RHODANESE ACTIVITY: A SIMPLE AND RELIABLE TAXONOMIC TOOL FOR GRAM-NEGATIVE BACTERIA

B. LÁNYI

National Institute of Hygiene, POB 64, H-1966, Budapest, Hungary

SUMMARY. The thiosulphate:cyanide sulphurtransferase (rhodanese) test of Vandenberg, Bawdon and Berk (1979) has been simplified and 2469 strains from a wide variety of sources representing different biochemical, serological or phage-pattern entities were tested. The percentages of rhodanese-producing strains were: *Escherichia coli* 98%, *Shigella flexneri* serovars 1–5% X and Y 0%, other shigellae 73–100%, *Yersinia* spp. 0%, *Salmonella* subgenera I–IV 0%, *Citrobacter freundii* 16%, *Klebsiella* 37%, *Enterobacter* 4%, *Hafnia alvei* 61%, *Proteus* spp. 0%, *Pseudomonas* spp. 98–100%. Rhodanese production by *S. flexneri* serovar 6 supports the view that this group of bacteria should be removed from *S. flexneri* and placed in another species of *Shigella*.

INTRODUCTION

Rhodanese (thiosulphate:cyanide sulphurtransferase, E.C.2.8.1.1) is a constitutive intracellular enzyme that catalyses the formation of thiocyanate from cyanide and thiosulphate. It was demonstrated in photosynthetic bacteria (Yoch and Lindstrom, 1971) and in *Pseudomonas aeruginosa* (Schook and Berk, 1978). A practical test for the detection of rhodanese in bacteria has been developed by Vandenberg *et al.* (1979), who screened the lysozyme-lysed cells of 411 strains belonging mainly to the family Enterobacteriaceae.

The present paper gives an account of rhodanese activity in 2469 bacterial strains tested by a simplified procedure.

MATERIALS AND METHODS

Organisms. Reference strains from the Hungarian National Collection of Medical Bacteria, Budapest (Lányi and Konkoly Thege, 1978) and fresh isolates were used (see table). The cultures represented different biochemical, serological or phage-pattern entities and were isolated from a wide variety of sources.

Test procedure. A modification of the method of Vandenberg *et al.* (1979) was used and the bacteria were grown for 20 h at 37°C on nutrient agar containing 5% (v/v) ox blood, except that some pseudomonads were incubated at 30°C for optimum growth.

A fresh solution of lysozyme (Reanal, Budapest) 5 mg/ml in water was made at 2-day intervals and stored at 4°C. Equal parts of the lysozyme solution and a 20mM stock solution of disodium ethylenediaminetetraacetate (EDTA) were mixed each day and distributed as 0.5-ml portions in 100 × 10 mm tubes. Several loopfuls of growth from the blood agar were transferred to the lysozyme-EDTA mixture to make a heavy suspension corresponding visually to a standard containing *c.* 3×10^{10} cells/ml. The maximum number of strains tested in one experiment was 60. The tubes were incubated at 25°C for 60 min.

The substrate mixture was prepared each day from stock solutions of 0.125M sodium thiosulphate (2 volumes), 0.1M Tris adjusted to pH 8.0 (1 volume), and 0.25M potassium cyanide (1 volume). The potassium cyanide stock solution was stored at 4°C for not longer than 1 week. The substrate mixture (0.5 ml) was added to the lysozyme-treated bacteria by means of a Cornwall dispenser. The mixtures were shaken, incubated in a waterbath at 37°C for 15 min, and shaken again.

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The reagent for the detection of thiocyanate was freshly prepared from 4 volumes of 0.1M ferric nitrate in 13% nitric acid and 1 volume of 38% (v/v) formaldehyde and 0.5 ml was added to each tube. The tubes were inspected immediately and a positive reaction was indicated by an instantaneous, distinct, slowly-fading brownish-red colour varying in strength with individual strains. Weakly positive reactions were indicated by a pale orange colour and reactions considered negative by honey yellow or faint yellow.

