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Low-Molecular-Weight Thiols in Streptomycetes and Their Potential Role as Antioxidants

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The intracellular low-molecular-weight thiols present in five gram-positive *Streptomyces* species and one *Flavobacterium* species were analyzed by high-performance liquid chromatography after fluorescence labeling with monobromobimane. Bacteria were chosen to include penicillin and cephalosporin β-lactam producers and nonproducers. No significant amount of glutathione was found in any of the streptomycetes. Major intracellular thiols in all strains examined were cysteine, coenzyme A, sulfide, thiosulfate, and an unknown thiol designated U17. Those streptomycetes that make β-lactam antibiotics also produce significant amounts of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), a key intermediate in their biosynthesis. In *Streptomyces clavuligerus*, a potent producer of β-lactams, the level of ACV was low during the early phase of growth and increased rapidly toward the end of exponential growth, paralleling that of antibiotic production. These and other observations indicate that ACV does not function as a protective thiol in streptomycetes. U17 may have this role since it was the major thiol in all streptomycetes and appeared to occur at levels about 10-fold higher than those of the other thiols measured, including ACV. Purification and amino acid analysis of U17 indicated that it contains cysteine and an unusual amine that is not one of the common amino acids. This thiol is identical to an unknown thiol found previously in *Micrococcus roseus* and *Streptomyces griseus*. A high level of ergothioneine was found in *Streptomyces lactamdurans*, and several unidentified thiols were detected in this and other streptomycetes.

Numerous species of *Streptomyces* and one known species of *Nocardia* synthesize sulfur-containing penicillin, cephalosporin, and cephamycin antibiotics (2). Early investigators of the biosyntheses of these β-lactam compounds noticed the close structural relationship (28) between a key intermediate in the pathway, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) and reduced glutathione, γ-L-glutamyl-L-cysteinyl-glycine (GSH) (Fig. 1). GSH is the predominant thiol in eucaryotic organisms, in which it plays an important role in protecting cells against oxygen toxicity (17). It is found in most gram-negative bacteria but has not been detected in several major classes of gram-positive bacteria (13, 14, 26). Conflicting results were reported for the presence of GSH in streptomycetes. In one study, GSH could not be detected in cell extracts of *Streptomyces griseus* (14). In another study, low levels of GSH were found in *Streptomyces lactamdurans* (syn. *Nocardia lactamdurans*) (9). One purpose of the present study was to establish whether streptomycetes are able to produce GSH. Another was to determine whether ACV or other low-molecular-weight thiols play an antioxidant role in streptomycetes similar to that of GSH in other organisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 1326 were obtained from D. A. Hopwood, Norwich, United Kingdom (10). These species, along with *Streptomyces clavuligerus* ATCC 27064 (DSM 738), *S. lactamdurans* ATCC 27382 (NRRL 3802), *Streptomyces jumonjinensis* ATCC 29864, and *Flavobacterium* sp. strain SC 12.154 (Squibb) (30) were maintained on 2% Bacto agar (Difco) containing 1% glycerol (Fisher), 0.5% yeast extract (Difco), and 0.5% malt extract (Difco). Trypticase soy broth (TSB; BBL Becton Dickinson Microbiology Systems) was chosen for growth of these strains in liquid culture since it contains very small amounts of GSH (see Results). Starter cultures were prepared by removing cells from agar plates and culturing them at 28°C in medium containing 3% TSB supplemented with 1% glycerol. Bulk cultures were inoculated with 1% of the starter in 500 ml of TSB medium without glycerol (except for the *Flavobacterium* species, for which 1% glycerol was used) and were shaken in 2-liter Erlenmeyer flasks at 200 rpm and 28°C until the desired cell density was reached. Certain species of *Streptomyces*, particularly *S. coelicolor*, tend to clump during culture. To disperse the cell clumps, 100 ml of TSB medium inoculated with *S. coelicolor* was cultured in a 250-ml Erlyenmeyer flask containing a stainless steel spring spanning the interior of the flask. Even with this precaution, it was often impossible to obtain adequate dispersion of the cells to obtain a meaningful A_{650} measurement (see Table 1). Cells were harvested by centrifugation at 4°C for 15 min at 5,000 × *g*. The unwashed cells were either extracted immediately or frozen on dry ice and stored at −70°C until they were extracted. Samples of conditioned medium were similarly frozen and stored until the samples were prepared for analysis.

Cell extraction for thiol analysis. The cell extraction procedure was a slight modification of the procedure described by Fahey and Newton (15). Frozen cells (200 to 400 mg) were transferred to preweighed screw-top 3-ml glass vials and reweighed to obtain the wet weight. One milliliter of warm (60°C) 50% aqueous acetonitrile containing 20 mM Tris-HCl (Sigma) (pH 8.0) and 2 mM monobromobimane (mBBr; Calbiochem) was added to the vial, and the sample was sonicated with a Branson sonicator equipped with a microtip for ~20 s at 60°C. The extract was maintained in a 60°C water bath for 15 min in the dark and then acidified by

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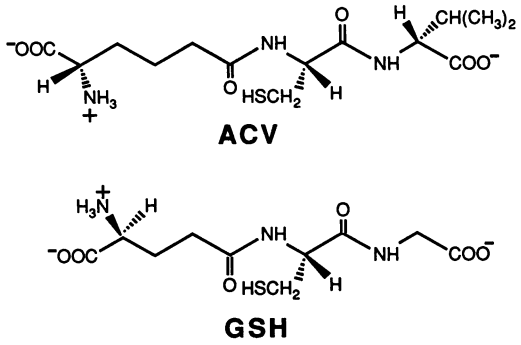

FIG. 1. Structures of ACV and GSH.

