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# Reagent for the Detection of Protein Thiocarboxylates in the Bacterial Proteome: Lissamine Rhodamine B Sulfonyl Azide

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***Abstract:*** Protein thiocarboxylates are involved in the biosynthesis of thiamin, molybdopterin, thioquinolobactin, and cysteine. Sequence analysis suggests that this post-translational modification is widely distributed in bacteria. Here we describe the development of lissamine rhodamine B sulfonyl azide as a sensitive click reagent for the detection of protein thiocarboxylates and describe the use of this reagent to detect PdtH, a putative protein thiocarboxylate involved in the biosynthesis of the pyridine dithiocarboxylic acid siderophore, in the *Pseudomonas stutzeri* proteome.

## Introduction

Protein thiocarboxylates function as sulfide donors in the biosynthesis of thiamin,[1] molybdopterin,[2] thioquinolobactin,[3] cysteine,[4] and eukaryotic transfer RNA (tRNA) modification[5] (Figure 1). These proteins are characterized by their small size (<100 amino acids), by the presence of a flexible GlyGly sequence at the thiocarboxylate-containing carboxy terminus, and by a $\beta$-grasp structure. Sequence analysis using the biochemically characterized protein thiocarboxylates ThiS, MoaD, QbsE, and CysO in the Pfam database (http://pfam.sanger.ac.uk/) suggests that ThiS-like proteins are widely distributed and may play a role in several additional biosynthetic pathways. Therefore, a reagent to selectively detect protein thiocarboxylates in any bacterial proteome would be of use in identifying systems for further biochemical characterization.

Selective protein thiocarboxylate labeling in proteomes is a challenging problem because the thiocarboxylate functional group is present at low concentrations in an environment containing many other nucleophiles. After surveying various possibilities, we decided to explore the "click reaction" between thiocarboxylates and electron-deficient sulfonyl azides to form N-acyl-sulfonamides as our labeling strategy.[6−8] Recently, this reaction was used to label purified ubiquitin thiocarboxylate with PEG-sulfonyl azide and biotin-sulfonyl azide, suggesting that the reaction has the needed selectivity for a proteomics study.[9] Here we describe the development of a simple, fast, and sensitive fluorescence-based method to detect new thiocarboxylate-containing proteins. The method utilizes lissamine rhodamine B sulfonyl azide (LRSA, **7**) as the fluorescent label and has been applied to tag pure overexpressed protein thiocarboxylates as well as protein thiocarboxylates in a bacterial proteome (Figure 2).

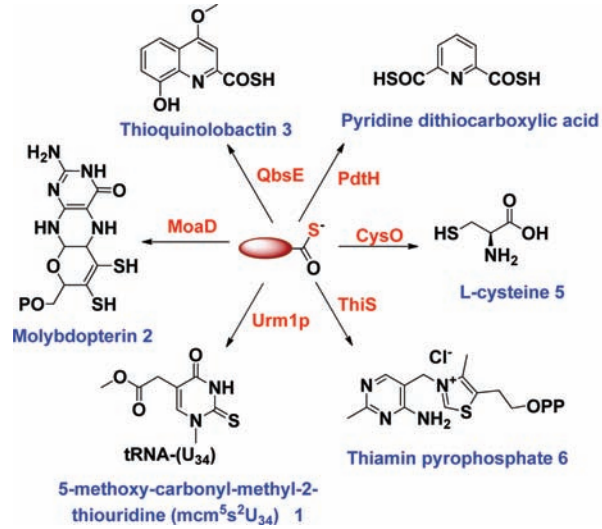


***Figure 1.*** Thiocarboxylate-containing proteins in biosynthetic pathways. The functions of ThiS, MoaD, QbsE, Urm1p, and CysO have been biochemically characterized. The function of PdtH is a proposed function based on sequence analysis.

