BACTERIAL PATHOGENICITY AND CHARACTERISATION

Carbon source utilisation by *Bordetella bronchiseptica*

BALBINA J. PLOTKIN and D.A. BEMIS*

Department of Microbiology, College of Osteopathic Medicine, Midwestern University, Downers Grove, IL and

*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA

Bordetella bronchiseptica isolates utilised tricarboxylic acid cycle intermediates – succinate, citrate, α -ketoglutarate, fumarate, lactate and oxalo-acetate; the organic acids pyruvate, acetate and lactate; and the amino acids proline, glutamate, glutamine and tyrosine – as sole sources of carbon and energy. The inability of *B. bronchiseptica* isolates, representing the three phase types and from different animal hosts, to utilise carbohydrates and sugar alcohols as sole carbon and energy sources was confirmed and extended. The influence of the carbon substrate on doubling time, piliation, flagellation, motility, capsule production and adherence to mammalian cells was also measured.

Introduction

Bordetella bronchiseptica is a gram-negative, obligately aerobic bacillus that colonises the ciliated respiratory epithelium of a broad range of animals. It is the aetiological agent of kennel cough in dogs and atrophic rhinitis in swine, and has been implicated as a cause of respiratory tract disease in several other animal species, including man [1–4]. Although the survivability of *B. bronchiseptica* in low-nutrient conditions has been investigated [5, 6], there is little information on the carbon and energy requirements of the organism, or on whether carbon source requirements and ultrastructural characteristics vary between strains from different host species.

It has been proposed that *B. bronchiseptica* is asaccharolytic [7–12]. However, this classification is based on the singular finding that it is unable, aerobically, to use glucose as a sole carbon and energy source. In addition to the lack of information on the utilisation of sugars and sugar alcohols for growth, the literature conflicts with regard to the ability of *B. bronchiseptica* to utilise amino acids or organic acids as sole sources of carbon and energy for growth [9–12]. Ulrich and Needham [12] reported that the organism required a mixture of amino acids for growth, whereas Proom [9] found that some isolates were capable of using pyruvate or glutamate as sole carbon and energy sources.

In the present study a defined medium was developed to determine the carbon and energy source utilisation patterns of 17 clinical isolates of *B. bronchiseptica* from various animal sources and representing the three phase types [13–14]. The effect of carbon source on cell surface properties was also examined.

Materials and methods

Bacterial strains

The 17 clinical isolates of *B. bronchiseptica* used for this study were obtained, grown and stored as described previously [13]. These isolates included: four smooth phase (SP) organisms from dogs (2) and rats (2); 10 intermediate phase (IP) isolates from dogs (4), guinea-pig (1), cat (1) and swine (4); and three rough phase (RP) organisms from dog, cat and guinea-pig. Each isolate was stored at -70°C until used. After thawing, the isolates were passaged no more than four times.

Growth conditions and growth rates

A defined growth medium (DGM) was developed (Table 1) and used for liquid cultures or solidified by the addition of Bacto Agar (Difco) 1.5% w/v. The carbohydrates and sugar alcohols (dextrose, mannose, galactose, maltose, mannitol, rhamnose, sorbitol, inositol, fructose, salicin, xylose, lactose, raffinose and sucrose) and organic and amino acids (citrate, succinate α -ketoglutarate, malate, fumarate, oxalo-acetate, lactate, acetate, pyruvate, lysine, glutamic acid sodium salt, phenylalanine, arginine, asparagine, tyrosine, histidine, glutamine, tryptophan, aspartic acid sodium

Received 27 Aug. 1997; revised version accepted 12 Jan. 1998.

Corresponding author: Dr B.J. Plotkin.

Table 1. Defined medium for the growth of *B. bronchiseptica* and testing of carbon source utilisation

Salts stock solution*	
MgSO ₄ ·7H ₂ O	10.0 g
FeSO ₄ ·7H ₂ O	0.5 g
NaCl	0.5 g
MnSO ₄ ·7H ₂ O	2.0 g
H ₂ O†	250 ml
Phosphate stock solution	
KH ₂ PO ₄	2.0 g
K ₂ HPO ₄	1.0 g
H ₂ O	100 ml
Yeast nitrogen base without amino acids and ammonium sulphate (YNB) stock solution	
YNB	6.7 g
H ₂ O	100 ml
Ammonium chloride stock solution	
NH ₄ Cl	15 g
H ₂ O	100 ml
Complete medium (100 ml; pH 7.0)	
Salts stock solution	2 ml
Phosphate stock solution	10 ml
Yeast nitrogen base stock solution	1 ml
Ammonium chloride stock solution	2 ml
H ₂ O	85 ml

* Phosphate and ammonium chloride stock solutions were autoclaved, the remaining stock solutions were filter sterilised.

