To the Graduate Council:

I am submitting herewith a dissertation written by Balbina Jean Plotkin entitled "A Study of the Adherence of Bordetella bronchiseptica to Mammalian Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

T. C. Montie, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

A STUDY OF THE ADHERENCE OF *BORDETELLA BRONCHISEPTICA* TO MAMMALIAN CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Balbina Jean Plotkin

December 1982

ACKNOWLEDGEMENTS

The author is grateful for the support and editorial assistance of Dr. David A. Bemis, and for the advice offered by Drs. T. C. Montie, J. M. Becker, D. Doyle, C. J. Wust, and B. T. Rouse.

She would also like to thank Drs. K. V. Thomas and G. Merkel and other friends too numerous to name for their unfailing support.

ABSTRACT

The adherence of Bordetella bronchiseptica smooth, intermediate, and rough phase isolates to Hamster Lung Fibroblasts (HLF), as well as to a variety of mammalian cells, was investigated and the mechanism of adherence to HLF was studied. Isolates representing the three phases adhered to the HLF in a manner that was rapid and reproducible. Adherence occurred within 5 min. and increased to a stationary level of adherence by 30 min. Adherence was not appreciably affected by pH or growth substrate. Carbon source did not affect piliation or flagellation of the isolates. Piliation and adherence of the rough and intermediate phase isolates were, however, affected by stage of growth; the level of adherence and percent of bacterial cells with pili increased with age of culture. Piliation and adherence of the smooth phase isolate were similar throughout its growth cycle. The level of adherence increased with bacterial concentration and temperature. Adherence of the isolates to other cell lines and freshly isolated epithelial cells was similar to that observed for the HLF except that the level of adherence to freshly isolated cells was more variable than that observed for cell lines. Although the three phases were similar with respect to the number of adherent bacteria per cell, attachment of all rough and intermediate phase isolates tested (n=13) regardless of animal species from which the isolates were obtained was inhibited and reversed by citrate. EGTA also inhibited adherence of these two phases, except when an excess of the divalent cations Ca^{+2}, Sr^{+2}, Cd^{+2}, or Mn^{+2} were present. Adherence of smooth phase isolates (n=4) was inhibited and

reversed by N-acetylglucosamine (GlcNAc). The entire configuration of this molecule appeared to be important in blocking attachment since N-acetylgalactosamine, N-acetymannosamine, N-actylcysteine, and glucosamine blocked adherence to a lesser extent than N-acetylglucosamine. Also, mannoseamine and galactosamine had no blocking effects. The GlcNAc appeared to act by binding to the bacterial adhesin and not to the HLF since it was demonstrated that smooth phase bacteria bound [^{3}H]-GlcNAc in a manner that was specific and saturable, and that bacteria preincubated with GlcNAc then washed free of the unbound amino sugar were nonadherent whereas HLF similarly treated still bound bacteria to their surface. The bacterial adhesin(s) of all three phases of B. bronchiseptica were heat labile proteins which could be removed from the bacterial surface by relatively gentle agitation in a homogenizer. The pili of B. bronchiseptica were also heat labile and were removed by brief agitation in a homogenizer. The results suggested that B. bronchiseptica adhered to eukaryotic cells by at least two mechanisms which may in part have been mediated by pili.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

APPENDIX

LIST OF FIGURES

FIGURE PAGE

APPENDIX

PART I

ADHERENCE OF SMOOTH, INTERMEDIATE, AND ROUGH PHASE ISOLATES OF *BORDETELLA BRONCHISEPTICA* TO MAMMALIAN CELLS

CHAPTER I

INTRODUCTION

It is a generally accepted premise that the capacity of a microorganism to cause an infectious disease resides in its ability to (1) enter into or onto host tissues, (2) multiply in/and or on host tissue, and (3) damage the host (3,19,27,39,50,51). The first two steps taken together are referred to as colonization (27,50,52). Since colonization is a requirement for the production of an infectious disease, the microbial products responsible for colonization can be considered as determinants of pathogenicity or virulence factors. Lack of any bacterial virulence factor may result in attenuation. The multifactorial nature of virulence also means that a single component essential to virulence may be present on a strain that is attenuated or avirulent. For example, Escherichia coli which produces toxin but does not possess surface factors responsible for colonization are not capable of producing an infectious disease (52). Conversely, E. coli which possesses the K88 antigen but are nontoxigenic are also avirulent (52). Adhesins are the surface components that promote the adherence of bacteria to host epithelial surfaces (9,50). Surface components can also assist in the resistance to host phagocytic defense mechanisms (9,50,51).

It is believed that for Bordetella brochiseptica, adherence is an integral step in its colonization (6). B. bronchiseptica has been observed by a number of investigators to closely associate with the respiratory epithelia (5,6,60). Further, bacteria associated with

respiratory tissues, from animals with bordetellosis, could not be removed from these tissues by washing (35). Bemis and Kennedy (6) also demonstrated that isolates representing the smooth and intermediate phases of B. bronchiseptica adhered to ciliated cells in culture. Although B. bronchiseptica displays a marked predilection for the ciliated cells of the respiratory tract, as do other members of the genus Bordetella and various species of Mycoplasma, B. bronchiseptica is also capable of adhering to established cell lines (16,26).

The original grouping of B. bronchiseptica was with B. pertussis and B. parapertussis in the genus Hemophilius. Later, it was placed in the genus Bordetella, a genus coined by Moreno-Lopez (1962) to honor Jules Bordet, since the symptoms and pathology produced in animals, as well as its gram and oxidase reactions, are similar to that of B. pertussis and B. parapertussis (37). Prior to its placement in the genus Bordetella, bronchiseptica was reclassified several times. First described by Galli-Valerio (1896), the species was isolated and identified as Bacillus bronchicanis by Ferry in 1910 (14,17). In the ensuing years, it was renamed six times: Bacterium bronchisepticus, Evan (1918); Alcaligenes bronchisepticus, Bergy (1925); Brucella bronchiseptica, Topley and Wilson (1929); Alcaligenes bronchicanis, Hampt (1935); and Haemophilus bronchisepticus, Wilson and Miles (1946) (7,13,24,43).

Smooth-rough variation in colonial morphology is another feature common to the members of the genus Bordetella. As a result of this variation, the following group designations have been proposed for B. bronchiseptica: Phase I (or smooth phase) organisms are hemolytic,

nonmotile, piliated and produce a smooth colinial morphology. Further, Phase I organisms are unstable and dissociate readily to rough phase organisms both *in vivo* and *in vitro* (4,24,38). Rough phase organisms are nonhemolytic, highly motile and produce rough colonies. Rough phase organisms are variable with respect to piliation (4,38). Intermediate phase organisms are also nonhemolytic, motile, and variable with respect to piliation. However, they produce smooth colonies (4).

The usual correlation between colonial morphology and virulence is that of smooth colony-virulent character and rough colony-avirulent character. This has been used successfully to categorize a number of gram negative organisms including *B. pertussis* and *Neisseria gonorrhoeae* (8,31,32). However, the relationship between colonial morphology and virulence in *B. bronchiseptica* is uncertain. In 1956, Nakase (38) found Phase I *B. bronchiseptica* isolates to be pathogenic for mice, while Phase IV (rough) isolates were avirulent. In contrast, Bemis (5) and Goodnow (24) reported that smooth, intermediate and rough colony types demonstrated similar virulence in the canine and swine respiratory tracts, respectively.

A correlation between piliation, adherence, and virulence has also been reported for some pathogens. Type 1 *Neisseria gonorrhoeae* that are piliated are virulent and also adhere to mammalian cells (45,57). Conversely, Type 4 *N. gonorrhoeae* are both avirulent, nonpiliated, and relatively nonadherent (34). A positive correlation between piliation, adherence, and virulence has also been shown for *Escherichia coli* and *B. pertussis* (1,32,56). However, the relationship between piliation, adherence, and virulence in *B. bronchiseptica* is uncertain since there

is little information concerning its adherence or cell surface properties. Furthermore, avirulent mutants deficient in specific surface properties are not currently available (4,24). Adherence of isolates representing the three phases of B. bronchiseptica was studied using various mammalian cells. The effect of growth, in the presence of various carbon sources, and culture age on the relative piliation of the three phases was also determined.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Growth

Seventeen isolates of B. bronchiseptica were used (Table 1). These represented the three phases (smooth, intermediate, and rough) and were from different animal species. All cultures were maintained on Brucella Agar (Difco Laboratories, Detroit, MI). The formulation of this medium is given in the Appendix Table A-1. Smooth phase isolates undergo smooth-rough colony variation, therefore, care was taken when subculturing to ensure the selection of smooth colonies. All initial inocula were made from cultures grown on Brucella Agar for 48 hours.

Bacterial inocula for the adherence assay were prepared by inoculating 15 x 125 mm Kimax test tubes (Kimble Products, Owen, IL), containing 5 ml of Brucella Broth, with inocula prepared as described above. After growth overnight at 37°C on a tube rotator (60 revolutions per min.), a portion of each culture was added in a dropwise fashion to 5 ml of fresh medium and the absorbance adjusted to 0.05 at 540 nm. This wavelength was used for all bacterial density adjustments. These tubes were again incubated on the rotor at 37°C until an absorbance of 1.65 was obtained for the culture (usually between 4 and 5 hours). The bacteria were then harvested by centrifugation (12,000 x g, 30 min., 5°C), the supernatant was decanted and the pellet was suspended to an O.D. of 0.1 in Dulbecco's phosphate buffered saline (PBS) pH 7.2.

TABLE 1

BORDETELLA BRONCHISEPTICA ISOLATES: THEIR SOURCE OF ISOLATION AND PHASE DESIGNATION

Isolate[a]	Host Species	Phase
110H	dog	smooth
Rat 1	rat	smooth
Rat 2	rat	smooth
BB	dog	smooth
87	dog	intermediate
SHGP-1	guinea pig	intermediate
BTS	pig	intermediate
7-8 NADL	pig	intermediate
Manhattan BB	dog	intermediate
S. Madrid	pig	intermediate
D-1	dog	intermediate
495	pig	intermediate
Ct Madrid	cat	intermediate
9340 ATCC	dog	intermediate
110NH	dog	rough
Columbus	cat	rough
ILG	guinea pig	rough

[a]All isolates were provided by D. A. Bemis, The University of Tennessee, Knoxville, TN.

It was determined by both standard plate count and total bacterial count, using a Petroff-Hausser counting chamber (Hausser Scientific, Blue Bell, PA) that an O.D. of 0.1 was equivalent to 1×10^8 colony forming units per ml. The growth curve for B. bronchiseptica in Brucella Broth is presented in Fig. A-1 in the Appendix.

Preparation of Mammalian Cells Used for Adherence Studies

A variety of mammalian cells were tested to determine the host range and tissue specificity of B. bronchiseptica adherence. Buccal cells were obtained from a human volunteer, a specific pathogen-free dog and a Golden Syrian Hamster, by gently scraping the inside of the cheek with a microspatula. These epithelial cells were immediately suspended in PBS. The cells were washed free of salivary secretions by sequentially suspending and centrifuging at least three times (45 x g, 10 min.). After washing, the cells were suspended in PBS, counted, and diluted with PBS to a concentration of 5×10^5 cells per ml.

The cell lines used for these studies included: pig kidney, Georgia bovine kidney, bovine turbinate, Maden Darby canine kidney, bovine embryo kidney, and hamster lung fibroblasts (HLF). All cell lines, with the exception of HLF, were obtained from Dr. D. A. Brian, The University of Tennessee. The HLF were obtained from the American Type Culture Collection (Rockville, MD). McCoy's 5A medium with glutamate and 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) was used for the growth and maintenance of HLF. All other cell lines were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY) supplemented

with 0.03% $NaHCO_3$, 50 µg/ml gentamicin and 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) buffer.

For the adherence assay, the cell lines were trypsinized (0.25%) for 10 min. at room temperature. The cells were then suspended in fresh medium, containing serum to neutralize the trypsin, and diluted with this medium to a concentration of 5×10^7 cells per ml. Twenty ml. of this suspension were used to seed glass coverslips (2.5 x 1.0 mm) contained in a 100 mm square plastic petri dish (Lab Tek, South Bend, IN). The cell suspension was incubated at 37°C in 5% CO_2 in air. When the resultant cell monolayers reached confluency (usually by 48 hr.), the coverslips were used in the adherence assay.