## Materials and Methods

Lissamine rhodamine B sulfonyl chloride (LRSA, **7**), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), potassium phosphate, ferrous ammonium sulfate, $\beta$-mercaptoethanol, and sterile disposable polyethylene terephthalate G copolymer (PETG) flasks, with vented closure, were purchased from Fisher Scientific

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**10.1021/ja1034107** 

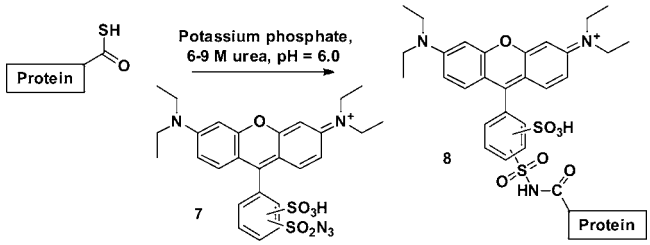


***Figure 2.*** Protein thiocarboxylate labeling using a "click reaction" with lissamine rhodamine B sulfonyl azide (LRSA, **7**).

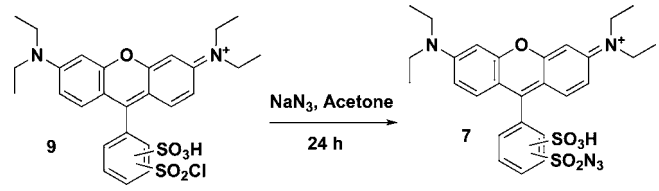


***Figure 3.*** Synthesis of lissamine rhodamine B sulfonyl azide (LRSA, **7**).

(Fairlawn, NJ). Sodium azide, urea, adenosine triphosphate (ATP), Tris, bacterial protease inhibitor cocktail, propanedithiol, and tris(2-carboxyethyl)phosphine (TCEP) were from Sigma-Aldrich (St. Louis, MO). Bacterial protease inhibitor cocktail contained AEBSF (serine protease inhibitor), EDTA (metalloprotease inhibitor), bestatin (aminopeptidase inhibitor), pepstatin A (acid protease inhibitor), and E-64 (cysteine protease inhibitor). Isopropyl $\beta$-D-thiogalactopyranosid (IPTG) was from Lab Scientific Inc. (Livingston, NJ). Luria-Bertani medium (LB) was from EMD Biosciences (Gibbstown, NJ), and Difco nutrient broth was from BD (Franklin Lakes, NJ). *Pseudomonas stutzeri* KC (ATCC 55595), *Streptomyces coelicolor* (ATCC 10147), *Saccharopolyspora erythrea* (ATCC 11635), *Streptomyces griesus* (ATCC 23345), and *Streptomyces avermitilis* (ATCC 31267) were purchased from American Type Culture Collection (Manassas, VA). *Burkolderia xenovorans* LB400 was a gift from Dr. James Tiedje (Michigan State University, East Lansing, MI), and *Rhodococcus* sp. RHA1 was provided by Dr. Lindsay Eltis (University of British Columbia, Vancouver, Canada). Chitin beads and the pTYB1 vector were obtained from New England Biolabs (Ipswich, MA). Protein concentrations were determined using the Bradford assay. All fluorescence gel images were scanned using a Typhoon 9400 or Typhoon trio instrument (excitation, 532 nm green laser; emission, 580 nm band-pass filter (580 BP 30)) from GE Healthcare Biosciences (Piscataway, NJ). Sonication used a Sonicator 3000 from Misonix Inc. (Farmingdale, NY). Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on an Esquire LC-00146 instrument (Bruker, Billerica, MA). One-dimensional sodium dodecyl sulfide−polyacrylamide gel electrophoresis (SDS−PAGE) analysis was done using a Hoefer SE 250 mini-vertical gel electrophoresis unit. Two-dimensional gel analysis was done using a Bio-Rad IEF protean cell with ReadyStrip IPG strips (pH 4.0−7.0). Econo-pac 10 DG desalting columns were from Bio-Rad (Hercules, CA). Dialysis used a Novagen D-tube Maxi dialyzer (EMD Biosciences), molecular weight cutoff = 3.5 kDa. Labeling reactions were carried out in the dark to prevent fluorophore photobleaching. Samples were desalted using the $CHCl_3$/methanol precipitation technique unless otherwise mentioned. For this method, 100 $\mu$L of the sample was treated with 400 $\mu$L of methanol and vortexed, 100 $\mu$L of $CHCl_3$ was added, and the sample was vortexed again. Next, 300 $\mu$L of water was added, and the samples were centrifuged at 16 000 relative centrifugal force (rcf) for 2 min. The proteins precipitated at the interface. The top phase was removed without disturbing the interface, and 400 $\mu$L of methanol was added. The sample was again vortexed and centrifuged for 2 min at 16 000 rcf to obtain the desalted protein as a pellet. The supernatant was decanted, and the pellet was air-dried and redissolved in the appropriate buffer. NanoLC-MS/MS analysis was done at the Proteomics and Mass Spectrometry Facility, Cornell University (Ithaca, NY). NanoLC was carried out on an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). The in-gel trypsin-digested peptides were injected using a Famous autosampler onto a C18 PepMap trap column (5 $\mu$m, 300 $\mu$m × 5 mm, Dionex) for in-line desalting and then separated on a PepMap C-18 RP nano column, eluting with a 30-min gradient of acetonitrile (10% to 40%) in 0.1% formic acid at 275 nL/min. The nanoLC was connected to a 4000 Q Trap mass spectrometer (ABI/MDS Sciex, Framingham, MA) equipped with a Micro Ion Spray Head II ion source.