† Distilled de-ionised water.

salt, cysteine, proline, methionine, glycine, alanine, valine, leucine, isoleucine, serine and threonine) were tested for their ability to act as the sole carbon and energy source for growth. All of the inorganic chemicals were obtained from Fisher Scientific (Pittsburgh, PA, USA). The amino acids and other organic chemicals were obtained from Sigma. All the amino acids used were of the L-configuration and the carbohydrates were of the D-configuration. The yeast nitrogen base without amino acids and ammonium chloride was from Difco. Each of the carbon and energy sources was filter-sterilised, then added aseptically to DGM at 0.02, 0.125, 0.25, 0.5 and 1.0 g/L of DGM (pH 7.0). Growth was determined by an increase in absorbance (A) and colony count after incubation (37°C; shaking culture; initial A₅₄₀ of 0.01) for at least 72 h. Ability to utilise a carbon source was defined as growth after three consecutive transfers (0.005–0.02 ml of inoculum/5 ml of DGM; A₅₄₀ equivalent of 0.01) through DGM containing the carbon source. Brucella Broth or Agar (Difco) was used as the growth control.

Growth rates for representatives of each phase type – isolate 110H (SP), 87 (IP) and 110NH (RP) – were determined by standard techniques, i.e., from the change in A₅₄₀ and viable cell count in cultures in their second transfer through DGM. All experiments were done in triplicate and repeated with different batches of defined media, and results expressed as means and SEM.

Phase switching (dissociation)

The effect of carbon and energy source on the stability of the phase type of the *B. bronchiseptica* isolates was

determined. The criteria used to assess phase dissociation were colonial morphology and motility [13]. Isolates were inoculated on to DGM agar with a carbon and energy source (1.0 mg/ml), or Brucella agar as the control. Colonial morphology was determined by examination of 100 colonies on each agar plate (five plates total) at random, with a dissecting microscope and indirect illumination [13]. Motility was assessed by microscopic observation of a hanging drop wet mount preparation.

Cell-surface properties

Isolates in the late logarithmic stage of growth on the various carbon sources were examined by transmission electron microscopy for the presence of pili and flagella. Bacteria were grown overnight in either Brucella broth or in DGM with a carbon and energy source (1.0 mg/ml of DGM). Samples (0.1 ml) were placed on a collodion-coated copper grid and stained with phosphotungstic acid 0.2%, pH 7.2. Because of the peritrichous arrangement of the flagella and pili, the degree of piliation and flagellation per cell was estimated for 50 bacterial cells by counting only the pili and flagella that extended from the edge of the cell in the plane of the collodion support. In addition, isolates 110H, 87 and 110NH, representative of the three phase types (SP, IP and RP, respectively), grown on the various carbon sources, were assessed for their piliation and flagellation at different stages in the growth cycle (early logarithmic, late logarithmic and mid-stationary – mid-point between initiation of stationary phase plateau and start of decline in viability).

The ability of strains 110H, 87 and 110NH to produce capsule in response to specific sources of carbon and energy was determined by the standard method of light microscopic examination of negatively stained (nigrosin) cell suspensions. They were grown overnight on DGM agar containing a carbon source (1.0 mg/ml) and on Brucella agar. Determination of the degree of capsulation was made on encoded preparations to avoid bias. A non-capsulate strain of *Escherichia coli* was used as the negative control and a capsulate strain of *Klebsiella pneumoniae* was the positive control for determination of capsule formation.

