Distribution of Thiols in Microorganisms: Mycothiol Is a Major Thiol in Most Actinomycetes

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Mycothiol [2-(*N*-acetylcysteinyl)amido-2-deoxy- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-*myo*-inositol] (MSH) has recently been identified as a major thiol in a number of actinomycetes (S. Sakuda, Z.-Y. Zhou, and Y. Yamada, *Biosci. Biotech. Biochem.* 58:1347–1348, 1994; H. S. C. Spies and D. J. Steenkamp, *Eur. J. Biochem.* 224:203–213, 1994; and G. L. Newton, C. A. Bewley, T. J. Dwyer, R. Horn, Y. Aharonowitz, G. Cohen, J. Davies, D. J. Faulkner, and R. C. Fahey, *Eur. J. Biochem.* 230:821–825, 1995). Since this novel thiol is more resistant than glutathione to heavy-metal ion-catalyzed oxidation, it seems likely to be the antioxidant thiol used by aerobic gram-positive bacteria that do not produce glutathione (GSH). In the present study we sought to define the spectrum of organisms that produce MSH. GSH was absent in all actinomycetes and some of the other gram-positive bacteria studied. Surprisingly, the streptococci and enterococci contained GSH, and some strains appeared to synthesize it rather than import it from the growth medium. MSH was found at significant levels in most actinomycetes examined. Among the actinobacteria four *Micrococcus* species produced MSH, but MSH was not found in representatives of the *Arthrobacter*, *Agromyces*, or *Actinomyces* genera. Of the nocardioforms examined, *Nocardia*, *Rhodococcus*, and *Mycobacteria* spp. all produced MSH. In addition to the established production of MSH by streptomycetes, we found that *Micromonospora*, *Actinomadura*, and *Nocardia* spp. also synthesized MSH. Mycothiol production was not detected in *Propionibacterium acnes* or in representative species of the *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus*, and *Clostridium* genera. Examination of representatives of the cyanobacteria, purple bacteria, and spirochetes also gave negative results, as did tests of rat liver, bonito, *Candida albicans*, *Neurospora crassa*, and spinach leaves. The results, which indicate that MSH production is restricted to the actinomycetes, could have significant implications for the detection and treatment of infections with actinomycetes, especially those caused by mycobacteria.

Glutathione (GSH) is considered to play a key role in protecting cells against oxygen toxicity, but its production among prokaryotes appears to be largely restricted to the cyanobacteria and the purple bacteria (4, 5, 17, 19). Most of the bacteria that lack glutathione, including many strictly aerobic species, produce other low-molecular-weight thiols, and we have sought to determine whether one or more of these thiols might play a role analogous to that of GSH. In the halobacteria, an aerobic subgroup of the archaeobacteria, γ -glutamylcysteine was found to be the major low-molecular-weight thiol (20) and appears to function similarly to glutathione (26, 27).

Among gram-positive bacteria, certain streptomycetes produce δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (ACV), a tripeptide similar to glutathione, which serves as an intermediate in penicillin and cephalosporin biosynthesis, but its pattern of production appeared to be inconsistent with a function analogous to GSH (19). However, streptomycetes were found to produce another cysteine derivative in larger amounts than ACV (19). This proved to be 2-(*N*-acetylcysteinyl)amido-2-deoxy- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-*myo*-inositol (15), a compound first extracted and identified from *Streptomyces* sp. strain AJ 9463 as its disulfide by Sakuda and coworkers (23),

who established the absolute stereochemistry as shown in Fig. 1. It was later isolated and identified from *Mycobacterium bovis* by Spies and Steenkamp (25). The latter authors gave it the trivial name mycothiol, for which we propose the abbreviation MSH. An unusual property of MSH is that it undergoes copper ion-catalyzed autoxidation at a markedly slower rate than does GSH which itself autoxidizes much more slowly than cysteine (15). Thus, MSH may have an analogous role to that of GSH in protecting cells against oxidative damage. To assess the distribution of MSH production in nature, we have conducted a survey of gram-positive bacteria, other eubacteria, and several eucaryotes to ascertain their MSH contents. The results reported indicate that MSH production is confined to actinomycetes and show that mycobacteria, including *Mycobacterium tuberculosis*, produce high levels of this unusual thiol.

FIG. 1. Structure of MSH.