To test the adherence of B. bronchiseptica to tissues, thick sections (approximately 2 mm) of the following tissues were obtained from specific pathogen-free dogs at necropsy (obtained from D. Bemis): trachea, cornea, lung, bladder, and intestine. The tissues were washed by agitation in PBS and then placed in fresh PBS.

Adherence Assay

A modification of the method described by Gibbons and van Haute (19) was used to measure adherence to buccal epithelial cells. One ml. of the bacterial suspension and 1.0 ml. of the epithelial cell suspension were combined and incubated on a tube rotor (60 rpm) for 30 min. at 37°C. Control tubes containing epithelial cells alone were also prepared. After incubation, the cells were washed free of nonadherent bacteria by suspension and centrifugation five consecutive times (45 x g, 10 min.). Smears of the washed cells were air dried, fixed with 1:1,

v/v, acetone/ethanol and stained with 1% aqueous crystal violet (Difco Laboratories, Detroit, MI). The number of adherent bacteria per epithelial cell was determined by microscopic examination. A minimum of 300 buccal cells were examined. All experiments were done in triplicate and repeated at least two times. The percentage of viable mammalian cells was determined before and after each assay by trypan blue dye exclusion. Only those preparations with greater than 98% viability were used. Since buccal cells have adherent autochthonous bacteria, all preparations which had a mean number of adherent bacteria than five per cell before assay were discarded. The mean number of adherent bacteria on the control cells (incubated with PBS alone) was subtracted from all experimental values.

To test B. bronchiseptica adherence to established cell lines, the cells were grown to confluent layers on coverslips, the coverslips were then washed in three changes of PBS and placed in a bacterial suspension containing 1×10^8 CFU per ml. Controls containing cell lines in PBS alone were also prepared. After incubation with the bacteria (30 min., 37°C), the monolayers were washed three times in PBS, fixed, stained with crystal violet, and examined by light microscopy. The number of adherent bacteria per cell was determined for a minimum of 50 cells per coverslip. All assays were done in duplicate and repeated at least once. Cell viability was determined before and after incubation with the bacteria by trypan blue dye exclusion.

The dog tissues were treated in a manner similar to that of the established cell lines. The thick sections were washed, then

incubated with 1 x 10^8 CFU/ml of B. bronchiseptica. After incubation (30 min., 37°C), the tissues were washed free of nonadherent bacteria, and processed for indirect immunofluorescence. Control tissues were incubated with PBS alone.

Indirect Immunofluorescence Technique

Pooled antiseria (0.1 ml) to B. bronchiseptica isolates 110H, 87, and 110NH, obtained from D. A. Bemis, was placed on both the mucosal and serosal surfaces of the dog tissues. After 30 min. incubation at room temperature in a covered petri dish, the tissues were washed in five changes of PBS, then stained with FITC-Protein A (0.25 mg/ml) (Pharmacia Fine Chemicals, Sweden) for 30 min. at room temperature. The tissues were washed in five changes of PBS, then examined by epi-fluorescent microscopy (Nikon Fluophot, Nikon, Inc., Japan). Tissue was considered to have adherent B. bronchiseptica if bright apple green fluorescing bacilli were observed only on the test tissues and not on the control tissues.

The Effect of Bacterial Growth Conditions on Adherence, Piliation, and Flagellation

B. bronchiseptica isolates were grown in either mineral medium (Table 2) containing the carbon compound to be tested or in Brucella Broth. The carbon compounds tested for utilization are listed in Table A-2 (Appendix). These included carbohydrates, amino acids, amino sugars, and organic acids. Test tubes with 5 ml of mineral medium containing either 0.5 mg/ml, 0.25 mg/ml, or 0.125 mg/ml, of the carbon source to be tested, or 5 ml of Brucella Broth, were

TABLE 2

COMPOSITION OF MINERAL MEDIUM USED TO SUPPORT THE GROWTH OF B. BRONCHISEPTICA

Compound	g/l
$MgSO_4 \cdot 7H_2O$	0.8
$FeSO_4 \cdot 7H_2O$	0.04
$NaCl_2$	0.04
$MnSO_4 \cdot 7H_2O$	0.16
KH_2PO_4	2.0
K_2HPO_4	1.0
Yeast Nitrogen Base[a]	6.7
NH_4Cl	3.0

[a]Difco Laboratories, Detroit, MI.

inoculated as described above. Cultures were incubated no more than 80 hrs. at 37°C on a tube rotator (60 rpm). When growth occurred (as evidenced by an absorbance of 0.1 or greater, and an increase in CFU), the bacteria were transferred to fresh homologous media. B. bronchiseptica was considered to be unable to utilize a carbon compound if no growth occurred after 80 hrs. of incubation. After inoculation into fresh homologous media, the bacterial cultures were grown to an absorbance of 1.65 and then divided in half. Half of the culture was centrifuged at 12,000 x g for 30 min. to determine the effect of centrifugation on adherence, piliation, and flagellation. The pellet was suspended in PBS and adjusted to an O.D. of 0.1 for use in the HLF adherence assay. A drop of this suspension was processed for electron microscopy (EM) as described below. The other half of the culture, which had not been centrifuged, was also processed for EM and adherence to HLF.

Electron Microscopy

For transmission electron microscopy, bacteria were placed on collodian coated copper grids stained with 0.2% aqueous phosphotungstic acid, pH 7.2, and examined on an RCA4 Transmission Electron Miscoscope. Using the criteria of Duguid (11,12), pili were identified as filaments between 0.2 and 10 μ in length and between 4.0 and 7.0 nm in width. Flagella were identified based on size and periodicity of structure (8,24). The number of pili and flagella observed to enter the edge of a bacterium were counted and the percent of bacteria with pili and/or flagella was calculated. For each determination a minimum of 50 bacteria were examined.

Adherence of Escherichia coli

The relationship between the HLF model system and other model systems for the study of bacterial adherence was determined by examining the ability of E. coli to adhere to HLF. Six strains of E. coli (D6, C1, D557, D5, D166, D163) were obtained from Dr. A. J. Schaffer, Northwestern University Medical School, and displayed either mannose sensitive or mannose resistant adherence to vaginal epithelial cells or guinea pig erythrocytes. These strains were inoculated into 5 ml of Luria Broth (Difco Laboratories, Detroit, MI). After incubation (18 hrs., 37°C) on a tube rotator (60 rpm), the bacteria were harvested by centrifugation (10,000 x g, 30 min., 5°C) and the bacterial pellet suspended to an O.D. of 0.2 (1 x 10 CFU/ml) in PBS containing 0.7% mannose or in PBS alone. The bacterial suspension was then used in the HLF adherence assay as described above.

Statistical Analysis

The standard error of the mean (SEM) was calculated for each test. Statistical significance between means was determined by the Student, Newman, Keuls test (55). A P value of 0.05 or less was reported as significant.

CHAPTER III

RESULTS

B. bronchiseptica Adherence

Seventeen bacterial isolates obtained from dogs, cats, pigs, rats, and guinea pigs were tested for their ability to adhere to HLF. All *B. bronchiseptica* adhered to HLF regardless of source of isolation or phase of the isolate (Tables 3 and 4). The adherence of isolates 110H, 110NH, and 87 (which represent smooth, rough, and intermediate phases, respectively) to established cell lines and buccal cells was not significantly different. Adherence to other established cell lines was not significantly different from adherence to HLF with regard to both number of bacteria per cell and the reproducibility of the adherence assay, as denoted by the small error of the mean. Although there was little variability in adherence to HLF and other cell lines, attachment of isolates to buccal cells was highly variable (SEM ± 9 to ± 14).

Isolates 110H, 87, and 110NH adhered to thick sections of tissue from the bladder, trachea, lung, cornea, and intestine as determined by indirect fluorescent microscopy. The three isolates adhered not only to tissue which they normally infect or colonize (i.e., trachea) but also to bladder, intestine, lung, and cornea. The bacteria were also distributed evenly over both the mucosal and serosal surfaces of all the dog tissues examined.

TABLE 3

EFFECT OF HOST SPECIES ON ADHERENCE OF *B. BRONCHISEPTICA* TO HAMSTER LUNG FIBROBLASTS

Host Species	Number of Adherent Bacteria[a]
Dog (N=7)[b]	30±2
Rat (N=2)	28±1
Guinea Pig (N=2)	30±1
Pig (N=4)	29±2
Cat (N=2)	30±2

[a] Mean ± SEM (Standard Error of the Mean) per cell.
[b] Number of isolates per each host species.

TABLE 4

ADHERENCE OF SMOOTH, INTERMEDIATE, AND ROUGH PHASE
B. BRONCHISEPTICA TO ESTABLISHED CELL LINES AND
FRESHLY ISOLATED EPITHELIAL CELLS

Cell Type	Number of Adherent Bacteria		
	Smooth (110H)	Intermediate (87)	Rough (110NH)
Human Buccal	30±9[a]	30±11	35±14
Canine Buccal	25±10	27±13	30±12
Hamster Cheek Pouch Epithelium	28±12	32±10	27±10
Hamster Lung Fibroblasts	28±2	28±1	30±2
Pig Kidney	27±3	28±2	28±3
Georgia Bovine Kidney	25±1	29±1	27±2
Maden Darby Canine Kidney	26±1	27±3	29±3
Bovine Embryo Kidney	27±1	29±2	30±3
Bovine Turbinate	25±1	30±1	30±3

[a]Mean ± SEM (Standard Error of the Mean); n=4.

Effect of Bacterial Growth Conditions on Piliation, Flagellation, and Adherence

Experiments were designed to determine how changes in carbon source and age of bacterial cells affect the number of pili, or flagella observed on the bacterial surface as well as the ability of the bacteria to adhere to HLF. The seventeen isolates grew in mineral medium only when one of the following amino acids were present: glutamate, proline, tryosine, glutamine, or when other organic acids such as citrate, succinate, malate, lactate, acetate, α-ketoglutarate, oxaloacetate, or pyruvate, were provided as a carbon source. Growth in mineral medium occurred at all concentrations of the carbon source tested (from 0.125 mg/ml to 0.5 mg/ml) and the bacteria reached the same maximal density (2×10^9 CFU/ml) at the greatest carbon concentration tested as that obtained in Brucella Broth. Of the 23 carbohydrates and six amino-sugars tested, none could be used for growth by B. bronchiseptica.

The 17 isolates were examined by electron microscopy for the presence of pili and flagella, after growth to late log phase in mineral media containing the various carbon sources. All of the smooth phase isolates grown in mineral medium were peritrichously piliated. Flagella were only rarely seen on these isolates. The intermediate and rough phase isolates generally were less piliated (less than eight pili per cell) than the smooth phase and isolates and nearly always peritrichously flagellated. Carbon source did not affect either piliation or flagellation.

Isolates 110H, 87, and 110NH were grown in Brucella Broth to determine the effect of culture age on piliation and flagellation

(Table 5). The smooth phase isolate was piliated throughout the growth cycle. Eighty-two (82), 98, and 82% of the bacteria in early log, late log and mid-stationary phases of growth, respectively, were piliated. No flagella were observed on the bacteria until the late log phase (3% bacteria flagellated). The number of piliated intermediate phase bacteria increased from 25% in early log phase to 54% in late log phase before decreasing to approximately 2% in the mid-stationary phase. Unlike the smooth and intermediate phase isolates, no pili were observed on the rough phase isolate in early log phase, although by late log phase, 34% of the bacteria were piliated and the number of piliated bacteria increased to 54% by mid-stationary phase. Both the intermediate and rough phase isolates were flagellated in early and late log phases. The number of intermediate phase bacteria possessing flagella decreased to 34% by mid-stationary phase. Photomicrographs of representative late log phase smooth, intermediate, and rough phase bacteria can be found in the Appendix.

The capacity of bacteria grown on various carbon sources to to adhere to HLF was determined. The 17 isolates adhered equally well to HLF, regardless of carbon source, and the number of adherent bacteria was similar to that obtained when the bacteria were grown in Brucella Broth.