The effect of carbon and energy source on adherence of *B. bronchiseptica* isolates to hamster lung fibroblasts was determined as described previously [15]. Organisms in early logarithmic, late logarithmic and mid-stationary phases of growth were pelleted, then suspended in phosphate-buffered saline, pH 7.2, and incubated for 30 min at 37°C with hamster lung fibroblasts (Don Line) in monolayer culture on coverslips. After incubation, the monolayers were washed free of non-adherent bacteria, fixed and stained with crystal violet 1% w/v. The number of adherent

bacteria/fibroblast for 50 fibroblasts/coverslip was counted. Each experiment was done three times in triplicate.

Results

Carbon sources sustaining growth and their effect on colonial morphology

Carbon and energy requirements for growth of the 17 isolates of *B. bronchiseptica*, regardless of host species, were met by four of the 20 amino acids (glutamate, glutamine, proline and tyrosine) and all the other organic acids tested (succinate, citrate, α -ketoglutarate, acetate, fumarate, pyruvate, malate, lactate and oxaloacetate). None of the carbohydrates or sugar alcohols tested (dextrose, mannose, galactose, maltose, mannitol, rhamnose, sorbitol, inositol, fructose, salicin, xylose, lactose, raffinose and sucrose) supported growth.

Changes in colonial morphology of IP and RP isolates consistent with phase dissociation were not observed on DGM containing any of the organic acids, even through repeated laboratory passage. The level of phase dissociation for the SP isolate 110H to the rough phase was not significantly different from that reported for this isolate after growth on Brucella agar [13]. Thus, spontaneous dissociation of SP colonies to RP colonies was not inhibited or enhanced by the cultural conditions tested. No back-revertants (rough-to-smooth colonies) were detected, a finding which confirms a previous observation that the IP and RP isolates are stable colonial morphologies [13].

Comparison of generation times for smooth (110H), intermediate (87) and rough (110NH) phase isolates, during growth in DGM containing various carbon sources, showed that doubling times varied with both the isolate and the carbon source tested (Table 2). Overall, the fastest doubling times were for isolates

grown in Brucella broth (0.82 h, isolate 110H; 1.4 h, isolate 87; 0.99 h, isolate 110NH). With respect to the single carbon and energy sources, there was a considerable range in generation times measured (1.9–6.9 h, isolate 110H; 1.7–5.2 h, isolate 87; 1.4–4.5 h, isolate 110NH). However, final cell concentrations for all isolates grown in DGM were similar to those measured for the same isolates grown in Brucella broth (10^{8-9} bacteria/ml of medium).

Influence of growth conditions on cell surface properties

The effect of carbon and energy source on the production of capsule was assessed. The relative amounts of capsulation varied with the source of carbon and energy for isolates 87 and 110H, but not for isolate 110NH, as shown in Table 2.

Bacteria from the late logarithmic growth stage were assessed for the presence of pili and flagella by electron microscopy and negatively stained preparations. Regardless of carbon source, all 17 isolates displayed the pattern of piliation and flagellation typical for their respective phase type, with patterns and numbers of appendages similar to those observed for controls grown in Brucella broth. The extent of piliation and flagellation of the isolates representing the three colonial phase types when grown in Brucella broth is shown in Table 3. When present, both flagella and pili were distributed peritrichously on the bacterial cell surface. Piliation of strains 87 (IP) and 110NH (RP) was dependent on the age of the culture. The extent of piliation of the smooth phase isolates throughout their growth cycle, as exemplified by isolate 110H, exceeded that expressed by either rough or intermediate phase isolates.

Adherence to hamster lung fibroblasts of two of the three phase types varied with culture age, as exemplified by the results presented for isolates 87

Table 2. Effect of single sources of carbon and energy on mean generation time (MGT) and capsule production of *B. bronchiseptica* isolates