using 50 μl of 5 N methanesulfonic acid (Fluka). The entire sample was transferred to a preweighed 1.5-ml microcentrifuge tube, and the protein and cellular debris were pelleted by centrifugation (14,000 × *g*) for 10 min in an Eppendorf microcentrifuge. The supernatant was transferred to a separate microcentrifuge tube for storage at −70°C. The supernatant was diluted 4- to 100-fold in aqueous 10 mM methanesulfonic acid prior to injection onto the high-performance liquid chromatography (HPLC) column. The amount of thiol found in the supernatant fraction was based on the residual dry weight of the cell pellet from that extract. The residual dry weight was determined by drying the cell pellet in a vacuum oven (40°C) until a constant weight was obtained. The residual dry weight for *S. lividans* was found to be 20 to 40 mg or 70% ± 11% ($n = 8$) of the total dry weight of a cell pellet. The total dry weight was obtained by drying a cell pellet of equal wet weight without prior extraction with warm 50% acetonitrile. Control samples were prepared by substituting 5 mM *N*-ethylmaleimide (NEM; Sigma) for the mBBr in the extraction buffer; the extract was incubated for 5 min at 60°C, mBBr was added to a final concentration of 2 mM, and the extract was incubated for an additional 15 min at 60°C. Further processing of control samples was identical to that of thiol samples.

Preparation of medium for thiol and disulfide analysis. Conditioned medium for *S. clavuligerus* was analyzed for thiols by reacting the medium with 5 mM mBBr in 50 mM Tris-HCl (pH 8.0) as described for tissue culture medium by Calabro-Jones et al. (7). The medium was also analyzed for disulfides plus thiols as described by Calabro-Jones et al. (7) for disulfides, except that the pretreatment of the medium with NEM was omitted. Briefly, the medium was incubated with 10 mM dithiothreitol (DTT) (high purity; Calbiochem) in 50 mM Tris-HCl (pH 8.0) and subsequently reacted with 27 mM mBBr. The labeled medium was mixed with one volume of 4 M sodium methanesulfonate containing 0.2 M methanesulfonic acid, and proteins were removed by centrifugation (14,000 × *g*) for 5 min in an Eppendorf microcentrifuge. The hydrophobic bimane derivatives were removed by extracting the acidified samples two times with an equal volume of dichloromethane. Recovery of cysteine, GSH, and ACV was determined by the addition of the thiols to a concentration of 10 μM in the medium prior to the addition of mBBr for thiol samples or DTT for disulfide plus thiol samples. The samples were analyzed by the same HPLC protocols described below for cell extracts.

HPLC analysis of thiol-mb derivatives. Cell extracts were routinely analyzed for thiols as their bimane 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 (15). Briefly, for HPLC method 1, a reversed-phase column was used 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 identities 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 (15). In this study, the major thiol in the streptomycetes coeluted with a reagent peak at 17 min by using method 1, so we were unable to obtain a quantitative analysis of the unknown. U17 did elute at a unique time by using method 2 chromatography, and quantitative data were easily obtained. To obtain a second analysis for U17, we utilized method 3 (15), a perchlorate ion-pairing protocol designed for aminothiols, as modified by Smoluk et al. (33), to analyze all extracts containing U17. The use of these three programs provided independent analysis by at least two HPLC protocols for all thiols found in this study with the exception of CoA and ergothioneine, which could be adequately measured by only one of these methods.

The best analysis for ACV (kindly provided by Gistbrocades, Delft, Holland) was obtained by HPLC method 1, with which the bimane derivative eluted at 35 min, well separated from other thiol and background peaks. ACV-mb could also be quantitated by using method 2 (retention time, 18 min), although some minor nonthiol background peaks were occasionally found in the NEM control samples at this retention time. Method 3 was not useful for quantitative analysis of ACV-mb as the derivative eluted during the column regeneration portion of the protocol when the baseline was irregular (retention time, 25 min). When δ-(L-α-aminoadipyl)-L-cysteine (AC; generously furnished by Gistbrocades) was converted to AC-mb and analyzed by HPLC, it was found to coelute with GSH by all three HPLC protocols. Only background level peaks were observed at the retention time of GSH and AC, and these established an upper limit for the amounts of these thiols in extracts obtained from streptomycetes.

Purification of U17 from streptomycetes. Purification of the mBBr derivative of the unknown thiol U17 was undertaken by using a large scale version of the method of Newton and Fahey (25). Available samples of stationary phase cells from *S. clavuligerus* (25.6 g [wet weight]) and *S. lividans* (9.4 g [wet weight]) were combined to obtain a single cell pellet rich in U17. This was extracted in 250 ml of 50% aqueous acetonitrile containing 25 mM methanesulfonic acid (60°C). The 250-ml acid extract was neutralized with 250 ml of 200 mM Tris-HCl–5 mM diethylenetriaminepentaacetic acid (Sigma)–1 mM DTT (pH 8.0). This extract was loaded at 3 ml/min onto a column (25 by 70 mm) of 2-mercaptopyridine thiol-activated thiol propyl Sepharose 6B, prepared as described by Axén et al. (5) as modified by Newton and Fahey (25). The binding capacity of the thiol affinity column was approximately 1 mmol of total thiol. The column was washed with 350 ml of 25% acetonitrile in 100 mM Tris-HCl (pH 8) and then with 350 ml of aqueous 50 mM Tris-HCl (pH 8). The bound thiols were eluted with 210 ml of 3 mM DTT in 50 mM Tris-HCl (pH 8.0). The eluent contained 0.29 mmol of total thiol and was derivatized with 0.37 mmol of mBBr for 30 min at room temperature in the dark. The eluent was washed three times with 100 ml of dichloromethane in a separatory