**Synthesis of Lissamine Rhodamine B Sulfonyl Azide (LRSA, 7).** Lissamine rhodamine B sulfonyl chloride (53 mg, 92 mmol; compound **9**, Figure 3) was dissolved in 10 mL of acetone in a round-bottomed flask wrapped in aluminum foil. Next, 29 mg of sodium azide (446 mmol, 5 equiv) was added, and the solution was stirred at room temperature for 24 h. The solvent was removed *in vacuo*, and the residue was redissolved in dichloromethane. The resulting solution was washed with water, dried over anhydrous $MgSO_4$, and filtered, and the solvent was removed *in vacuo* to give LRSA (**7**; 44.6 mg, 83%). A 15 mM stock solution in dimethylsulfoxide (DMSO) was prepared and stored in 1 mL aliquots at −20 °C in the dark. $^1$H and $^{13}$C NMR spectra of the two regioisomers (2, 4-) and chemical shift values are shown in the Supporting Information, Figures 1s and 2s. High-resolution ESI-MS (positive mode): $m/z$ = 584.14 (predicted mass, 584.16; Supporting Information, Figure 3s).

**Overexpression and Purification of ThiS Thiocarboxylate (ThiSCOSH) and ThiS DTT Thioester (ThiSCODTT).** The *thiS* gene from *Thermus thermophilus* was inserted into pTYB1 (between the *Nde*I and *Sap*I restriction sites; forward primer, ggttagcatatg-gtgtggcttaacggggagccc; reverse primer, ggtggttgctcttccgcaacccccct-gcatcagggccacc). The protein was overexpressed in *Escherichia coli* BL21(DE3) as follows: 2 L cultures were grown at 37 °C in LB medium to an $OD_{600}$ of 0.6. The temperature was then reduced to 15 °C, and the cultures were induced with IPTG to a final concentration of 1 mM. Further growth was carried out at 15 °C for 12−16 h with constant agitation. The cultures were harvested by centrifugation and lysed by sonication on ice in 20 mM Tris, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.8. The samples were then loaded onto a column of chitin beads (20 mL) at a flow rate of 0.5 mL/min and washed with 300 mL of 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.8, at a flow rate of 2 mL/min. Intein-mediated cleavage of the protein was carried out at 4 °C for 48 h with 30 mL of 50 mM DTT to give ThiSCODTT or with 30 mL of 50 mM $Na_2S$ to yield ThiSCOSH. The proteins were buffer-exchanged by dialysis into 100 mM potassium phosphate, pH 8.0, and stored at −80 °C in 30% glycerol. (No reducing agent was added to the frozen stocks, as this would reduce the sulfonyl azide during protein labeling.)