Carbon source*	Smooth phase (isolate 110H)		Intermediate phase (isolate 87)		Rough phase (isolate 110NH)	
	MGT (h) (SEM)	Capsule†	MGT (h) (SEM)	Capsule†	MGT (h) (SEM)	Capsule†
Brucella broth (control)	0.82 (0.01)	++	1.4 (0.02)	++	0.99 (0.02)	+
Succinate	2.1 (0.01)	++	1.7 (0.01)	++	1.4 (0.02)	+
Citrate	1.9 (0.01)	+	1.8 (0.01)	++++	1.6 (0.01)	+
α -Ketoglutarate	2.2 (0.01)	++	1.8 (0.01)	++	1.5 (0.02)	+
Acetate	2.4 (0.02)	+	2.0 (0.01)	+	2.3 (0.01)	+
Fumarate	2.7 (0.02)	+	2.1 (0.02)	+	2.0 (0.02)	+
Pyruvate	3.0 (0.02)	+++	3.0 (0.01)	+++	3.0 (0.03)	+
Malate	2.8 (0.01)	+	3.6 (0.01)	++	2.8 (0.01)	+
Lactate	5.7 (0.02)	+	3.6 (0.03)	+	4.2 (0.01)	+
Oxaloacetate	3.8 (0.01)	+	4.0 (0.02)	+	4.5 (0.01)	+
Tyrosine	2.6 (0.01)	+	1.8 (0.01)	+	2.1 (0.03)	+
Proline	4.6 (0.02)	++	2.7 (0.01)	++	3.3 (0.02)	+
Glutamate	3.6 (0.01)	++	3.5 (0.01)	+++	3.7 (0.01)	+
Glutamine	6.9 (0.03)	++++	5.2 (0.03)	+	4.1 (0.03)	+

* All single carbon sources were tested at 1.0 mg/ml.

† Relative amounts of capsule observed microscopically.

Table 3. Effect of stage of growth in Brucella broth on cell surface properties of *B. bronchiseptica*

Phase morphotype	Growth phase*	Number of pili/cell† (range)	Percentage of bacteria piliated	Number of flagella/cell† (range)	Percentage of bacteria flagellated	Motility‡	Adherence‡
Smooth (isolate 110H)	Early logarithmic	11 (1–40)	96	0 (–)	0	(–)	28 (2)
	Late logarithmic	13 (3–45)	98	3 (1–3)	3	(+)	29 (2)
	Mid-stationary	9 (1–20)	82	4 (3–7)	4	(+)	28 (1)
Intermediate (isolate 87)	Early logarithmic	3 (1–3)	24	3 (1–6)	100	(+)	25 (3)
	Late logarithmic	5 (1–6)	54	5 (1–9)	100	(+)	29 (1)
	Mid-stationary	1 (–)	2	3 (1–6)	34	(+)	11 (3)
Rough (isolate 110NH)	Early logarithmic	0 (–)	0	3 (1–6)	100	(+)	3 (2)
	Late logarithmic	2 (1–4)	34	3 (1–6)	100	(+)	19 (2)
	Mid-stationary	4 (1–6)	34	3 (1–6)	100	(+)	29 (3)

*Determined from A₅₄₀ readings.

†Mean results from two experiments. A total of 50 bacteria were surveyed for each time point. Calculation of mean numbers of pili and flagella includes only those cells possessing pili or flagella.

‡Mean number of adherent bacteria per hamster lung fibroblast (SEM).

§Presence or absence of microscopically detected translocational movement in a hanging drop wet mount preparation.

(IP) and 110NH (RP) (Table 3). The level of adherence of the smooth phase isolates, as shown by strain 110H, was similar at all phases of growth. All organisms of a particular phase type showed similar variations, or not, according to growth stage regardless of carbon source or animal host of origin. Adherence appeared to be related to the level of pilus expression. The finding that early logarithmic cultures of isolate 110NH exhibited low levels of adherence, despite the lack of observable pili, may be indicative of the level of sensitivity of direct observation, the presence of an alternative adherence mechanism [15], or both.

Rough and intermediate phase isolates were flagellate throughout their growth cycle as shown by isolates 110NH and 87, respectively (Table 3). In contrast, flagella were not observed on the smooth phase isolate, 110H, until the late logarithmic phase of growth and then only appeared on *c.* 3% of the organisms. This observation is consistent with the rate of smooth-to-rough phase switching that has been reported previously [13]. For all isolates, the presence of flagella correlated positively with motility.

Discussion

Previous studies have reported that in a complex broth medium containing glucose, *B. bronchiseptica* (phase type unreported) grew aerobically, increasing the pH of the medium, but that no growth was observed anaerobically in the presence of glucose, lactose, maltose, saccharose, dextrose or mannitol [7]. The use of a defined medium in this study supports and extends previous hypotheses that *B. bronchiseptica* is asaccharolytic.