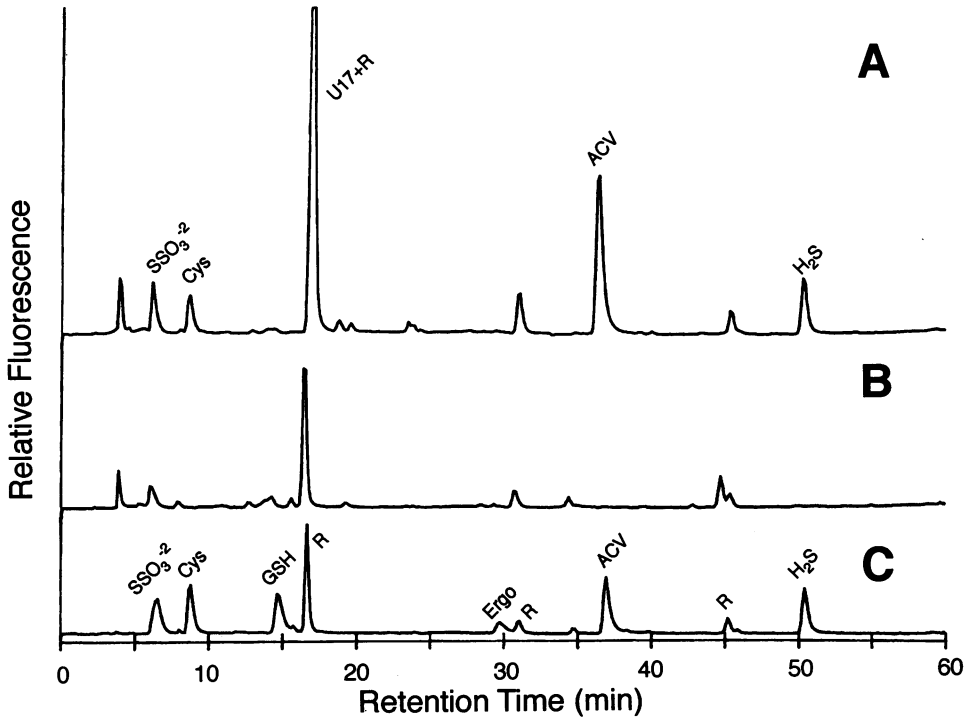

FIG. 2. HPLC chromatograms obtained by using method 1 and cell samples prepared from 19-h cultures of *S. clavuligerus*. (A) Cell extracts derivatized directly with mBBr to convert all thiols to fluorescent thiol-mb derivatives; (B) cell extracts reacted with NEM to block thiols prior to reaction with mBBr in order to identify fluorescent nonthiol components; (C) a known mixture of thiol-mb standards containing 100 pmol of each derivative. Ergo, ergothioneine; SSO_3^{2-}, thiosulfate; H_2S, inorganic sulfide; R, mBBr-derived reagent peak; U17, unknown thiol eluting at 17 min.

funnel to remove unreacted mBBr and the bimane derivatives of 2-mercaptopyridine and DTT. The aqueous phase was reduced to dryness by lyophilization, and the dried residue was redissolved in 1 N HCl.

The unknown, U17-mb, was separated from other thiol derivatives by preparative HPLC on a Vydac C_{18} semipreparative column (10 by 250 mm; catalog no. 218TP152022). The column was operated at ambient temperature and a flow rate of 4.2 ml/min, and the eluent was monitored by fluorescence. The elution buffers were aqueous 10 mM HCl (A buffer) and Fisher HPLC-grade methanol (B buffer), and a gradient of 15 to 30% B buffer over 30 min was used to separate the thiol derivatives. U17-mb eluted at 33 min and was manually collected. The HPLC solvent was removed by lyophilization, and the sample was resuspended in water for structural studies. HPLC analysis of purified U17-mb showed a single, symmetrical peak with HPLC methods 1 and 2 (results not shown). This material was assayed found to be >95% pure on the basis of the fluorescence.

Amino acid analysis of U17. Two samples were subjected to acid hydrolysis followed by amino acid analysis. The first sample was 300 nmol of the bimane derivative of glutathione (GS-mb) which was hydrolyzed for 40 min in 6 N HCl at 155°C in vacuo. The second sample contained approximately 300 nmol of U17, on the basis of GS-mb fluorescence, to which 150 nmol of the internal standard of norleucine was added. The unknown sample was split into four equal aliquots and hydrolyzed under the same conditions for 0, 20, 40, and 60 min, respectively. After hydrolysis, the samples were desiccated over NaOH in a vacuum desiccator and redissolved in 0.2 M sodium citrate (pH 2.2) for amino acid analysis.

Samples were analyzed by cation-exchange chromatography with postcolumn ninhydrin detection by using a modified Pickering Laboratories (Mountain View, Calif.) amino acid analyzer system. The system consisted of a Spectra Physics model 8700 gradient HPLC and model S200 UV-Vis detector. A Pickering 10-μm-diameter cation-exchange column (3 by 250 mm; catalog no. 1193250) was operated at 50°C with a dual-solvent sodium citrate gradient as recommended by the manufacturer for the 60-min hydrolysate program. The amino acids were measured by using a Pickering Laboratories postcolumn ninhydrin reactor system and visible detection at 570 and 470 nm. The amino acid calibration standards (Beckman Instruments) were run at 2 nmol per amino acid.

RESULTS

Quantitation of ACV and other low-molecular-weight thiols was accomplished by HPLC separation of the mBBr derivatives. Figure 2C illustrates the separation of the ACV derivative from those of other common biological thiols by using HPLC method 1 of Fahey and Newton (15). Figure 3C shows the separation obtained by using HPLC method 2, a procedure that permits determination of CoA content. To examine the thiols present in *S. clavuligerus*, cultures were grown in TSB and harvested in the late exponential growth phase, conditions which support active cephamycin C biosynthesis (31, 37). TSB was selected as the growth medium because it is free of glutathione and therefore eliminates the