**Labeling of ThiSCOSH with LRSA.** Frozen aliquots of ThiSCOSH and ThiSCODTT (both 184 $\mu$M) were thawed and buffer-exchanged into 50 mM potassium phosphate, 6 M urea, pH 6.0. Both proteins (50 $\mu$L) were then treated with LRSA (2.5 equiv, 1.5 $\mu$L of 15 mM stock in DMSO). The samples were incubated at room temperature in the dark for 15 min. TCEP (6 $\mu$L of 250 mM solution in 1 M potassium phosphate, pH 6.0) was then added, and the samples were analyzed by SDS−PAGE (15% Tris-glycine) and imaged on a Typhoon 9400 instrument (see Figure 4, below).

**Time Course for the Labeling Reaction.** ThiSCOSH was buffer-exchanged into 50 mM potassium phosphate containing 6 M urea at pH 6.0. The resulting protein (100 $\mu$L, 93 $\mu$M) was incubated with LRSA (3 equiv, 2 $\mu$L of 15 mM stock made in DMSO). Aliquots of 10 $\mu$L were taken at 0, 5, 10, 15, 30, 45, 60, and 75 min and treated with SDS−PAGE sample buffer (10 $\mu$L) containing 50 mM TCEP, analyzed by SDS−PAGE (15% Tris-glycine), and imaged on a Typhoon 9400 instrument (see Figure 4, below).

**Specificity and Detection Limits of the Labeling Chemistry.** *E. coli* BL21(DE3) was grown in 75 mL of LB at 37 °C to an $OD_{600}$ of 1.6. The cells were harvested by centrifugation, lysed by sonication on ice in 4 mL of 50 mM potassium phosphate, 300 mM NaCl, 2 mM TCEP, pH 8.0, and centrifuged to obtain the proteome, which was buffer-exchanged into 50 mM potassium phosphate, 9 M urea, pH 6.0, using an Econo-pac 10 DG desalting column. Three 90-$\mu$L samples of this extract were prepared containing ThiSCOSH (in 100 mM potassium phosphate, 30% glycerol, pH 8.0) at final concentrations of 11 $\mu$M, 1.1 $\mu$M, and 110 nM. Each sample was treated with 20 $\mu$L of LRSA (15 mM stock in DMSO) for 15 min at room temperature in the dark, followed by treatment with 25 mM TCEP for another 30 min in the dark to cleave any sulfenamides formed between LRSA and cysteine thiols.[10,11] The samples were desalted by $CHCl_3$/methanol precipitation and then dissolved in 50 $\mu$L of 50 mM potassium phosphate, 9 M urea, pH 6.0. An equal volume of SDS−PAGE sample buffer was added, and the samples were analyzed on a 16% Tris-tricine gel and imaged on a Typhoon trio instrument (see Figure 5, below).

**Protein Thiocarboxylate Detection in the *P. stutzeri* KC Proteome.** *P. stutzeri* KC was maintained on a nutrient broth agar plate at 4 °C, and a colony from this plate was used to inoculate a 100 mL culture of defined rich media (DRM) medium[12] in a sterile disposable PETG flask with vented closure. The culture was grown at 30 °C for 48 h with shaking. The culture was harvested by centrifugation. The *P. stutzeri* KC cell-pellet was resuspended in 3 mL of 50 mM potassium phosphate, 300 mM NaCl, 2 mM TCEP, pH 8.0, containing 6.5 mg/mL of bacterial protease inhibitor cocktail. The sample was lysed by sonication on ice and clarified by centrifugation. The resulting proteome was then treated with $Na_2S$ (9 mM), ATP (18 mM), and $MgCl_2$ (6 mM) and incubated at room temperature for 6 h to reconstitute protein thiocarboxylate formation (see Figure 6, below). The sample was dialyzed into 50 mM ammonium acetate and freeze-dried for storage. For the labeling reaction, the sample was dissolved in 1 mL of 50 mM potassium phosphate, 6 M urea, pH 6.0. An aliquot (100 $\mu$L) of this denatured proteome was treated with 20 $\mu$L of LRSA (15 mM stock in DMSO) for 15 min at room temperature in the dark and then with 12 $\mu$L of TCEP (250 mM dissolved in water) and further incubated at room temperature for 30 min in the dark. The sample was precipitated by $CHCl_3$/methanol and subjected to 2D gel analysis (pH 4−7, 7 cm IEF strip, active rehydration at 50 V for 12 h at 20 °C. Four step focusing (20 °C): S1: 250 V, 15 min, S2: 4000 V, linear voltage ramp, 2 h, S3: 4000 V, rapid voltage ramp, 20000Vh, S4: 500 V, hold. Current limit/gel: 50 $\mu$A). The lower molecular weight fluorescent spot, imaged on a Typhoon trio, was excised from the gel and subjected to nano-LC-MS/MS analysis.

