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Utilization of *d*-tartaric acid by *Salmonella paratyphi B* and *Salmonella java*: comparison of anaerobic plate test, lead acetate test and turbidity test

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SUMMARY

d-Tartrate dehydrase of *Salmonella java* is an oxygen-sensitive enzyme active in cultures incubated under the poorly aerated conditions of static culture but not in fully aerated shaken cultures nor on plates incubated aerobically. On plates of *d*-tartrate minimal agar incubated anaerobically the enzyme or the degradation products of *d*-tartrate are exported from *d*-tartrate-positive cells and are available to *d*-tartrate-negative bacteria. This may give misleading growth results when *d*-tartrate-positive and *d*-tartrate-negative strains are tested for growth on the same plate of *d*-tartrate minimal agar.

The lead-acetate test terminated at 24 h, the 24 h turbidity test and the ability to grow on *d*-tartrate minimal agar within 48 h differentiated 53 *S. paratyphi B* strains that were negative in each of the three tests from 76 *S. java* that were positive in each of the tests. An intermediate group of eight strains utilized *d*-tartrate in Difco bacto-peptone water to give a positive lead acetate reaction at 2 days, were stimulated to a varying degree by *d*-tartrate in Oxoid peptone water within the same period of incubation and grew poorly on *d*-tartrate minimal agar. These latter strains may be deficient in a permease controlling uptake of *d*-tartrate or export of *d*-tartrate dehydrase.

Inability to utilize *d*-tartrate is unlikely to be the single character accountable for the reputed enhanced pathogenicity of *S. paraptyphi B* when compared with *S. java*. Indications for the existence of an enzyme, complementary to and mutually exclusive with *d*-tartrate dehydrase, that has a positive correlation with pathogenicity are discussed.

INTRODUCTION

Since *dextro*-tartaric acid was recognized by Brown, Duncan & Henry (1924) as a useful organic acid for differentiating salmonella it has been incorporated in most typing schemes that use biochemical reactions. The most important of the serotypes containing strains that differ in their reaction with *d*-tartrate is *Salmonella paratyphi B*. On the basis of reaction with *d*-tartrate, slime wall production and pathogenicity, Kristensen & Kauffmann (1937) recognized *S. paratyphi B* as being *d*-tartrate-negative and slime wall-positive; and *S. java* as being *d*-tartrate-positive and slime wall-negative; and the latter serotype was considered to have reduced pathogenicity for man.

Over the years several tests have been developed for detecting utilization of *d*-tartaric acid. First, the lead acetate test, using Difco bacto-peptone water as the base medium, in which undecomposed tartaric acid is detected by the formation of a bulky precipitate on the addition of lead acetate. Brown, Duncan & Henry (1924) recommended that the test be terminated at 48 h, though later authors extended the period of incubation to 14 days and included observation of enhanced growth and changes in pH as indicators of *d*-tartrate utilization (Kauffmann, 1969).

Because the lead acetate test proved unreliable as a test for fermentation of tartaric acid by *S. typhimurium*, due to different results being obtained in replicate tests on the same strain and the emergence of fermenting mutant bacilli in some test cultures, Alfredsson *et al.* (1972) introduced a second method, the turbidity test, to assess ability to utilize tartaric acids. In this latter test fermentation is indicated by a significantly greater amount of growth in a 24 h culture in Oxoid peptone water with added tartaric acid than in control cultures without acid. This 24 h turbidity test proved reliable in typing the *d*- and *l*-tartrate reaction of 4102 strains of *S. typhimurium* (Anderson *et al.* 1978; Barker, Old & Sharp, 1980). Oxoid peptone water rather than Difco bacto-peptone water was specified for the turbidity test because mutative fermentation by a genotypically *d*-tartrate non-fermenting strain occurred earlier and more often in the Difco-based medium. Conversely, Oxoid peptone water is not suitable as a base medium for the lead acetate test because the sodium chloride content leads to the formation of a bulky precipitate of lead chloride in the absence of *d*-tartaric acid (Alfredsson *et al.* 1972).

