SPECIATION WITHIN THE GENUS BRUCELLA

IV. Fermentation of Carbohydrates

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Formation of acid in qualitative fermentation tests has been utilized as a differential criterion within the family *Parvobacteriaceae*. Brucellae are nonfermentative under these conditions; however, it was demonstrated nearly thirty years ago by McAlpine and Slanetz (1928) that glucose is utilized in broth cultures of *Brucella* species. More recently, McCullough and Beal (1951) have shown that several carbohydrates are oxidized by resting suspensions of brucellae.

These reports suggested to us that the qualitative demonstration of carbohydrate fermentation by brucellae was primarily a technical problem which should be amenable to solution. Our working hypothesis was that conditions which minimize deaminations and maximize the visualization of glycolysis would be most suitable for qualitatively revealing fermentation. Furthermore we anticipated that the fermentative abilities of different strains of brucellae would be related to their species designation.

This report describes a test, based on the above hypothesis, which demonstrates the fermentative abilities of brucellae and which reveals species differences in fermentation patterns. The test employs a peptone-free, phosphate buffered basal medium containing agar and a relatively sensitive pH indicator and requires a massive inoculum.

MATERIALS AND METHODS

Inocula. Working suspensions of brucellae in sterile distilled water were obtained by harvesting the growth on forty-eight hour supplemented Albimi brucella agar as in our earlier studies (Pickett *et al.*, 1953). The suspensions were used within twenty-four hours of the time of harvest, and their concentrations were adjusted, if necessary, to contain between 10 and 30 billion cells per 0.1 ml inoculum.

Basal fermentation medium. The different potential ingredients and the other variables which were examined, together with results obtained, are summarized below. Usually, only one or two strains of each species were employed for exploratory examinations of each variable. Additional strains were included in the final examination of these factors. The medium finally adopted consisted of carbohydrate, 1 g; cresol red, 2 mg; and agar, 200 mg per cent in 0.01 M Sorensen's phosphate, pH 7.6. Eight-tenths ml of the basal medium was dispensed into a 12 or 13 mm tube and sterilized at 120 C for 15 min; 0.1 ml of filter sterilized, 10 per cent, carbohydrate solution, and 0.1 ml of inoculum were then added. The inoculated fermentation tubes were incubated aerobically at 35 C. Initially, readings were taken after 1, 2, 4, 6, and 10 days' incubation; however, upon progressive improvements in the basal medium a final reading could be made at the second day with strains of *Brucella melitensis* and *Brucella suis*, and at the third or fourth day with most strains of *Brucella abortus*. Occasionally more than 4 days' incubation was required when unduly small inocula were used, or with strains of *B. abortus* which only slowly fermented mannose and rhamnose.

RESULTS

Development of basal medium. A consideration of likely pH indicators led to an examination of neutral red, bromcresol purple, bromthymol blue, phenol red, and cresol red. Of these, only phenol red and cresol red were satisfactory and, qualitatively, gave comparable results. Cresol red was slightly more sensitive and therefore permitted earlier reading of tests on strains of *B. melitensis* and *B. suis*; however, it had the disadvantage that, with some strains of *B. abortus*, control tubes showed an initial acidity which required several additional days' incubation before a final reading could be made.

Sorensen's phosphate buffers, varying in pH

from 6.5 to 8.3 and in final molarity from 0.001 to 0.05, were examined. Satisfactory results were obtained with concentrations from 0.007 to 0.015 M at pH 7.6 to 7.8. The substitution of dipotassium for disodium phosphate and the addition of 0.5 per cent sodium chloride, 0.1 mg per cent nicotinamide, 0.1 mg per cent thiamin, or 0.4 mg per cent hemin did not appear to influence the results obtained in fermentation tests.

Since oxidative degradation of carbohydrate was not desired, 0.1 to 0.5 per cent agar was added to effect partial exclusion of oxygen from the medium. With cresol red indicator, 0.2 per cent agar was optimal, while with phenol red 0.2 to 0.5 per cent agar gave satisfactory results. With both indicators, either anaerobic incubation or rubber stoppering of the tubes led to false positive tests. Addition of 0.05 per cent sodium thioglycolate was also unsatisfactory since this compound inhibited fermentation of glucose by *B. melitensis*. Some increase in the ratio of volume to surface area was effected by the use of 10 mm tubes, but the results were not significantly better than those obtained with 12 mm tubes, and the smaller tubes introduced a hazard of self-infection during mixing of inoculum with medium.