**Growth Conditions for Other Organisms.** Typical growth conditions for *B. xenovorans* LB400, *S. coelicolor*, *S. griesus*, *S. erythrea*, *S. avermitilis*, and *Rhodococcus* sp. RHA1 are shown in Table 1s (Supporting Information). All the cultures were harvested by centrifugation and lysed by sonication (in the case of *S. avermitilis* and *S. griesus*, this step was preceded by homogenization). The labeling protocol was the same as that used for the *P. stutzeri* KC proteome.

**Iron Dependence of Protein Thiocarboxylate Labeling in the *P. stutzeri* Proteome.** *P. stutzeri* KC (ATCC 55595) from a nutrient broth agar plate, maintained at 4 °C, was used to inoculate DRM medium (100 mL)[12] in a sterile disposable PETG flask with vented closure. This low-iron culture was grown, with shaking, at 30 °C for 48 h and harvested by centrifugation. For iron-rich conditions, nutrient broth was used to grow the bacteria at 30 °C to an $OD_{600}$ of 1.0. The resulting cell-pellets were suspended in 3 mL of lysis buffer (50 mM potassium phosphate, 300 mM NaCl, 2 mM TCEP, pH 8.0, containing 6.5 mg/mL of bacterial protease inhibitor cocktail), lysed by sonication on ice, and clarified by centrifugation. To reconstitute protein thiocarboxylate formation, $Na_2S$, ATP, and $MgCl_2$ were added (final concentrations 9 mM, 18 mM, and 6 mM, respectively), and the samples were incubated at room temperature for 6 h. As controls, thiocarboxylate reconstitution was omitted from one low-iron sample, and a second was blocked by iodoacetic acid alkylation. For the latter sample, freshly prepared iodoacetic acid (in 1 M potassium phosphate, pH 8.0) was added to the sample to a final concentration of 100 mM. All samples were further incubated at room temperature for 1 h, buffer-exchanged by dialysis into 50 mM ammonium acetate, and freeze-dried. The residue thus obtained was dissolved in labeling buffer (50 $\mu$L of 50 mM potassium phosphate, 6 M urea, pH 6.0), 2 $\mu$L of LRSA (15 mM stock in DMSO) was added, and the samples were incubated in the dark at room temperature for 15 min. Equal volumes of 50 mM TCEP in 100 mM potassium phosphate, pH 8.0, were added to the samples, which were further incubated in the dark for 30 min. The samples were then precipitated with $CHCl_3$/methanol and redissolved in 50 mM potassium phosphate, 6 M urea, pH 6.0. Equal volumes of SDS−PAGE sample buffer containing 50 mM TCEP were added, and the samples were analyzed on a 16% Tris-tricine gel imaged on a Typhoon 9400 instrument.

## Results

**Synthesis of Lissamine Rhodamine B Sulfonyl Azide (LRSA, 7).** LRSA (**7**) was obtained in high yield, in a single step, from a readily available starting material (Figure 3). The product consisted of two regio-isomers (2, 4-) as shown and was characterized by $^1$H- and 13C-NMR and high resolution ESI-MS.

**ThiSCOSH and ThiSCODTT Production and Labeling.** The *T. thermophilus thiS* gene was cloned into pTYB1, an *E. coli* cloning and expression vector designed for the in-frame insertion of a target gene adjacent to the *Sce* VMA intein/chitin binding domain (55 kDa). This results in the fusion of the C-terminus of the target protein to the N-terminus of the intein tag.[13] Cleaving the overexpressed ThiS-intein with sulfide or DTT yielded the thiocarboxylate (ThiSCOSH) or the thioester (ThiSCODTT). LRSA labeled ThiSCOSH but not ThiSCODTT, confirming the expected thiocarboxylate specificity of the labeling reaction. Approximately 70% labeling was achieved in 15 min (Figure 4).