The relationship between bacterial growth stage and adherence is shown in Table 6. The number of adherent bacteria per cell varied from 28 to 29 throughout the smooth phase growth cycle. Adherence of the intermediate phase organism during logarighmic phase of growth varied

TABLE 5

EFFECT OF GROWTH PHASE ON PILIATION OF SMOOTH, INTERMEDIATE, AND ROUGH PHASE B. BRONCHISEPTICA ISOLATES[a]

Phase	Growth Phase	Age of Culture[b]	Number of Pili/cell[c]	Percent Bacteria Piliated
Smooth (110H)	Early Log	7	+++	96
	Late Log	10	+++	98
	Mid-Stationary	18	+++	82
Intermediate (87)	Early Log	7	+	24
	Late Log	10	++	54
	Mid-Stationary	18	+	2
Rough (110NH)	Early Log	7	-	0
	Late Log	10	+	34
	Mid-Stationary	18	++	54

[a]Brucella Broth was the medium used for growth.
[b]Hours post inoculation.
[c]Number of pili/cell (N=50), +++ = Heavily piliated (>8 pill/cell); ++ = Moderately piliated (3-8 pili/cell); + = Lightly piliated (<3 pili/cell); - = Non-piliated.

TABLE 6

RELATIONSHIP BETWEEN B. BRONCHISEPTICA GROWTH PHASE AND CAPACITY TO ADHERE TO HAMSTER LUNG FIBROBLASTS

Growth Phase	Culture Age[a]	Number of Adherent Bacteria: Smooth (110H)	Intermediate (87)	Rough (110NH)
Early Log	7	28±1[b]	25±3	7±1
Mid-Log	8.5	29±1	27±1	19±2
Late Log	10	29±2	29±1	27±1
Mid-Stationary	18	28±1	11±3	29±3

[a]Hours post inoculation.
[b]Mean + SEM (Standard error of the mean); n=4.

from 25 to 29 adherent bacteria. By stationary phase, the number of adherent bacteria had decreased to 11 bacteria per fibroblast. Adherence of the rough phase isolate, 110NH, increased progressively with the age of the culture. In early log phase of growth, an average of seven bacteria were adhered per fibroblast, but by stationary phase the number of adherent organisms had increased to 29 per fibroblast.

Parameters of Adherence

These experiments were designed to determine the effect of various incubation conditions on adherence of smooth, intermediate and rough phase isolates to HLF. To determine the effect of temperature on adherence, adherence assays were carried out at incubation temperatures of 4, 20, 30, 37 or 45°C. At the temperatures listed, optimum attachment was observed at 30 and 37°C. At and below 20°C the level of adherence declined with decrease in temperature to a low of 5 to 6 adherent bacteria at 4°C. At 45°C, there was a decrease in the viability of the HLF during the assay (100 to 37% viable). To assess the effect of pH, bacteria representing the three phases were suspended in PBS which had been previously equilibrated with either HCl or NaOH to a pH of 4, 5, 6, 7.2, or 8. No significant differences in adherence were noted.

The effect of incubation time on adherence was determined for the three phases (Figure 1). All isolates initially adhered at the same rate. At 60 min., the intermediate phase isolate, 87, adhered to a slightly greater extent than the smooth or rough phase isolates (44 bacteria/HLF vs. 34 bacteria/HLF). The level of adherence

Figure 1. Effect of incubation time on the adherence of smooth (●), intermediate (■), and rough (▲) phase isolates of B. bronchiseptica to Hamster Lung Fibroblasts. Incubation was done at 37°C.

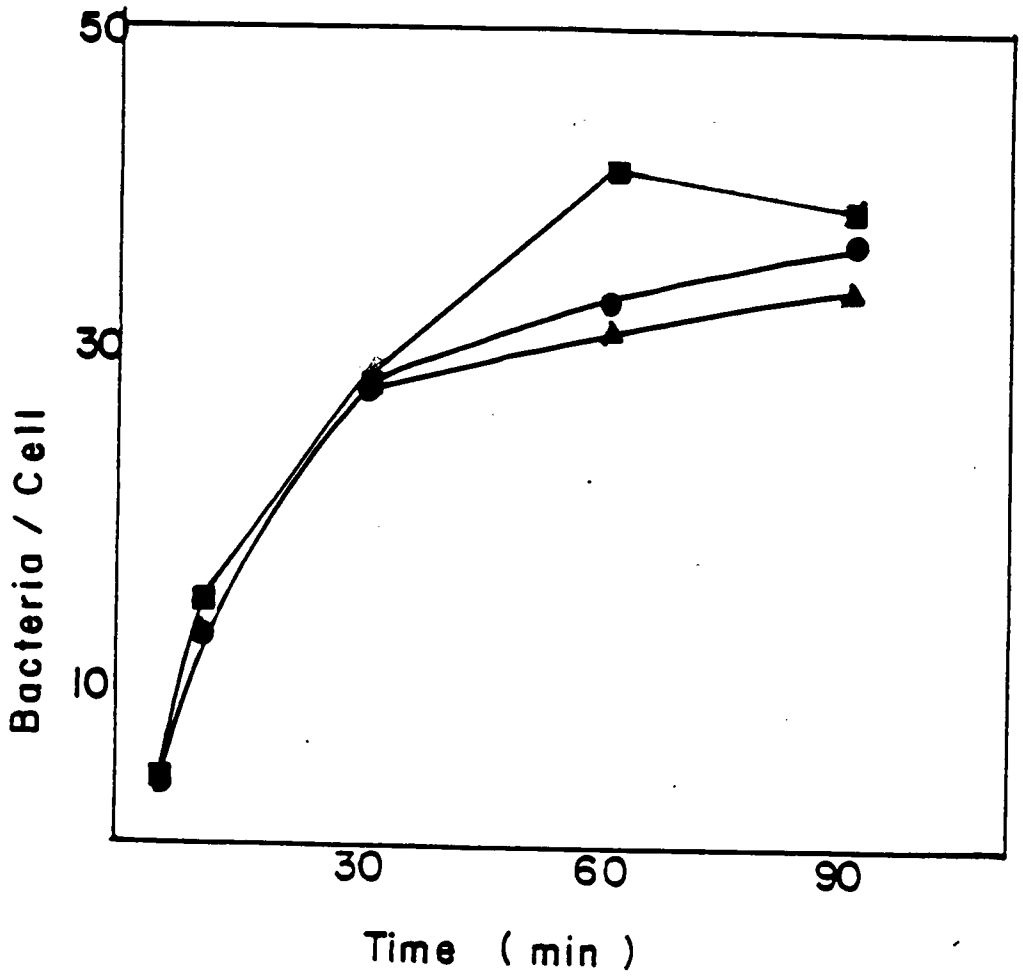

Figure 1.

declined slightly (by 7%) after 60 min.; however, there was also a decrease in the number of adherent fibroblasts after 60 min. The decline in the number of fibroblasts was also observed when fibroblasts were incubated in PBS alone.

The relationship between adherence and bacterial concentration was examined by adding increasing concentrations of either smooth, intermediate, or rough phase (110H, 87, and 110NH, respectively) bacteria to the HLF (Figure 2). With increasing concentrations of bacteria, the number of adherent bacteria increased by approximately 15 bacteria/HLF for every ten fold increase in bacterial concentration between the ranges of 1×10^6 to 1×10^9 CFU per ml. At concentrations greater than 1×10^9 CFU/ml, the bacterial suspensions autoagglutinated.

The effect of centrifugation on adherence, piliation and flagellation was also determined. No differences were observed between the adherence and relative piliation and flagellation of centrifuged and non-centrifuged bacteria.

Adherence of Escherichia coli

Six strains of E. coli adhered to HLF to the same extent (29±2 adherent E. coli per HLF). This level of adherence was similar to that observed with the uroepithelial cell model system, in which 31 bacteria adhered per cell (20). Mannose, which had been shown to inhibit three of the E. coli strains (D6, C1, D163) from adhering to both erythrocytes and uroepithelial cells also completely inhibited their adherence to HLF. The three strains whose adherence is mannose insensitive in other systems (D557, D166, D5) were also unaffected by mannose in the HLF model system.

Figure 2. The effect of varying concentrations of *B. bronchiseptica* smooth (110H, ●), intermediate (87, ■), and rough (110NH, ▲) phases on their adherence to Hamster Lung Fibroblasts. Incubation was done at 37°C for 30 min.

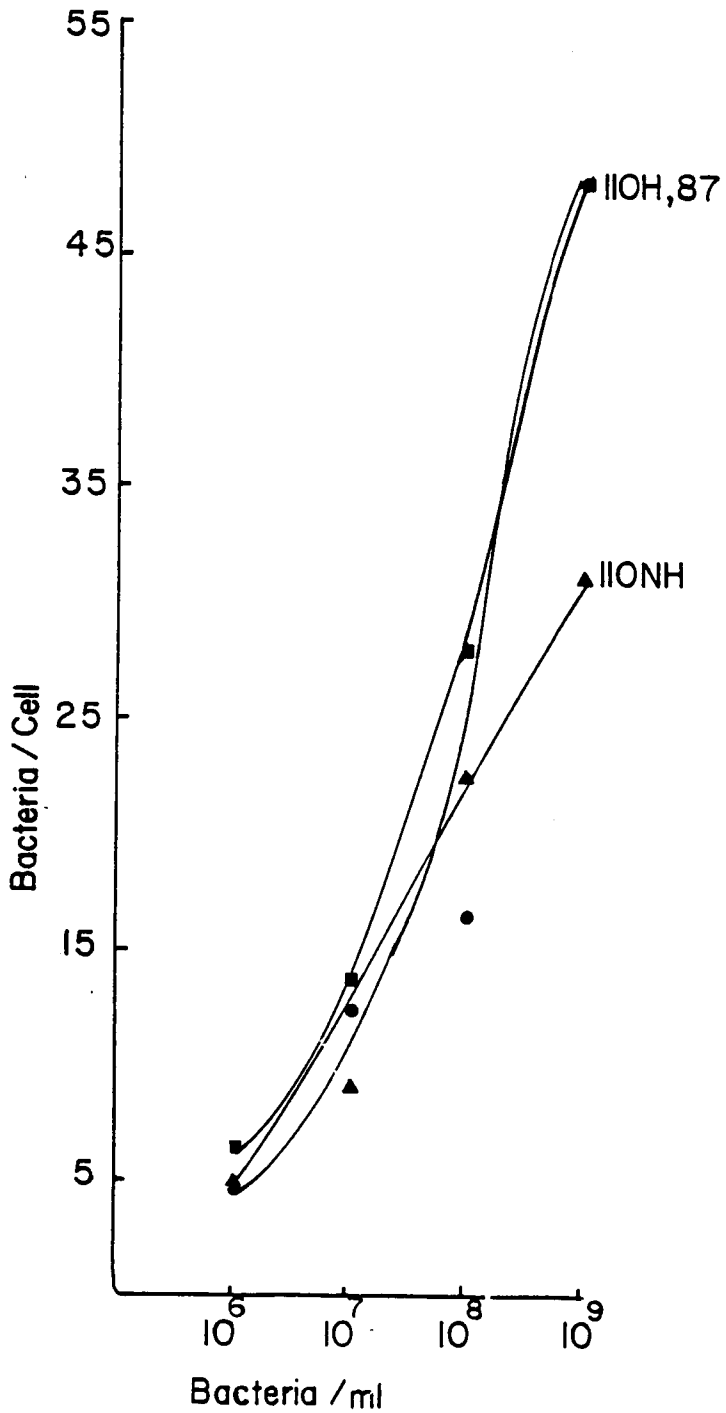
55
45
35
25
15
5
Bacteria / Cell
IIOH,87
IIONH
10^6
10^7
10^8
10^9
Bacteria / ml

Figure 2.

CHAPTER IV

DISCUSSION

This study describes a reproducible system for assaying the adherence of B. bronchiseptica to tissue culture cells. In addition to providing a large population of homogeneous cells, monolayer cell cultures circumvent several problems encountered in the use of tracheal ring cultures and buccal cells. Factors which must be taken into consideration in the use of tracheal ring cultures include: (1) physiological status of the host; (2) relatively small tissue mass; (3) mixed cell population; and (4) associated cell secretions. Many of these factors must also be evaluated when freshly isolated buccal epithelial cells are used in the study of bacterial adherence. However, in addition to the factors mentioned for tracheal ring cultures, buccal epithelial cells have adherent bacteria which could alter cell receptivity, and make differentiation of the added bacteria from those already present difficult (18). The mean number of adherent bacteria per cell was not significantly difficult for the epithelial cells and monolayer cell cultures tested; however, the SEM calculated for adherence to buccal epithelial cells was greater than that calculated for adherence to cell monolayers. These results further support the hypothesis stated above that cell monolayers provide a more reproducible model system for the study of adherence than buccal cells or organ culture model systems. HLF were selected for further study because they were obtained from an animal species which is susceptible to infection (personal

observation, data now shown) and from the organ system usually infected by B. bronchiseptica.

