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# Import and Metabolism of Glutathione by *Streptococcus mutans*

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**Glutathione (γ-GluCysGly, GSH) is not found in most gram-positive bacteria, but some appear to synthesize it and others, including *Streptococcus mutans* ATCC 33402, import it from their growth medium. Import of oxidized glutathione (GSSG) by *S. mutans* 33402 in 7H9 medium was shown to require glucose and to occur with an apparent $K_m$ of 18 ± 5 μM. GSSG, GSH, *S*-methylglutathione, and homocysteine-glutathione mixed disulfide (hCySSG) were imported at comparable rates (measured by depletion of substrate in the medium), as was the disulfide of γ-GluCys. In contrast, the disulfide of CysGly was not taken up at a measurable rate, indicating that the γ-Glu residue is important for efficient transport. During incubation with GSSG, little GSSG was detected in cells but GSH and γ-GluCys accumulated during the first 30 min and then declined. No significant intracellular accumulation of Cys or sulfide was found. Transient intracellular accumulation of D/L-homocysteine, as well as GSH and γ-GluCys, was observed during import of hCySSG. Although substantial levels of GSH were found in cells when *S. mutans* was grown on media containing glutathione, such GSH accumulation had no effect on the growth rate. However, the presence of cellular GSH did protect against growth inhibition by the thiol-oxidizing agent diamide. Import of glutathione by *S. mutans* ATCC 25175, which like strain 33402 does not synthesize glutathione, occurred at a rate comparable to that of strain 33402, but three species which appear to synthesize glutathione (*S. agalactiae* ATCC 12927, *S. pyogenes* ATCC 8668, and *Enterococcus faecalis* ATCC 29212) imported glutathione at negligible or markedly lower rates.**

Bacteria import peptides composed of two to eight residues by means of a number of different multiprotein uptake systems or permeases (14). Of the bacterial permeases, those of *Escherichia coli*, *Lactococcus lactis*, and *Salmonella typhimurium* are the best studied (6, 7). In these organisms, there are individual permeases that have high affinity for dipeptides, tripeptides, dipeptides and tripeptides, or oligopeptides. Among the bacterial peptide permeases (14), there seems to be no discrimination of the specific amino acids of the transported peptides. However, switching the stereochemistry of $C_\alpha$ from L to D or modifying the C-terminal carboxylate or N-terminal amine of transported peptides significantly reduces the rate of transport. One transport system which does seem to recognize peptide residue side chains has been reported to exist in *Enterococcus faecalis*; this system transports only peptides that possess an N-terminal Asp or Glu (13).

In 1978, we reported that glutathione (γ-GluCysGly, GSH) is not synthesized by most gram-positive bacteria (4), apparent exceptions being *Streptococcus agalactiae* and *L. lactis* (previously *Streptococcus lactis*). However, some of the gram-positive bacteria appeared to acquire GSH by import of another form of GSH from the growth medium. Uptake of glutathione by *Streptococcus mutans* was later studied by Thomas (16), who found that total cellular thiol content, and radioactivity from labeled GSH or oxidized GSH (GSSG), increased with the same kinetics. A careful study of *L. lactis* subsp. *cremoris* by Wiederholt and Steele (17) established that strain Z8 efficiently accumulates GSH when grown in medium supplemented with GSH but is unable to synthesize it, whereas strain C2 can neither import nor synthesize GSH. Species of *Peptostreptococcus* and *Fusobacterium* have been shown to markedly increase their production of $H_2S$, apparently derived by import of glutathione from the growth medium (2). Finally, cellular accumulation of radioactivity from radiolabeled GSH or GSSG added to the incubation medium has been demonstrated in *Streptococcus pneumoniae*, and a mutant in which the apparent transport of glutathione is blocked has been found (9).

In a recent report (10), we provided evidence for accumulation of GSH through transport and synthesis of GSH by streptococci and enterococci, but the occurrence of these processes appeared to be species dependent and even, for some species, strain dependent. Such strain dependence appears most variable for *L. lactis*, where different strains can synthesize GSH, accumulate GSH by import, or do neither (4, 17). In the present research, we expand on our studies of streptococci in order to gain insight into the nature of the glutathione species transported, the fate of the glutathione once it enters the cell, and the function of glutathione in the cell.

## MATERIALS AND METHODS

**Reagents.** D/L-Homocysteine (hCySH), GSSG, and *S*-methylglutathione (GSMe) were purchased from Sigma, [glycine-2-$^3$H]GSH was purchased from DuPont, and iodomethane was purchased from Aldrich. Sources for other reagents and thiols are listed elsewhere (12). All inorganic compounds were of reagent grade or higher quality.

**Media.** Trypticase soy broth (TSB; BBL) and Todd-Hewitt broth (TH; Difco) were prepared from dehydrates and autoclaved. Middlebrook 7H9 broth (7H9; Difco) was prepared from the dehydrate and filter sterilized. YTS was prepared by supplementing TH with yeast extract (0.5%; Difco) and sucrose (0.25%) and autoclaving. Glutathione-depleted TH and YTS (dpTH and dpYTS) were prepared by treatment (37°C, 4 h) with γ-glutamyl transpeptidase (1 U/ml; Sigma) before autoclaving. 7H9-Glc was prepared by supplementing 7H9 with glucose (1%) and filter sterilizing. Medium supplemented with GSH in a bound reducible form (GSX) was prepared by addition of GSH from a fresh concentrated stock solution to dpTH and incubating it at room temperature for at least 48 h. Thiol analysis with monobromobimane (mBBr) revealed that residual GSH was <3% of the initial value but that ≥80% of the initial GSH was present as GSX which was released as GSH upon reaction with dithiothreitol (DTT).

