Rapid Test for Determining the Intracellular Rhodanese Activity of Various Bacteria

PETER A. VANDENBERGH, ROGER E. BAWDON, AND RICHARD S. BERK

Departments of Immunology and Microbiology, and Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201

A simple, reproducible technique with potential taxonomic application was developed for the rapid detection of rhodanese activity in gram-negative and gram-positive bacteria. The method requires suspension of the growth from three colonies in a solution of lysozyme and ethylenediaminetetraacetic acid for 60 min. After cell lysis, the presence of rhodanese activity is determined colorimetrically by measuring the amount of thiocyanate formed from thiosulfate and potassium cyanide by use of ferric nitrate. By this technique, a survey of 411 bacterial strains revealed the presence of rhodanese in all test strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter*, *Bordetella*, *Shigella*, and *Citrobacter*. No activity was detected in *Salmonella*, *Klebsiella*, *Enterobacter*, *Serratia*, or *Proteus* species. Randomly selected strains which did not exhibit rhodanese activity were confirmed to be rhodanese-negative by assay of mechanically disrupted cells harvested from 500 ml of growth medium.

Rhodanese, also known as thiosulfate:cyanide sulphurtransferase (E.C. 2.8.1.1), was originally described by Lang (4). The enzyme catalyzes the formation of thiocyanate from cyanide and thiosulfate according to the following equation: $S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$. Rhodanese was first isolated by Sörbo in 1953 in crystalline form from bovine liver (7). It has since been found in all mammalian tissues except blood and muscle, the highest being in the adrenals and liver. Various plant and microbial sources also demonstrate rhodanese activity. A comprehensive survey of many different organisms for the presence of rhodanese activity has been lacking, and it was initially thought that the enzyme is primarily restricted to various species of thiobacilli and photosynthetic bacteria (11). However, previous studies by Hall and Berk (1), and Schook and Berk (6), suggest that the activity is constitutively present and may be more widespread than previously suspected. Since intracellular rhodanese activity cannot be detected by conventional diagnostic techniques employing growing or resting cell suspensions (i.e., Analytical Products Inc.), a rapid screening technique was developed. This technique employed lysis of the cells in three picked colonies by ethylenediaminetetraacetic acid (EDTA)—lysozyme preparations and subsequent rhodanese assay of the lysed-cell supernatant. The accuracy of the results obtained by this type of cell lysis exhibited 100% correlation with the results obtained with mechanically disrupted broth-grown cells. Therefore, the following survey of mechanically lysed cells and EDTA-lysozyme lysed cells was

undertaken to determine the degree of distribution of the enzyme in various bacteria in the hope that it may be of potential taxonomic and diagnostic significance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used for this survey are listed in Tables 1 and 2. The sources of cultures of these strains are identified in Acknowledgments. The assay of intracellular rhodanese activity was performed by two different methods employing extracts obtained either by mechanical disruption of cells (cell-free extracts), using a French Press or by lysozyme-EDTA treatment. Cell-free extracts prepared from 61 organisms mechanically disrupted were initially assayed for rhodanese activity. The organisms used in this study were routinely cultured in 500 ml of a peptone-Trypticase soy broth (BBL Microbiology Systems) on a shaker for 24 h at 35°C. After 24 h of growth, these cells were harvested by centrifugation at $10,000 \times g$ for 30 min and washed twice with 0.85% saline. The washed cells were suspended in 25 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) and disrupted three times with a French Press at 16,000 lb/in².