To further evaluate the specificity and sensitivity of the labeling reaction, ThiSCOSH was added to the *E. coli* BL21(DE3) proteome in varying concentrations, and the labeling reaction was carried out under denaturing conditions (9 M urea, Figure 5). Labeled ThiSCOSH could clearly be seen at 17 pmol but not at 1.7 pmol on a 1D SDS−PAGE gel. This places the sensitivity of the labeling reaction at approximately 10 pmol.

**Detection of Protein Thiocarboxylates in a Bacterial Proteome.** The proteomes from several bacterial sources (*P. stutzeri*, *S. coelicolor*, *S. avemitilis*, *S. griesus*, *B. xenovorans*, *Sa. erythrea*, and *Rhodococcus* sp. RHA1) were treated with sodium sulfide, ATP, and $Mg^{2+}$ to reconstitute protein thiocarboxylate formation (Figure 6) and labeled with LRSA followed by analysis by 1D and 2D SDS−PAGE. The *P. stutzeri* KC (Figure 7) and *S. coelicolor* proteomes showed strong protein labeling in the lower molecular weight region characteristic of ThiS-

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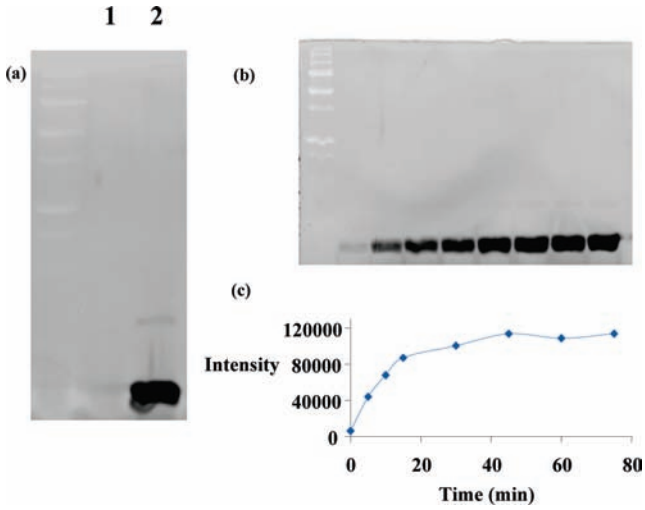


***Figure 4.*** Reaction of ThiSCODTT and ThiSCOSH with LRSA. (a) ThiSCOSH (lane 2) is labeled with LRSA. ThiSCODTT (lane 1) does not react. (b) Time-dependence of the ThiSCOSH labeling reaction. Samples were analyzed after 0, 5, 10, 15, 30, 45, 60, and 75 min. (c) Time course for ThiSCOSH labeling quantitated using GE ImageQuant 5.1 software. PMT voltage: 300 V (Typhoon 9400).

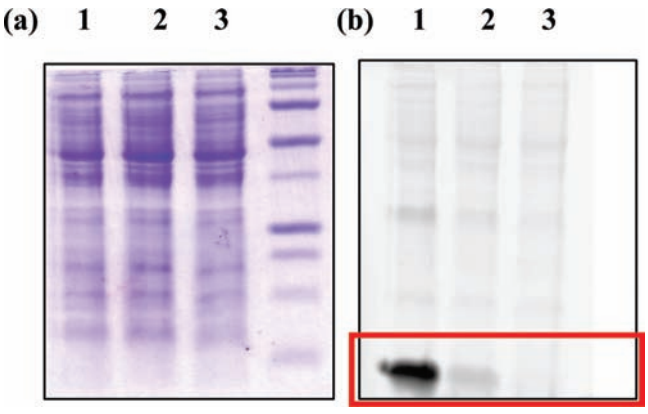


***Figure 5.*** Sensitivity of labeling of ThiSCOSH in the presence of the *E. coli* BL21 (DE3) proteome. Lanes 1, 15 $\mu$L proteome, 170 pmol ThiSCOSH; lane 2, 15 $\mu$L proteome, 17 pmol ThiSCOSH; and lane 3, 15 $\mu$L proteome, 1.7 pmol ThiSCOSH. (a) Coomassie staining and (b) fluorescent image. PMT voltage: 400 V (Typhoon trio).