**Bacterial strains and culture conditions.** All strains with ATCC numbers were obtained directly from the American Type Culture Collection. *Streptococcus pyogenes* UCSD 20 was a patient isolate furnished by Charles Davis, UCSD Medical Center, and was characterized by standard methods. All liquid cultures

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were grown at 37°C with gentle swirling in a New Brunswick model G25 incubator.

**Preparation of glutathione-homocysteine mixed disulfide (hCySSG).** hCySH (0.3 mmol) was added to a solution (20 ml) of GSH (0.1 mmol) before addition of $NH_4OH$ (to pH 8). The solution was then bubbled with $O_2$ (10 h) before the solution was neutralized with HCl and dried under reduced pressure. The dry residue was resuspended (0.1% trifluoroacetic acid [TFA]–$H_2O$), and the insoluble homocystine was removed by centrifugation. The supernatant was again dried, resuspended, and centrifuged before the mixed disulfide was purified by reversed-phase, high-pressure liquid chromatography (HPLC; Beckman Ultrasphere 5-μm octyldecyl silane column in 0.1% TFA–$H_2O$, eluted with 0.1% TFA–methanol).

**Preparation of $^3$H-GSMe.** GSH (3.3 μmol) and $^3$H-GSH (2 μl, 22 μM, as supplied by the manufacturer) were dissolved in 250 μl of dimethyl formamide containing 10 μl of iodomethane and 70 mM Tris-Cl (pH 8.0), and the solution was incubated for 10 min at room temperature. The reaction was quenched by addition of 730 μl of 0.1% TFA. A fraction of the reaction mixture was analyzed by HPLC on a Beckman IP Ultrasphere 5-μm column eluted with aqueous acetonitrile containing 0.1% TFA and indicated complete conversion of GSH. Scintillation counting of the fractions revealed that 90% of the radioactivity eluted in the same position as GSMe. The quenched reaction solution served as a stock solution of $^3$H-GSMe.

**Medium and cellular thiol and disulfide levels during import of glutathione derivatives.** Stationary-phase cells from culture in dpTH were collected by centrifugation and resuspended in one-third to one-half of the original volume of the desired medium. Zero-time samples (5 ml) were removed immediately into preiced centrifuge tubes. The cell incubation mixture was transferred to a shaking incubator (37°C), and 5-ml samples were removed at the specified times. All samples were chilled for 20 min on ice before centrifugation (10 min, 3,000 × *g*). For analysis of low-molecular-weight thiol (RSH) levels in the medium, a 1-ml sample of supernatant was mixed with 28 μl of mBBr (100 mM in acetonitrile) plus 30 μl of Tris-Cl (1 M, pH 8.0) and incubated for 15 min at room temperature. The solution was acidified with methanesulfonic acid (10 μl, 5 N) and analyzed by HPLC. For analysis of RSH and low-molecular-weight disulfide (RSS) levels in the medium, the supernatant (1 ml) was mixed with 10 μl of DTT solution (100 mM) and 30 μl of Tris-Cl (1 M, pH 8.0) and then incubated for 20 min at room temperature before reaction with mBBr as described above.

For analysis of cellular RSH levels, a cell pellet was resuspended in 1 ml of water, transferred to a preweighed tube, and pelleted in a microcentrifuge. A 250-μl aliquot of 50% aqueous acetonitrile containing 2 mM mBBr and 20 mM Tris-Cl (pH 8) was added to the cell pellet, and the suspension was heated for 15 min at 60°C. Methanesulfonic acid (10 μl, 5 N) was added to the mixture, the sample was centrifuged, and the supernatant was removed. A measured sample of the supernatant was dried (Speed-Vac), resuspended in twice the volume of 10 mM methanesulfonic acid, and analyzed by HPLC. The tube containing the pellet was dried in a vacuum oven at 40°C for at least 24 h before it was weighed to obtain the cellular residual dry weight (RDW). A second control sample was prepared by replacing mBBr with *N*-ethylmaleimide in the initial extraction; the sample was then reacted with 2 mM mBBr and processed as described above.

For analysis of the cellular RSS content, duplicate 5-ml samples of the cell incubation mixture were removed and kept on ice until *N*-ethylmaleimide in acetonitrile (15 μl, 0.5 M) and Tris-Cl (150 μl, 1 M, pH 8) were added. The suspensions were incubated for 20 min at room temperature and pelleted by centrifugation. The pellets were resuspended in water, transferred to preweighed tubes, and repelleted. One pellet was used to check that all thiols had been alkylated; 250 μl of 50% aqueous acetonitrile containing Tris-Cl (20 mM, pH 8) and mBBr (5 mM) was added, the suspension was heated at 60°C for 15 min, and the mixture was centrifuged. The supernatant was processed for thiol analysis, and the pellet was processed for RDW, as described above. The second sample of pelleted cells was processed for analysis of RSS content; 250 μl of 50% aqueous acetonitrile containing DTT (3 mM) and Tris-Cl (20 mM, pH 8) was added, the suspension was heated at 60°C for 15 min, mBBr (18.75 μl, 100 mM in acetonitrile) was added to the mixture, and heating was continued for 15 min. After addition of 10 μl of 5 N methanesulfonic acid, the suspension was centrifuged; the supernatant and pellet were analyzed for thiol content (RSS equivalents) and for RDW, respectively, as described above.