Procedures in which KCN was handled were performed in a cabinet equipped with exhaust ventilation. The tubes and flasks containing the remainder of the KCN reagent were discarded by immersing them in potassium permanganate solution (c. 15 g in 3 L of tap water).

RESULTS

Reproducibility of the rhodanese test

Distinct positive and negative tests were always reproducible. Negative tests, characterised by a honey-yellow colour, were less constant and the strength of the yellow colour varied. Thus the differentiation between these and weak-positive reactions needed some practice.

The suspension of many strains, mainly of *Pseudomonas*, *Klebsiella* and *S. sonnei*, became slimy and to give an evenly distributed colour reaction, the suspensions needed vigorous shaking.

The number of bacteria suspended in the lysozyme-EDTA mixture was critical and in the range 10^{11} – 5×10^9 cells/ml rhodanese production was readily detectable. The positive reactions became fainter at lower counts and below 10^9 /ml there was a tendency towards weakly positive or even negative tests.

The duration of the lysozyme-EDTA treatment was not critical but the colour reaction was sensitive to the amount of EDTA in the system; at concentrations higher than the optimum, the brownish-red colour quickly faded or even failed to appear.

There was no spontaneous production of thiocyanate; if rhodanese-producing bacteria were boiled before testing, the colour of the test was pale yellow.

Vandenbergh *et al.* (1979) regarded as negative an absorbance of <0.1 at 460 nm. To establish that our visual readings were valid, supernates obtained by centrifugation after completing the reaction by the procedure described here were read in a Spekol (Carl Zeiss, GDR) spectrophotometer at 460 nm. Positive and negative tests differed significantly in absorbance values and there was a smaller but measurable difference between the pale-yellow and honey-yellow negative reactions. The honey-yellow colour is probably associated with a minor degree of thiocyanate formation but, in practice, it is more feasible to regard the honey-yellow colour as negative because it is difficult to distinguish from the pale-yellow colour of a "true-negative" reaction.

Distribution of rhodanese activity

The table shows that the presence or absence of rhodanese activity was a characteristic of the majority of isolates within a given taxon. Exceptions were *Shigella*, *Citrobacter*, *Klebsiella* and *Hafnia*, among which some strains were positive and some negative.

Among the strains of *Shigella*, rhodanese activity was distributed according to definite subentities of the genus. *S. flexneri* strains, except *S. flexneri* serovar 6, were uniformly negative. The rest of shigellae were positive, and only some old laboratory strains of *S. boydi* serovars 3 and 11, and *S. dysenteriae* serovars 3, 5, 8 and 10 failed to produce rhodanese.

Rhodanese production was characteristic of *E. coli*, except for one fresh isolate (serogroup O25), and seven laboratory strains: O17:K16:H18, O23:K18:H15, O56:K:H⁻, O96:K:H19, O158:K:H20 (one strain of each) and O138:K81:H14 (two strains). *E. coli* strains with O antigens identical with, or related to, *S. flexneri* antigens were uniformly rhodanese-positive.

In the genus *Pseudomonas*, most strains were strongly positive. Thus 94 strains of unclassified fluorescent pseudomonads (Ajello and Hoadley, 1976; Lányi, Hoadley and Ajello, 1979), one strain each of *P. aminovorans*, *P. aureofaciens*, *P. cepacia*, *P. maltophilia*, *P.*