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MATERIALS AND METHODS

Materials. Unless otherwise stated, organic reagents and biochemicals were as described elsewhere (7, 18). All inorganic reagents were from Fisher Scientific, except as noted. Trypticase soy broth (TSB) was provided by BBL Laboratories. Todd-Hewitt medium (Difco) was depleted of GSH by addition of type 1 bovine kidney γ -glutamyltranspeptidase (Sigma) to 1 U/ml and incubation at 37°C for 3 h before sterilization by autoclaving.

Bacterial strains and culture conditions. All bacterial strains designated UCSD were obtained as patient isolates from the University of California, San Diego, Medical Center and identified in the Medical Center Diagnostic Microbiology Laboratory by standard diagnostic procedures. (These cultures are available from Charles Davis.) *Enterococcus faecalis* JH2-2 was obtained from Marilyn Roberts, University of Washington, *Staphylococcus aureus* RN450 was obtained from Richard Novick, Public Health Institute, New York, N.Y., and *Micromonospora floridensis* JCM 3265 and *Micromonospora fulvopurpurea* JCM 5696 were obtained from Wang Yue, National University of Singapore. Because it contains very small amounts of GSH, TSB was chosen as the growth medium; aerobic species were incubated in TSB at 37°C, unless otherwise stated. Typically, 1-liter cultures were shaken in 2-liter Erlenmeyer flasks at 220 rpm and 28°C until late log or early stationary phase growth. A stainless steel spring spanning the interior of the flask was often used to disperse cell clumps. Cells were harvested by centrifugation for 15 min at $5,000 \times g$ at 4°C. The unwashed

cells were either extracted immediately or frozen on dry ice and stored at -70°C until they were extracted.

Actinomyces israelii ATCC 10049, *Clostridium perfringens* UCSD 29, and *Propionibacterium acnes* UCSD 28 were cultured in anaerobic jars at 37°C in TSB. *Anabaena* sp. strain ATCC 29151 was grown photoautotrophically as described previously (27). *Agromyces ramosus* ATCC 25173 and *Actinomadura hibisca* ATCC 53557 were cultured in brain heart infusion medium (Difco) at 30 and 26°C, respectively. *Enterococcus faecalis* ATCC 29212 and *Streptococcus mutans* ATCC 33402 were cultured on GSH-depleted Todd-Hewitt medium at 37°C. *Mycobacterium smegmatis* MC²6, a gift from W. Jacobs (Albert Einstein College of Medicine, New York, N.Y.), was cultured at 37°C on Middlebrook 7H9 medium containing 0.05% Tween 80 and 0.4% glucose; all other mycobacteria were cultured on Lowenstein-Jensen (Difco) agar slants at 37°C. *Nocardiosis mutabilis* ATCC 31520 was cultured in malt extract broth (Difco) at 26°C. *Micromonospora carbonacea* ATCC 27114, *Micrococcus agilis* ATCC 966, and *Rhodococcus roseus* ATCC 144 were cultured in TSB at 26°C. *Micrococcus luteus* ATCC 4698 and *Micrococcus kristinae* ATCC 27570 were cultured in TSB at 30°C. Cultures of *Neurospora crassa* bd mycelia, grown on Vogel 2% glucose minimal media for 48 h at room temperature, were kindly provided by S. Brody, University of California, San Diego. Rat liver samples, obtained from three anesthetized Fisher female rats, were stored on ice until extracted (<1 h). Bonito (*Sarda chiliensis*) was taken from local San Diego waters, and the tissues were

