SPECIES METABOLIC PATTERNS IN MORPHOLOGICALLY SIMILAR GRAM NEGATIVE PATHOGENS

MARGARET E. MEYER AND H. S. CAMERON

School of Veterinary Medicine, University of California, Davis, California

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The morphological, cultural, biochemical, and nutritional characteristics of many of the species of microorganisms that are classified within the genera of *Moraxella, Neisseria, Brucella,* and *Bordetella* are so similar that identification of small gram negative organisms is both tedious and difficult. This impressive similarity that exists between species, and even between genera, is probably indicative of a close biological relationship, but it has created taxonomic problems.

Henricksen (1952) discusses the lack of distinguishing characteristics between *Moraxella* and *Neisseria*, and suggests that the two genera may be closely related. There is, in fact, no cultural or biochemical feature that clearly delineates these two genera. When *Bergey's Manual* (Breed *et al.*, 1948) created the genus *Moraxella*, it was placed in the tribe *Haemophileae*. Its relationship to other genera in this tribe, particularly *Haemophilus*, has not been clearly established.

Proom (1955), on the basis of nutritional evidence, supported the proposal of Moreno-Lópes that *Brucella bronchisepticus, Haemophilus pertussis*, and *Haemophilus parapertussis* be classified into the genus *Bordetella*. In view of the fact that this newest classification of these species will appear in the forthcoming seventh edition of *Bergey's Manual*, that nomenclature will be followed in this paper.

Many of the difficulties attendant upon identifying and classifying the small gram negative pathogens seemingly arise from a lack of information concerning the general metabolism of each species. The physiological studies reported herein were undertaken to determine if the basic metabolic pattern of selected species within the genera of *Brucella, Moraxella, Neisseria*, and *Bordetella*, that are culturally difficult to differentiate one from the other, would display enough characteristic metabolic differences to aid in identifying the species, and to perhaps help determine their taxonomic relationships.

MATERIALS AND METHODS

The strains of organisms selected for investigation of their metabolic patterns were those which conformed completely to species identification tests as described in *Bergey's Manual* (1948) and by Wilson and Miles (1955). The cultures of *Brucella* used for study were *B. abortus* strains 19, 1004, and 6; *B. suis* strains 55, 56, and 57; *B. melitensis* strains 83, 4103, and 103. All were smooth cultures that have been maintained as stock cultures in this laboratory, and easily speciated by appropriate techniques.

Cultures of *Moraxella bovis* and *Neisseria catarrhalis* were obtained from the stock culture collection of the Department of Microbiology, School of Veterinary Medicine, Davis. *Bordetella bronchisepticus* and *Bordetella pertussis* were obtained from the Cutter Laboratories, Berkeley, California, and are the strains used by them for the commercial production of bacterins.

The organism referred to as "ovine organism" is a small, gram negative, somewhat pleomorphic, coccobacillus that was isolated from the epididymis of rams by Buddle and Boyes (1953) and identified by them as a rough variant of *Brucella melitensis*. Organisms isolated by P. C. Kennedy in Davis, California, by W. T. K. Hall in Australia, and M. B. Buddle in New Zealand, were used for this study.

The medium used was tryptose agar (Difco) dispensed in either Blake bottles or Roux flasks; for the ovine organism it was enriched with 5 per cent whole bovine blood. Bordet-Gengou medium with 30 per cent sheep blood added was used for *B. pertussis*. The media were inoculated with a saline suspension of the desired organism, and a 24 hr incubation period at 37 C was allowed for *M. bovis, N. catarrhalis, B. bronchisepticus*, and 48 hr for the *Brucella* species, *B. pertussis*, and the ovine organism. The cells were harvested, washed, suspended in Sorenson's M/15 phosphate buffer at pH 7.0, and the cell concentration

TABLE 1

Comparative rates of oxidative utilization of amino acid substrates by gram negative pathogens

