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Occurrence of Glutathione in Bacteria

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Glutathione and soluble thiol content were examined in a broad spectrum of bacteria. Significant soluble thiol was present in all cases. The thiol compound was glutathione in most of the gram-negative bacteria but not in most of the gram-positive bacteria studied. Glutathione was absent in four anerobes and one microaerophile but was present in a blue-green bacterium. The glutathione content of *Escherichia coli* increased significantly during transition from exponential to stationary phase.

Glutathione has long been thought to occur in all living cells (4, 14, 17, 19, 26), but the fundamental role of this compound has not been clearly identified. The recent isolation of *Escherichia coli* mutants defective in glutathione biosynthesis (2, 15) represents an important advance in the effort to identify the function of glutathione in cells. While attempting to determine whether changes in the glutathione thiol-disulfide status analogous to those observed with *Neurospora crassa* (13) also occur in bacteria, we were surprised to find that glutathione is not detectable in *Bacillus cereus* T. To establish the generality of this phenomenon we undertook a survey of the occurrence of glutathione in bacteria.

MATERIALS AND METHODS

Unless otherwise indicated, cells were grown at 37°C in Trypticase soy broth (BBL), except that *Streptomyces griseus* (from E. Bird) was grown in this medium at 30°C. V. Gage furnished cultures of *Bacillus subtilis trpC2, Enterobacter aerogenes* 62-1, *Micrococcus luteus* (*lysodeikticus*), *Staphylococcus aureus* b1-01, *Streptococcus agalactiae* ATCC 12927, and *Lactobacillus casei* subsp. *rhamnosus*. The latter organism was grown at 37°C on 3% (wt/vol) lactobacillus broth AOAC from Difco. Cultures of *Beneckea* (*Vibrio*) *alginolytica* 86 (4), *Beneckea* (*Pseudomonas*) *natriegens* 108 (5), and *Photobacterium vibriofitscheri* were provided by P. Baumann via K. Nealson, and these three organisms were grown at 27°C in a medium containing 1% (wt/vol) yeast extract (Difco), 1% (wt/vol) peptone (Difco), and 0.5% (wt/vol) glucose in sterile seawater. *E. coli* W3110 was supplied by J. Abelson and was grown aerobically at 37°C on M9 medium (21) or anerobically under a 95% N_2-5% CO_2 atmosphere at 25°C in the basal medium of Lester and DeMoss (18). Frozen cells and fresh cultures (11) of *Pseudomonas fluorescens* ATCC 25289 were furnished by R. Doolittle. *Nostoc muscorum* 7119 cultures were grown under nitrogen-fixing conditions (25) and were furnished by N. Weare. *B. cereus* T was obtained from T. Hashimoto and was grown on Trypticase soy broth or on modified G medium (16). Spores of *B. cereus* were prepared from cells grown in modified G medium and were purified by the method of Sacks and Alderton (23). *Acinetobacter calcoaceticus* BD413 was obtained from E. Juni, and *Chromobacterium violaceum* was obtained from E. Crawford. *Alcaligenes faecalis, Micrococcus corallinus, Micrococcus roseus, Serratia marcescens, Staphylococcus epidermidis, Streptococcus lactis,* and *Streptococcus salivarius* were obtained from Carolina Biological Supply. *Chromatium vinosum* was grown (6) and supplied by T. Kakuno. Cell growth was monitored by measurement of the absorbance at 650 nm or turbidimetrically with a Klett-Summerson photoelectric colorimeter, using a no. 66 filter. In general, cultures were harvested by centrifugation at an absorbancy at 650 nm of 1.0 to 1.5 or at approximately 100 Klett units and were extracted without washing. A number of bacteria were obtained as frozen cell samples. These included: *Myxococcus xanthus* MD-1 (22), furnished by M. Dworkin; *Azotobacter vinelandii* OP, provided by C. McKenna; *Clostridium pasteurianum* and *Clostridium kluyveri,* from G. Novelli; *Desulfovibrio vulgaris* NCIB 8303 (10) and *Rhodopseudomonas sphaeroides* (9), supplied by R. Bartsch. Some bacteria containing glutathione exhibited a declining level upon being stored frozen in excess of 1 year, but in no case was glutathione completely lost.