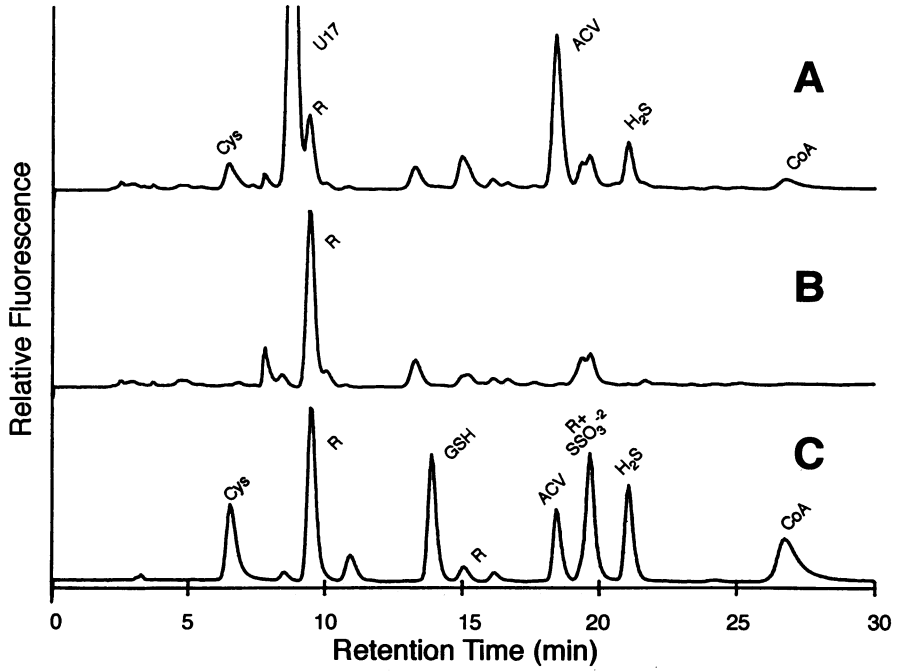

FIG. 3. HPLC chromatograms obtained by using method 2 and cell samples prepared from 24-h cultures of *S. clavuligerus*. (A, B, and C) Samples were prepared as described in the legend to Fig. 2. R, mBBr-derived reagent peak.

possibility that cells could accumulate glutathione from the medium (13). Derivatization and analysis of *S. clavuligerus* samples prepared in this fashion produced the chromatograms shown in Fig. 2A and 3A. The chromatograms from control samples in which the cell extract was reacted with NEM to block thiol groups before derivatization with mBBr are shown in Fig. 2B and 3B. These chromatograms allow identification of fluorescence peaks that are not derived from thiols, but it should be noted that thiosulfate is only partially blocked by NEM. GSH is clearly not present in extracts of *S. clavuligerus* whereas ACV, cysteine, CoA, sulfide, and thiosulfate were present in substantial amounts. Surprisingly, the thiol present in largest amount proved to be an unknown thiol, not corresponding to any of the known biological standards in our possession (15). This was not immediately apparent by using HPLC method 1 since the unknown and a reagent-derived peak nearly coelute. However, treatment of *S. clavuligerus* extracts with NEM greatly reduced the peak at 17 min, indicating that a thiol, designated U17, is present in addition to the reagent-derived component (Fig. 2). Analysis by HPLC method 2 unambiguously demonstrated the presence of a major unknown thiol derivative eluting at 8 min and assumed to be U17 (Fig. 3).

Quantitative data for the thiol levels in *S. clavuligerus* are

TABLE 1. Thiols in β-lactam-producing bacteria and related strains[a]

Organism	β-lactam producer	A_{650}	Thiol content (μmol/g [residual dry weight][b])							Other-content
			Cys	U17	ACV	GSH + AC	SSO_3^{2-}	CoA	H_2S	
S. clavuligerus	+	4.5	0.78	5.4	0.95	<0.01	1.2	0.84	1.3	U23-0.6[c]
S. jumonjinensis	+	2.0	1.3	6.7	0.70	<0.09	0.77	1.9	0.83	U12-0.4[d]
S. lactamdurans	+	1.4	0.93	3.7	0.039	<0.08	0.85	0.52	0.84	Ergo-8.7[e]
		5.3	1.3	5.9	0.050	<0.04	0.40	1.1	1.3	Ergo-23[f]
		6.3[g]	1.1	6.0	0.063	<0.04	0.36	1.1	1.5	Ergo-26[h]
S. lividans	−	3.4	0.58	6.6	<0.01	<0.06	0.36	1.0	1.3	U12-0.5
		6.6	0.11	5.5	<0.01	<0.05	0.34	0.20	0.40	U12-0.09
S. coelicolor	−	48h[g]	0.56	2.8	≤0.02	<0.02	0.52	0.52	0.75	
Flavobacterium sp. strain 12.154	+	0.25	1.0	<0.02	<0.01	4.0	0.11	0.32	0.75	
		0.52	1.7	<0.02	<0.01	6.2	≤0.05	0.33	0.60	
		2.2	1.0	<0.01	<0.01	5.5	≤0.05	0.33	0.59	

[a] Results are the averages of duplicate samples.
[b] Approximately 70% of the total dry weight. If no thiol peak was found, results are listed as "<." If a small peak above the NEM background was found, the results are listed as "≤."
[c] The other unknown in this sample was U12-0.5.
[d] Other unknowns in this sample were U19-0.3 and U23-0.07.
[e] Ergo, ergothioneine. Other unknowns in this sample were U12-0.2, U19-0.7, and U23-0.07.
[f] Other unknowns in this sample were U12-1.8, U19-0.70, and U23-0.26.
[g] A_{650} either approximate or unmeasurable; see Materials and Methods.
[h] Other unknowns in this sample were U12-0.3, U19-1.0, and U23-0.4.

given in Table 1. By using the fluorescence factor for the GS-mb standard, the amount of U17 was calculated to be 5.4 μmol/g (residual dry weight), which corresponds to about 1 mM in hydrated cells. The corresponding concentrations for thiosulfate and sulfide were about 200 μM, while those for Cys, ACV, and CoA were in the 100 to 200 μM range. Two thiol components having retention times of 12 and 23 min using HPLC method 1 were also detected but did not match any of the thiols previously characterized (15). These thiols were designated U12 and U23 and were quantitated assuming that their fluorescence yield was the same as that for the mBBr derivative of GSH. The AC derivative was prepared and examined to test whether either of these unknowns might be AC. However, the retention times for AC-mb were the same as for GS-mb by all three HPLC programs used, showing that neither of the unknowns is AC. Examination of the chromatogram for *S. clavuligerus* revealed that AC is present at less than 0.01 μmol/g (residual dry weight).