TABLE

Results of a modified rhodanese test on 2469 strains of gram-negative bacteria

Genus	Species or subgenus	Number of strains tested	Number of strains that were rhodanese				Percentage positive
			positive		negative		
			+	(+)	(-)	-	
<i>Escherichia</i>	<i>coli</i> *	426	418	0	3	5	98
<i>Shigella</i>	<i>boydi</i>	33	31	0	0	2	94
	<i>dysenteriae</i>	30	22	0	2	6	73
	<i>flexneri</i> serovar 6	19	19	0	0	0	100
	<i>flexneri</i> other	86	0	0	0	86	0
	<i>sonnei</i>	94	94	0	0	0	100
<i>Yersinia</i>	<i>enterocolitica</i>	153	0	0	0	153	0
	<i>pseudotuberculosis</i>	21	0	0	0	21	0
<i>Salmonella</i>	subgenus I	254	0	0	157	97	0
	subgenus II	15	0	0	1	14	0
	subgenus III	64	0	0	32	32	0
	subgenus IV	1	0	0	0	1	0
<i>Citrobacter</i>	<i>freundii</i>	85	12	2	35	36	16
<i>Edwardsiella</i>	<i>tarda</i>	1	0	0	1	0	0
<i>Klebsiella</i>	various	119	22	22	23	52	37
<i>Enterobacter</i>	various	54	0	2	36	16	4
<i>Serratia</i>	<i>marcescens</i>	3	0	0	0	3	0
<i>Hafnia</i>	<i>alvei</i>	51	12	19	14	6	61
<i>Proteus</i>	<i>vulgaris</i>	100	0	0	0	100	0
	<i>mirabilis</i>	100	0	0	0	100	0
	<i>morgani</i>	156	0	0	0	156	0
	<i>rettgeri</i>	42	0	0	0	42	0
	<i>inconstans</i>	82	0	0	0	82	0
<i>Pseudomonas</i>	<i>aeruginosa</i>	262	261	1	0	0	100
	<i>fluorescens</i>	40	40	0	0	0	100
	UFP†	94	92	2	0	0	100
	other‡	84	79	3	0	2	98

+ = Reaction colour brownish-red; (+) pale orange; (-) honey yellow; - pale yellow.

* Includes eight anaerogenic strains ("Alkalescens-dispar").

† Unclassified fluorescent pseudomonads—see text.

‡ See text.

Percentages in italics derived from <20 strains.

pseudomallei, *P. putrefaciens*, *P. stutzeri*, *P. syringae* and *P. testosteroni*, 22 strains of unidentified fluorescent and 51 strains of unidentified non-fluorescent pseudomonads were positive. In contrast, *P. alcaligenes*, strain NCTC10367 and *P. diminuta*, strain NCTC8545, failed to produce detectable amounts of rhodanese.

Subtle differences in the "negative" reaction were also of some value. Thus all strains tested of *S. flexneri* (except serovar 6), *Yersinia* spp. and *Proteus* spp. gave a pale-yellow colour, whereas some strains of *Citrobacter*, *Klebsiella* and *Enterobacter* and the majority of strains of *Salmonella* gave a honey-yellow colour.

DISCUSSION

Vandenbergh *et al.* (1979) detected rhodanese activity in *E. coli* (132 strains), *Shigella* (37 strains), *Citrobacter* (10 strains), *Acinetobacter* (24 strains) and *P. aeruginosa* (30 strains), but not in *Salmonella* (21 strains), *Klebsiella* (92 strains) and *Proteus* (44 strains).

We have devised a simplified test, and by the use of smaller test volumes, higher concentrations of bacteria and some reagents, and visual instead of photometric reading, we have obtained reproducible results that agree in the most part with those of Vandenbergh *et al.* (1979). Exceptions among *S. flexneri*, *C. freundii* and *Klebsiella* may be attributed to the testing

of a greater variety of strains in the present study. Whether or not rhodanese-positive and rhodanese-negative *Klebsiella* strains belong to defined subentities of the genus, remains to be investigated.

From the results of Vandenberg *et al.* (1979) and of the present study, it may be suggested that rhodanese production is an important factor in the characterisation of higher taxons of the Enterobacteriaceae and other gram-negative bacteria. The test may be useful to distinguish between anaerogenic *E. coli* and *S. flexneri*, between *Salmonella* and rhodanese-positive *Citrobacter* or between rhodanese-producing *Klebsiella* and other Enterobacteriaceae.

The difference in rhodanese production between *S. flexneri* serovar 6 and other *S. flexneri* strains is especially interesting. On the basis of biochemical and genetic properties, it has been suggested (see Petrovskaya and Bondarenko, 1977) that *S. flexneri* 6 should be removed from the species *S. flexneri* and considered as one of the serovars of *S. boydi*. Rhodanese activity, which seems to be constant in *S. flexneri* 6, is a new character supporting this view.

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