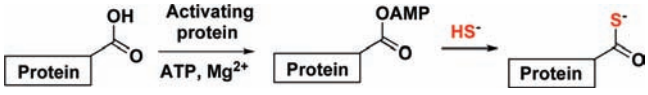


***Figure 6.*** Strategy used to reconstitute protein thiocarboxylate formation prior to LRSA labeling.

COSH orthologues (~10 kDa). *S. avemitilis* showed weak labeling, and *B. xenovorans* LB400, *S. griesus*, *Sa. erythrea*, and *Rhodococcus* sp. RHA1 did not show any labeling (data not shown). The labeled protein in the *P. stutzeri* KC proteome was isolated, subjected to LC-MS/MS analysis (Supporting Information, Figures 4s and 5s), and identified as PdtH, a ThiSCOSH orthologue proposed to function as the sulfide donor in the biosynthesis of the pyridine dithiocarboxylic acid siderophore (**4**, Figure 1).

The *P. stutzeri* KC proteome was also isolated from cells grown under iron-rich and iron-depleted conditions and treated with LRSA. Labeling of the protein assigned as PdtH was observed only in the iron-depleted proteome. The proteome produced under iron-rich conditions did not show labeling. In addition, proteome not subjected to thiocarboxylate reconstitution or proteome in which the thiocarboxylate was alkylated with iodoacetic acid did not show labeling (Figure 8).

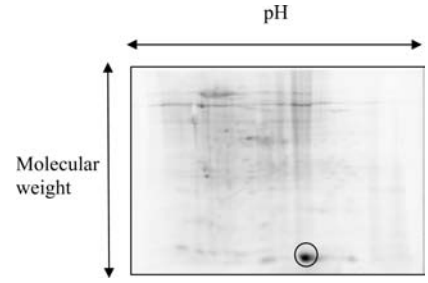


***Figure 7.*** 2D gel analysis of *P. stutzeri* KC proteome labeled with LRSA, 7 cm IEF strip, pH 4−7. PMT voltage: 400 V (Typhoon trio). The circled spot was identified as PdtH.

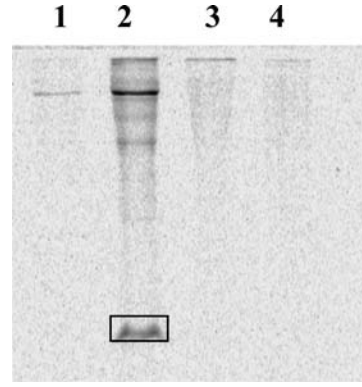


***Figure 8.*** Labeling of the *P. stutzeri* KC proteome with LRSA. Lane 1, proteome produced in iron-limiting medium without thiocarboxylate reconstitution; lane 2, proteome produced in iron-limiting medium with thiocarboxylate reconstitution; lane 3, lane 2 sample treated with iodoacetic acid prior to labeling; and lane 4, proteome produced in iron-rich medium with thiocarboxylate reconstitution. PMT voltage: 250 V (Typhoon 9400).

## Discussion

The involvement of protein thiocarboxylates in an increasing number of biosynthetic pathways involving sulfide chemistry suggested that a sensitive labeling strategy would be of use in assays for protein thiocarboxylate formation and in screening bacterial proteomes for protein thiocarboxylate formation. Here we describe the development of lissamine rhodamine B sulfonyl azide (LRSA, **7**) as our first generation reagent to address this problem. This reagent rapidly labels ThiSCOSH under mild conditions (Figure 4). The reagent also labels ThiSCOSH in the presence of the *E. coli* proteome. In this experiment, less than 10 pmol of labeled protein could be detected (Figure 5). Under normal conditions, protein thiocarboxylates are not expected to accumulate in the proteome. Therefore, to maximize their concentration, the proteome was treated with sulfide and ATP (Figure 6) prior to labeling with LRSA. This strategy assumes that high concentrations of sulfide can replace the physiological sulfide donor. This may not always be the case, and the possibility remains that we have not yet identified optimal conditions for maximizing thiocarboxylate concentrations in the proteome. This may be an important factor in limiting the sensitivity of our thiocarboxylate detection strategy.