d-Tartaric acid is utilized by *S. typhimurium* in media in tubes incubated statically in poorly aerated conditions but not under the highly aerobic conditions of continuously shaken cultures. Furthermore, *S. typhimurium* is not able to utilize *d*-tartaric acid incorporated in minimal agar as the sole source of carbon and energy when the plates are incubated aerobically (Alfredsson *et al.* 1972). However, when incubated for 2 days under anaerobic conditions, growth of a *d*-tartrate-positive strain is clearly visible. This new, third, test for *d*-tartrate utilization has been used to differentiate 137 strains of *S. paratyphi B*/*S. java*, and the results are compared with those obtained in the lead acetate test and turbidity tests.

MATERIALS AND METHODS

Bacteria

The 137 cultures of *S. paratyphi B* or *S. java* included 95 from France in the years 1976 (23 cultures), 1977 (38) and 1981 (34) and 42 from Scotland in the years 1982 (19) and 1983 (23). Sixty-seven were obtained from man (faeces 50, blood 12, urine 2, pus 2 and bile 1), 49 from the environment, 12 from food, 2 from an avian source and 7 from unspecified sources. Eight cultures from frog legs and 3 from human faeces were monophasic (phase 1). Ninety-seven cultures belonged to one of 18 phage types, 28 were not typable and 12 cultures were not phage typed.

Organic acid

d-(+)-Tartaric acid $(CHOH.COOH)_2$. AnalaR grade, was from British Drug Houses Ltd. It was prepared as a 10% w/v solution in deionized water and neutralized with NaOH. The solution was autoclaved at 121 °C for 15 min.

Inocula for tests

Stock cultures were maintained on Dorset's egg slopes at ambient temperature. The test strain was grown on Oxoid nutrient agar for 18–24 h at 37 °C. The bacteria from the mass growth were suspended in saline solution (0·85% NaCl) to a density of about 10^9 bacteria per ml. Test media received inocula of about 0·05 ml of the saline suspension.

Procedure for tests

Anaerobic plate test

Minimal agar medium was that of Davis & Mingioli (1950) and contained, per litre of deionized water: 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0·1 g $MgSO_4.7H_2O$ and 12 g Difco 'certified' bacto-agar. Sterile *d*-tartrate solution was added to sterile medium to give a final concentration of 0·5%. The medium was poured in amounts of 20 ml in plastic Petri dishes, 8·5 cm diameter. Saline suspensions of bacteria of up to 12 strains were inoculated with a twelve-pronged inoculator in spots in equivalent positions, 2 cm apart, on plates of minimal agar medium with *d*-tartrate. Incubation at 37 °C for 48 h was in an atmosphere of 10% carbon dioxide and 90% hydrogen in an anaerobic jar (McIntosh & Fildes or Baird & Tatlock).

Lead acetate test

Difco 'certified' bacto-peptone (10 g/l) was autoclaved at 121 °C for 15 min. A solution of *d*-tartrate (10% w/v) was added to the base medium to give a final concentration of 1% (pH 7·2), and the medium was dispensed in amounts of 8 ml in cotton-wool-stoppered test-tubes (1·0 × 12 cm). Cultures were tested for *d*-tartrate utilization, detected by the formation of a small precipitate on the addition of a saturated aqueous solution of lead acetate in the proportion of 0·1 ml per 1 ml of culture. A bulky precipitate of lead tartrate indicated a negative reaction. Readings were made at 1, 2 and 6 days, either on independent cultures or on samples of 1 ml removed from the test culture.

Turbidity test

Cultures were examined for stimulation of growth in Oxoid peptone water (10 g/l) as described by Alfredsson *et al.* (1972). *d*-Tartrate (10%) was added to the peptone water to a final concentration of 10 g/l and the pH was adjusted to pH 7·0 or, as indicated, to 7·4. The medium was dispensed in 8 ml amounts in cotton-wool-stoppered test-tubes (1·0 × 12 cm).