The reported ability of *B. bronchiseptica* to utilise carbon sources other than saccharides for growth is not as clear cut. Studies on the nutritional requirements of *B. bronchiseptica* indicated a high level of variability between isolates in their ability to utilise particular carbon sources [9–12]. Proom [9] reported

that, whereas most isolates tested could grow in a mixture of amino acids or citrate or lactate, only two 'old laboratory strains' were capable of growing with glutamate, α -ketoglutarate, succinate or pyruvate as carbon sources. The findings in the present study, with low passage number isolates of *B. bronchiseptica*, were analogous to those with the laboratory passaged strains of Proom with regard to their ability to utilise some of the organic acids other than amino acids. The range of carbon sources supporting growth was also expanded from Proom's initial work. Furthermore, no variability was observed between isolates from different hosts or of different phase types in their ability to use a particular carbon and energy source for growth. However, growth rates did vary according to isolate and carbon source, although final cell concentration was similar to that after growth in Brucella broth. These findings confirm those of Bemis *et al.* [13] on 50 strains of *B. bronchiseptica* in that there is remarkably little variability between isolates in some of their physiological characteristics, including colony dissociation rate of smooth phase isolates, haemagglutination pattern, antibiotic susceptibility and nitrate reduction.

None of the isolates exhibited an absolute requirement for glutamic acid, as had been previously reported [9]. Furthermore, glutamine, proline or tyrosine, in addition to glutamic acid, supported growth. *B. bronchiseptica* had been reported to deaminate serine, glycine, alanine and leucine [10]. However, none of these amino acids was able to support the growth of any of the 17 isolates tested. Thus, there does not appear to be a direct correlation between deaminase activity and substrate utilisation for growth, as was suggested by Rowatt [10].

An effect of carbon source on colonial phenotypes and phase dissociation was not discernible. However, carbon source and growth stage did influence the expression of cell surface structures including capsule, flagella and pili. While these results are in agreement with the findings of Lee *et al.* [16], that pili are

ubiquitous in this species, Bemis *et al.* [13] reported that the intermediate and rough phase organisms were variable with respect to pilus production. The discrepancies that exist in the literature as to the ultrastructural properties of *B. bronchiseptica* may be explained by the different conditions under which *B. bronchiseptica* has been grown [13, 15–19]. Thus, the caution expressed by Peppler and Schrumpf [19] on the influence of growth media on antigenic variation in *B. bronchiseptica* is well founded. These culture-influenced alterations in cell surface structures could potentially affect host–parasite interactions.

Pili are considered to mediate *B. bronchiseptica* adherence to mammalian cells [15, 17–18]. Expression of these surface structures was to some extent affected by stage of growth. However, the carbon source did not appear to alter expression of pili as compared to control organisms grown in Brucella broth. Pili were not detected on rough phase organisms in early logarithmic phase. The lack of pili detectable by electron microscopy raises the possibility that, at least for the rough phase isolates, adherence may be mediated *via* surface structures other than pili. However, it must be cautioned that the level of sensitivity of detection of pili by electron microscopy cannot preclude their possible involvement in adherence to mammalian cells. It has been reported by us and others [18, 20] that multiple haemagglutinins are likely to exist within this species, and that there are at least two mechanisms for adherence to hamster lung fibroblasts [15]. Further studies will be needed to determine if the antigenic characteristics of the pili and receptors to which they bind are influenced by growth substrate.

The role of capsule or flagella as virulence factors for *B. bronchiseptica* has not been defined. Of the surface structures examined, the expression of capsular material was most affected by the carbon source available. However, there appeared to be no correlation between the presence of observable capsule, or amount of capsulation and generation time, stage of growth, or adherence to hamster lung fibroblasts. Bemis *et al.* [13] reported that capsule was not readily demonstrable on the organisms examined, whereas Nakase [21, 22] reported phase I organisms to be both capsulate and flagellate. This conflict may best be attributed, as in the case of piliation, to differences in culture conditions. The findings of the present study confirm those of Bemis *et al.* [13] that smooth phase isolates can be non-flagellate, although this property is growth-stage dependent, and flagellation is probably a reflection of the number of smooth phase isolates that have undergone dissociation to rough phase isolates in culture.

A culture medium has been developed to assess the influence that carbon substrates have on the growth

and surface properties of *B. bronchiseptica*. The study of the biological characteristics of *B. bronchiseptica* and its virulence factors will lead to an increased understanding of the factors which allow for the broad host range of this organism and variation in its pathogenicity.