**Medium depletion assays.** Stationary-phase cells grown in dpTH were collected by centrifugation (3,000 × *g*, 10 min) and resuspended in 7H9-Glc at ~100 times the original cell density. An aliquot of the cell suspension was diluted 4- to 49-fold with 7H9-Glc, depending on the concentration of disulfide to be analyzed. Disulfide stock solution was prepared in 7H9-Glc at twice the desired assay concentration. Equal aliquots of the disulfide stock solution and the diluted cell suspension were equilibrated in a water bath (>15 min, 40°C); the assay was started by adding the disulfide solution to the cell suspension. Over the course of 10 to 30 min, aliquots (0.5 to 1 ml) were removed at specific times and a portion was gently pushed through a 0.2-μm-pore-size filter fitted to a syringe; the filtrate was collected and stored on ice. An aliquot (2 μl) was diluted into 200 μl of 1 mM DTT in 20 mM Tris-Cl (pH 8.0), and the reaction was allowed to proceed for 15 min at 20°C. The solution was then treated by addition of mBBr to 3 mM and incubation for 15 min at 20°C before addition of methanesulfonic acid to 100 mM. For filtrates of samples that contained less than 50 μM disulfide, the filtrate was diluted only 10-fold into the DTT solution. The derivatized thiols were analyzed by HPLC, and the rate of depletion was calculated from the change in measured thiol content with time. Cells from a measured volume of the initial cell suspension were collected by centrifugation and dried in a tared tube in a vacuum oven (40°C) for at least 24 h. The weight of the pellet was used to establish the cell dry weight present in the diluted cell suspensions.

TABLE 1. Effect of GSX in the growth medium on cellular GSH content

| Species | Medium | GSH (μmol/g [RDW]) |
|---|---|---|
| Grown on medium with ≤1 μM GSX | | |
| *S. pyogenes* ATCC 8668 | dpTH | 20 |
| *S. pyogenes* UCSD 20 | TSB | <0.04 |
| *S. pneumoniae* ATCC R6 39937 | dpYTS | 0.03 |
| *S. mutans* ATCC 25175[a] | TSB | 0.9 |
| *S. mutans* ATCC 33402[a] | dpTH | 0.13 |
| Grown on medium with ~30 μM GSX | | |
| *S. pyogenes* UCSD 20 | TH | 3.9 |
| *S. pneumoniae* ATCC R6 39937 | YTS | 0.6 |
| *S. mutans* ATCC 25175 | TH | 5.3 |
| *S. mutans* ATCC 33402 | TH | 3.3 |

[a] Data from reference 10.

**Uptake of $^3$H-GSH and $^3$H-GSMe.** Cell suspensions (25 ml), prepared in 7H9-Glc as described above for the import assays, were mixed with 375 μl of the $^3$H-GSH or $^3$H-GSMe stock solutions (3 mM in 25% aqueous dimethyl formamide). As needed, GSH (0.25 ml, 5 mM) and DTT (0.25 ml, 1 M) were also added. The cultures were incubated at 37°C with shaking, and 5-ml samples were removed onto ice at specified times. After centrifugation, the supernatants were removed, mixed with scintillation cocktail (ScintiVerse; Fisher), and counted with a Beckman LS1701 scintillation counter. The cell pellets were extracted with 50% aqueous acetonitrile for 15 min at 60°C; the mixture was added to scintillation cocktail and counted.

**Growth rate studies.** Stationary-phase *S. mutans* 33402 was diluted 10- to 20-fold into dpTH or into TH with additions of GSMe, diamide, and/or GSH as indicated. Cell suspensions were shaken in a New Brunswick incubator at 37°C, and growth was monitored by periodic determination of the $A_{600}$ value.

**HPLC analysis of thiols.** Thiols were derivatized with mBBr and analyzed by HPLC according to the general protocols presented elsewhere (5, 12). HPLC methods 1 and 2 (5) were used with the described instrumentation (12).

## RESULTS

**Some streptococci import GSX by an energy-dependent process.** Table 1 provides representative data for several streptococci grown in media of low GSX content (dpTH, TSB, and dpYTS) and in media of moderate GSX content (TH and YTS). For *S. pyogenes* ATCC 8668 grown in dpTH, the cellular GSH content was so high that uptake of the entire medium content of GSX was inadequate to accommodate the result, leading to the conclusion that cellular synthesis of GSH must occur, as reported previously for other species (10). In contrast, *S. pyogenes* UCSD 20 grown in TSB does not contain detectable GSH unless grown on media having significant GSX content, indicating that it imports GSX from the medium and converts it to GSH. The data in Table 1 also show that one strain of *S. pneumoniae* and two strains of *S. mutans* accumulate GSH by import of GSX.