TABLE 1. Thiols produced by actinomycetes

Strain	Thiol ($\mu\text{mol/g}$ [residual dry weight])					
	MSH	Cys	GSH	H ₂ S	CoA	Other ^a
Actinobacteria						
<i>Arthrobacter globiformis</i> ATCC 8010	<0.01	0.11	<0.01	0.4	0.34	1U18, 0.28
<i>Micrococcus agilis</i> ATCC 966	0.9	<0.01	<0.01	0.4	0.47	
<i>Micrococcus luteus</i> UCSD 22	3.3	0.11	<0.01	0.5	1.1	
<i>Micrococcus luteus</i> ATCC 4698	3.6	<0.02	<0.02	0.3	1.1	NAC, 0.48
<i>Micrococcus kristinae</i> ATCC 27570	0.60	<0.01	<0.01	0.10	0.50	
<i>Agromyces ramosus</i> ATCC 25173	<0.03	<0.04	<0.03	0.15	0.38	
<i>Actinomyces israelii</i> ATCC 10049	<0.02	0.22	<0.02	0.04	0.17	
<i>Corynebacterium diphtheriae</i> UCSD 21	0.18	0.015	<0.01	0.039	0.19	1U22, 0.14
Nocardioforms						
<i>Nocardia asteroides</i> UCSD 3	3.5	<0.06	<0.05	0.35	0.66	1U15, 0.01
<i>Nocardia brasiliensis</i> UCSD 1	2.8	<0.03	<0.03	0.47	1.0	4'Ppant, 0.03
<i>Rhodococcus roseus</i> ATCC 144	3	0.2	<0.03	0.3	0.15	
<i>Mycobacterium avium</i> NJH9151	2.7	<0.02	<0.02	1.8	0.7	
<i>Mycobacterium avium</i> NJH1854-4	4.6	<0.03	<0.02	1.8	1.0	
<i>Mycobacterium chelonae</i> UCSD 102	6.6	<0.05	<0.02	0.8	5	
<i>Mycobacterium fortuitum</i> UCSD 101	9.4	<0.03	<0.02	0.9	3	
<i>Mycobacterium smegmatis</i> MC ² 6	19	0.09	<0.02	0.9	3	
<i>Mycobacterium tuberculosis</i> ATCC 25618	12	<0.02	<0.02	1.1	3	
<i>Mycobacterium tuberculosis</i> PZA ^R UCSD 100	9.0	<0.02	<0.02	0.66	1.0	
Actinoplanetes						
<i>Micromonospora carbonacea</i> ATCC 27114	1.5	0.20	<0.01	3.8	0.3	TS, 2.0; NAC, 0.4
<i>Micromonospora floridensis</i> JCM 3265	14	0.9	<0.01	2.5	0.91	TS, 1.8; NAC, 2.4
<i>Micromonospora fulvopurpurea</i> JCM 5696	2.4	0.6	<0.01	2.3	0.58	TS, 1.0; NAC, 0.9
<i>Actinomadura hibisca</i> ATCC 53557	1.5	0.43	<0.01	1.0	0.17	NAC, 0.21; 1U16, 2.2
<i>Nocardiosis flava</i> ATCC 29533	2.7	1.1	0.22	0.74	0.39	1U17.5, 0.4; NAC, 0.09; TS, 0.20
<i>Nocardiosis mutabilis</i> ATCC 31520	0.44	0.06	<0.02	0.29	0.9	1U22, 0.45; 1U16, 0.4
Streptomycetes^b						
<i>Streptomyces clavuligerus</i> ATCC 270674	5.4	0.78	<0.01	1.3	0.8	ACV, 0.95; 1U23, 0.6
<i>Streptomyces coelicolor</i> A3(2)	2.8	0.56	<0.02	0.75	0.5	ACV, <0.02
<i>Streptomyces jumonjinensis</i> ATCC 29864	6.7	1.3	<0.09	0.83	1.9	ACV, 0.7; 1U12, 0.4
<i>Streptomyces lactamdurans</i> ATCC 27382	5.9	1.3	<0.08	1.3	1.1	ACV, 0.05; Ergo, 23
<i>Streptomyces lividans</i> 1326	6.6	0.58	<0.06	1.3	1.0	ACV, <0.01; 1U12, 0.5
<i>Propionibacterium acnes</i> UCSD 28	<0.03	0.08	<0.02	0.28	0.4	

^a 1UX, unknown thiol eluted at X min by HPLC method 1; 4'Ppant, 4'-phosphopantetheine; ACV, δ -(L- α -amino-adipyl)-L-cysteiny-D-valine; Ergo, ergothioneine; NAC, N-acetylcysteine; TS, thiosulfate.

^b The data are from reference 19.

kept on dry ice until they were extracted. Spinach leaves (*Spinacia oleracea* [family *Chenopodiaceae*]) were obtained from a local supermarket and were extracted as described below for tissues.