Extracts were prepared in boiling 80% ethanol and assayed for reduced and oxidized glutathione (GSH and GSSG, respectively) with a cycling enzyme assay, utilizing glutathione reductase as previously described (13). The thiol content of the ethanol extract was determined by titration with Ellman's reagent (12). The residue from the ethanol extraction was dried at 100°C and weighed; all results are expressed as a ratio to this residual dry weight. Failure to detect glutathione cannot result from inhibition of the glutathione assay, since each assay was internally calibrated with authentic glutathione. Good recovery was obtained when authentic glutathione was added to *B. subtilis* before extraction, so that modification or degradation of glutathione during extraction appears unlikely. Since glutathione reductase is quite specific (27), ap-

proximate equivalence of the glutathione and thiol content of extracts is strong evidence for the presence of glutathione. However, where the thiol content greatly exceeds the glutathione content, the possibility that some thiol other than GSH is giving a low but finite activity in the glutathione assay cannot be rigorously excluded. Finally, when low glutathione content was found for bacteria grown in media containing glutathione the possibility that glutathione was derived from the medium must be considered. As much as 0.1 μmol of glutathione per g was found associated with *B. cereus* grown in medium containing 33 nmol of added GSH or 16 nmol of added GSSG per ml.

RESULTS

Glutathione was detected in only a few of the gram-positive bacteria studied (Table 1) but was found in most of the gram-negative bacteria examined (Table 2). The low level found for *L. casei* can be attributed to glutathione from the growth medium, which had a total content of 40 nmol/ml. On the other hand, *S. agalactiae* and *S. lactis* definitely appear to produce glutathione, since the total found associated with the cells was much greater than the total available in the growth medium. The presence of glutathione was considered uncertain in one gram-positive species (*S. aureus*) and in four gram-negative species (*B. alginolytica, B. natriegens, S. marcescens,* and *M. xanthus*), because the thiol content significantly exceeded the glutathione content.

The glutathione content was assayed in *C. vinosum* (≤0.2 μmol/g), in *R. sphaeroides* (≤0.1 μmol/g), and in the blue-green bacterium *N. muscorum* (2.5 μmol/g). Only the latter can be clearly identified as making glutathione.

TABLE 1. *Total glutathione content and total thiol content for gram-positive bacteria*

Species	GSH + ½GSSG (μmol/g)	Total thiol (μmol/g)
Anaerobic and microaerophilic bacteria		
Clostridium pasteurianum	≤0.01	>1[a]
Clostridium kluyveri	≤0.01	>1[a]
Lactobacillus casei	0.05	2
Facultative anaerobes		
Bacillus cereus	≤0.01	2
Bacillus cereus (spores)	≤0.02	
Bacillus subtilis	≤0.01	3
Staphylococcus aureus	0.5 ± 0.2	3
Staphylococcus epidermidis	≤0.02	2
Streptococcus agalactiae	3.2 ± 0.5	2-4
Streptococcus lactis	4.6 ± 0.4	5
Streptococcus salivarius	≤0.03	3
Aerobes		
Streptomyces griseus	≤0.02	2-3
Micrococcus corallinus	≤0.02	3
Micrococcus luteus	≤0.02	3
Micrococcus roseus	≤0.01	2-3

[a] Values declined during storage of the frozen cells, so only a lower limit is given.