*S. mutans* ATCC 33402 was chosen for more detailed studies of glutathione import by streptococci. Media containing various levels of GSX were prepared by supplementing dpTH medium with GSH and allowing it to stand 48 h at room temperature. Analysis of the medium revealed that ≥98% of the GSH had been converted to other forms but that >80% of the original GSH could be recovered by treatment of the medium with DTT, implying that most of the GSH had been converted to forms (GSX) that could be cleaved to release GSH. Log-phase cells were resuspended in this medium, and the cellular GSH level was determined after a 200-min incu-

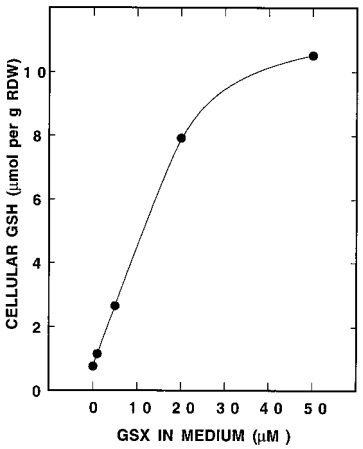


FIG. 1. GSH levels of *S. mutans* ATCC 33402 grown in dpTH medium containing various levels of GSX.

bation at 37°C (Fig. 1). The results show that low micromolar concentrations of GSX in the growth medium suffice to produce a substantial intracellular GSH content.

To elaborate the details of glutathione uptake, a less complex incubation medium was sought. Cells were grown to stationary phase in dpTH medium and resuspended in 7H9 minimal broth. Incubation at 37°C in this medium supplemented with 10 μM GSSG or 1% Glc resulted in no accumulation of cellular GSH (<0.1 μmol per g [RDW]). However, when both GSSG and Glc were present in the medium, cellular GSH levels of ~14 and ~5 μmol per g (RDW) were measured after 1- and 2-h incubations, respectively. To test whether a better GSS transport substrate might be formed in dpTH medium through reaction with GSH, a stock solution was prepared by incubating 0.5 mM GSH in dpTH medium and used to introduce GSX in the transport studies. Cellular accumulation of GSH from medium containing 10 μM GSX was about one-sixth of that from medium containing 10 μM GSSG. Thus, no derivative of glutathione appears to be formed at substantial levels in TH medium which serves as an especially good substrate for the transport system.

**GSH is a good substrate for uptake and is metabolized by *S. mutans*.** Uptake of GSH was compared with that of GSSG. Since micromolar levels of GSH oxidize rapidly in aerobic solution, uptake of GSH was examined at 37°C in 7H9-Glc medium in the presence of 3 mM DTT to keep the GSH reduced. The cellular contents of GSH after a 40-min incubation were 4 and 36 μmol per g (RDW) for uptake in 5 and 50 μM GSH and were 6 and 42 μmol per g (RDW) for uptake in 5 and 50 μM GSSG. Thus, uptake of GSH and uptake of GSSG by *S. mutans* ATCC 33402 occur at comparable rates, in accord with earlier observations with *S. mutans* GS-5 (16).

A representative time course of GSH and GSS loss from medium to which 40 μM GSH had been added is shown in Fig. 2 together with data for the appearance of GSH in cells. Half of the GSH was converted to GSS by the time the incubation with cells was initiated. During the initial 30 min, the amount of glutathione that disappeared from the medium was ~3-fold greater than the amount of GSH that appeared in the cell. At 2 h, the medium GSH and GSS plus cellular GSH accounted for only ~15% of the original glutathione. This loss could not be ascribed to degradation of glutathione in the medium since no accumulation of γ-GluCys, CysGly, or Cys, the expected products of peptidase activity, was detected in the medium.

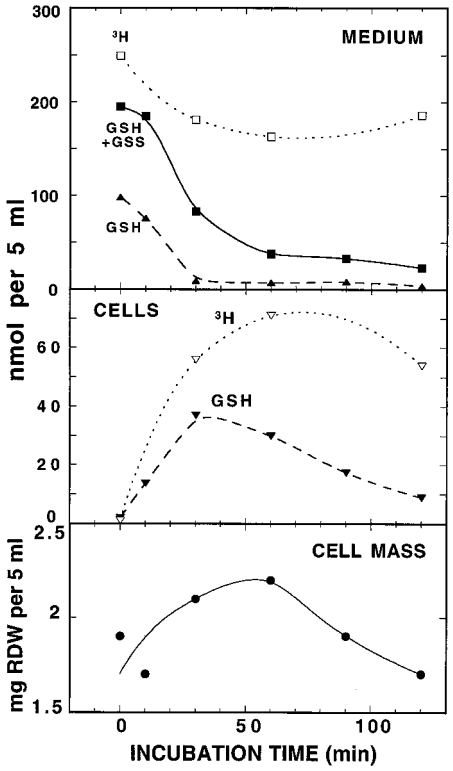


FIG. 2. Uptake from 7H9-Glc measured after addition of 40 μM GSH (GSH + GSS in medium [■], GSH in medium [▲], GSH in cells [▼], and RDW of cells [●]). In a separate experiment, uptake of radiolabel in 7H9-Glc was measured after addition of 50 μM $^3$H-GSH ($^3$H in medium [□] and $^3$H in cells [▽]).

To probe further the fate of imported glutathione, we measured radiolabel levels during uptake of 50 μM GSH labeled with [$^3$H]glycine (Fig. 2). Loss of $^3$H from the medium was substantially less pronounced than loss of GSH plus GSS. However, accumulation of $^3$H in cells exceeded that of GSH during the initial hour of incubation, after which the cellular $^3$H level began to fall. This finding indicates that the cells were metabolizing GSH and releasing glycine and/or $^3$H-labeled products of glycine metabolism. From about 30 to 90 min, the import and export of $^3$H-labeled material are roughly balanced.