Bacteria in smooth, intermediate, and rough phases were able to adhere to various dog tissue cells, which had been obtained from organs not normally involved in infection. This apparent lack of specificity of adherence is rather at odds with the viscerotropism displayed by these organis in vivo (5,24). However, differences in adherence patterns in vivo vs. in vitro are not unusual. B. pertussis and Mycoplasma sp., both of which are respiratory tract pathogens and specific in their preferred site of colonization in vivo, can adhere to a variety of cell types in vitro including erythrocytes and cell monolayers (16,26,32). Perhaps this is due to the relative degree of exposure of these receptors in the natural state; i.e., receptors may be masked or unmasked by components of the normal physio-chemical environment which are not maintained in vitro.

Evidence that binding affinity may be related to a microbe's colonization pattern are provided by studies that examined the interaction between Mycoplasma gallisepticum and various sialylo-glycoproteins (22). M. gallisepticum, M. synoviae, and M. pneumoniae are able to agglutinate erythrocytes and bind to mammalian cell surfaces through neuraminidase-sensitive cell surface receptors, however, they do not display the same host and organ specificity (10). M. gallisepticum and M. synoviae,which are primarily associated with upper respiratory disease and synovitis, respectively, have a host specificity for poultry, whereas M. pneumoniae, the etiological agent of primary atypical pneumonia, is the only proven human pathogen (10,52).

These differences in organ and host specificity suggest that they may not bind to identical cell surface receptors (22). This hypothesis is supported by evidence that although M. gallisepticum can interact with a number of sialic acid moieties, certain sialyl glycoproteins are preferred, with the binding affinity of M. gallisepticum being a function of the number of terminal sialic acid residues and the structure of the sialic acids at the receptor site (22). Preliminary evidence that binding affinity may play a role in specificity at the site of colonization displayed by B. bronchiseptica is presented in Part II where N-acetylated amino sugars were shown to vary in their ability to inhibit B. bronchiseptica adherence. However, for all of the above mentioned studies, before the correlation between binding affinity and host-organ specificity can be made, a comparison of the number of high affinity receptors present at the site of infection to that present in other organs and hosts is necessary.

For a number of microbes such as Escherichia coli, Streptococcus mutans, and Mycoplasma pneumonia adherence is purported to be a virulence factor since mutants that can not adhere also do not cause disease (8,10,18,41). All of the B. bronchiseptica tested to date were able to attach to both HLF and human buccal cells. Since mutants of B. bronchiseptica that are either non-infectious or non-adherent have not yet been described, no definitive correlation can be made between the ability of B. bronchiseptica to adhere and its pathogenicity. It was observed that a rough phase isolate, 11ONH, although infectious (5) and able to adhere to HLF, did not adhere to ciliated cells in culture (6). These apparent discrepancies may be due to differences in bacterial culture methods or adherence assay methods.

Adherence of B. bronchiseptica to HLF was affected by the temperature of incubation, and length of incubation. The observation that B. bronchiseptica adhered less at 4°C than at 37°C suggests that fluidity of the fibroblast cell membrane may be important in adherence (48). However, enzymatic processes do not appear to play a role in adherence since bacterial adherence was not altered when either the fibroblasts or bacteria were fixed with acetone/ethanol or formalin, respectively (Part II). In the present study, adherence of all B. bronchiseptica strains was not significantly different over a pH range of 4 to 8. In this regard, B. bronchiseptica adhesin(s) appears to be different from that of Neisseria gonorrhoeae or Escherichia coli. Mardh and Westrom (33) noted that adherence of piliated strains of N. gonorrhoeae to vaginal epithelial cells was greater at pH 4.5 than 5.5 or 7.2. In uropathogenic E. coli, where piliation appears to be related to adherence, the bacteria have also been shown to adhere more avidly to mammalian cells at pH 4.5 (48). Furthermore, purified E. coli pili attachment to Vero cells was optimal at pH 4 to 5 (46).

Pili were detected on all strains of B. bronchiseptica studied. Yokomizo (60) reported that the smooth phase organisms he worked with were piliated and the rough phase organisms were either rarely piliated or nonpiliated. Bemis also reported that smooth phase organisms were piliated, intermediate phase organisms variable with respect to piliation and rough phase organisms were either rarely piliated or nonpiliated (4). The differences between these reports and the data presented here might be due to differences in growth conditions. It was observed here that rough phase isolates

were less piliated than smooth phase isolates and are nonpiliated in early log growth stage. The effect of growth conditions on piliation has been well documented for other microbes. For example, culture age (32,56), shake vs. stationary cultures (1), and carbon source (6,39) have all been demonstrated to have a profound effect on piliation of Escherichia coli, and B. pertussis, respectively. Svanborg-Eden (56) reported that E. coli in late log growth stage adhered to a greater extent than did E. coli in early log growth phase and that E. coli grown in the presence of glucose did not adhere. In this study, an association between culture age, the level of adherence as well as with the relative piliation of B. bronchiseptica intermediate and rough phase isolates was noted. Further, there was a positive relationship between the level of adherence and the degree of piliation. In addition, the smooth phase isolate was piliated and adhered at the same level throughout its growth cycle. These results suggest a positive relationship between piliation and adherence. However, nonpiliated rough phase bacteria also adhered though to a lesser extent than piliated rough phase bacteria. This could indicate another mechanism for adherence or a low level of piliation that was not detected. Adherence which is not pili mediated has been observed in other microbes. Swanson (57) and McGee (34) observed that nonpiliated Neisseria gonorrheae adhered to epithelial cells at a lower level than that observed for piliated gonococci. A hypothesis, which deals with the phenomenon of non-pili mediated adherence, has been postulated by Heckels (25). He suggests that the role pili play in N. gonorrhoeae adherence is one of reducing the net negative charge on the bacterial cell surface, thus allowing

for better bacteria - cell interaction while other adhesins may be responsible for the majority of the observed adherence. A similar situation may now be postulated for B. bronchiseptica adherence. It is not known how many pili-receptor contacts are necessary to allow for bacterial binding; however, it is interesting to note that while the level of adherence of the rough and intermediate phase isolates was similar to that of the smooth isolate, they were less piliated, suggesting that amount of piliation may not be related to the ability to attach.

Although no effect on piliation or adherence was demonstrated by the B. bronchiseptica substrate utilization studies, the observed ability of B. bronchiseptica to utilize various organic acids refutes Proom's observation (44) that citrate and glutamate are the only organic acids that B. bronchiseptica could metabolize, and supports the observation of Fukumi (15) that pyruvate can be utilized for growth. The results also extend and confirm the findings of Proom (44) and Snell (54) that B. bronchiseptica can not utilize carbohydrates for growth.

Although the receptor sites on the HLF were not saturated by the highest bacterial concentration tested (1×10^9 CFU/ml), when adherence was examined over time the number of bacteria bound was lower than would be predicted by the saturation curve. Similar observations have been made for B. pertussis (32), Escherichia coli (56), Mycoplasma pneumoniae (16), and Streptococcus mutans (18) where after an initial increase, the level of adherence remained constant over time. As with all of the above organisms, the receptor sites available for adherence of B. bronchiseptica could not be saturated by the highest bacterial concentration tested.

This study has shown that the three phases of B. bronchiseptica adhered to HLF in a manner that was rapid, reproducible, and subject to a variety of parameters. Adherence was altered by varying the bacterial growth conditions or the conditions under which the bacteria and fibroblasts were incubated.

PART II

ADHERENCE OF *BORDETELLA BRONCHISEPTICA* SMOOTH, INTERMEDIATE, AND ROUGH PHASE ISOLATES TO HAMSTER LUNG FIBROBLASTS

CHAPTER I

INTRODUCTION

Adherence to mucosal surfaces is recognized as an important step in colonization and infection (27,52). The ability of a pathogen to bind to mammalian cells appears to be mediated by surface components on both the bacterium and the mammalian cell. Studies to probe the nature of the structures involved in the attachment process have focused on compounds which inhibit adherence presumably by competing with identical or related substances on the cell surface (29,40,41). Recent studies suggest that sugar residues, amino sugars, and/or glycolipids on the surface of mammalian cells serve as the receptor determinants for certain human and animal bacterial pathogens (30,40,47). Mannose or its derivatives specifically inhibit the adherence of Escherichia coli to epithelial cells (41). Further, some mannose-insensitive E. coli have been postulated to bind to globotetrasylceramide, a glycolipid found on the surface of P^+ human erythrocytes (30). An amino sugar which has been shown to be involved in adherence is N-acetylneuraminic acid which mediates the binding of Mycoplasma pneumoniae to mammalian cells (16).

Although B. bronchiseptica adheres to a variety of cell types, as shown in Part I, little is known about the surface structures which mediate the bacterial-cell interaction. A compound capable of inhibiting and reversing bacterial adherence is generally considered to be an essential component of the mammalian cell receptor to which the bacterial adhesin binds (27,29,40,50). The purpose of this

study was to investigate the effect of various compounds on the adherence of B. bronchiseptica to Hamster Lung Fibroblasts.

CHAPTER II

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Seventeen isolates of B. bronchiseptica, which have been described in Part I, were used. These isolates represented the three phases (smooth, intermediate, and rough) and were from various animal sources. All initial inocula were made from cultures grown on Brucella agar for 48 hours.

Bacterial inocula for the adherence assay were prepared as described previously (Part I). Briefly, test tubes containing 5 ml of Brucella Broth were inoculated with 48 hr. cultures of each isolate. After growth overnight, a portion of each culture was used to adjust 5 ml of fresh medium to an O.D. of 0.05 (540 nm). These tubes were incubated until an O.D. of 1.65 was obtained. The bacteria were then harvested by centrifugation and the bacterial pellet suspended to an O.D. of 0.1 in Tris buffered saline (TBS) containing 0.4 M Trizma Base (Sigma Chemical Co., St. Louis, MO), 0.14 M NaCl, 0.1 mM $CaCl_2$, and 0.2 mM $MgCl_2$, pH 7.2.

Preparation of Mammalian Cells Used for Adherence Studies

Hamster Lung Fibroblasts (HLF), Don Line, were obtained from the American Type Culture Collection (Rockville, MD). The HLF were grown in McCoy's 5A with glutamate and 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY). For the adherence assay, the HLF were grown to confluency on glass coverslips, as described in Part I.

The human buccal cells were obtained and prepared as described in Part I. Briefly, after the cells were obtained, and washed free of salivary secretions, they were suspended in TBS to a concentration of 5×10^5 cells per ml of TBS.

Adherence Assay

Adherence of B. bronchiseptica to HLF was tested as described previously (Part I). Briefly, confluent monolayers of HLF were incubated (30 min., 37°C) with a bacterial suspension (1×10^8 CFU/ml) after which the monolayers were washed free of nonadherent bacteria, fixed, stained, and examined by light microscopy. The number of bacteria per cell was determined for a minimum of 50 cells on each of two coverslips. All experiments were repeated at least two times.

A modification of the method described by Gibbons and van Haute (19) was used to measure B. bronchiseptica adherence to buccal cells. As previously described (Part I), B. bronchiseptica were grown to late log phase and suspended to a concentration of 1×10^8 CFU/ml of TBS. One ml of the bacterial suspension was added to an equal volume of buccal cells (5×10^5 cells/ml of TBS). After incubation (30 min., 37°C), the cells were washed free of nonadherent bacteria, fixed, stained, and examined by light microscopy. The number of adherent bacteria was determined. All experiments were done in triplicate and repeated at least two times; a minimum of 300 cells were examined.