A total of 411 organisms were screened by the second cellular disruption procedure. These organisms were routinely grown for 18 h at 35°C on trypticase soy agar plates containing 5% sheep's blood (BBL Microbiology Systems). Three colonies were removed from the plate with a sterile loop and standardized to a cell concentration of 1.5×10^9 cells per ml at 440 nm. Lysozyme and potassium-EDTA (final concentrations, 0.5 mg/ml and 10 mM, respectively) were added to the test tube (2). The cells were incubated in this solution for 60 min at 25°C, and cell mixtures were routinely monitored for release of nucleic acid at 260 nm to

TABLE 1. *Rhodanese activity in mechanically lysed cells of various bacteria*"

Organism	Sp. act. (μ mol of CNS formed/mg of protein)	Source of culture
<i>Bacillaceae</i>		
<i>Bacillus megaterium</i>	800	Clinical isolate
<i>Bacillus subtilis</i>	1,800	Clinical isolate
<i>Micrococcaceae</i>		
<i>Staphylococcus aureus</i> ATCC 25923	0	ATCC
<i>Staphylococcus epidermidis</i>	0	Clinical isolate
<i>Micrococcus luteus</i>	0	Clinical isolate
<i>Streptococcaceae</i>		
Group B, beta-hemolytic streptococcus	0	Clinical isolate
Group D, streptococcus	0	Clinical isolate
<i>Pseudomonadaceae</i>		
<i>Pseudomonas aeruginosa</i> ATCC 17933	200	ATCC
<i>Pseudomonas aeruginosa</i> ATCC 17934	1,300	ATCC
<i>Pseudomonas aeruginosa</i> ATCC 19660	400	ATCC
<i>Pseudomonas fluorescens</i>	600	Clinical isolate
<i>Pseudomonas maltophilia</i>	600	Clinical isolate
<i>Pseudomonas pseudocaligenes</i>	700	Clinical isolate
<i>Pseudomonas reptilovora</i>	500	Clinical isolate
<i>Pseudomonas stutzeri</i>	300	Clinical isolate
Gram-negative aerobic rods of uncertain affiliation		
<i>Alcaligenes faecalis</i>	1,200	Clinical isolate
<i>Bordetella bronchiseptica</i>	2,300	Clinical isolate
Gram-negative facultatively anaerobic rods of uncertain affiliation		
<i>Chromobacterium violaceum</i>	700	Clinical isolate
<i>Yersinia enterocolitica</i>	0	Clinical isolate
<i>Neisseriaceae</i>		
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i>	3,400	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain 1-71	2,500	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain 1-77	2,000	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain a-83	2,000	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain 1-101	3,200	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain A	2,300	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain B	2,000	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain C	2,200	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain D	1,800	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain E	1,700	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain F	1,500	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain G	1,700	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain H	600	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain I	1,900	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratum</i> strain 1-104	2,800	U. of Michigan
<i>Acinetobacter calcoaceticus</i> subsp. <i>lwoffii</i>	1,700	Clinical isolate
<i>Acinetobacter calcoaceticus</i> subsp. <i>lwoffii</i>	2,100	Clinical isolate
<i>Acinetobacter calcoaceticus</i> subsp. <i>lwoffii</i>	800	Clinical isolate
<i>Moraxella lacunata</i>	1,000	Clinical isolate
<i>Enterobacteriaceae</i>		
<i>Escherichia coli</i>	800	Clinical isolate
<i>Shigella boydii</i>	1,200	Clinical isolate
<i>Shigella flexneri</i>	200	Clinical isolate
<i>Shigella sonnei</i>	500	Clinical isolate

TABLE 1. (continued)

Organism	Sp. act. (μ mol of CNS formed/mg of protein)	Source of culture
<i>Edwardsiella tarda</i>	400	Clinical isolate
<i>Citrobacter freundii</i>	300	Clinical isolate
<i>Salmonella enteritidis</i>	0	Clinical isolate
<i>Salmonella arizonae</i>	0	Clinical isolate
<i>Klebsiella pneumoniae</i>	0	Clinical isolate
<i>Klebsiella ozaenae</i>	0	Clinical isolate
<i>Enterobacter aerogenes</i>	0	Clinical isolate
<i>Enterobacter cloacae</i>	0	Clinical isolate
<i>Enterobacter hafniae</i>	0	Clinical isolate
<i>Serratia liquefaciens</i>	0	Clinical isolate
<i>Serratia marcescens</i>	0	Clinical isolate
<i>Serratia rubidae</i>	0	Clinical isolate
<i>Proteus mirabilis</i>	0	Clinical isolate
<i>Proteus morganii</i>	0	Clinical isolate
<i>Proteus vulgaris</i>	0	Clinical isolate
<i>Proteus rettgeri</i>	0	Clinical isolate
<i>Proteus inconstans</i>	0	Clinical isolate
<i>Proteus alcalifaciens</i>	0	Clinical isolate

^a Mechanical disruption by the French Press. Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CNS, thiocyanate.

ascertain the lytic effectiveness of the lysozyme-EDTA mixture.