**GSSG, CySH, and $H_2S$ do not accumulate during import of GSSG.** To test whether some of the missing glutathione is in the form of GSSG accumulating in the cells, we examined the cellular content of GSH and GSS during uptake in 200 μM GSSG. The GSH/GSS ratio remained 50 ± 15 ($n$ = 4) during the first 30 min of import as the cellular GSH content increased from an initial value of ~2 to ~30 μmol per g (RDW). Thus, no significant amount of GSSG accumulates in the cells. CySH and $H_2S$ levels were not significantly elevated during import of GSSG, remaining below 0.4 μmol per g (RDW) in all determinations. We also measured γ-GluCys and found the cellular level to be about 1/10 of the GSH level during the first 30 min of import with GSSG. Imported GSH is apparently degraded to some extent via removal of the glycine residue.

**hCySH and GSH are incorporated during transport with hCySSG.** hCySSG was examined as a substrate in an attempt to assess whether both components of a glutathione disulfide are imported (Fig. 3). Initially almost all of the medium components were in the disulfide form; the GSS and total hCys

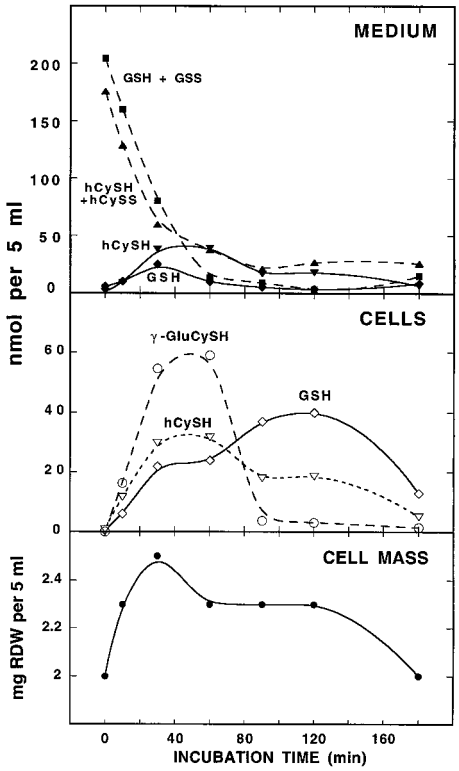


FIG. 3. Uptake from 7H9-Glc after addition of 40 μM hCySSG. (Top) RSH plus RSS and RSH levels in medium; (middle) RSH levels in cells; (bottom) RDW of cells.

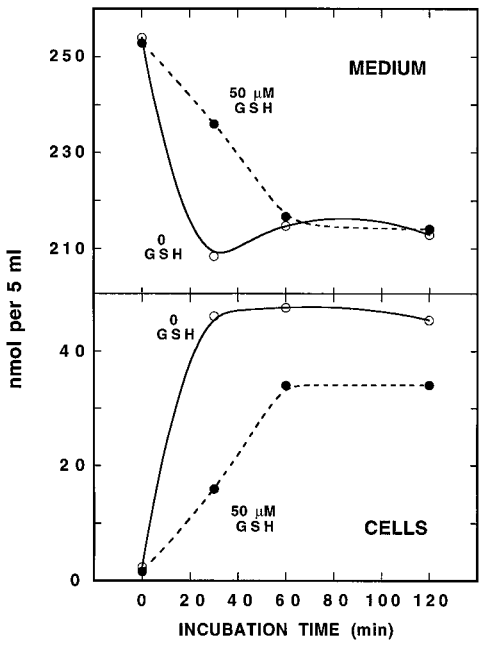


FIG. 4. Uptake of $^3$H from 7H9-Glc after addition of 50 μM $^3$H-GSMe with (●) or without (○) 50 μM GSH in medium and cells. (Top) $^3$H levels in medium; (bottom) $^3$H levels in cells.

disulfide (hCySS) contents were roughly equal, consistent with hCySSG as the dominant medium component. Both components were lost from the medium at a rapid rate, and GSH and hCySH appeared in the cell at similar rates. However, the rate of appearance in the cell was roughly an order of magnitude lower than that of loss from the medium. The predominant thiol in the cell was γ-GluCySH, presumably formed by hydrolytic cleavage of glycine from GSH. The rate of appearance in the cell of γ-GluCySH combined with that of GSH accounted for half of the glutathione lost from the medium.

GSH and hCySH appear in the medium at rates comparable to or somewhat greater than those for appearance in cells. To test whether import of hCySH might occur by reduction of the mixed disulfide and import of hCySH, we examined uptake of 50 μM hCySH in the presence of 3 mM DTT. This is slightly more than the maximum medium level of hCySH determined in Fig. 3, but after a 40-min incubation the cells had accumulated only 1.3 μmol of hCySH per g (RDW), just 5% of the level found in the uptake with hCySSG. Thus, we conclude that the two components of the mixed disulfide are imported simultaneously. It is apparent that the hCySH is rapidly metabolized to other unidentified products once it is in the cell.

It is also clear that the metabolism of the cell was changing during the course of the import study. Import and hydrolysis of GSH appeared to slow dramatically after the first hour of incubation, the cellular GSH level increased slowly during the next hour, and it then declined during the third hour of incubation.

Although comparable amounts of thiol appeared in cells and in the medium, the concentrations were quite different. At 30 min, the RDW of cells in 5 ml of medium was 2.5 mg. Assuming the wet weight to be an order of magnitude larger, we estimate the hCySH and GSH levels in the cell to be ~1 mM at 30 min, compared with 4 to 6 μM in the medium. Thus, a ~200-fold gradient of intracellular over extracellular thiol concentration is established and is maintained during the remaining 2.5 h of incubation.