Substrates at 5 mg Concentration	Comparative $QO_2(N)$ values*							
	Brucella abortus	*Brucella melitensis*	*Brucella suis*	Ovine organism	*Moraxella bovis*	*Neisseria catarrhalis*	*Bordetella bronchi-septicus*	*Bordetella pertussis*
D-Alanine	83	398	129	200	200	400	230	222
L-Alanine	72	151	50	35	90	350	250	212
B-Alanine	0	20	5	0	17	0	25	0
L-Arginine	40	0	100	0	0	0	0	0
D-Asparagine	8	104	0	0	0	0	131	0
L-Asparagine	198	85	20	200	278	261	150	41
D-Aspartic Acid	0	50	10	96	42	57	95	60
L-Aspartic Acid	110	237	20	136	135	37	170	180
DL-Citrulline	60	30	140	0	0	89	25	0
D-Glutamic Acid	0	60	0	85	78	0	170	103
L-Glutamic Acid	300	170	52	190	270	286	260	380
Glycine	20	40	14	0	101	144	65	0
D-Histidine	0	0	5	0	19	0	0	0
L-Histidine	0	20	21	0	45	92	0	0
L-Leucine	0	0	0	20	10	0	207	0
L-Lysine	30	20	69	0	0	0	0	0
DL-Ornithine	70	20	230	0	0	204	0	0
DL-Phenylalanine	0	0	0	17	0	0	270	0
L-Proline	40	145	14	0	165	109	175	350
L-Serine	43	60	66	200	235	500	230	98
D-Threonine	0	0	0	0	30	0	161	0
L-Threonine	0	0	0	0	75	400	167	0
DL-Tryptophan	0	0	0	0	30	10	128	0
L-Tyrosine	0	0	0	0	25	0	400	0
D-Valine	0	0	0	0	0	0	205	0
L-Valine	0	0	0	0	0	0	220	0

* Endogenous rates subtracted.

adjusted on a Coleman Junior Model spectrophotometer. Cellular nitrogen was determined by a micro-Kjeldahl technique. Complete details for cell preparations were described earlier by Cameron and Meyer (1953, 1955).

The substrates were prepared by dissolving chemically pure amino acids in the buffer and adjusting the solutions to pH 7.0 by the addition of sodium hydroxide. Conventional manometric techniques were employed to determine oxygen uptake (Umbreit, Burris, and Stauffer, 1945). Each flask contained 1.0 ml cells, 0.5 ml of substrate containing 5 mg of the desired amino acid, 1.4 ml buffer, and 0.1 ml of alkali. Endogenous uptake rates were determined for each experiment, all exogenous respiration rates were determined in duplicate, and all experiments were repeated on several harvestings of cells from various lots of media.

RESULTS AND DISCUSSION

The figures given in table 1 are QO_2N values with the endogenous rates subtracted. The three species of microorganisms that comprise the genus *Brucella* are notably homogeneous, and species identification cannot be determined from growth or morphologic characteristics. Dye bacteriostasis, agglutinin adsorption, and bio-chemical techniques are the only enabling methods of differentiating the species. However, since the three species of *Brucella* are biochemically similar, the tests are necessarily ones of degree rather than kind. Such tests, therefore, have been the subject of intense study and controversy. Renoux (1952) believes that because of the homogeneous nature of the genus, it should not be speciated, but instead, the organisms be considered varieties of a single species.

The metabolic pattern of the members of the

genus *Brucella*, when on amino acid substrates, is evidence that some distinct differences do exist between the species. The metabolism of *B. suis* varies from *B. abortus*, and *B. melitensis* in its utilization of L-glutamic acid, L-asparagine, L-arginine, DL-citrulline, and DL-ornithine. L-Proline was utilized only by *B. melitensis*. The features distinguishing the species are few, but it is believed that they are definitive. Since the *Brucella* species display a loose but none the less apparent host preference, show quantitative biochemical differences, and have characteristic metabolic patterns, it is felt that the present species classification should be retained.