Enzyme assay conditions. The assay for rhodanese was based on the method of Sörbo (8). The reaction mixture contained 1 ml of 0.125 M sodium thiosulfate, 0.5 ml of 0.25 M potassium cyanide, and 0.5 ml of 0.1 tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0). Added to this reaction mixture was 1.5 ml of the lysed cell mixture or 0.5 ml of the French cell-prepared extracts. The reaction mixture was incubated at 37°C for 5 min, and the reaction was stopped with the addition of 0.5 ml of 38% formaldehyde. The production of thiocyanate was determined colorimetrically by the addition of 2.5 ml of 1% ferric nitrate (in 13% nitric acid) reagent, followed by centrifugation at 20,000 $\times$ g for 5 min and measurement of absorbance at 460 nm. One rhodanese unit was defined as that amount of enzyme which formed 1 μ mol of thiocyanate during the 5-min incubation period. The reaction was linear with time during the 5-min incubation period. Enzyme-free controls containing all of the above mentioned reagents never exhibited spontaneous production of thiocyanate.

For the study employing mechanically disrupted cells, the supernatants were assayed three times to determine the validity of the results. For a given culture to be considered positive for rhodanese activity, its cell-free extracts had to form a ferric nitrate-thiocyanate complex giving a minimum absorbance reading of 0.1 or greater at 460 nm.

Protein determination. In the cell-free extract survey, protein determinations were based on the pro-

cedure of Koch and Putnam (3) with crystalline bovine serum albumin used as the standard.

RESULTS

Initial studies on *Acinetobacter* and *P. aeruginosa* strains indicated that rhodanese activity was constitutively present regardless of the growth medium employed and was associated with the intracellular soluble fraction of the cell. Activity could never be detected extracellularly in the broth after growth or in resting cells. Therefore, subsequent rhodanese assays were performed on cell-free extracts prepared from disrupted cultures. Table 1 itemizes the rhodanese activity in the cell-free extracts of 61 different strains of bacteria grown on peptone-Trypticase soy broth and mechanically disrupted. The greatest activity was detected in the two *Acinetobacter* species followed by *Bordetella bronchiseptica*. All strains of *Pseudomonas*, *Shigella*, *Escherichia*, and *Citrobacter* demonstrated less activity. However, no activity was detected in the cell-free extracts of the gram-positive cocci or in the *Serratia*, *Enterobacter*, *Klebsiella*, *Salmonella*, and *Proteus* species.

Table 2 itemizes rhodanese activity in the lysed cells of several hundred *Enterobacteriaceae* strains. In this survey, only the *Escherichia*, *Shigella*, and *Citrobacter* species dem-

TABLE 2. Intracellular rhodanese activity of lysozyme-EDTA-lysed cells of various bacteria