**A thioether of glutathione is also transported.** To test whether a nonreducible derivative of GSH could also be transported, we prepared $^3$H-GSMe and examined the uptake of $^3$H from medium containing 50 μM $^3$H-GSMe in the absence and presence of 50 μM GSH (Fig. 4). With no added GSH the radioactivity in the medium dropped sharply over the first 30 min, corresponding to a rate of 330 cpm per min, but then remained constant over the following 90 min. However, HPLC analysis of the medium at the end of the 120-min incubation revealed that only 43% of the $^3$H was associated with GSMe, as opposed to 88% for the starting GSMe. Thus, the disappearance of GSMe from the medium appears to continue well beyond the initial 30-min period but the $^3$H in the medium remains constant as the result of the accumulation of other labeled material, presumably cellular metabolites of GSMe. Assuming that all of the $^3$H-GSMe that disappeared from the medium by 120 min was imported by the cell, the mean rate was 240 cpm per min, only 25% lower than the rate calculated from total loss of $^3$H from the medium during the first 30 min.

In the presence of 50 μM GSH, the initial rate of $^3$H loss from the medium was reduced by ~60% (Fig. 4). This inhibition of GSMe uptake by GSH demonstrates that the same transport system is used for GSMe and GSH import and implies that the two compounds bind with similar affinities to the transport binding site. Based on the rates of $^3$H depletion in the medium during the initial 30 min, import of GSMe occurs at a rate only slightly (~25%) lower than that for import of GSH.

Since GSH inhibits GSMe uptake, the reverse would also be expected. This was tested qualitatively by showing that the GSH content of *S. mutans* following a 40-min incubation with

TABLE 2. Initial rate of depletion of 200 μM GSSG from 7H9-Glc by different bacteria

| Species | Medium GSH + GSS depletion rate {μM $min^{-1}$ (mg [dry wt]/ml)$^{-1}$} |
|---|---|
| *S. mutans* ATCC 33402 | 2.0 ± 0.8[a] |
| *S. mutans* ATCC 25175 | 2.0 ± 0.8 (3[b]) |
| *S. agalactiae* ATCC 12927 | 0.4 ± 0.3 (3) |
| *S. pyogenes* ATCC 8668 | 0.1 ± 0.3 (3) |
| *E. faecalis* ATCC 29212 | −0.1 ± 0.5 (3) |

[a] Based on concentration dependence in 10 independent experiments.
[b] Number of independent experiments.

50 μM GSSG 7H9-Glc at 37°C was decreased from 36 to 11 μmol per g (RDW) by the presence of 25 μM GSMe in the incubation mixture.

**Kinetics of GSSG import and rates for related disulfides.** Measurements of the depletion of GSSG from the medium by *S. mutans* were made in a fashion that allowed the rate to be determined during the first 10 to 30 min of incubation at 37°C in 7H9-Glc. Double-reciprocal plots of velocity versus concentration were reasonably linear for measurements with a given batch of cells. These yielded an apparent $K_m$ value for GSSG of 18 ± 5 μM, using rate measurements made on three different days with GSSG varying from 7 μM to 1 mM. However, the maximal velocities varied substantially, indicating that different batches of cells produced different levels of transport activity. For this reason, comparisons of rates measured with different substrates were meaningful only if made on the same day with the same batch of cells.

A comparison of medium disulfide depletion rates for GSSG (120 μM) and the symmetrical disulfides of γ-GluCys (90 μM) and CysGly (125 μM) measured with the same batch of cells yielded values of 0.64 ± 0.09, 0.62 ± 0.03, and <0.05 μM disulfide per min ($n$ = 3 for each), respectively. Thus, the glutamyl residue appears to be critical for recognition by the transport system. As noted above, comparisons of GSH with GSSG and of GSH with GSMe indicated that these three substrates are imported at similar rates.

It was important to assess whether import of glutathione occurs in streptococci other than *S. mutans* 33402, and especially in streptococci that are apparently able to synthesize glutathione. Rates of medium depletion of 200 μM GSSG were compared for several species and strains of streptococci as shown in Table 2. *S. mutans* 25175 imported glutathione at a rate equivalent to that of strain 33402, but *S. agalactiae* and *S. pyogenes* showed little or no ability to take up GSSG. The latter two species both appear to produce glutathione, whereas both strains of *S. mutans* do not (10). Import by *Enterococcus faecalis* ATCC 29212, another gram-positive bacterium apparently able to synthesize glutathione (10), was undetectable (Table 2).

**Import of glutathione protects *S. mutans* against growth inhibition by the thiol oxidant diamide.** It has been established for organisms that synthesize GSH that intracellular GSH provides protection against thiol-reactive toxins, including diamide, a thiol oxidant which penetrates cells and reversibly inhibits growth (8). To test whether similar protection occurs for cellular GSH acquired by import of GSX from the growth medium coupled with intracellular reduction, we examined the ability of diamide to inhibit growth of *S. mutans* in dpTH medium, where the low GSX level in the medium severely limits cellular GSH levels. This was compared with the inhibition by diamide of growth in dpTH medium to which 50 μM GSH was added and allowed to oxidize, a medium producing substantial intracellular levels of GSH (Fig. 1). Significant inhibition of growth was not observed in either medium in the presence of diamide up to 0.5 mM. However, in 2 mM diamide, the exponential growth rate in dpTH was reduced by a factor of 0.46 ± 0.06 ($n$ = 3), whereas the exponential growth rate in supplemented medium was reduced by a factor of 0.76 ± 0.14 ($n$ = 3). Since the GSX-supplemented medium contained <2 μM GSH initially and since only low micromolar levels of GSH accumulate in the medium in the absence of diamide, inactivation of diamide by GSH in the supplemented medium cannot be the mechanism of protection. Thus, cellular GSH derived from the medium must provide protection against thiol oxidants.