TABLE 2. *Total glutathione content and total thiol content for gram-negative bacteria*

Species	GSH + ½GSSG (μmol/g)	Total thiol (μmol/g)
Anaerobes		
Desulfovibrio vulgaris	≤0.02	2
Facultative anaerobes		
Beneckea alginolytica	1.5 ± 0.5	6
Beneckea natriegens	0.7 ± 0.2	5
Serratia marcescens	0.2 ± 0.1	2
Enterobacter aerogenes	1.2 ± 0.4	1.5
Chromobacter violaceum	3.0 ± 1	3
Photobacterium vibriofitscheri	5.2 ± 0.4	7
Escherichia coli	27.0 ± 2	30
Escherichia coli (anaerobically grown)	7.0 ± 1	5-7
Aerobes		
Alcaligenes faecalis	25.0 ± 5	23
Myxococcus xanthus	0.8 ± 0.2	2-3
Pseudomonas fluorescens	1.6 ± 0.2	1.3
Acinetobacter calcoaceticus	6.0 ± 1	5-7
Azotobacter vinelandii	8.0 ± 1	5-7

The presence or absence of glutathione in bacteria does not seem to depend upon the growth conditions or the stage of harvesting. Glutathione was present in *E. coli* grown aerobically or anaerobically, although the level was somewhat lower in the latter case (Table 2). Glutathione was present at all stages of growth in *E. coli,* but the level was found to increase in stationary phase as observed previously (1). Similarly, no substantial glutathione was found in *B. cereus* harvested in exponential phase or early in stationary phase. Nor was there significant glutathione in these cells when grown in Trypticase soy broth medium, containing traces of glutathione, or in modified G medium, lacking glutathione.

DISCUSSION

The present survey represents the most comprehensive effort to date to establish the distribution of glutathione in bacteria. A brief note by Miller and Stone (20) describes the only other attempt to survey a rather wide spectrum of bacteria. They determined the soluble thiol content as a measure of the glutathione content. It is evident from the present results that this is not a generally valid approach. However, it is interesting that they failed to detect thiol in cocci and bacilli.

In a recent study of sporulation in *B. cereus,* Cheng et al. (7) found that glutathione associated with the vegetative cells disappeared during spore formation. The glutathione was considered an important source of the cysteine incorporated

into spore coat protein (3). The present results indicate that this glutathione is not produced by the *B. cereus* cells. Since the media used contained yeast extract, which is ordinarily rich in glutathione, it is possible that the glutathione found under these conditions was derived from the medium (A. I. Aronson, personal communication).

Another recent study reported the purification of glutathione reductase from *C. vinosum* (8). This appears to be in conflict with the present failure to find significant glutathione levels in this organism. The reductase had a K_m for GSSG of 7×10^{-3} M, a value two orders of magnitude greater than that found for other glutathione reductases (29). Moreover, this value is greater than the GSH content of most cells. Since GSSG must be present in the cell in much lower levels than GSH, it seems highly improbable that GSSG is the natural substrate for this enzyme. It is more probable that another disulfide having a higher affinity for the enzyme is the normal substrate. If this prediction proves correct, the structure of this disulfide will be of considerable interest. Tests indicate that it cannot be cystine, oxidized lipoic acid, or oxidized lipoamide (8).

The present findings support and extend conclusions about the role of glutathione derived from the studies of *E. coli* mutants blocked in glutathione synthesis (2, 15). Since a wide variety of bacteria lack glutathione, it is clear that this tripeptide is not specifically required for essential processes such as protein synthesis, nucleic acid synthesis, fatty acid synthesis, fermentative metabolism, or amino acid transport. Apontoweil and Berends (2) compared the effects of a wide range of chemical agents on the glutathione-defective mutants of *E. coli* and on their parent strains. They found generally that the mutants were more sensitive. These observations support the view that glutathione, although not essential for laboratory growth, confers protection against chemical challenge.

An important aspect of the present results is the finding that all bacteria examined had significant levels of soluble thiol. The structure and function of such thiols in bacteria lacking glutathione is an important subject for further study. Setlow and Setlow (24) have found coenzyme A to be present at 1.45 μmol/g in *B. megaterium* during exponential growth, so that coenzyme A is a major contributor to the thiol pool. They have also shown that little coenzyme A is in the disulfide form in vegetative cells but most of it is in the disulfide form in the spores. Thus, whereas sporeforming bacteria appear to lack glutathione, other soluble thiols such as coenzyme A may undergo thiol-disulfide changes upon transition to and from the dormant state, similar to the changes in glutathione found with *N. crassa* (13).

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