TABLE 1

Carbohydrates which do not distinguish species of Brucella

Carbohydrates	Strains Positive/Strains Tested		
	B. abortus	*B. melitensis*	*B. suis*
Adonitol	16/16	12/12	12/12
Arabinose	12/12	8/8	8/8
Erythritol	12/12	8/8	8/8
Fructose	11/11	8/8	8/8
Galactose	4/4	4/4	4/4
Ribose	4/4	5/5	11/11
Sorbose	11/11	8/8	8/8
Xylose	10/10	6/6	6/6
Glycerol	17/17	9/14	11/13
Sucrose	15/16	5/8	11/11
Cellobiose	0/8	0/4	0/4
Dulcitol	0/12	0/8	0/8
Inulin	0/6	0/7	0/10
Lactose	0/13	0/8	0/9
Mannitol	0/13	0/12	0/12
Melezitose	0/12	0/8	0/8
Melibiose	0/12	0/8	0/8
Raffinose	0/13	0/11	0/12
Salicin	0/10	0/8	0/8
Sorbitol	0/8	0/4	0/4
Starch	0/4	0/4	0/4

TABLE 2

Differential fermentation tests on 91 strains of typical and slightly atypical brucellae

Species	Strains Positive/Strains Tested					
	Glucose	Inositol	Maltose	Mannose	Rhamnose	Trehalose
B. abortus	44/44	44/44	0/44	40/44	44/44	0/44
B. melitensis	14/14	0/14	0/14	0/14	0/14	0/14
B. suis	33/33	0/33	33/33	33/33	0/33	33/33

Initially it was assumed that a trace of exogenous organic nitrogen would be required for adaptive enzymes, and hence Albimi peptone C, Albimi peptone M, Difco infusion broth, and Difco yeast extract were examined at levels from 10 to 500 mg per cent. The higher concentrations were, as anticipated, not admissible, and at no level did any of these substances frankly improve the results obtained in fermentation tests with either glucose or sucrose. Occasionally fermentation was improved slightly by 50 mg per cent of yeast extract, but the results suggested that the massive inocula provided adequate available nitrogen for adaptive enzymes.

Finally, no obvious qualitative or significant quantitative differences in fermentation tests were observed (1) when inocula were harvested

TABLE 3

Fermentation tests on frankly atypical strains of brucellae

Species According to Urea and Serological Tests	Strains	Glucose	Inositol	Maltose	Rhamnose	Trehalose
B. abortus	A19, A20, A45, A102, A103	+	+	−	+	−
B. melitensis	A90	+	−	−	−	−
B. suis	M8, S19, S25, S86, S87	+	−	+	−	+

from 2 per cent peptone M (Albimi) agar or trypticase soy (BBL) agar rather than from supplemented Albimi brucella agar, (2) when cell suspensions were held 24 to 48 hours at 4 C before use, or (3) with concentrations of carbohydrate varying between 0.5 and 2.0 per cent.

Fermentation tests. Twenty-seven different carbohydrates were screened, and those which had no differential value for speciation purposes are listed in table 1. Adonitol and sorbose were fermented slowly by all three species while glycerol was fermented slowly by *B. abortus*, and both slowly and irregularly by *B. melitensis* and *B. suis*. Sucrose was, similarly, fermented slowly and irregularly by *B. abortus* and *B. melitensis*, but rapidly and regularly by *B. suis*.

Carbohydrates that distinguish the species of *Brucella* are presented in table 2 which includes glucose, even though it is not differential, as a routine positive control. Table 2 encompasses only strains for which there was no occasion to question the species designation; therefore this table shows the fermentation patterns which obtain for the three species. Tables 3 and 4 show the fermentation patterns which were found with frankly atypical strains and with species-intermediate strains. Eleven of the strains listed in table 4 had recently been received from Dr. Cruickshank and had already been shown by him to be *B. abortus-B. melitensis* intermediates (Cruickshank, 1954, and *personal communication*); the remaining 7 strains had also been examined, by us (Pickett *et al.*, 1953), in some detail. The serological data for strains B49 through B206 are those of Cruickshank (1954, *personal communication*), while the serological, sensitivity, and urea test data for strains M6, M36, M40, M47, and M48 are those of Pickett *et al.* (1953).