## DISCUSSION

We begin with a discussion of the GSX transport process itself. This is a highly efficient process, capable of importing low micromolar amounts of GSX from the growth medium and achieving steady-state concentrations of GSH in the cell orders of magnitude greater than the total glutathione content of the medium. Glutathione substrates recognized by the transport system include GSH, GSSG, hCySSG, and GSMe, all of which are imported with similar levels of efficiency. Thus, the substrate affinity is not markedly influenced by modifications at the thiol group of glutathione. The system also transports the symmetrical disulfide of γ-GluCys at a rate similar to that of GSSG, but the symmetrical disulfide of CysGly was not transported at a measurable rate. Thus, the glutamyl residue appears to be critical to the transport process.

Quantitative measurements of the import of glutathione were hampered by an apparent variability in the maximal rate, which may depend on the timing of growth and harvest of the cells. Some unidentified factor appears to influence the rate of glutathione transport. The rates measured here for *S. mutans* (Table 2) are in the range reported for other peptide transport systems in streptococci (13, 16) and other bacteria (14). It should be borne in mind that the measurements were made with cell densities severalfold above those for stationary-phase cells but generally similar to those used in the cited studies.

Next we consider the fate of the glutathione once it is imported. Import of GSH itself is followed by rapid degradation (Fig. 2). By the time cellular GSH reached its peak value at 30 min, two equivalents of this amount had been taken up and metabolized, and the $^3$H-metabolite(s) derived from the $^3$H-Gly of $^3$H-GSH had been largely excreted back into the medium. Metabolism of $^3$H-Gly presumably begins with its cleavage from $^3$H-GSH, which would produce γ-GluCys as the other product. This was not specifically determined in the experiments with GSH as the substrate but was found in subsequent studies with hCySSG (Fig. 3) and GSSG. It is curious that we did not detect CysGly, the product of removal of the γ-glutamyl residue of GSH. It may be that cleavage of CysGly is so rapid, relative to its formation, that the steady-state concentration is too low to detect.

Another thiol metabolite of glutathione that was found not to accumulate is Cys. The level of Cys remained below 0.4 μmol per g (RDW) during import of GSSG despite the large turnover of cellular GSH. Apparently the cells store Cys or S in some other form or excrete it from the cell. Since Cys undergoes very rapid heavy metal ion-catalyzed autoxidation, much faster than GSH or other thiols accumulated at substantial levels in cells (3, 11, 15), streptococci may have evolved efficient mechanisms to keep the cellular Cys level low.

The reduction of GSSG occurs with, or very quickly after, its import into the cell, as no evidence for marked accumulation

of GSSG within the cell was found. Thomas (16) found that anaerobic incubation of *S. mutans* with GSSG or cystine in the presence of glucose led to marked accumulation of total cellular thiol content but little increase in the thiol level of the medium, ascribed to active transport of the disulfides and intracellular reduction. However, the opposite result was found for lipoic acid or cystamine. This is the expected result since the reduced forms of the latter compounds, as well as lipoic acid itself, rapidly cross cellular membranes by passive diffusion. In this case, the cells thus serve as a catalyst for reduction of medium disulfides at the expense of glucose, with the bulk of the thiol accumulating in the large volume of the medium compared with that of the cells.

The complexity of the metabolism of glutathione derivatives is illustrated by the results for hCySSG import (Fig. 3). By 60 min, all of the original 40 μM disulfide had disappeared from the medium, which then contained 2 μM GSH and 8 μM hCySH. Also, during this initial 60 min the RDW increased 25% before it declined to the original value over the ensuing 2 h. This change reflects synthesis and destruction of polymeric material, not low-molecular-weight components which would have been extracted by warm 50% acetonitrile. These changes paralleled variations in cellular glutathione metabolism. Initially import of glutathione and its intracellular cleavage to γ-GluCys occurred at similar rates, both higher than the cleavage rate of γ-GluCys, leading to an accumulation of γ-GluCys within the cell. However, between 60 and 90 min, the γ-GluCys level fell sharply and the GSH level increased, even though the import of glutathione from the depleted medium must have dropped dramatically. We must conclude that conversion of GSH to γ-GluCys becomes dramatically slower around 60 min, either through inhibition or degradation of the responsible enzyme. It seems likely that transfer of cells from dpTH to 7H9-Glc medium containing glutathione disulfides promotes changes in cellular metabolism which include synthesis and degradation of protein spanning the 2- to 3-h time course covered by the present experiments and are responsible for the variations in RDW and GSH metabolism. To characterize GSX transport in a more quantitative fashion, it would clearly be desirable to identify ways to prepare and maintain the cells in a more metabolically stable state during the interval of measurement.