RESULTS

Anaerobic plate test

Growth of strains able to utilize *d*-tartrate as sole source of carbon and energy was clearly visible at 2 days when the inoculum was a saline suspension transferred by a prong inoculator to the surface of *d*-tartrate minimal agar and the plate was incubated in an anaerobic jar. Growth was not seen on similar plates incubated aerobically. *d*-Tartrate-negative strains failed to show growth in 2 days on anaerobically incubated plates even when the area of the inoculum was close to that of a *d*-tartrate-positive strain. However, when these plates were re-incubated and examined at 4 days the area of the inoculum was covered by a ghost-like growth that could be interpreted as ability to utilize the *d*-tartrate. Subculture from this area failed to grow when plated on *d*-tartrate minimal agar in the absence of a *d*-tartrate-positive strain.

When read at 2 days the anaerobic plate test classified the 137 strains into 76 that were *d*-tartrate-positive, 53 that were *d*-tartrate-negative and 8 intermediate strains, growth of which appeared faintly visible at 2 days and clearly visible at 6 days on plates without a *d*-tartrate-positive strain.

When inoculated in close proximity to a *d*-tartrate-positive strain, each of the 53 strains of *S. paraptyphi B* that failed to grow in 2 days showed clearly visible 'ghost' growth at 4 days. Conversely, each of the 76 strains of *S. java* and the 16 mutant strains of *S. paratyphi B* isolated in Difco bacto-peptone with *d*-tartrate was able to induce this 'ghost' growth in a strain of *S. paratyphi B*. The eight intermediate strains were unable to induce growth and were themselves induced to give an earlier appearance of growth when inoculated near a *d*-tartrate-positive strain. This cross-feeding between *d*-tartrate-positive and *d*-tartrate-negative bacteria suggests either that the enzyme is exported from producing cells, and is active in the medium in degrading *d*-tartrate to a form in which it can be utilized by cells that do not themselves produce *d*-tartrate dehydrase, or that the *d*-tartrate-positive bacteria break down the *d*-tartrate internally and export the degradation products which are then available to allow a limited number of divisions of the *d*-tartrate-negative bacteria.

Lead acetate test

Sixteen strains of *S. java*, known to be *d*-tartrate-positive in static culture, were incubated for 24 h in continuously shaken cultures of Difco bacto-peptone water with *d*-tartrate and tested by the addition of lead acetate. The formation of a bulky precipitate indicated that each strain was unable to utilize the *d*-tartrate under fully aerobic conditions.

The formation of a small precipitate on the addition of lead acetate to cultures incubated under static conditions for 24 h indicated complete utilization of *d*-tartrate by 76 strains. Of the 61 cultures considered negative by the formation of a bulky precipitate in 24 h static cultures, 53 were negative when the test was repeated on fresh cultures incubated for 2 days and 6 days, and 8 were positive after incubation for 2 days. These reactions were reproducible in repeated tests.

When the 53 *d*-tartrate-negative strains were cultured in Difco bacto-peptone water with *d*-tartrate and serially subcultured at 14-day intervals, 16 strains

Table 1. *Results of representative turbidity tests showing the effect of initial pH on the amount of growth of strains of* Salmonella java *and* Salmonella paratyphi B *grown in Oxoid peptone water without and with* d-*tartrate for* 24 *h at* 37 °*C under aerobic static conditions*

	Amount of growth (turbidity) in Oxoid peptone water					
	Initial pH 7·0			Initial pH 7·4		
Strain number	Without *d*-tartrate	With *d*-tartrate	Stimulation of growth by *d*-tartrate (%)	Without *d*-tartrate	With *d*-tartrate	Stimulation of growth by *d*-tartrate (%)
			S. java (1)*			
54/76	0·20	1·25	+525	0·21	1·25	+495
1069/82	0·32	1·27	+296	0·28	1·27	+353
			S. java (2)*			
53/76	0·24	1·21	+404	0·28	0·36	+28
2472/82	0·28	1·32	+371	0·22	0·23	+4
			S. java (3)*			
2225/82	0·25	0·31	+24	0·21	0·20	−5
2828/83	0·17	0·28	+64	0·17	0·19	+12
			S. paratyphi B (4)*			
22/77	0·35	0·38	+8	0·34	0·32	−6
3290/83	0·16	0·16	0	0·17	0·16	−6