Two other antibiotic-producing streptomycetes, *S. jumonjinensis* and *S. lactamdurans*, produce U17, cysteine, CoA, thiosulfate, and sulfide at levels comparable to those for *S. clavuligerus* (Table 1). However, the level of ACV in *S. lactamdurans*, was an order of magnitude lower than that found in *S. clavuligerus* and *S. jumonjinensis*. Thiol U12 was detected at a similar level and U23 at a lower level than that found in *S. clavuligerus*. An additional unidentified thiol, U19, was detected in *S. jumonjinensis* and *S. lactamdurans*. *S. lactamdurans* also produced ergothioneine at quite high levels.

Thiol analysis of two streptomycetes, *S. lividans* and *S. coelicolor*, that do not produce β-lactam antibiotics showed that they do not contain measurable amounts of ACV (Table 1). Both species produce large amounts of U17 and smaller amounts of cysteine, CoA, thiosulfate, and sulfide. Thiol U12 was found in *S. lividans*. When authentic ACV was added at 0.5 μmol/g of residual dry weight to *S. lividans* cells during the extraction, 98% ± 13% was recovered, showing that the failure to detect ACV in this organism does not result from loss or degradation of ACV under the conditions for extraction and analysis. Analogous studies using cysteine and CoA provided recoveries of 99% ± 18% and 70% ± 24%, respectively, with *S. lividans* after correction for the endogenous amounts of these thiols. The good recoveries of thiols found with *S. lividans* are taken as indicating that thiol loss during extraction and analysis is not likely to represent a problem with any of the bacteria studied here.

None of the streptomycetes studied exhibited peaks at the retention time for the GSH derivative that were significantly above the noise level of the chromatograms. Upper limits for the GSH content are indicated in Table 1 and correspond to cellular concentrations around 10 μM or lower. Recovery experiments with *S. lividans* showed that 100% ± 6% of GSH was recovered when added at 0.5 μmol/g (residual dry weight) during the extraction. We conclude that GSH either is not synthesized by these organisms or is produced at concentrations at least two orders of magnitude lower than that in GSH-producing organisms (see below).

We have included in Table 1 a thiol analysis for the gram-negative unicellular bacterium *Flavobacterium* sp. strain 12.154, which produces small amounts of cephalosporin antibiotics. Note that in this organism, U17 and ACV were not detected but GSH was found at concentrations similar to those of U17 in the streptomycetes.

A more detailed study of *S. clavuligerus* was undertaken to determine how the intracellular thiol levels vary during the growth cycle. Figure 4C shows that ACV is barely detectable in the early stages of the growth cycle, begins to accumulate after 10 h, reaches a maximum level of about 2.5 μmol/g (residual dry weight) in the late exponential phase, and then decreases rapidly in the stationary state. The U17 content remained essentially constant throughout the growth phase but increased two- to threefold in the late stationary phase. Cysteine, CoA, and thiosulfate levels varied by up to threefold, but the patterns were not the same (Fig. 4B and D). The amount of sulfide was about 1 μmol/g (residual dry weight) throughout exponential growth but increased twofold in stationary phase (data not shown). The unidentified thiol U23 remained at a low concentration during exponential growth and increased eightfold in the stationary phase (Fig. 4D).

The growth medium was also monitored for thiol components during growth of *S. clavuligerus*. In the extracellular environment, thiols are rapidly oxidized to disulfides, and such oxidation may continue during manipulation and storage of the medium samples. For this reason, disulfide components were reduced with DTT before labeling with mBBr. The bulk of the contaminating DTT derivatives and other hydrophobic components were contaminants that remained and contributed significantly to the background noise level in the HPLC analysis. Nevertheless, a comparison of the chromatograms obtained by using HPLC method 1 for the reduced, derivatized sample (Fig. 5A), for the NEM control sample (Fig. 5B), and for a standard mixture of thiols in Tris buffer submitted to the same procedure (data not shown) demonstrated that adequate analyses for ACV, GSH, and Cys could be obtained. GSH and Cys in the medium proved to be <2 and <5 μM, respectively, in all samples analyzed. The ACV levels measured are shown in Fig. 6 as a function of growth time. HPLC method 1 did not give satisfactory analyses for U17 because of the large reagent-derived peak that nearly coelutes with U17. A satisfactory analysis was obtained by using HPLC method 2 (chromatography not shown), providing the data for U17 as a function of growth shown in Fig. 6. When samples were derivatized with mBBr without prior reduction with DTT, the amounts of ACV and U17 found were much smaller, showing that the thiol form represents less than 10% of the total amount present for each of these components. Recovery experiments conducted by adding 10 μM thiol standard to the medium sample demonstrated that >90% of Cys, GSH, and ACV were recovered by using the protocol either for analysis of thiol alone or for analysis of thiol plus disulfide.

The amounts of ACV and U17 in the growth medium increased over the growth cycle and represent substantial quantities in the stationary phase (Fig. 6). After 42 h, the cell content corresponds to 1.7 g (residual dry weight) per liter so that each liter of cell suspension contains 0.9 μmol of ACV thiol contributed by the cells and 35 μmol of ACV as thiol plus disulfide components in the medium. The cell content of U17 per liter of culture was 22 μmol in the thiol form, whereas the medium contained 14 μmol/liter of U17 as thiol and disulfide forms. Thus, <3% of the ACV is in the cells in the stationary phase, whereas about 60% of U17 is retained in the cells.