Cell extraction for thiol analysis. The cell extraction procedure is a slight modification of the procedure described by Fahey and Newton (7). Freshly harvested or frozen cells (~200 mg) were transferred to preweighed 1.5-ml microcentrifuge tubes and reweighed to obtain wet weight. One milliliter of warm (60°C) 50% aqueous acetonitrile containing 20 mM Tris-HCl (Sigma) (pH 8.0) and 2 mM monobromobimane (mBB; Molecular Probes) was added to each tube, and the samples were vortexed. Fibrous plant and animal tissues were suspended in extraction buffer using a Tekmar Tisumizer at maximum speed for 1 min. Extracts were maintained in a 60°C water bath for 15 min in the dark and then acidified with 5 μ l of 5 N methanesulfonic acid (Fluka). The protein and cellular debris were pelleted by centrifugation for 10 min in an Eppendorf microcentrifuge. The supernatants were transferred to separate microcentrifuge tubes and stored at -70°C. Supernatants were diluted 2- to 10-fold in aqueous 10 mM methanesulfonic acid prior to injection into the high-performance liquid chromatography (HPLC) column. The amount of thiol in the supernatant fraction is expressed on the basis of dry weight of the residual cell pellet from each extract, which was determined by drying the cell pellet in a vacuum oven (40°C) until a constant weight was obtained. These residual dry weights were found to be 70 to 80% of the dry weight obtained from cells that had not been extracted. Control samples were prepared by substituting 5 mM *N*-ethylmaleimide (Sigma) for mBB in the extraction buffer, incubating for 5 min at 60°C, adding mBB to a final concentration of 2 mM, and incubating for an additional 15 min at 60°C. Further processing of control samples was identical to that of thiol samples. A second control cell sample was also extracted in 50% acetonitrile containing 25 mM methanesulfonic acid as described above. The acid supernatant fraction was analyzed for total thiol by thiol titration (3). This amount of thiol was found to be consistent with the total of the fluorescent HPLC derivatives.

GSH analysis of growth media. Total glutathione was measured in TSB and Todd-Hewitt media by diluting samples 1:2 with aqueous buffer (100 mM Tris-Cl [pH 8.0]) containing 4 mM dithiothreitol and incubating at room temperature for 15 min. For thiol analysis, mBB was added to a final concentration of 5 mM, and the sample was incubated at 60°C for 15 min, after which methanesulfonic acid was added to 100 mM. A separate thiol-blocked control was prepared by addition of *N*-ethylmaleimide to a final concentration of 5 mM, incubation at 60°C for 10 min, and treatment as above with mBB and methanesulfonic acid. The samples were diluted 1:2 with 10 mM methanesulfonic acid for HPLC analysis.

HPLC analysis of thiol-mB derivatives. Duplicate samples of cell extracts were routinely analyzed for thiols IRSH as their biman derivatives (RS-mB) by at least two different HPLC protocols developed in this laboratory. The chromatographic conditions used in these protocols, the sources for reagents, the preparation of thiol-mB standards, and the HPLC equipment used have been described in detail elsewhere (7, 18). Briefly, in HPLC method 1 we used a reversed-phase column with aqueous sodium acetate-methanol gradients to separate most low-molecular-weight thiol derivatives normally encountered in biological extracts but not those of highly charged thiols such as coenzyme A (CoA). To confirm the identity and amounts of thiols found by using method 1, we also used method 2, a tetrabutylammonium phosphate ion pairing protocol designed for the separation of CoA-mB derivatives (7).

Structure of mycothiol from *Mycobacterium smegmatis*. The major low-molecular-weight thiol from *Mycobacterium smegmatis*, whose HPLC retention time was identical to that for MSH, was isolated, converted to the mBB derivative, purified by preparative HPLC, and examined by ¹H and ¹³C nuclear magnetic resonance spectroscopy by methods described previously (15, 16).

RESULTS

Table 1 presents the results of thiol analyses of 23 species of the order *Actinomycetales* included in the present study and also summarizes results published previously (19) for five *Streptomyces* species. The microorganisms tested span the main subgroups of the actinomycete classification as described by Goodfellow (10). Table 1 also provides results obtained with one representative each of the corynebacteria and the propionibacteria. Three of the four genera of actinobacteria tested did not produce MSH, but MSH was found in four *Micrococcus* species. All other actinomycetes tested produced MSH, as did *Corynebacterium diphtheriae*, but no MSH was found in *P. acnes*. Mycobacteria contained the highest level of MSH, and *Mycobacterium smegmatis* was the top producer of all. MSH was previously found by Spies and Steenkamp (25) in *Mycobacterium bovis* and tentatively identified in *Mycobacterium tuberculosis* H37RV, but these investigators did not quantify MSH content. In order to confirm the HPLC assignment for MSH from mycobacteria, we isolated MSH from *Mycobacte-*