TABLE 4

The characteristics of 18 species-intermediate strains of brucellae

Strains	Agglutinated by:		Sensitive to:						Urea	Fermentations				
	A*	M*	T	F	V	P	A	C		I	Mn	Ml	R	T
M6	+	−	−	−	−	−	−	+	−	+	+	−	−	−
M36	+	−	−	−	−	−	−	+	−	+	−	−	−	−
M40	+	−	−	−	−	−	−	+	−	+	−	−	−	−
A105	+	−	±	−	−	−	−	+	−	+	−	−	±	−
A106	+	−	±	−	−	−	−	+	−	+	−	−	±	−
B49	+	−	±	−	−	−	−	+	−	+	−	−	±	−
B55	+	−	±	−	−	−	−	+	−	+	±	−	±	−
B75	+	−	±	±	±	−	−	+	−	+	−	−	±	−
B77	+	−	±	−	−	±	−	+	−	+	±	−	±	−
B76	+	−	±	+	+	−	+	+	−	+	−	−	±	−
B32	−	+	+	−	−	−	+	−	−	+	+	−	+	−
B129	−	+	+	−	−	−	+	−	−	+	+	−	+	−
B131	−	+	+	−	−	−	+	−	−	+	+	−	+	−
B153	−	+	+	−	−	−	+	−	−	+	+	−	+	−
B158	−	+	+	−	−	−	+	−	−	+	+	−	+	−
B206	−	+	+	−	−	−	+	−	−	+	+	−	+	−
M47	−	+	−	−	−	−	−	+	+	+	−	−	−	−
M48	−	+	−	−	−	−	−	+	+	+	−	−	−	−

* Agglutination tests: A—absorbed *B. abortus* serum, M—absorbed *B. melitensis* serum.

Key: Sensitivity tests—T thionin, F basic fuchsin, V crystal violet, P pyronin, A azure A, C carbamate. Fermentation tests—I inositol, Mn mannose, Ml maltose, R rhamnose, T trehalose.

DISCUSSION

The most important observation during the course of this investigation, we think, was the uniformity of fermentation patterns shown by the 102 typical and atypical strains of brucellae which were examined. No irregularities were found in their reactions toward inositol, maltose, rhamnose, and trehalose; and only 4 (of 49) strains of *B. abortus* failed to ferment mannose. The nine slightly atypical strains in table 2 and the eleven frankly atypical strains of table 3 had given irregular results in dye sensitivity tests (Pickett *et al.*, 1953), but all these, as well as the typical strains, gave consistent fermentation patterns with four of the five differential carbohydrates. This is in contrast to frequent irregularities which are encountered when only thionin and basic fuchsin sensitivity tests and hydrogen sulfide production are employed for determination of species; and occasional irregularities even when crystal violet, pyronin, carbamate, nitrite, and urea tests are included (Pickett *et al.*, 1953; Pickett and Nelson, 1954). Furthermore, we have found that these latter tests may give unreliable results, even with typical strains, if the inocula are not standardized in respect to concentration. In fermentation tests, on the other hand, satisfactory results are obtained with inocula which may vary from 75×10^8 to 500×10^8 cells, and therefore the

bacterial suspensions need not be standardized photometrically before use. We have concluded, in fact, that failure to use inocula of known concentration accounts for the discrepancies obtained by other workers, particularly in thionin and urea tests, and hence their conclusion that only types or varieties rather than species should be recognized in the genus *Brucella*. Furthermore, Renoux's (1952) argument that strains of brucellae differ only quantitatively may no longer be tenable since frank qualitative differences were observed under our experimental conditions.

The consistent fermentation patterns obtained here with strains which gave frankly atypical results in dye sensitivity tests should also be emphasized, and the inadequacy of serological data noted. Five of these (table 3) were acutely dye sensitive strains of *B. abortus*, one was a sensitive strain of *B. melitensis*, and five were resistant strains of *B. suis*.

The only frankly irregular fermentation patterns observed during this investigation were those associated with strains which appear to be truly intermediate in respect to species. These patterns, therefore, substantiate their other atypical characteristics. Strains M6 through M40 (table 4), a *B. abortus-B. melitensis* group, are *B. abortus* antigenically, *B. melitensis* in sensitivity tests, and *B. abortus-B. melitensis* intermediates in fermentation tests, being glucose and inositol positive but rhamnose negative. Strains A105 through B76, another *B. abortus-B. melitensis* group, are also *B. abortus* antigenically, slightly sensitive *B. melitensis* in dye tests, and, again, *B. abortus-B. melitensis* intermediates in fermentation tests, being frankly inositol positive but giving only weak or negative tests with mannose and rhamnose. Strains B32 through B206 represent a quite different *B. abortus-B. melitensis* group, being frankly *B. abortus* in both sensitivity and fermentation tests but just as frankly *B. melitensis* antigenically. Finally, strains M47 and M48, formerly described (Pickett *et al.*, 1953) as strains of *B. melitensis* giving atypical urea tests, are frankly inositol positive and appear, therefore, to represent rare *B. abortus-B. melitensis-B. suis* intermediates.

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SUMMARY

Procedures were developed for qualitative fermentation tests with brucellae. Five carbohydrates, inositol, maltose, mannose, rhamnose, and trehalose, distinguish the species. Data were presented which show the reliability of these tests and their value for the identification of atypical strains and for the characterization of species-intermediate strains.

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