To further characterize the major thiol from streptomycetes, we isolated a pure sample of the bimane derivative of U17 from a combined sample of stationary-phase cells from *S. clavuligerus* and *S. lividans*. In the first step, a cell extract (2.9 g [residual dry weight]) containing approximately 25 μmol of U17 was applied to a thiol affinity column (thio-Sepharose 6B activated as a mixed disulfide with 2-mercap-

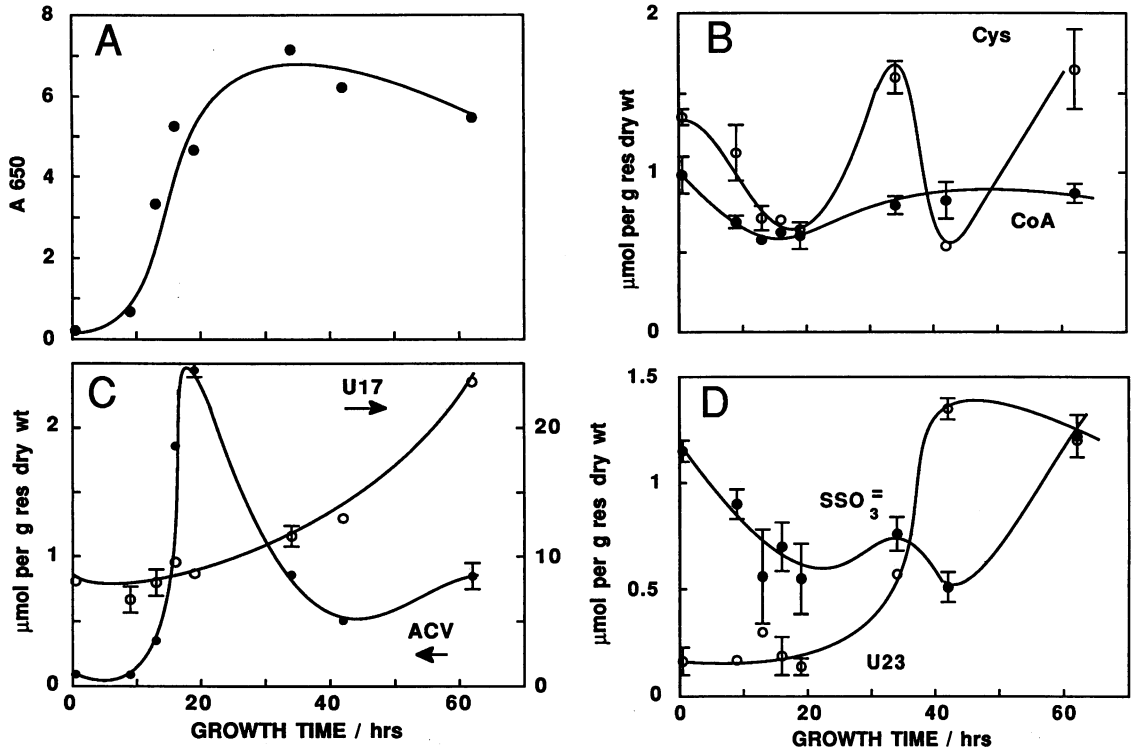

FIG. 4. Intracellular thiol levels in *S. clavuligerus* during growth. (A) Cell density monitored spectrophotometrically (A_{650}); (B) cysteine (○) and CoA (●); (C) unknown thiol (○) eluting at 17 min by using HPLC method 1 (right-hand axis) and ACV (●) (left-hand axis; (D) thiosulfate (●) and unknown thiol (○) eluting at 23 min by using HPLC method 1. res, residual.

topyridine). The column was washed free of unbound material and eluted with DTT. The eluent contained 13 µmol of U17 contaminated with DTT, 2-mercaptopyridine, and minor amounts of cellular thiols. The thiols were derivatized with mBBr, and the hydrophobic bimanes, including unreacted mBBr and the derivatives of DTT and 2-mercaptopyridine, were removed by extraction with dichloromethane. U17-mb was separated from the other ionic thiol-mb deriv-

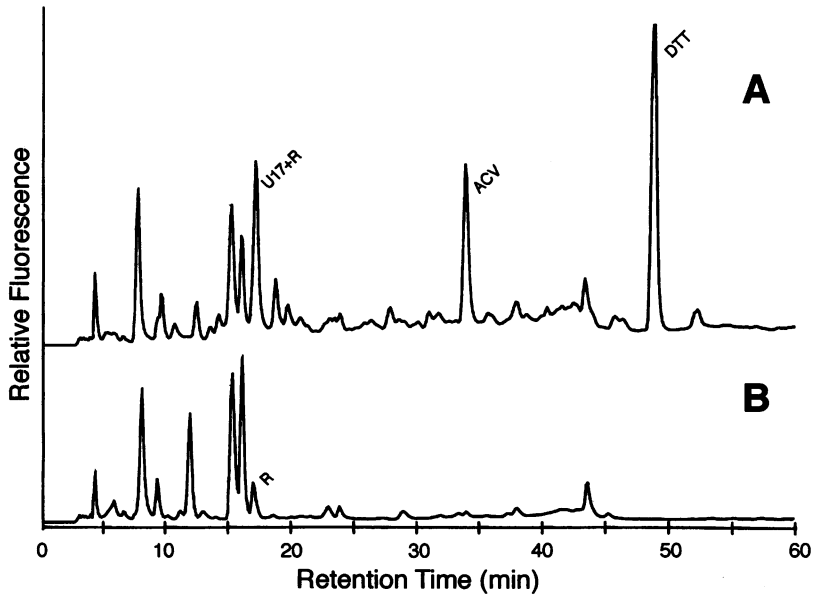

FIG. 5. HPLC chromatograms obtained by using method 1 for analysis of medium samples obtained at 42 h in the *S. clavuligerus* growth experiment for which the results are shown in Fig. 4. (A) The medium was reduced with DTT prior to labeling with mBBr so derivatives of both thiols and disulfides are present. (B) The medium was reduced with DTT, reacted with NEM, and finally treated with mBBr to identify fluorescent impurities not corresponding to thiols. R, mBBr-derived reagent peak.

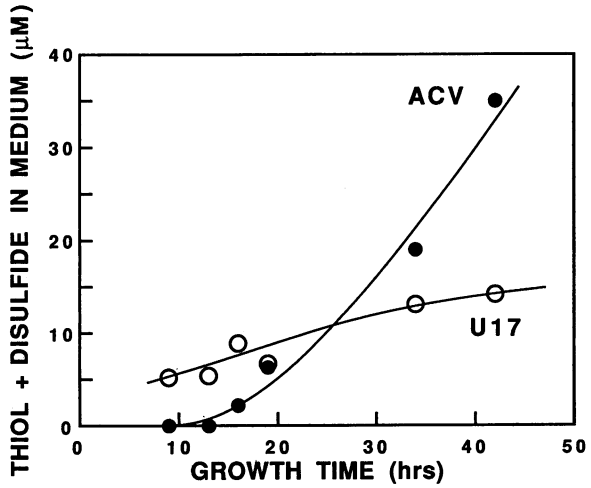

FIG. 6. Levels of ACV and U17 present in culture medium as thiol plus disulfide (level calculated as twice that of the symmetrical disulfide) during growth of *S. clavuligerus*. Medium samples were from the same experiment for which the results are shown in Fig. 4.

atives by preparative reverse-phase HPLC to give 8 μmol of material that was >95% pure by analytical HPLC.