Chemicals

Various compounds were tested for their ability to inhibit B. bronchiseptica adherence. The compounds used were as follows:

arabinose, xylose, galactose, sorbose, fructose, glucose, rhamnose, fucose, mannose, cellobiose, maltose, sucrose, lactose, trehalose, mellobiose, raffinose, glycerol, inositol, mannitol, dulcitol, starch, lysine, arginine, histidine, aspartic acid, asparagine, glutamine, glutamate, cysteine, methionine, phenylalanine, tyrosine, tryptophane, proline, glycine, alanine, valine, leucine, isoleucine, serine, and threonine. These carbohydrates and amino acids were tested at 0.2 mM and 2.0 mM concentrations. The sodium salts of the following organic acids were also tested at 0.2 mM and 2.0 mM concentrations: malate, oxaloacetate, citrate, succinate, acetate, fumarate, pyruvate. The disodium salts of EGTA and EDTA were tested at concentrations of 10 mM, 5 mM, 1 mM, 0.5 mM, 0.25 mM, 0.1 mM, and 0.05 mM. N-acetylglucosamine (GlcNAc) was tested at concentrations of 0.5 M, 0.25 M, 0.125 M, 0.0625 M, and 0.0312 M. The following amino sugars were tested at concentrations of 0.5 M and 0.25 M: N-acetyl-muramic acid, N-acetyl-D-mannosamine, N-acetylcysteine, glucosamine, galactosamine, and mannosamine. All of the above chemicals were purchased from Sigma (St. Louis, MO) with the exception of the amino sugars which were purchased from Calbiochem - Behring (La Jolla, CA).

Bacterial Treatment Procedures

B. bronchiseptica isolates 110H, 110NH, or 87 were suspended in TBS, containing one of the above compounds, to a concentration of 1×10^8 CFU/ml. The suspension was kept at room temperature for 5 min. before addition of the fibroblasts. In addition, some suspensions were treated with either citrate or GlcNAc and centrifuged (12,000 x g), after 5 min. incubation, to remove unbound

citrate or GlcNAc. After the supernatant fluid was decanted, the pellet was suspended in TBS alone to a concentration of 1 x 10^8 CFU/ml, before using in the adherence assay.

Bacteria were treated with trypsin (0.25%) or protease K (20 µg/ml) (Sigma Chemical Co., St. Louis, MO) for 10 min. at 25°C to remove or digest bacterial surface proteins. Enzyme treatments were done in the presence of chloramphenical (200 µg/ml) to inhibit the regeneration of bacterial cell surface proteins. After treatment, the bacteria were washed free of the enzyme (12,000 x g, 30 min.). The bacterial pellet was then suspended in TBS with chloramphenical (1 x 10^8 CFU/ml) and a portion was removed for electron microscopy (EM). The EM was done (as described in Part I) to determine the effect of the enzyme treatments on piliation and flagellation. It was initially determined that enzyme treatment had no effect on bacterial viability, and chloramphenical did not reduce fibroblast viability.

Additional bacterial treatments were carried out to determine whether nonviable bacteria and bacteria whose cell surface components had been sheared off could adhere to HLF. These treatments included: heat inactivation (60°C, 60 min.), formalin inactivation (0.5%), and homogenization (20 min., at full speed in a Virtis homogenizer). The homogenization was done in the presence of chloramphenical (200 µg/ml) to inhibit regeneration of bacterial surface components. Following homogenization or formalin inactivation, the bacteria were washed by centrifugation (12,000 x g) to either separate the bacteria from the surface components that had been sheared from the cells or to remove excess formalin. The bacteria were then suspended in TBS (1 x 10^8 CFU/ml)

with 200 µg/ml of chloramphenical and a portion removed for examination by EM. Pili were isolated from the bacteria which had been homogenized by subjecting the homogenized supernatant to a series of high speed centrifugations (100,000 x g, 6 hrs, 5°C). The final pellet was suspended in TBS containing 0.1 M $MgCl_2$. The precipitate obtained after overnight incubation at 4°C was suspended in TBS and dialyzed against distilled deionized water. The dialysate was kept frozen at -20°C until examination by EM and use in the adherence assay.

Fibroblast Treatment Procedures

To examine the role of fibroblast surface components on *B. bronchiseptica* adherence, HLF were enzymatically treated prior to use in the adherence assay with either fucosidase (1 U/ml, 10 min.), neuraminidase (2 U/ml, 10 min.), or trypsin (0.25%, 5 min.). The treatments were carried out at 25°C. In addition to the enzyme treatments, fibroblasts which had been fixed with 1:1 v/v, acetone/ethanol were also used. This treatment was done to determine whether fibroblast viability affected adherence. The fibroblasts were also incubated with 2 ml of a preparation containing isolated pili for 10 min. prior to use in the adherence assay. After enzyme treatment, fixation, or incubation with the pili preparation, the fibroblasts were washed in three changes of TBS and used in the adherence assay.

Reversal of Adherence

Sodium citrate and GlcNAc were tested for their ability to cause adherent bacteria to release from the HLF. The adherence assay was performed as usual; however, instead of fixing the HLF,

they were placed in either sodium citrate (0.2 mM) or GlcNAc (0.25 M), and removed at 5 min. intervals. HLF were washed, fixed, and examined in the usual manner. Controls were incubated in TBS alone.

Cations were tested for their ability to block EGTA inhibition of adherence. The adherence assay was performed as usual except that modified TBS was used ($MgCl_2$, and $CaCl_2$ were excluded and 0.1 mM EGTA was added). To this reaction mixture was added 0.5 mM of the cation to be tested. These included the chloride salts of calcium, manganese, strontium, magnesium, lead, sodium, lithium, cesium, cadmium, potassium, barium, iron, mercury, zinc, and tin as well as zinc sulfate and ferric sulfate. Bacterial viability was not affected in the modified TBS. Controls also included HLF which were incubated with bacteria in TBS without EGTA.

Binding of Radio-Labeled GlcNAc to B. bronchiseptica

Isolates 110H, 87, and 110NH were grown as described above and then harvested by centrifugation and suspended to a concentration of 1×10^7 CFU/ml in TBS. The bacteria were mixed with tritiated GlcNAc [40 µl of 5.11×10^{-5} M GlcNAc (2.94 Ci/mM)] in plastic 13 x 75 mm test tubes and incubated at either 37°C or 40°C. For time course binding, portions of the suspension were removed after 1 min. and every 5 min. thereafter. Saturation binding was determined after incubation for 35 min. using various concentrations of GlcNAc. Competition studies were done using unlabeled GlcNAc or glucose (100 times ^{3}HGlcNAc concentration). Unbound GlcNAc was removed by filtration (0.45 µm millipore filters, New Brunswich, NY) of TBS and placed in 5 ml of Bray's

solution (Research Products Itern., Elk Grove Village, IL). The amount of radiolabel bound to the bacteria was determined by scintillation count (Searle Scintillation Counter, Iscap/300).

B. bronchiseptica isolates 110H and 87 that had been either treated with protease K or homogenized, as described above, were also examined for their ability to bind radiolabeled GlcNAc. Furthermore, the effect of homogenization on bound [^{3}H] GlcNAc was determined by homogenizing isolates 110H and 87 for 10 min. prior to filtration.

Statistical Analysis

The standard error of the mean (SEM) for each test was calculated. Statistical significance between means was determined by the Student, Newman, Keuls test (55). A p value of 0.05 or less is reported as significant.

CHAPTER III

RESULTS

Isolates 110H, 87, and 110NH were incubated with HLF in the presence of various carbohydrates, amino acids, and other organic acids. Adherence of these isolates in the presence of the twenty-three carbohydrates was equal to that of the TBS controls. Nineteen of the twenty amino acids tested also had no effect on adherence of B. bronchiseptica to HLF. However, glutamate (Table 1) inhibited adherence of the intermediate (87) and rough (110NH) phase isolates by 92% as compared to TBS controls. Adherence of the smooth phase isolate (110H) was unaffected by glutamate.

Incubation of the isolates with other organic acids also resulted in a decrease in the adherence of the intermediate and rough phase isolates, but not the smooth. As shown in Table 1, EGTA was the most effective as it completely inhibited adherence to HLF. The other organic acids also produced quantitative reductions in adherence to HLF. Of the organic acids tested, pyruvate was the least effective, resulting in a 20% inhibition of adherence with either 110NH or 87.

Ability of Cations to Overcome the Effect of EGTA on Adherence

Various cations were tested for their ability to overcome EGTA inhibition of adherence of 87 and 110NH (Table 2). This was done by adding the cations to be tested to a reaction mixture containing bacteria, HLF, and EGTA. The divalent cations, calcium, cadmium,

TABLE 7

EFFECT OF CATIONIC CHELATORS ON THE ADHERENCE OF B. BRONCHISEPTICA ROUGH AND INTERMEDIATE PHASE ISOLATES TO HAMSTER LUNG FIBROBLASTS

Chelator	Number of Adherent Bacteria			
	Intermediate (87)		Rough (110NH)	
TBS (control)	28[a]	(0)[b]	28	(0)
Glutamate	2	(92)	2	(92)
Oxaloacetate	1	(95)	1	(95)
Citrate	4	(85)	4	(85)
Malate	4	(85)	4	(85)
Succinate	6	(80)	6	(80)
Acetate	8	(72)	8	(72)
Fumarate	13	(54)	13	(54)
Pyruvate	22	(20)	22	(20)
EGTA	0	(100)	0	(100)
EDTA	20	(27)	20	(27)

[a]Mean number of adherent bacteria; SEM = 2.

[b]Percent inhibition: $100 \times \frac{\text{number of adherent bacteria in test}}{\text{number of adherent bacteria in control}}$.

TABLE 8

EFFECT OF CATIONS ON EGTA INHIBITION OF *B. BRONCHISEPTICA* ROUGH AND INTERMEDIATE PHASE ADHERENCE TO HAMSTER LUNG FIBROBLASTS

	Number of Adherent Bacteria	
	Intermediate	Rough
EGTA (control)	0[ab]	0
Calcium	29	28
Manganese	28	29
Strontium	28	28
Cadmium	29	28
Magnesium	0	0
Sodium	0	0
Potassium	0	0

[a]Mean number of adherent bacteria.
[b]SEM = 2.

strontium, and manganese restored the adherence of both isolates after it had been abolished by EGTA. Mangesium, sodium, and potassium did not affect the EGTA inhibition of adherence. Five of the cations tested (zinc, mercury, iron, and lead, and copper) caused autoagglutination of 87 and 110NH as well as 110H. Four other cations tested (lithium, cesium, tin, and barium) caused a substantial decrease (approximately 70% decreased) in the number of adherent fibroblasts.

Citrate Reversal of Adherence

The purpose of these experiments was to determine whether citrate, a compound that was able to inhibit the adherence of the rough and intermediate phase isolates, could also cause adherent bacteria to release from the HLF. The results are shown in Figure 1. Although the citrate had no effect on the adherence of the smooth phase isolate, it caused the intermediate and rough phase isolates (87 and 110NH, respectively) to be released from the fibroblasts. Half of the rough phase bacteria was still bound after 10 min., while half of the intermediate phase bacteria was adherent after 16 min. Citrate (0.2 mM) inhibited and reversed the adherence of thirteen additional rough and intermediate phase isolates. However, intermediate or rough phase isolates incubated with citrate and washed free of unbound citrate by centrifugation still adhered to HLF. The number of adherent smooth phase bacteria and bacteria adhering to the HLF in TBS controls remained constant for the duration of the assay.

Effect of N-acetylated Compounds and Amino Sugars on Adherence

Isolates 110H, 87 and 110NH were incubated with various N-acetylated compounds prior to their exposure to the HLF. The effects

Figure 3. Effect of 0.2 mM citrate on B. bronchiseptica bound to HLF. Symbols: (●) 110H, smooth phase isolate; (■) 87, intermediate phase isolate; (▲) 110NH rough phase isolate.