FIG. 2. Variation of MSH content with stage of growth of *Mycobacterium smegmatis* in Middlebrook medium at 37°C. Duplicate cultures were evaluated in each of three separate experiments. Open symbols, A_{600} ; closed symbols, MSH content. The data are means with ranges (bars).

rium smegmatis and verified that its structure was identical to that shown in Fig. 1 from its ¹H and ¹³C NMR spectra (15).

The data of Table 1 were obtained with cells grown to late log or early stationary phase. It was previously reported that the MSH (U17) content of *Streptomyces clavuligerus* increased twofold from early log phase to stationary phase (19). For *Mycobacterium smegmatis* a relatively constant level of MSH was found from early-log- to mid-log- to stationary-phase cells (Fig. 2). The reproducibility of the measurements is also illustrated in Fig. 2, which shows the results of analyses of parallel cultures from three separate growth experiments. Analyses of parallel cultures on a given day gave a maximum deviation of $\pm 10\%$ from the mean. The maximum variation for cultures on different days was seen in early log phase, in which values ranged a maximum of $\pm 30\%$ from the mean. The mean and standard deviation for the seven determinations at different growth points shown in Fig. 2 was $19 \pm 3 \mu\text{mol/g}$ (residual dry weight). The data in Tables 1 and 2 are derived from two or more replicate cultures and are expected to be reliable to $\pm 15\%$. However, substantially greater variations with growth phase and growth medium composition are possible for all of the thiols studied.

When present, MSH was usually the predominant thiol, but the level of CoA was frequently only slightly lower. Hydrogen sulfide was measured at appreciable levels in most species and probably reflects sulfide extracted from iron-sulfur proteins rather than free sulfide. Actinoplanetes contained high sulfide levels and significant levels of thiosulfate. Several other thiols were detected at lower levels, including a number of unknown thiols identified by the symbol 1UX, with *X* equal to the retention time of the peak in minutes with HPLC method 1. GSH was undetectable in nearly all species included in Table 1. The only exception was *Norcardiopsis flava*, which appeared to have a low level of GSH.

Our earlier examination of eubacteria other than actinomycetes and of eucaryotes (6, 17) failed to disclose a major biman-labeled thiol with elution time comparable to that of MSH. However, low levels could easily have been overlooked, and we undertook a reexamination of selected organisms to ascertain whether minor amounts of MSH might be present. Table 2 presents the results obtained. The gram-positive clostridiales are the group considered to be most closely related to