Organism	No. of strains examined	Rhodanese reaction	Source of culture
<i>Enterobacteriaceae</i>			
<i>Escherichia coli</i> ATCC 25922	1	+	ATCC
<i>Escherichia coli</i> ATCC 19413	1	+	ATCC
<i>Escherichia coli</i>	130	+	Clinical isolates
<i>Shigella boydii</i>	15	+	Clinical isolates
<i>Shigella dysenteriae</i>	3	+	Clinical isolates
<i>Shigella dysenteriae</i> ATCC 13313	1	+	ATCC
<i>Shigella flexneri</i>	8	+	Clinical isolates
<i>Shigella sonnei</i>	10	+	Clinical isolates
<i>Citrobacter diversus</i> ATCC 27156 ^a	1	+	ATCC
<i>Citrobacter diversus</i>	2	+	Clinical isolates
<i>Citrobacter freundii</i>	7	+	Clinical isolates
<i>Arizona hinshawii</i>	1	—	Clinical isolates
<i>Salmonella typhi</i> ATCC 19430 ^a	1	—	ATCC
<i>Salmonella enteritidis</i> ATCC 13076	1	—	ATCC
<i>Salmonella enteritidis</i>	2	—	Clinical isolates
<i>Salmonella typhimurium</i> ATCC 14028	1	—	ATCC
<i>Salmonella typhimurium</i>	14	—	Clinical isolates
<i>Salmonella choleraesuis</i> ATCC 13312 ^a	1	—	ATCC
<i>Klebsiella pneumoniae</i> ATCC 13883 ^a	1	—	ATCC
<i>Klebsiella pneumoniae</i>	45	—	Clinical isolates
<i>Klebsiella oxaenae</i>	5	—	Clinical isolates
<i>Enterobacter aerogenes</i>	21	—	Clinical isolates
<i>Enterobacter cloacae</i> ATCC 23355	1	—	ATCC
<i>Enterobacter cloacae</i> ATCC 13047	1	—	ATCC
<i>Enterobacter hafniae</i>	4	—	Clinical isolates
<i>Enterobacter agglomerans</i>	5	—	Clinical isolates
<i>Serratia marcescens</i> ATCC 8100	1	—	ATCC
<i>Serratia marcescens</i>	8	—	Clinical isolates
<i>Proteus mirabilis</i>	30	—	Clinical isolates
<i>Proteus morganii</i> ATCC 25830 ^a	1	—	ATCC
<i>Proteus rettgeri</i> ATCC 9250	1	—	ATCC
<i>Proteus rettgeri</i>	2	—	Clinical isolates
<i>Proteus vulgaris</i> ATCC 13315 ^a	1	—	ATCC
<i>Proteus vulgaris</i>	5	—	Clinical isolates
<i>Proteus inconstans</i> ATCC 25825	1	—	ATCC
<i>Proteus inconstans</i>	3	—	Clinical isolates
<i>Neisseriaceae</i>			
<i>Acinetobacter calcoaceticus</i> ATCC 15308	1	+	ATCC
<i>Acinetobacter calcoaceticus</i> ATCC 17905	1	+	ATCC
<i>Acinetobacter calcoaceticus</i> ATCC 9957	1	+	ATCC
<i>Acinetobacter calcoaceticus</i> ATCC 15150	1	+	ATCC
<i>Acinetobacter calcoaceticus</i>	20	+	Clinical isolates
<i>Moraxella bovis</i> ATCC 19575	1	+	ATCC
<i>Moraxella lacunata</i>	1	+	Clinical isolates
<i>Spirillaceae</i>			
<i>Campylobacter fetus</i> ATCC 29428	1	—	ATCC
<i>Pseudomonadaceae</i>			
<i>Pseudomonas aeruginosa</i> ATCC 10145 ^a	1	+	ATCC

TABLE 2. (continued)

Organism	No. of Strains examined	Rhodanese reaction	Source of culture
<i>Pseudomonas aeruginosa</i> ATCC 17933	1	+	ATCC
<i>Pseudomonas aeruginosa</i> ATCC 17934	1	+	ATCC
<i>Pseudomonas aeruginosa</i> ATCC 19660	1	+	ATCC
<i>Pseudomonas aeruginosa</i>	26	+	Clinical isolates
<i>Pseudomonas fluorescens</i> ATCC 15553	1	+	ATCC
<i>Vibrionaceae</i>			
<i>Aeromonas hydrophila</i>	2	—	Clinical isolates
Rods of uncertain affiliation			
<i>Alcaligenes</i> species	5	+	Clinical isolates
<i>Bacillaceae</i>			
<i>Bacillus subtilis</i>	1	+	Clinical isolate
<i>Streptococcaceae</i>			
Group A streptococcus	1	—	ATCC
Group B streptococcus	1	—	Clinical isolate
Group C streptococcus	1	—	ATCC
Group D streptococcus	1	—	Clinical isolate
Group F streptococcus	1	—	Clinical isolate
Group G streptococcus	1	—	ATCC