With hCySSG as the substrate, the corresponding thiol forms are an important component of the thiol produced in the medium by *S. mutans* (Fig. 3). The thiol exported by some species of *Peptostreptococcus* and *Fusobacterium* (2) is hydrogen sulfide. Under anaerobic conditions, *Peptostreptococcus micros* utilizes glutathione to produce and excrete substantial quantities of $H_2S$, with import of glutathione being the rate-limiting step. This is considered to be an important source of the $H_2S$ production associated with oral bacteria but was found to be a major process in only 7 of 37 different species of oral bacteria surveyed (2). No significant $H_2S$ production was detected in the present studies with *S. mutans.*

Finally, we consider the function which imported glutathione plays in *S. mutans*. Its rapid metabolism within the cell indicates that it may serve as a nutrient source. However, under normal growth conditions on media having modest glutathione content, *S. mutans* accumulates significant levels of cellular GSH, and we wondered if this serves any protective function for *S. mutans* as it does in organisms that synthesize it. Accumulation of GSH is not necessary for growth in standard medium, but this is also true for *Escherichia coli*, where GSH is thought to play a role in protecting against thiol-reactive toxins (1). When growth was challenged with the thiol-oxidizing agent diamide, conditions leading to accumulation of GSH did protect against growth inhibition. Thus, the accumulation of GSH in *S. mutans* may contribute to its ability to withstand oxidative challenge.

Import of GSSG was as efficient in *S. mutans* ATCC 25275 as in strain 33402 (Table 2). However, *S. agalactiae* ATCC 12927, *S. pyogenes* ATCC 8668, and *E. faecalis* ATCC 29212 all had markedly lower or negligible rates of glutathione uptake (Table 2). The latter group, unlike *S. mutans*, appears to be able to synthesize GSH, and so uptake from the growth medium would not be required to attain the protection against oxidants afforded by GSH.

It seems clear that the uptake and metabolism of glutathione in *S. mutans* is a complex process involving multiple metabolic pathways, at least some of which are modified by the incubation conditions. Further elaboration of the specific processes involved and elucidation of the factors which regulate glutathione metabolism in streptococci are topics for further study.

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## REFERENCES

1. **Apontoweil, P., and W. Berends.** 1975. Isolation and initial characterization of glutathione-deficient mutants of *Escherichia coli* K 12. Biochim. Biophys. Acta **399:**10–22.
2. **Carlsson, J. T., J. T. Larsen, and M.-B. Edlund.** 1993. *Peptostreptococcus micros* has a uniquely high capacity to form hydrogen sulfide from glutathione. Oral Microbiol. Immunol. **8:**42–45.
3. **delCardayré, S. B., K. P. Stock, G. L. Newton, R. C. Fahey, and J. E. Davies.** 1997. Coenzyme A disulfide reductase: purification and characterization of the enzyme component of the primary thiol/disulfide redox system in *Staphylococcus aureus*. J. Biol. Chem., in press.
4. **Fahey, R. C., W. C. Brown, W. B. Adams, and M. B. Worsham.** 1978. Occurrence of glutathione in bacteria. J. Bacteriol. **133:**1126–1129.
5. **Fahey, R. C., and G. L. Newton.** 1987. Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. Methods Enzymol. **143:**85–96.
6. **Foucud, C., E. R. S. Kunji, A. Hagting, J. Richard, W. N. Konings, M. Desmazeaud, and B. Poolman.** 1995. Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- and tripeptides. J. Bacteriol. **177:**4652–4657.
7. **Higgins, C. F., and M. M. Gibson.** 1986. Peptide transport in bacteria. Methods Enzymol. **125:**365–377.
8. **Kosower, N. S., and E. M. Kosower.** 1995. Diamide: an oxidant probe for thiols. Methods Enzymol. **251:**123–133.
9. **Kumaresan, K. R., S. S. Springhorn, and S. A. Lacks.** 1995. Lethal and mutagenic actions of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine potentiated by oxidized glutathione, a seemingly harmless substance in the cellular environment. J. Bacteriol. **177:**3641–3646.
10. **Newton, G. L., K. Arnold, M. S. Price, C. Sherrill, S. B. delCardayre, Y. Aharonowitz, G. Cohen, J. Davies, R. C. Fahey, and C. Davis.** 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J. Bacteriol. **178:**1990–1995.
11. **Newton, G. L., C. A. Bewley, T. J. Dwyer, R. Horn, Y. Aharonowitz, G. Cohen, J. Davies, D. J. Faulkner, and R. C. Fahey.** 1995. The structure of U17 isolated from *Streptomyces clavuligerus* and its properties as an antioxidant thiol. Eur. J. Biochem. **230:**821–825.
12. **Newton, G. L., and R. C. Fahey.** 1995. Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. Methods Enzymol. **251:**148–166.
13. **Payne, J. W., G. M. Payne, and T. M. Nisbet.** 1982. An anionic peptide transport system in *Streptococcus faecalis*. FEMS Microbiol. Lett. **14:**123–127.
14. **Payne, J. W., and M. W. Smith.** 1994. Peptide transport by micro-organisms. Adv. Microb. Physiol. **36:**1–80.
15. **Sundquist, A. R., and R. C. Fahey.** 1989. The function of gamma-glutamylcystine and bis-gamma-glutamylcystine reductase in Halobacterium halobium. J. Biol. Chem. **264:**719–725.
16. **Thomas, E. L.** 1984. Disulfide reduction and sulfhydryl uptake by *Streptococcus mutans*. J. Bacteriol. **157:**240–246.
17. **Wiederholt, K. M., and J. L. Steele.** 1994. Glutathione accumulation in lactococci. J. Dairy Sci. **77:**1183–1188.