TABLE 2. Thiols produced by organisms other than actinomycetes

Organism or tissue	Thiol ($\mu\text{mol/g}$ [residual dry weight])					
	MSH	Cys	GSH	H ₂ S	CoA	Other ^a
Eubacteria						
Gram positive (clostridiales)						
<i>Listeria monocytogenes</i> UCSD 8	<0.02	0.03	0.25	0.05	1.1	1U10, 0.03
<i>Staphylococcus aureus</i> UCSD 2	<0.01	0.02	<0.01	0.15	0.15	
<i>Staphylococcus aureus</i> RN450	<0.02	0.7	<0.02	0.19	1.1	
<i>Streptococcus mutans</i> ATCC 33402	<0.02	0.03	0.13	0.17	0.11	TS, 2.6; 1U15, 0.2
<i>Streptococcus mutans</i> ATCC 25175	<0.02	<0.04	0.9	<0.03	0.15	
<i>Streptococcus agalactiae</i> ATCC 12927	<0.05	<0.08	11	<0.05	2.1	
<i>Streptococcus thermophilus</i> ATCC 19258	<0.01	<0.02	5.7	<0.01	0.5	1U12.5, 1.8
<i>Enterococcus faecalis</i> ATCC 29212	<0.02	0.34	1.8	0.16	0.8	TS, 0.5; 1U13, 0.2
<i>Enterococcus faecalis</i> JH2-2	<0.03	0.04	5.1	0.4	0.15	TS, 0.06; 1U14, 0.6
<i>Enterococcus faecium</i> ATCC 6569	<0.02	0.03	1.6	0.06	0.04	TS, 0.08; 1U9, 1.4; 1U14, 0.2
<i>Bacillus subtilis</i> UCSD 11	<0.02	0.59	<0.02	0.08	0.19	
<i>Clostridium perfringens</i> UCSD 29	<0.01	0.03	0.24	2.2	0.21	1U24, 0.22
Gram negative and others						
<i>Anabaena</i> sp. strain ATCC 29151	<0.03	0.9	0.9	1.3	<0.02	
<i>Escherichia coli</i> UCSD 25	<0.01	0.02	4.5	0.01	0.6	1U12, 0.33; 1U20, 0.14
<i>Pseudomonas aeruginosa</i> UCSD 24	<0.01	0.8	6.3	0.4	1.3	1U12, 0.13
<i>Spirochaeta halophila</i> ^b ATCC 29478	<0.01	<0.1	<0.1	1.6	0.2	
Eucaryotes						
Rat liver	<0.01	0.7–1.3	16–25	0.1–0.14	0.24–0.3	1U6.5, 0.07
Bonito muscle	<0.04	0.13	2.2	1.5	<0.04	TS, 0.3; 1U17.5, 0.5
<i>Candida albicans</i> UCSD 4650A	<0.01	0.12	8	0.15	0.10	1U8.5, 0.08
<i>Neurospora crassa</i> bd mycelia	<0.03	0.29	16	0.32	0.25	1U12, 0.26
Spinach leaves	<0.03	4.4	4.8	0.45	<0.03	

^a See Table 1, footnote a.^b Based on the absence of a peak for mycothiol in the analysis published previously (17).

the actinomycetes (21); however, none of the 12 strains surveyed in this group produced detectable levels of MSH. Representative bacterial species belonging to the cyanobacteria, the purple bacteria, and the spirochetes also lacked MSH, as did typical animal, fungal, and plant samples (Table 2). Thus, it seems reasonably certain that MSH is not produced by organisms other than actinomycetes.

The present results with the clostridiales (Table 2) suggest that some species are able either to synthesize GSH or to import it from the growth medium, whereas others are not. This confirms a similar finding based upon GSH determination by an enzymatic assay (4) and more recently by HPLC (9). To permit assessment of the possible import of GSH, the media used for growth of these bacteria were reduced with dithiothreitol and then analyzed for thiols to measure the total glutathione (GSH plus GSS) present. The total glutathione level in TSB medium proved to be <0.5 μM , and that in GSH-depleted Todd-Hewitt medium was <0.2 μM .

DISCUSSION

The results of our survey of MSH production are summarized in the dendrogram in Fig. 3, which documents an important conclusion of this study: production of MSH is confined to actinomycetes that are members of the high-G+C gram-positive group of bacteria (21). MSH production was found in each of the seven major subgroups of the actinomycetes. Among producers, the level of MSH varied appreciably, from a low value of 0.18 $\mu\text{mol/g}$ in *Corynebacterium diphtheria* (32% of total thiol) to a value 100-fold higher in *Mycobacterium smegmatis* (81% of total thiol). Although the MSH content of *My-*

FIG. 3. Summary of MSH distribution in actinomycetes and other organisms (10). + and -, presence and absence of MSH, respectively. The numbers of strains are indicated in parentheses.

cobacterium smegmatis varied little with growth phase (Fig. 2), this finding cannot be generalized to other organisms or other thiols, which can show more marked variations with growth phase and nutritional status (19). Only one of the thirty organisms in Table 1 produced a measureable level of GSH, and the low value for *Nocardopsis flava* may indicate that this organism is able to import GSH from the medium (see below). Since MSH is even more resistant to chemical oxidation than GSH (15), its widespread occurrence in actinomycetes suggests that it functions as an antioxidant thiol in this group of bacteria.

Three of the four genera of actinobacteria tested did not have measurable levels of MSH. Whether this reflects limited ability to synthesize MSH among actinobacteria or unusual nutritional requirements for its synthesis in some genera is unclear. The finding of *N*-acetylcysteine at significant levels in some actinomycetes may be important because it represents a portion of the MSH molecule (Fig. 1) and could be an intermediate in the biosynthesis or degradation of MSH.