" Type or neotype strain. Abbreviation: ATCC, American Type Culture Collection, Rockville, Md. Symbols: +, Rhodanese produced; —, rhodanese not produced.

onstrated rhodanese activity. No rhodanese activity was detected in the *Salmonella*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Proteus* species. Also, the greatest activity was again associated with the *Acinetobacter* and *P. aeruginosa* strains.

DISCUSSION

The results described here suggest that intracellular rhodanese activity is found in a relatively large number of bacteria and can be detected rapidly and accurately by use of the EDTA-lysozyme cell lysis method. This procedure avoids the cumbersome and prolonged procedure of growing large numbers of cells and mechanically disrupting them after centrifugation. A number of functions have been associated with rhodanese. These functions include the role of cyanide detoxification (8), thiosulfate reductase activity (10), and a synthetic function involved in the formation of cysteine from sulfite (9). The presence or absence of rhodanese activity is a stable property which may have potential in differentiating the various genera of the family *Enterobacteriaceae* as well as other unrelated genera. At present, the *Enterobacteriaceae* can be divided into cyanide-sensitive and cyanide-resistant groups of bacteria, a division based mainly on the work of Møller (5). The cyanide-sensitive strains include *Escherichia*, *Shigella*,

Edwardsiella, *Salmonella*, and *Arizona* species. The cyanide-resistant strains include *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Providencia*, and *Aeromonas* species. Hence, our data seem to suggest a role for rhodanese other than cyanide detoxification based on the observation that it occurs in both cyanide-sensitive and cyanide-resistant members of the family *Enterobacteriaceae*. The presence or absence of activity in all members of a given genus so far examined suggests that this enzyme is a stable, heritable property which is not easily lost or promiscuously transferred from genus to genus and may be of value in future taxonomic studies as well as in diagnostic laboratories.

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REPRINT REQUESTS

Address reprint requests to: Dr. Richard S. Berk, Department of Microbiology and Immunology, Wayne State University School of Medicine, Detroit, MI 48201.

LITERATURE CITED

1. Hall, M. R., and R. S. Berk. 1968. Microbial growth on mercaptosuccinic acid. *Can. J. Microbiol.* 14:515-523.
2. Kaback, H. R. 1971. Bacterial membranes. *Methods Enzymol.* 22:99-120.

3. Koch, A. L., and S. L. Putnam. 1971. Sensitive biuret method for determination of protein in an impure system such as whole bacteria. *Anal. Biochem.* **44**:239-245.
4. Lang, K. 1933. Die Rhodanbildung in Tierkörper. *Biochem. Z.* **259**:243-256.
5. Möller, V. 1954. Diagnostic use of the Braun KCN test within the enterobacteriaceae. *Acta Pathol. Microbiol. Scand.* **34**:115-126.
6. Schook, L. B., and R. S. Berk. 1978. Nutritional studies with *Pseudomonas aeruginosa* grown on inorganic sulfur sources. *J. Bacteriol.* **133**:1377-1382.
7. Sörbo, B. H. 1953. Crystalline rhodanese. *Acta Chem. Scand.* **7**:1129-1136.
8. Sörbo, B. H. 1960. Rhodanese. *Enzymology.* **2**:334-337.
9. Torü, K., and R. S. Bandurski. 1967. Yeast sulfate-reducing system. *Biochim. Biophys. Acta* **136**:286-295.
10. Villarejo, M., and J. Westley. 1963. Rhodanese-catalyzed reduction of thiosulfate by reduced lipoic acid. *J. Biol. Chem.* **238**:1185-1186.
11. Yoch, D. C., and E. S. Lindstrom. 1971. Survey of photosynthetic bacteria for rhodanese (thiosulfate: cyanide sulfur transferase) activity. *J. Bacteriol.* **106**:700-701.