* (1) 2 of 25 strains of *S. java* positive on all occasions of testing; (2) 2 of 51 strains of *S. java* for which reaction was pH-dependent; (3) 2 of 8 intermediate strains (see text); (4) 2 of 53 strains of *S. paratyphi B* negative on all occasions of testing.

produced *d*-tartrate-positive mutants which grew when plated on *d*-tartrate minimal agar. Three strains produced mutants during growth in the third subculture, 9 during the fourth and 4 during the fifth subculture.

Turbidity test

An absorptiometer reading of 1·00 or over of a culture grown for 24 h under aerobic static conditions in Oxoid peptone water with *d*-tartrate indicates full utilization of the acid. A strain unable to utilize the *d*-tartrate is either inhibited or slightly stimulated when the amount of growth (turbidity) is compared with that in the same base medium without *d*-tartrate.

When strains of *S. java* and *S. paratyphi B* were tested for utilization of *d*-tartrate at initial pH 7·0 the growth was either stimulated by 200–500 % (76 strains) or the turbidity measurements were between 20 % less (i.e. inhibition of growth) and 30 % more than in tartrate-free medium (53 strains). Only eight strains gave an absorptiometer reading that indicated an intermediate degree of stimulation. Different results were obtained for several strains when the pH of the medium before inoculation was raised to 7·4. Under this condition of growth strains which at pH 7·0 gave a reading greater than 1·00 now gave readings that ranged from the same as that in tartrate-free medium to 1·00. This partial stimulation of growth

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Table 2. d-*Tartrate reaction of strains of* Salmonella java *and* S. paratyphi B *tested by the turbidity test in Oxoid peptone water* pH 7·0 *at* 24 *h, the lead acetate test in Difco bacto-peptone terminated at* 1 *day and* 2 *days and the test for growth at* 2 *days on* d-*tartrate minimal agar incubated anaerobically*

Turbidity test, 24 h	Lead acetate test		Anaerobic growth in 2 days on minimal agar with *d*-tartrate	Number of strains of stated reaction pattern
	1 day	2 days		
+	+	+	+	76
−	−	+	⊥	8
−	−	−	−	53

was reflected in the intermediate values obtained for the percentage stimulation calculated from a comparison with growth in the tartrate-free medium (i.e. percentage stimulation of 0–100). The results for some representative strains are given in Table 1.

This difference in response to the presence of *d*-tartrate in the medium at alkaline pH is the most likely factor to account for the variable results obtained when peptone water and *d*-tartrate solution were prepared to pH 7·2 (as recommended by Alfredsson *et al.* 1972). If the medium was autoclaved after adjustment of pH the process of sterilization rendered it more alkaline (pH 7·3–7·4). When tested under these conditions 51 of the 76 strains classed as *d*-tartrate-positive gave a negative reaction on at least one occasion in repeated tests.

When incubated in continuously shaken culture the level of turbidity in peptone water of the 16 strains tested greatly exceeded that in static culture. However, none was stimulated by *d*-tartrate by more than 100% under the more highly aerobic conditions.

Correlation of reaction in different tests

The 76 strains classified as *d*-tartrate-positive in any one test gave a similar reaction in the other two tests, and likewise the 53 *d*-tartrate-negative strains were also classified by each of the three tests (Table 2). The eight strains that gave a late positive reaction in the lead acetate test were either not stimulated or stimulated by less than 100% within the 24 h incubation period of the turbidity test, but sometimes showed enhanced growth to the full extent (i.e. absorptiometer reading > 1·00) in *d*-tartrate peptone water after incubation for 48 h. This late *d*-tartrate-positive reaction in liquid medium correlated with poor ability to utilize *d*-tartrate as sole carbon and energy source in minimal medium.