We have previously observed an unknown thiol from *S. griseus* and *Micrococcus roseus* that was designated U25 (14) by using an HPLC protocol no longer in use in our laboratory (14). To determine whether the previously isolated unknown, U25, is the same unknown as U17 currently found in the streptomycetes, an equal mixture of the bimane derivative purified from *M. roseus* with that purified from a combined cell pellet of *S. clavuligerus* and *S. lividans* was analyzed by using HPLC methods 1 and 2. The mixture gave a single, symmetrical peak with the two HPLC protocols used, and U25 and U17 are therefore presumed to be the same unknown thiol.

A sample of the purified U17-mb was examined for amino acid content by acid hydrolysis and amino acid analysis. U17-mb was mixed with norleucine (an internal standard) and was hydrolyzed at 155°C for 0, 20, 40, and 60 min. The fluorescence of U17-mb was rapidly lost during the first few minutes of acid hydrolysis, but the zero time sample (in 6 N HCl) retained its fluorescence. The only amine-containing peak observed for the zero time sample eluted at 28.4 min, the retention time of norleucine. The 20-, 40-, and 60-min hydrolysis samples showed two peaks in addition to norleucine that did not correspond to any of the 17 amino acids in the hydrolysate standard mix. The first peak was not completely resolved from norleucine, eluted at 28.7 min, and was stable with the time of acid hydrolysis. The second peak eluted at 29.6 min (between norleucine and tyrosine), and its size decreased with the time of hydrolysis. The maximum contamination by any other amino acid was 2% of the above two peaks, indicating that the U17-mb sample was essentially free from amino acid-containing contaminants.

A plausible product of hydrolysis of U17-mb is the hydrolysis product of Cys-mb. To examine this possibility, a 5-nmol sample of GS-mb was hydrolyzed for 40 min at 155°C and analyzed to reveal the presence of 4.7 nmol of glutamic acid, 4.3 nmol of glycine, and a ninhydrin-reactive component eluting at 28.6 min that was taken to be the hydrolysis product of Cys-mb. Assuming it represents 4.5 nmol, the apparent ninhydrin factor was calculated to be ~75% of the average for α-amino acids in the hydrolysate standard mix, which indicates an efficient conversion to a stable hydrolysis product of Cys-mb. Since the first peak obtained from U17-mb eluted at the same retention time, we conclude that U17 is a cysteine derivative. By using the fluorescence factor for GS-mb to quantitate U17-mb and the ninhydrin factor for the Cys-mb hydrolysis product described above, hydrolysis of 6.5 nmol of U17-mb produced 9.0 nmol of the Cys-mb hydrolysis product. Because the fluorescence factors of bimane derivatives of cysteine peptides can vary appreciably (16), these results do not clearly indicate whether U17 contains one or two cysteine residues.

The retention time of the second hydrolysis product of U17-mb (29.6 min) did not correspond to that of any of the 17 standard amino acids in our hydrolysate mix or to that of γ-aminobutyric acid (33.9 min), α-aminoadipic acid (19.6 min), α-aminopimelic acid (25.3 min), or 6-aminocaproic acid (39.7 min). Using an average ninhydrin color factor and extrapolating the hydrolysis data to zero time, we calculate that 6.5 nmol of the amine eluting at 29.6 min was obtained from the estimated 6.6 nmol of U17-mb. This suggests that U17 contains one equivalent of this amine component.

DISCUSSION

We comment first on the use of mBBr labeling combined with HPLC analysis for determining ACV and other thiol components. Banko et al. (6), Jensen et al. (21), and Zhang et al. (40) have used this approach successfully to assay the production of ACV from the constituent amino acids catalyzed by cell extracts. Orford et al. (29) reported that they were unable to obtain satisfactory analyses of ACV disulfide after reduction with DTT and labeling with mBBr because of interference from other fluorescent components. However, they appear not to have used an excess of mBBr over the amount of DTT used, and they did not extract their derivatized samples, which likely accounts for their unsatisfactory results. They did achieve satisfactory results using sodium borohydride to reduce disulfides, dansylaziridine to derivatize the thiols, and HPLC with fluorescence detection to measure the thiol derivatives. Two other HPLC methods for analysis of ACV have been reported by the same laboratory, one based on pulsed amperometric detection (12) and the other based on prederivatization with 9-fluorenylmethylchloroformate (FMOC) (32).

Derivatization with *o*-phthalaldehyde before HPLC analysis has been employed by several groups to measure ACV (9, 11, 36, 38). The fluorescent amine assays (*o*-phthalaldehyde and FMOC) have the advantage that they may be used to analyze the nonthiol components of ACV, L-α-aminoadipic acid, and D-valine, the amine-containing β-lactam antibiotics, and specific mixed disulfides involving ACV. However, the diversity of amines present in biological samples can cause the background noise level to be substantial for analysis of cell extracts or samples of growth medium.

The present method seems especially well suited for measuring cellular thiol components. Disulfide levels are generally low in cells, and the specificity of the mBBr labeling provides acceptably low levels of background peaks upon HPLC analysis. It can also be applied to determine the total concentrations of thiol and disulfide forms in the medium but cannot be used to measure concentrations of specific mixed disulfides in the medium. Analysis of medium samples is complicated by the high level of background noise when DTT is used as reductant, and this might be improved by use of sodium borohydride to reduce disulfides (23).