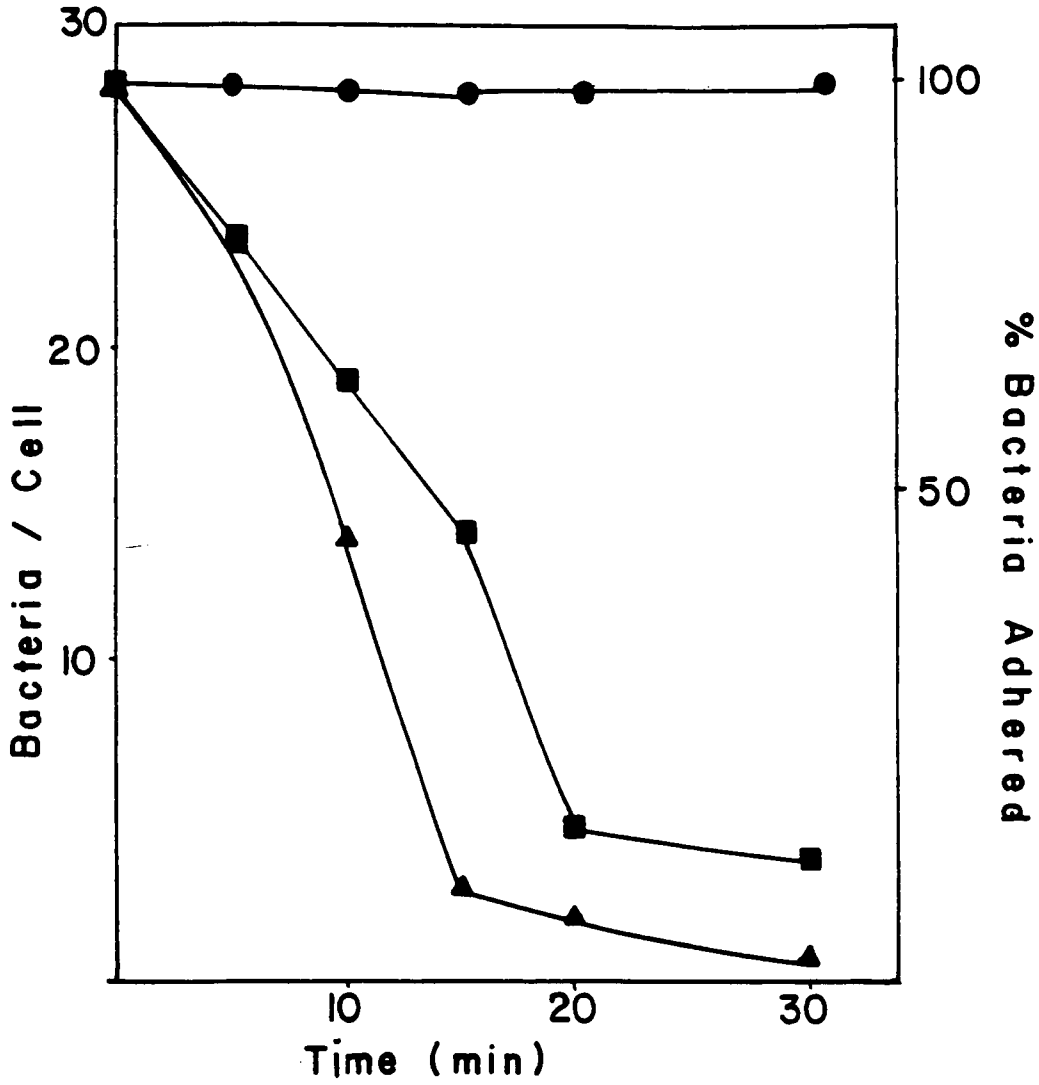
30
20
10
Bacteria / Cell
100
50
% Bacteria Adhered
10
20
30
Time (min)

Figure 3.

of these compounds on adherence are shown in Table 3. The most inhibitory compounds were GlcNAc, which inhibited 100% of 110H adherence and N-acetylmuramic acid, which inhibited 98% of 110H adherence. The N-acetylated compounds had no effect on adherence of rough and intermediate phase isolates. Additionally, smooth phase bacteria incubated with GlcNAc and washed free of unbound GlcNAc by centrifugation did not adhere to HLF. Rough and intermediate phase isolates similarly treated still adhered to HLF. All three isolates adhered to fibroblasts which had been incubated with GlcNAc and washed free of unbound GclNAc prior to use in the adherence assay. Other N-acetylated compounds such as N-acetylgalactosamine, N-acetylmannosamine, and N-acetylcysteine inhibited adherence by 50, 36, or 43%, respectively. Of the three amino sugars tested, only glucosamine inhibited adherence. It inhibited 50% of the smooth phase adherence.

The effect of various concentrations of GlcNAc on the adherence of isolate 110H is shown in Figure 2. GlcNAc at a concentration of 0.125 M inhibited 110H adherence to HLF by approximately 50%, while concentrations at or above 0.25 M completely inhibited adherence. GlcNAc at a concentration of 0.065 M did not effect adherence.

N-acetylglucosamine Reversal of Adherence

Fibroblasts with adherent bacteria were exposed to GlcNAc to determine if GlcNAc could reverse adherence. The data (Figure 3) indicate that adherence of the smooth phase isolate was reversed, whereas adherence of the rough and intermediate phase isolates, in the presence of GlcNAc was the same as the TBS controls. After approximately

TABLE 9

THE EFFECT OF AMINO SUGARS ON *B. BRONCHISEPTICA* SMOOTH PHASE ADHERENCE TO HAMSTER LUNG FIBROBLASTS

Compounds	Number of Adherent Bacteria	Percent Inhibition of Adherence[a]
TBS (control)	28 ± 1	0
N-acetylglucosamine	0	100
N-acetylmuramic acid	1 ± 1	96
N-acetylgalactosamine	14 ± 1	50
N-acetylmannosamine	18 ± 1	36
N-acetylcysteine	16 ± 1	43
Glucosamine	14 ± 1	50
Galactosamine	28 ± 1	0
Mannosamine	28 ± 1	0

[a] $100 \times \frac{\text{number of adherent bacteria in test}}{\text{number of adherent bacteria in control}}$.

Figure 4. Effect of concentration on GlcNAc's ability to inhibit the adherence of isolate 110H to HLF. Symbols: (●) 110H in TBS containing GlcNAc; (■) 110H in TBS alone.

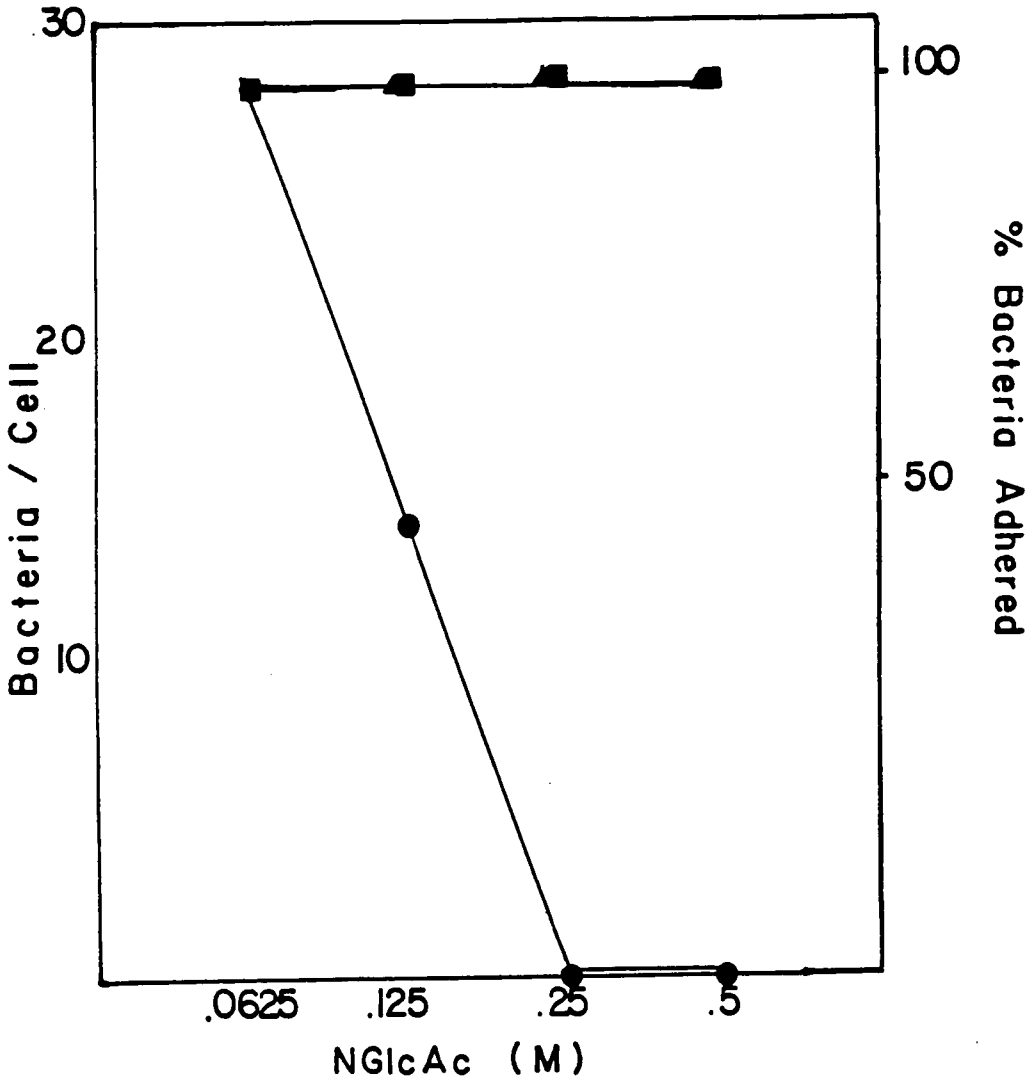
30
20
10
Bacteria / Cell
100
50
% Bacteria Adhered
.0625
.125
.25
.5
NGlcAc (M)

Figure 4.

Figure 5. Effect of N-acetylglucosamine (0.25 M) on B. bronchiseptica bound to HLF. Symbols: (▲) 110H, smooth phase isolate; (●) 87, intermediate phase isolate; (■) 110NH, rough phase isolate.

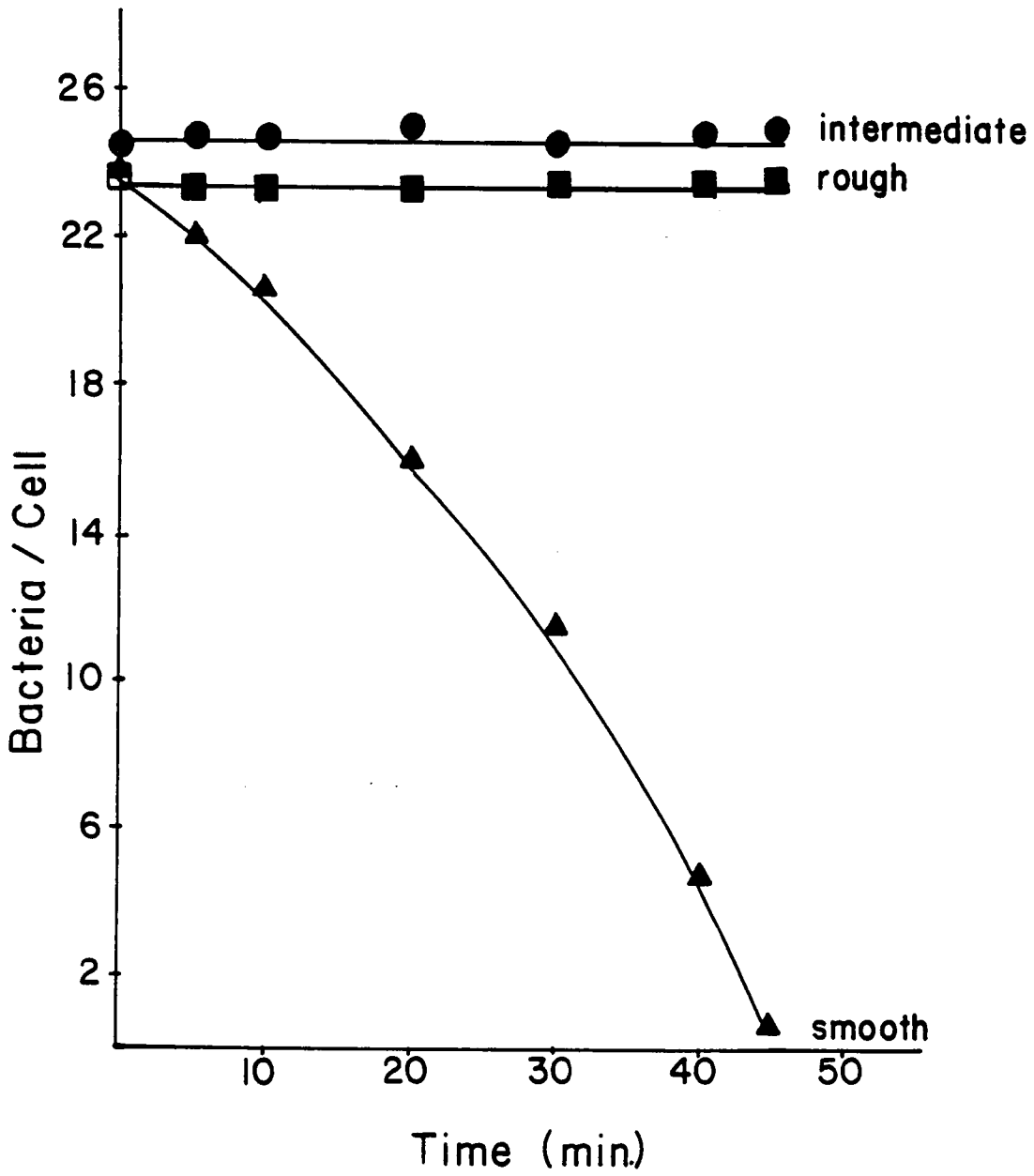
26
22
18
14
10
6
2
Bacteria / Cell
intermediate
rough
smooth
10
20
30
40
50
Time (min.)

