹⁴C]leucine incorporated into cold trichloroacetic acid-insoluble precipitates.
[d] These peptidoglycan values were not corrected for protein contamination, because very little [¹⁴C]leucine was incorporated into the protein fraction.

TABLE 3. *Peptidoglycan turnover during exponential growth of S. sanguis strains*

Chase time (h)	dpm ×10³															
	10558		M-5		Challis		Wicky		DL-1		DL-101		71X26		71X48	
	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]	PG[a]	Protein[b]
0	10.2	14.9	8.0	7.3	29.5	9.0	20.4	19.6	7.5	4.5	7.6	3.8	82.5	26.0	97.5	40.0
1	14.0	16.0	7.0	6.4	29.5	12.0	17.3	20.0	8.7	4.7	7.8	3.7	92.5	34.0	100.0	39.5
5			6.0	5.9	30.0		15.6	18.1	8.8	5.0	7.8	4.5	83.3	31.0	105.0	38.0
17	14.4	13.2	5.5	5.1	24.5	10.4	12.5	14.3	7.3	4.5	8.2	4.1	87.5	31.0	103.0	37.0

[a] Amount of L-[³H]lysine incorporated into a cold trichloroacetic acid-precipitable, hot trichloroacetic acid-insoluble, trypsin-resistant fraction, corrected for residual contamination by protein. PG, Peptidoglycan.
[b] Amount of L-[¹⁴C]leucine incorporated into a cold trichloroacetic acid-insoluble precipitate.

cell wall peptidoglycan in gram-positive cocci which divide in one plane (streptococci) is conserved, even when the organisms are exposed to adverse conditions. These results contrast with the loss of streptococcal surface components, such as membrane-associated lipoteichoic acid (15, 17, 21), the polysaccharide of *S. mutans* AHT that carries the serotype-specific determinant that is also known to be covalently linked to the cell wall peptidoglycan (9), and the rhamnose-containing, grouping polysaccharide from a strain of group B *Streptococcus* (6a). Although we did not specifically examine peptidoglycan turnover in the AHT strain of *S. mutans* or, for that matter, in the group B *Streptococcus*, in view of the evidence of the absence of wall peptidoglycan turnover in streptococci presented here and elsewhere, it seems unlikely that peptidoglycan turnover occurs in either of these bacteria. In the absence of peptidoglycan turnover, it is difficult to understand the mechanism by which non-peptidoglycan polysaccharides that are normally part of the cell wall are released to the supernatant growth medium. The presence of such polysaccharides in supernatant growth media could be due to their failure to become covalently linked to the peptidoglycan during the wall assembly process, to enzymatic hydrolysis of polysaccharides that had been incorporated into the cell wall, or to their occur-

rence in two forms, one covalently linked to peptidoglycan and the other a capsular type of polysaccharide.

The reasonably sensitive method used throughout most of these studies would have detected losses of acid-precipitable peptidoglycan fragments from cells grown under the variety of different conditions used. In addition, experiments designed to detect the possible release from exponentially growing cells of small amounts of peptidoglycan fragments that retained the ability to be precipitated by trichloroacetic acid were performed and failed to yield evidence of peptidoglycan turnover. Neither of the methods used was capable of detecting the presence of or release of either acid-soluble or -insoluble peptidoglycan turnover products that had rebound to and/or coprecipitated with cells. We have been unable to devise an experimental procedure capable of detecting such turnover products. Similarly, the presence of or loss of soluble, presumably precursor peptidoglycan similar to that shown to be present in several other bacterial species (16, 27, 31) would not have been included in our measurements.

In a few instances (Tables 2 and 3) small losses of [^{3}H]lysine from peptidoglycan fractions were observed after prolonged(e.g., 17-h) incubations. In nearly all cases, such as that observed in *S. sanguis* Wicky, decreases in [^{3}H]lysine in peptidoglycan were accompanied by nearly corresponding losses of [^{14}C]leucine in protein. Similar losses of cellular peptidoglycan accompanied fluoride-induced damage to several bacterial species, including two strains of *S. mutans* (20).

Roles for peptidoglycan hydrolase activities in the changes in shape (morphogenesis) of bacterial cells that occur during normal cell division cycles have been proposed (11). Changes in cell shape do occur during the division cycle of streptococci (14). Although it is thought that such changes in the shape of the rigid and protective wall require action of peptidoglycan hydrolase(s), release of peptidoglycan fragments from cells is not required. Clearly, it is possible that bonds in peptidoglycans can be hydrolyzed (or nicked) without the release of products from the cells. It is only when a sufficient number of bonds are hydrolyzed, perhaps in a localized area of the wall, that such products are released from the cells.

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