The present finding that some low-G+C gram-positive bacteria, such as streptococci and enterococci, contain GSH, is another interesting result from the present studies. In our first study of bacterial thiols we found that *Streptococcus agalactiae* and *Streptococcus lactis* had significant levels of GSH as measured by an enzymatic assay for GSH whereas *Streptococcus salivarius* did not contain detectable amounts (4). The results in Table 2 confirm that many streptococci and enterococci contain GSH. Some gram-positive bacteria have been shown to have a system for uptake of GSH from the growth medium (12, 28), and such transport might account for low levels of GSH. However, the TSB and GSH-depleted Todd-Hewitt media used for growth of streptococci and enterococci contained <0.5 and <0.2 μ M GSH, respectively, and at harvest the bacterial residual dry weight content was $\sim$ 0.2 g/liter. Thus, the maximum GSH levels achievable by import of all of the medium GSH are 2.5 and 1 μ mol/g (residual dry weight) in the TSB and Todd-Hewitt media, respectively. The values determined for *Streptococcus agalactiae*, *Streptococcus thermophilus*, and *Enterococcus faecalis* JH2-2 grown in TSB medium and for *Enterococcus faecalis* ATCC 29212 grown in Todd-Hewitt medium clearly exceed these limits, implying that these bacteria must have the capacity to synthesize GSH. The synthesis of GSH by some streptococci and enterococci, all low-G+C gram-positive bacteria, is the first clear case of GSH production by prokaryotes other than cyanobacteria or purple bacteria (17). The fact that some clostridiales have the ability to synthesize GSH whereas others do not suggests that the genes for GSH metabolism may have been acquired late in the evolutionary process, perhaps by horizontal gene transfer (13, 14, 24) from a host with GSH synthesis capacity colonized by these bacteria. It is pertinent to note that a GSH reductase activity has been found in extracts of *Streptococcus thermophilus* and a disulfide reductase gene from this organism has been shown to have substantial sequence identity to the GSH reductase from *Escherichia coli* (22).

Listeria monocytogenes and *Clostridium perfringens* also contained low levels of GSH. Whether this reflects their ability to make small amounts of GSH or to concentrate glutathione from the small pool present in the growth medium is unclear from the present results. Previous determinations failed to detect GSH in *Clostridium pasteurianum* (4, 6) and *Clostridium kluyveri* (4).

Thus, while GSH is clearly not made by actinomycetes, most of which make MSH, the ability to synthesize GSH appears to have been acquired by some members of the clostridiales, which do not make MSH. No organism was found to make both GSH and MSH in significant amounts. This is consistent

with the view that GSH and MSH serve as oxidation-resistant storage forms of cysteine (8, 15, 19) since synthesis of both thiols would be redundant. The potential importance of MSH in actinomycetes makes elaboration of its biosynthetic pathway and its role as a cofactor in enzymatic reactions important areas for further study.

The high level of MSH production by mycobacteria and its absence from eucaryotes has potential significance for detection and treatment of infection by mycobacteria. Thus, a sensitive assay for MSH might be used as a means of detecting the presence of tuberculosis and other mycobacterial infections. Such an assay could be an extremely important addition to our diagnostic armamentarium since the World Health Organization estimates that at least 8,000,000 new infections and 3,000,000 deaths per year are directly attributable to tuberculosis (11), making it the leading cause of death caused by a single infectious agent. Furthermore, the number of cases of tuberculosis in the United States increased more than 18% from 1985 to 1991. The Centers for Disease Control and Prevention calculated that this increase represented 39,000 infections in excess of the number expected on the basis of the pre-1985 downward trend (2). Although an assay for MSH would not be specific for mycobacteria, only a few other microorganisms that infect people make MSH and most are uncommon and cause completely different clinical syndromes (e.g., *Corynebacterium diphtheriae*). Only pulmonary nocardiosis caused by *Nocardia asteroides*, or less commonly by *Nocardia brasiliensis* and other *Nocardia* species, would be a likely cause of confusion, and infections with these actinomycetes are much less common than tuberculosis (1). An even more important aspect of MSH biochemistry could lie in the identification of new targets for antibiotic development. If MSH does play a role analogous to GSH and protects mycobacteria and other actinomycetes against oxygen toxicity, it may be possible to develop antibiotics which function by blocking MSH action but have no adverse effect upon infected animals which lack MSH.

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