Figure 5.

20 min., 50% of the smooth phase bacteria were released from the HLF, and by 45 min. 96% of the smooth phase organisms had been released.

GlcNAc was also tested on fourteen additional isolates to determine if it could inhibit and reverse their adherence. Adherence of all smooth phase isolates tested (n=3) was inhibited and reversed by GlcNAc (0.25 M), while adherence of the rough and intermediate phase isolates (n=11) was unaffected.

Effect of GlcNAc and Citrate on Adherence to Human Buccal Cells

Human buccal cells were tested to determine whether B. bronchiseptica adherence to a cell type, other than HLF could also be altered by GlcNAc or citrate. These experiments were done by incubating the seventeen isolates, to be tested, for 5 min. with either GlcNAc (0.25 M) or citrate (0.2 mM) before use in the adherence assay. The bacteria and human buccal cells were then incubated for 30 min. at 37°C. The results obtained paralleled those obtained for HLF (approximately 27±10 adherent bacteria per cell for all isolates). Adherence of all smooth phase isolates (n=4) was inhibited (100% inhibition) by GlcNAc; while adherence of rough and intermediate phase isolates (n=13) was unaltered. In contrast, citrate inhibited (100% inhibition) the adherence of all rough and intermediate phase isolates (n=13) did not affect the adherence of the smooth phase isolates (n=4).

Effect of Other Physical and Chemical Treatments on Adherence

The effect of various treatments on bacterial adherence is shown in Table 4. Both heat inactivation and homogenization in the presence of chloramphenical reduced the capacity of B. bronchiseptica to adhere to HLF by 97% or more. Proteast K treatment of the smooth, intermediate,

TABLE 10

THE EFFECT OF BACTERIAL PRETREATMENT ON THE ADHERENCE OF *B. BRONCHISEPTICA* TO HAMSTER LUNG FIBROBLASTS

Treatment	Smooth (110H)		Intermediate (87)		Rough (110NH)	
TBS (Control)	28[a]	(100)[b]	27	(100)	28	(100)
Heat[c]	0	(0)	1	(3)	0	(0)
Homogenization[d]	1	(3)	1	(3)	1	(3)
Protease K[e]	0	(0)	0	(0)	0	(0)
Trypsin[f]	28	(100)	27	(100)	28	(100)
Formalin[g]	28	(100)	27	(100)	28	(100)

[a] Mean number of adherent bacteria, SEM = 2.
[b] Percent of control.
[c] 60°C, 60 min.
[d] 20 min., full speed (Virtis Homogenizer).
[e] 20 mg/ml, room temperature.
[f] 0.25%, room temperature.
[g] 0.5%, overnight.

and rough phase isolates completely eliminated their ability to adhere. Trypsin or formalin treatment had no effect on adherence. As determined by electron microscopy, pili or flagella could not be detected after heat inactivation, homogenization, or protease K treatment, provided the latter two treatments were done in the presence of chloramphenical. Bacteria which were homogenized or protease K treated in the absence of the antibiotic possessed normal appearing pili and flagella, by 30 min. post-treatment, and could adhere to HLF. Bacteria which could adhere to HLF were also observed to be piliated. The presence of flagella was highly variable with regard to bacterial attachment, with the smooth phase isolate being rarely flagellated while rough and intermediate phase isolates were frequently flagellated.

Fibroblast Treatment

Isolates 110H, 87, and 110NH adhered as well to HLF which had been treated with neuraminidase (2 U/ml), fucosidase (1 U/ml), trypsin (0.25%), or acetone as they did to TBS controls.

The effect of bacterial homogenates containing isolated pili on adherence to HLF is shown in Table 5. The presence of pili in the homogenates was confirmed by EM. In addition to containing pili, membrane fragments and, in the case of 87 and 110NH, flagella were also observed. Homogenates obtained from either the smooth or intermediate phase isolates caused at least a two fold increase in number of adherent bacteria observed for all phases tested. However, homogenates obtained from the rough phase isolate (110NH) enhanced only rough phase adherence. When enhancement was observed, the pattern of adherent bacteria, instead of being single, uniformly distributed bacterial cells was more uneven and clumps of adherent bacteria were observed.

TABLE 11

THE EFFECT OF B. BRONCHISEPTICA SMOOTH, INTERMEDIATE, AND ROUGH PHASE HOMOGENATES ON ADHERENCE TO HAMSTER LUNG FIBROBLASTS[a]

Homogenate Preparations	Number of Adherent Bacteria					
	Smooth (1100H)		Intermediate (87)		Rough (110NH)	
Smooth Phase (110H)	64[b]	(226)[c]	63	(224)	63	(225)
Intermediate Phase (87)	65	(229)	84	(299)	64	(227)
Rough Phase (110NH)	26	(94)	27	(97)	65	(232)

[a]HLF were incubated with homogenates prior to use in the adherence assay.

[b]Mean number of adherent bacteria per cell; SEM = 2.

[c]Percent of TBS control; TBS control equal to 100% bacteria adhered.

Binding of Radiolabeled GlcNAc to B. bronchiseptica

The binding of radiolabeled GlcNAc to smooth and intermediate phase isolates is shown in Figure 4. GlcNAc did not bind to the rough phase isolate (110NH). GlcNAc bound to isolate 110H more rapidly (5.6 ng/min./1 x 10^7 CFU) than to the intermediate phase isolate (1.3 ng/min./1 x 10^7 CFU). Equilibrium binding of GlcNAc was apparent after 20 min. for the smooth phase isolate and after 10 min. for the intermediate phase isolate. The rate of GlcNAc binding at 4°C and 37°C was similar for both isolates. Additionally, the smooth phase isolate had a greater capacity to bind GlcNAc than did the intermediate phase isolate (Figure 5). The calculated maximum amount of GlcNAc bound to the smooth phase isolate was 60 ng/1 x 10^7 CFU while the maximum bound to the intermediate phase isolate was 15 ng/1 x 10^7 CFU. Saturation binding of GlcNAc was similar at 4°C for both isolates. The Scatchard plot of saturation binding was linear for both isolates (Figures 6 and 7). The apparent dissociation constant (K_D) of 110H was 0.18 pm with a calculated number of binding sites ('N') of 2.9 nm/1 x 10^7 CFU. The K_D of the intermediate phase isolate was 0.9 pm with an 'N' value of 0.7 nm/1 x 10^7 CFU. The number of GlcNAc molecules bound per bacterium was calculated at 1.5 x 10^7 molecules per smooth phase bacterium and 3.8 x 10^7 molecules bound per intermediate phase bacterium. The ability of unlabeled GlcNAc or glucose to compete with [^{3}H]-GlcNAc bound to the smooth phase bacterium is shown in Figure 8. GlcNAc (unlabeled) was able to effectively compete with bound [^{3}H]-GlcNAc for the bacterial binding sites with complete dissociation occuring after 62 min. Glucose did not cause dissociation of bound GlcNAc.

Figure 6. Time course binding of [^{3}H]-GlcNAc to B. bronchiseptica smooth and intermediate phase isolates. Binding was measured at both 4°C and 37°C for both the smooth (● , 4°C, ○ , 37°C) and intermediate phase (■ , 4°C, □ , 37°C) isolates.

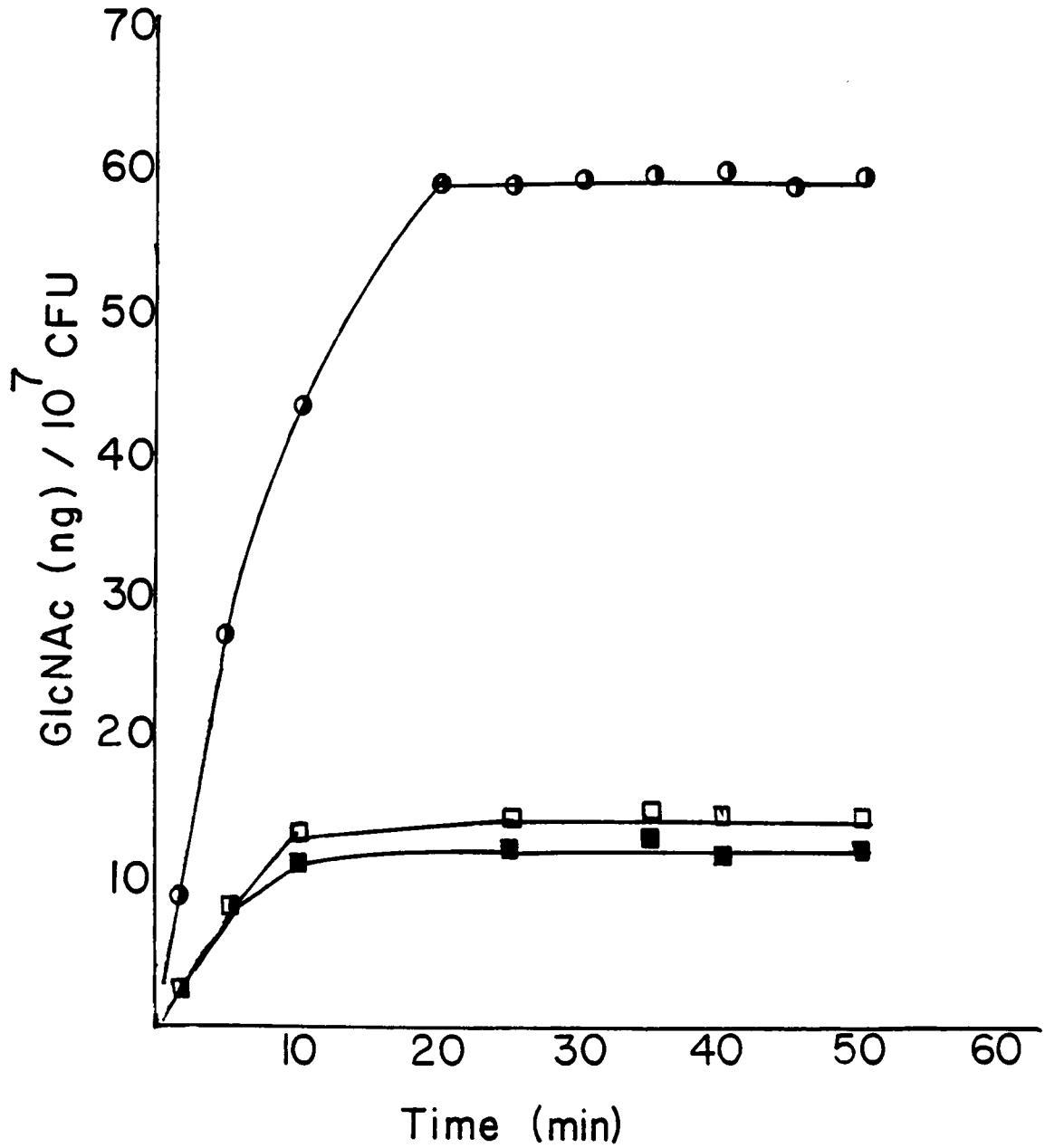

Figure 6.