Another important feature to note in the present results is that glutathione is not made in any of the five *Streptomyces* species examined. Fahey et al. have previously noted that gram-positive bacteria appear to lack the ability to synthesize GSH (13). Our results are in accordance with this view. In contrast, Castro et al. (9) reported the presence of GSH in extracts of *S. lactamdurans*. However, the yeast extract used in their growth medium is known to contain GSH (27), and it has been reported that some gram-positive bacteria can accumulate small amounts of GSH from the medium (13). We suppose this is the origin of the low levels of GSH detected in their analyses, a result that emphasizes the need to grow bacteria in GSH-free medium for these kinds of studies. That streptomycetes are unable to synthesize GSH implies that they lack GSH-related enzymes. This prediction was confirmed by the finding that *S. clavuligerus* lacks glutathione reductase and contains a disulfide reductase of broad specificity (1).

Since streptomycetes are strict aerobes but do not produce GSH, it seemed possible that they produce some other thiol that serves a function analogous to GSH. One role of GSH is to serve as a slowly autoxidizable reservoir of cysteine in cells (17). GSH also functions in gram-negative bacteria as a substrate for *S*-transferase and thioltransferase enzymes that play a role in protecting against oxygen toxicity (17). GSH is also absent in the aerobic archaebacterium *Halobacterium halobium* (27); in this organism, γ-glutamylcysteine is produced at millimolar concentrations and appears to have an antioxidant role (27, 34, 35). Since the structure of ACV is very similar to that of GSH (Fig. 1), it seemed possible that it might have an antioxidant function in streptomycetes. However, the present findings are not consistent with this view. If ACV were to function as a protective thiol analogous to GSH, then one might expect that it would be produced in substantial levels throughout the growth cycle and that it would be produced by all streptomycetes, not just those that produce β-lactam antibiotics. However, ACV production is confined to the late exponential phase of growth in *S. clavuligerus*, and no ACV was found in those streptomycetes that do not produce β-lactam antibiotics. Prokaryotes that produce GSH typically contain millimolar concentrations of this tripeptide, a level well in excess of the cellular cysteine level (26). In contrast, the level of ACV found in β-lactam-producing streptomycetes ranged from 10 to 200 μM and exceeded the cysteine level only toward the end of exponential growth (Fig. 4). Moreover, the bulk of the ACV produced was excreted into the medium toward the end of exponential growth. In GSH-producing bacteria such as *Escherichia coli*, GSH occurs at millimolar concentrations and accumulates in the stationary phase (4, 22). We conclude that ACV does not function effectively as a slowly autoxidizable reservoir of cysteine or as a protective thiol in the streptomycetes.

The present results are consistent with the view that ACV is produced principally as an intermediate for antibiotic biosynthesis. In the early exponential phase of growth (0 to 12 h), the ACV level stayed low and approximately constant. Subsequently, ACV accumulated rapidly to reach a maximum level at 18 h. The sharp rise in the ACV level in this period closely follows and slightly precedes the appearance of β-lactam antibiotics in the medium (37). At later times (40 h), the ACV level fell to an intermediate level. The overall pattern of ACV accumulation in *S. clavuligerus* is similar in most respects to that reported by Castro et al. for *S. lactamdurans* (9). We suppose the ACV profile reflects the combined effects of synthesis, conversion to β-lactam antibiotics, and excretion of ACV. Recent studies of the regulation of penicillin synthesis in *S. clavuligerus* support this view. They establish that synthesis of isopenicillin N synthase, the enzyme responsible for cyclization of ACV to isopenicillin N (37), commences at about the same time and increases in a manner similar to that seen here for ACV accumulation. What, if any, function the excretion of ACV by *S. clavuligerus* plays in the survival of the organism is not clear. It could result in part from lysis of the older parts of the mycelium, but this should have released into the medium a fraction of U17 larger than that observed. In contrast to the present finding, during growth of a cephalosporin C nonproducing mutant of *Cephalosporium acremonium*, 80% or more of the total ACV was found in the cells (39).

Other than ACV, the most conspicuous intracellular thiols present in streptomycetes were U17, CoA, cysteine, inorganic sulfide, and thiosulfate. *S. lactamdurans* also produced ergothioneine in large amounts. Ergothioneine is synthesized by fungi and bacteria and is concentrated to millimolar levels in many mammalian tissues from the diet (18, 24). Its function is unclear, but recent studies support an antioxidant role for this unusual thiol in animals (3, 20). Perhaps it has a similar role in *S. lactamdurans*, but its absence in the other organisms studied here indicates that this is not a mechanism generally used by streptomycetes. Several other unidentified thiols were detected, but none accumulated to a level approaching that of U17, which was found to be the dominant thiol in the streptomycetes.

U17 appears to be the same compound as an unknown thiol observed earlier in other actinomycetes. Thus, the major thiol compound from *M. roseus* and *S. griseus* was an unknown thiol designated U25 on the basis of the elution of its mBBr derivative at 25 min by using an earlier HPLC analysis method (14). Chromatography of a purified sample of this unknown showed that it coelutes with U17 from *S. clavuligerus* under the two chromatography methods employed here, so it seems likely that these unknowns are identical. Since *M. roseus* is classified in a group (the actinobacteria) different from that of the streptomycetes (19), U17 may not be restricted to the streptomycetes and could have a wider distribution among the actinomycetes.

Although ACV does not have an antioxidant function in the streptomycetes similar to that of GSH in organisms that produce GSH, the unknown thiol U17 may serve such a role. U17 was found in the actinomycetes at millimolar concentrations, at a level well above the cysteine level, and since U17 contains cysteine, it represents a potential reservoir of cysteine for protein synthesis and production of β-lactam antibiotics. Because millimolar concentrations of cysteine are thought to be toxic for aerobic bacteria because of its facile autoxidation which generates H_2O_2 (8), U17 may be a less toxic way to store cysteine. If U17 does serve such an antioxidant function, then the streptomycetes presumably produce one or more enzymes capable of maintaining it in a reduced state. We note that a novel disulfide reductase having broad specificity has recently been isolated from *S. clavuligerus* (1). The first step in testing the protective role of U17 is to fully elucidate its structure, and studies to achieve this goal are in progress.

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