DISCUSSION

Conditions under which *d*-tartrate is degraded by *S. java* are critical with respect to the oxygen concentration in the medium and the pH. *d*-Tartrate was utilized in poorly aerated static cultures and anaerobically incubated plates but not in highly aerated liquid culture in Difco bacto-peptone or Oxoid peptone water, nor

by bacteria growing on the surface of aerobically incubated agar plates. In this character the *d*-tartrate-specific enzyme of *S. java* resembles the oxygen-sensitive *d*-tartrate dehydrase of *Pseudomonas putida* described by Hurlbert & Jakoby (1965).

The influence of pH on *d*-tartrate utilization was conditioned to some extent by the base medium and had a greater effect in Oxoid peptone water than in Difco baco-peptone. Growth in peptone water without *d*-tartrate made the medium more alkaline. In the presence of *d*-tartrate cultures of *d*-tartrate-negative bacteria remained alkaline, whereas those of *d*-tartrate-positive bacteria converted to acidity. This change generally occurred within 24 h when the initial pH of the medium was 7·0, but was delayed in cultures of many strains when the inital pH was 7·4. In these latter cultures the turbidity reading at 48 h usually indicated full utilization of the acid. The effect of pH within the range 7·0–7·4 could be overcome by specifying an incubation period of 48 h. However, this might lead to the classification of genotypically *d*-tartrate-negative strains as *d*-tartrate-positive due to a non-specific increase in turbidity. Such an increase sometimes occurs following the outgrowth of fimbriate bacteria in the highly selective conditions of aerobic static liquid culture (Old & Duguid, 1970).

With adherence to the optimum conditions and the recommended period of incubation, most strains of *S. paratyphi B* and *S. java* reacted similarly with *d*-tartrate by each of the three test methods and were clearly differentiated into *d*-tartrate-negative and *d*-tartrate-positive types. The eight strains found to give an intermediate reaction were isolated in Scotland in 1982 and 1983 and were of phage type Dundee (6 strains), Dundee v. 1 (1 strain) and one of an unspecified phage type. They probably represent a clone and should be classified as *S. java*, since they are capable of utilizing the acid on prolonged incubation. This delay in utilization is probably due to a deficient permease system for the passage of *d*-tartrate into the cell or for the export of *d*-tartrate dehydrase. Such strains are possibly analogous to strains of the rhamnose-weak phenotype described by Akhy, Brown & Old (1984) in the rhamnose utilization system of *S. typhimurium*, and should be considered to be *d*-tartrate-weak.

Apart from enhanced pathogenicity for man, an unsatisfactory character to score, reaction with *d*-tartrate is the most reliable of the characters reported by Kristensen & Kauffmann (1937) for distinguishing strains of *S. paratyphi B* from *S. java*, because many strains of *S. java* were found to produce copious amounts of slime wall when grown on an appropriate medium such as glucose minimal agar (Nicholson & Barker, unpublished observation). Although mutation to *d*-tartrate utilization by *S. paratyphi B* was a rare event, such mutations did occur and *d*-tartrate-positive mutants were isolated from several strains, indicating that the gene for *d*-tartrate dehydrase is chromosomal. In *S. paratyphi B* this gene may be switched off, or it may code for either a non-functional enzyme or a functional enzyme with a specificity for some substrate other than *d*-tartrate. If this substrate has a function in establishing infection then the production of the dehydrase in its alternative form should confer enhanced pathogenicity on *d*-tartrate-negative cells. There would thus be a correlation between enhanced pathogenicity and inability to utilize *d*-tartrate and no single cell would exhibit both enhanced pathogenicity and ability to utilize *d*-tartrate at any one time. Most infections due

to *S. paratyphi B* or *S. java* in Britain and France are imported or sporadic and there is little opportunity to observe *in vivo* mutation in the *d*-tartrate character. Close scrutiny of an outbreak in a closed community may reveal whether such a mutation does occur and tracing of strains isolated from humans and the environment they pollute, such as sewage, may show whether the environment plays a part in selecting bacteria with a particular *d*-tartrate character.

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