The ovine organism displayed a metabolic pattern that distinguished it from the genus *Brucella* in its inability to utilize L-proline, DL-ornithine, L-arginine or DL-citrulline, and its high oxygen uptake rates on L-serine, and D-glutamic acid. The ovine organism, however, lacks other distinguishing features in its metabolic pattern, and it is still difficult to classify. Its identity from the other species included can be determined by its complete inability to utilize L-Proline. The metabolic pattern for the three strains of ovine organism, gathered from widely separated countries, were identical.

The reclassification of *Haemophilus pertussis* to *Bordetella pertussis* solved a difficult taxonomic problem. From the early work of Fildes (1923) to the recent work of Proom (1955) there has been recurrent criticism of the placing of the pertussis organism in the genus *Haemophilus*. However, until the genus *Bordetella* was created, there was no existing genus into which this organism could logically be placed. *B. pertussis* is fastidious in its growth requirements, which is apparently reflected in its amino acid metabolism. This aids in distinguishing it from other organisms included in this study. Jebb and Tomlinson (1951) reported upon the catabolic activity of *B. pertussis*, and the results reported here parallel their results.

The organism now classified as *Bordetella bronchisepticus* has long posed a problem to taxonomists as it has been variously classified in the genera of *Alcaligenes*, *Brucella*, and *Haemophilus*. The metabolic pattern of this organism on amino substrates clearly distinguished it from each of the species of *Brucella*. Proom (1955) observed that this organism was also nutritionally distinct from the Brucella group. In view of these differences it is justifiably removed from the genus *Brucella*. In identifying it from the other organisms included herein, its high rate of utilization of L-leucine, DL-phenylalanine, D- and L-valine, D- and L-threonine, L-tyrosine, and DL-tryptophan clearly delineates it from its morphological relatives. While this work was in progress, Rowatt (1955) reported upon the deamination of amino acids by the genus *Bordetella* and recorded the identical amino acid utilization for *B. bronchisepticus* and *B. pertussis* as reported here.

Henrikson's (1952) review details the similarity of the genera *Moraxella* and *Neisseria*. The amino acid utilization of the species chosen for this study would indicate that the two genera are indeed, as he suggested, closely related. The only observed metabolic difference was the utilization of DL-ornithine and DL-citrulline by *N. catarrhalis*.

Since the experimental conditions in this investigation were standardized for all species, the rates given in table 1 are not necessarily the maximum rates obtainable, as optimum concentrations of cells and substrates often vary with the species. However, some observations concerning the general metabolic behavior of this group of organisms, as well as the definitive metabolic species characteristics, may be drawn from these data. It may be seen that in the species investigated, all used D- and L-alanine, and L-glutamic acid. *B. suis* was the only microorganism that failed to utilize L-asparagine and L-aspartic acid, and only the *Brucella* genus displayed a low uptake rate on serine. Since these particular amino acids are also essential constituents of both the tricarboxylic acid cycle and the transaminating systems, further species and genus metabolic differences may be reflected in their utilization of substrates involved in the Krebs cycle.

It is concluded that, while several species of microorganisms classified in the genera *Moraxella*, *Neisseria*, *Bordetella* and *Brucella* are morphologically similar, the basic metabolic patterns on substrates of amino acids were characteristic for the particular species; and that metabolic studies of this nature may be valuable aids in identifying these organisms and evaluating their taxonomic positions.

SUMMARY

Selected species of microorganisms from the genera of *Brucella*, *Moraxella*, *Neisseria*, and *Bordetella* were subjected to standardized experi-

mental conditions for manometric studies to determine the basic metabolic patterns of resting cells on 26 amino acid substrates. A determination of the metabolic pattern may be helpful in the species identification of these morphologically similar microorganisms. The method may also prove of value in ascertaining general biological and taxonomical relationships.

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