References

- Goodnow RA. Biology of *Bordetella bronchiseptica*. *Microbiol Rev* 1980; **44**: 722–738.
- Woolfrey BF, Moody JA. Human infections associated with *Bordetella bronchiseptica*. *Clin Microbiol Rev* 1991; **4**: 243–255.
- Bauwens JE, Spach DH, Schacker TW, Mustafa MM, Bowden RA. *Bordetella bronchiseptica* pneumonia and bacteremia following bone marrow transplantation. *J Clin Microbiol* 1992; **30**: 2474–2475.
- Woodard DR, Cone LA, Fostvedt K. *Bordetella bronchiseptica* infection in patients with AIDS. *Clin Infect Dis* 1995; **20**: 193–194.
- Porter JF, Parton R, Wardlaw AC. Growth and survival of *Bordetella bronchiseptica* in natural waters and in buffered saline without added nutrients. *Appl Environ Microbiol* 1991; **57**: 1202–1206.
- Porter JF, Wardlaw AC. Long-term survival of *Bordetella bronchiseptica* in lakewater and in buffered saline without added nutrients. *FEMS Microbiol Lett* 1993; **110**: 33–36.
- Ferry NS. Etiology of canine distemper. *J Infect Dis* 1911; **8**: 399–420.
- Ferry NS, Noble A. Studies relative to the apparent close relationship between *Bact. pertussis* and *B. bronchisepticus*. I. Cultural agglutination and absorption reactions. *J Bacteriol* 1918; **3**: 193–208.
- Proom H. The minimal nutritional requirements of organisms of the genus *Bordetella* López. *J Gen Microbiol* 1955; **12**: 63–75.
- Rowatt E. Amino acid metabolism in the genus *Bordetella*. *J Gen Microbiol* 1955; **13**: 552–560.
- Snell JJS, Lapage SP. Carbon source utilization tests as an aid to the classification of non-fermenting gram-negative bacteria. *J Gen Microbiol* 1973; **74**: 9–20.
- Ulrich JA, Needham GM. Differentiation of *Alcaligenes faecalis* from *Brucella bronchisepticus* by biochemical and nutritional methods. *J Bacteriol* 1953; **65**: 210–215.
- Bemis DA, Greisen HA, Appel MJG. Bacteriological variation among *Bordetella bronchiseptica* isolates from dogs and other species. *J Clin Microbiol* 1977; **5**: 471–480.
- Bemis DA, Greisen HA, Appel MJG. Pathogenesis of canine bordetellosis. *J Infect Dis* 1977; **135**: 753–762.
- Plotkin BJ, Bemis DA. Adherence of *Bordetella bronchiseptica* to hamster lung fibroblasts. *Infect Immun* 1984; **46**: 697–702.
- Lee SW, Way AW, Osen EG. Purification and subunit heterogeneity of pili of *Bordetella bronchiseptica*. *Infect Immun* 1986; **51**: 586–593.
- Bemis DA, Wilson SA. Influence of potential virulence determinants on *Bordetella bronchiseptica*-induced ciliostasis. *Infect Immun* 1985; **50**: 35–42.
- Ishikawa H, Isayama Y. Bovine erythrocyte-agglutinin as a possible adhesin of *Bordetella bronchiseptica* responsible for binding to porcine nasal epithelium. *J Med Microbiol* 1988; **26**: 205–209.
- Peppler MS, Schrumpf ME. Phenotypic variation and modulation in *Bordetella bronchiseptica*. *Infect Immun* 1984; **44**: 681–687.
- Bemis DA, Plotkin BJ. Hemagglutination by *Bordetella bronchiseptica*. *J Clin Microbiol* 1982; **15**: 1120–1127.
- Nakase Y. Studies on *Hemophilus bronchisepticus* I. The antigenic structures of *Hemophilus bronchisepticus* from guinea pig. *Kitasato Arch Exp Med* 1957; **30**: 57–72.
- Nakase Y. Studies on *Hemophilus bronchisepticus* II. Phase variation of *Hemophilus bronchisepticus*. *Kitasato Arch Exp Med* 1957; **30**: 73–78.