The gels shown in Figures 5, 7, and 8 show staining of other proteins with LRSA. We currently do not understand the nature of this labeling. It is unlikely to be due to cysteine labeling

because the mercaptoethanol and TCEP treatment after labeling would reduce sulfenamides.[10,11] Labeling could be due to displacement of azide by hyper-reactive nucleophiles. Alternatively, our assumption that all thiocarboxylate-containing proteins are orthologues of ThiS (i.e., <10 kDa with the thiocarboxylate at the carboxy terminus) may not be correct, and it is possible that the thiocarboxylate post-translational modification also occurs on larger proteins.

Detection of protein thiocarboxylates in the *P. stutzeri* KC proteome was next chosen as a test system. This micro-organism produces the pyridine dithiocarboxylate siderophore (PDTC, **4**) under iron-limiting conditions.[14] The PDTC biosynthetic gene cluster encodes a ThiS orthologue (PdtH) likely to be involved in the formation of the siderophore thiocarboxylate. This putative functional assignment was confirmed by treating the *P. stutzeri* KC proteome prepared from cells grown in the presence of high and low contents of iron with LRSA. For the low-iron sample, a band with the expected molecular mass was labeled and identified by MS analysis as PdtH thiocarboxylate. This band was absent from the high-iron proteome as expected because the biosynthesis of pyridine dithiocarboxylic acid in *P. stutzeri* KC is repressed by iron[15] (Figure 8).

Our detection limit of less than 10 pmol of labeled protein converts to approximately 6000 copies of the protein thiocarboxylate per cell (*E. coli* cell volume $= 10^{-15}$ L, 1 OD $= 10^9$ cells/mL).[16,17] To place this number in context, *E. coli* has between 7000 and 72 000 ribosomes/cell and 370 000 pyruvate molecules/cell.[18,19] This demonstrates that the LRSA labeling strategy is likely to be most useful in surveying bacterial proteomes for highly expressed protein thiocarboxylates. However, the sensitivity of the method could, in principle, be increased by attaching a sulfonyl azide to a solid support via a cleavable linker. In this way, protein thiocarboxylate could be isolated and concentrated from large samples prior to SDS−PAGE analysis.

We have surveyed several other bacteria for protein thiocarboxylates. We have detected strong labeling of a small protein in *S. coelicolor*, a weakly labeled spot in *S. avermitilis*, and no protein thiocarboxylates in the proteomes of *B. xenovorans*, *Rhodococcus* sp. RHA1, *S. griesus*, and *S. erythrea*. Reasons for not noticing labeling in the cell-free extracts of these organisms could be low reconstitution of protein thiocarboxylates in the proteome with sulfide as the sulfur donor or nonexpression of thiocarboxylate-forming proteins under the growth conditions.

## Conclusions

Protein thiocarboxylates function as key intermediates in the biosynthesis of a variety of natural products. Lissamine rhodamine B sulfonyl azide labels protein thiocarboxylates to give stable fluorescent adducts that can be detected by SDS−PAGE. The reaction is selective and occurs under mild conditions with a detection limit of >10 pmol. To illustrate the applicability of this labeling strategy, LRSA was used to detect a protein thiocarboxylate in the *Pseudomonas stutzeri* proteome proposed to be involved in the biosynthesis of the pyridine dithiocarboxylic acid siderophore. This reagent will be of use in assays of protein thiocarboxylate formation as well as for the detection of protein thiocarboxylates in the bacterial proteome.

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**Supporting Information Available:** $^1$H NMR and ESI-MS data for LRSA, MS data for the identification of PdtH, and growth conditions for bacteria screened for protein thiocarboxylates. This material is available free of charge via the Internet at http://pubs.acs.org.

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