Figure 7. Saturation binding of [^{3}H]-GlcNAc to 1 x 10^7 CFU of smooth (◯) and intermediate (□) phase B. bronchiseptica at 37°C. The binding of [^{3}H]-GlcNAc was also measured at 4°C (● , smooth phase; ■ , intermediate phase).

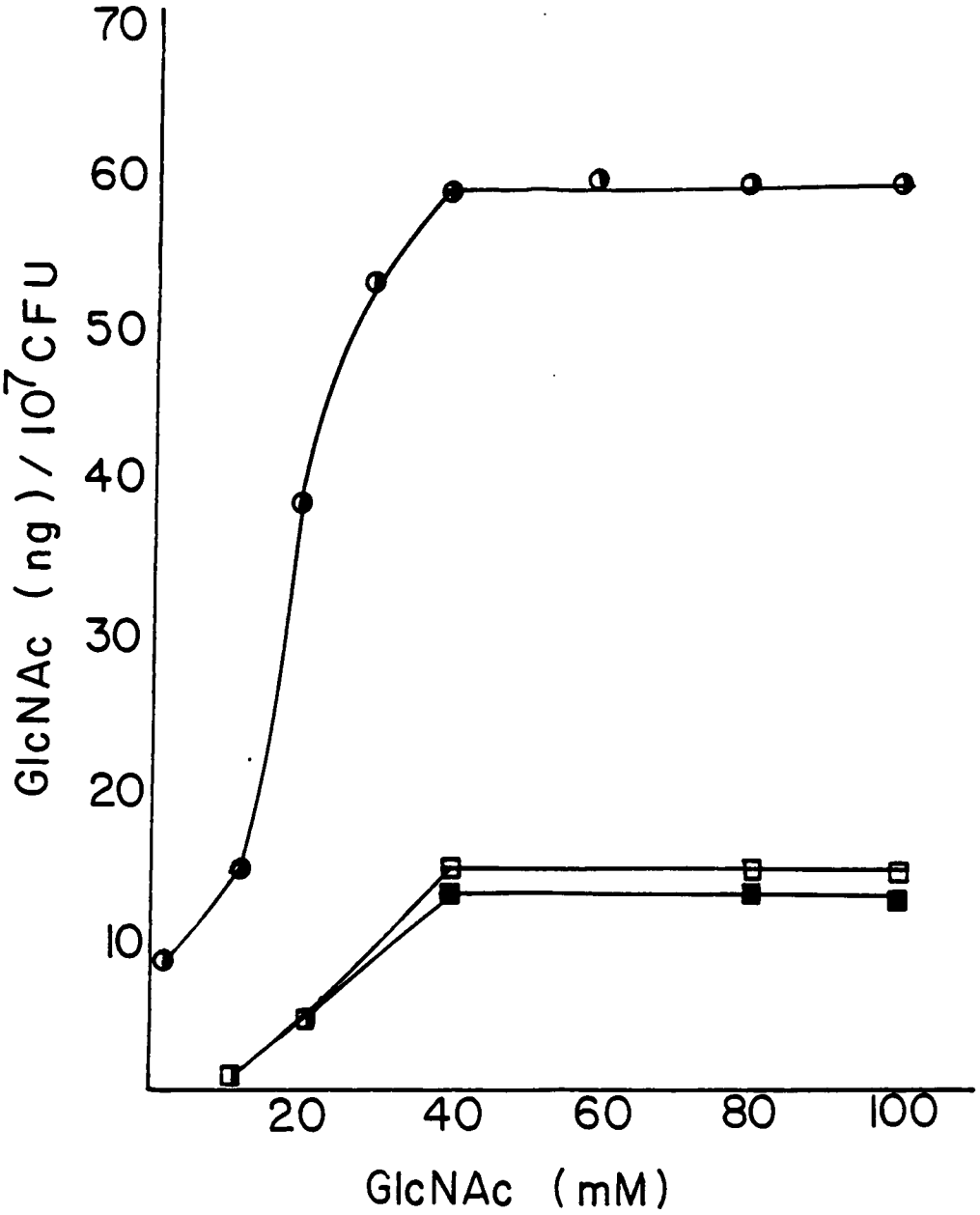

Figure 7.

Figure 8. Scatchard plot of [^{3}H]-GlcNAc binding to the smooth phase B. bronchiseptica isolate at 37°C (○) and at 4°C (●). B and B/F represent nanograms of GlcNAc bound and the ratio of bound to free GlcNAc, respectively, at equilibrium.

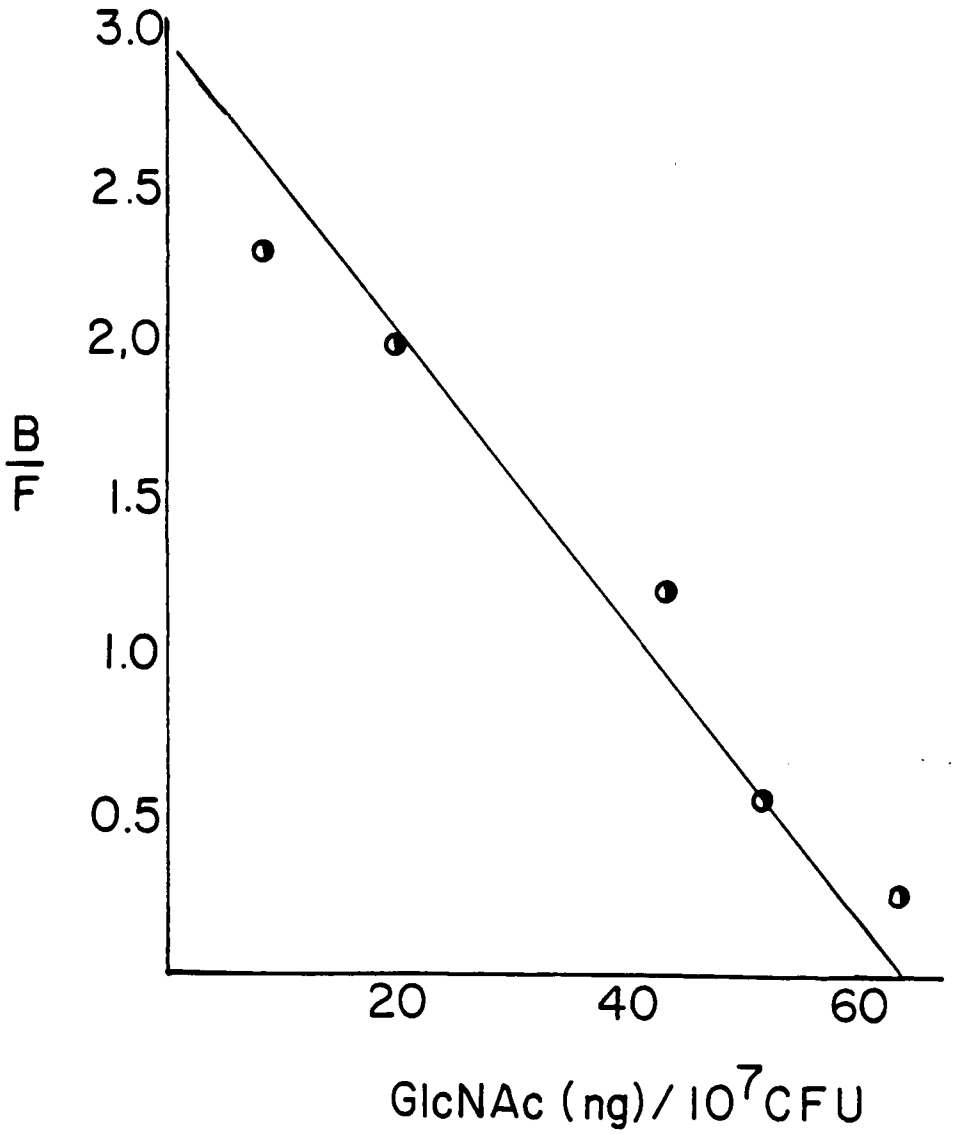

Figure 8.

Figure 9. Scatchard plot of [^{3}H]-GlcNAc binding to the intermediate phase B. bronchiseptica isolate of 37°C (□) and at 4°C (■). B and B/F represent nanograms of GlcNAc bound and the ratio of bound to free GlcNAc, respectively, at equilibrium.

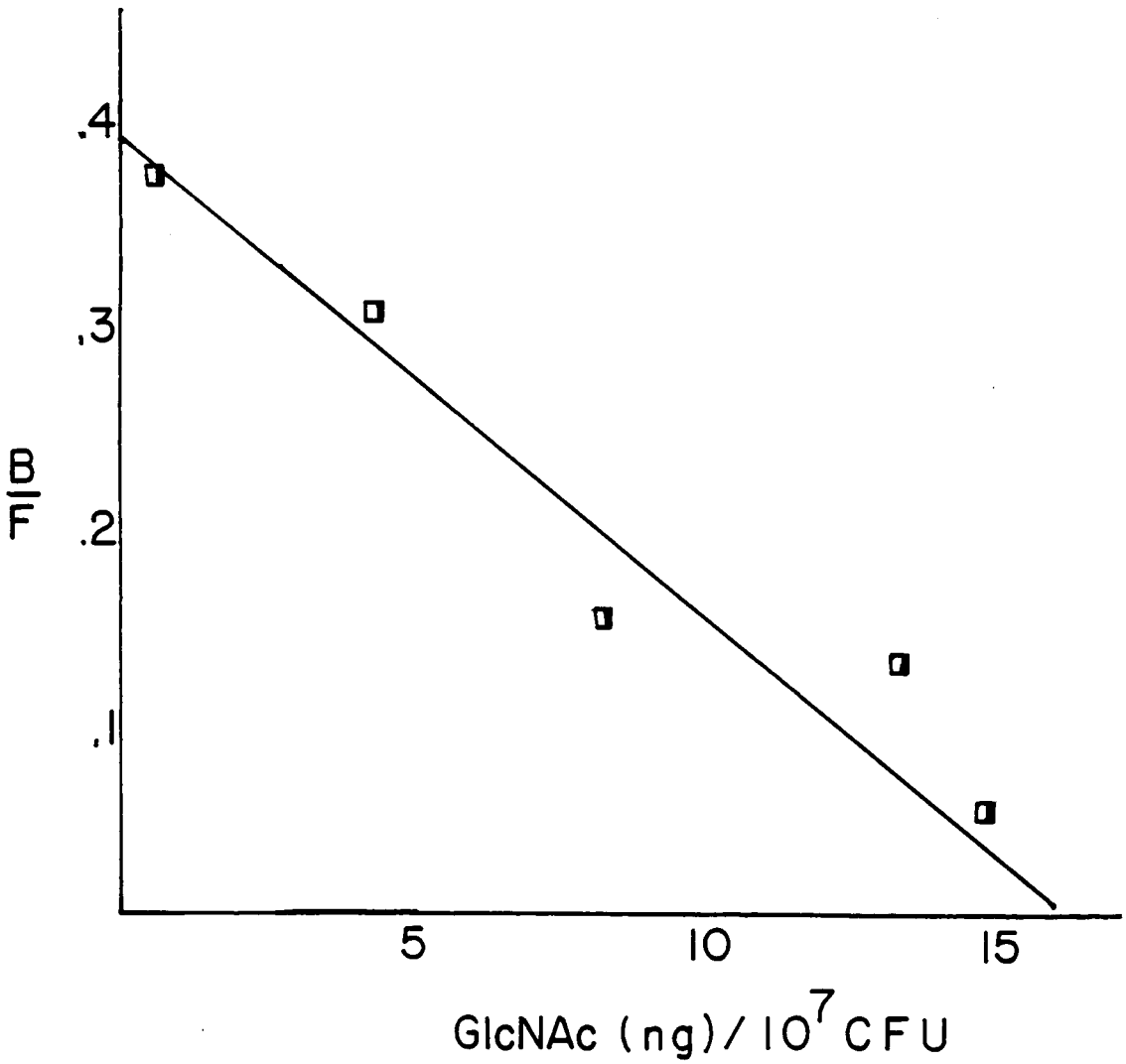

Figure 9.

Figure 10. Effect of unlabeled GlcNAc (○) and glucose (●) on the dissociation of [^{3}H]-GlcNAc - bacterial complex (smooth phase isolate) after association (35 min.). % B_0 = normalized counts bound = B/B_0 x 100 where B_0 is B at t = 0 (after time lag of 35 min.).

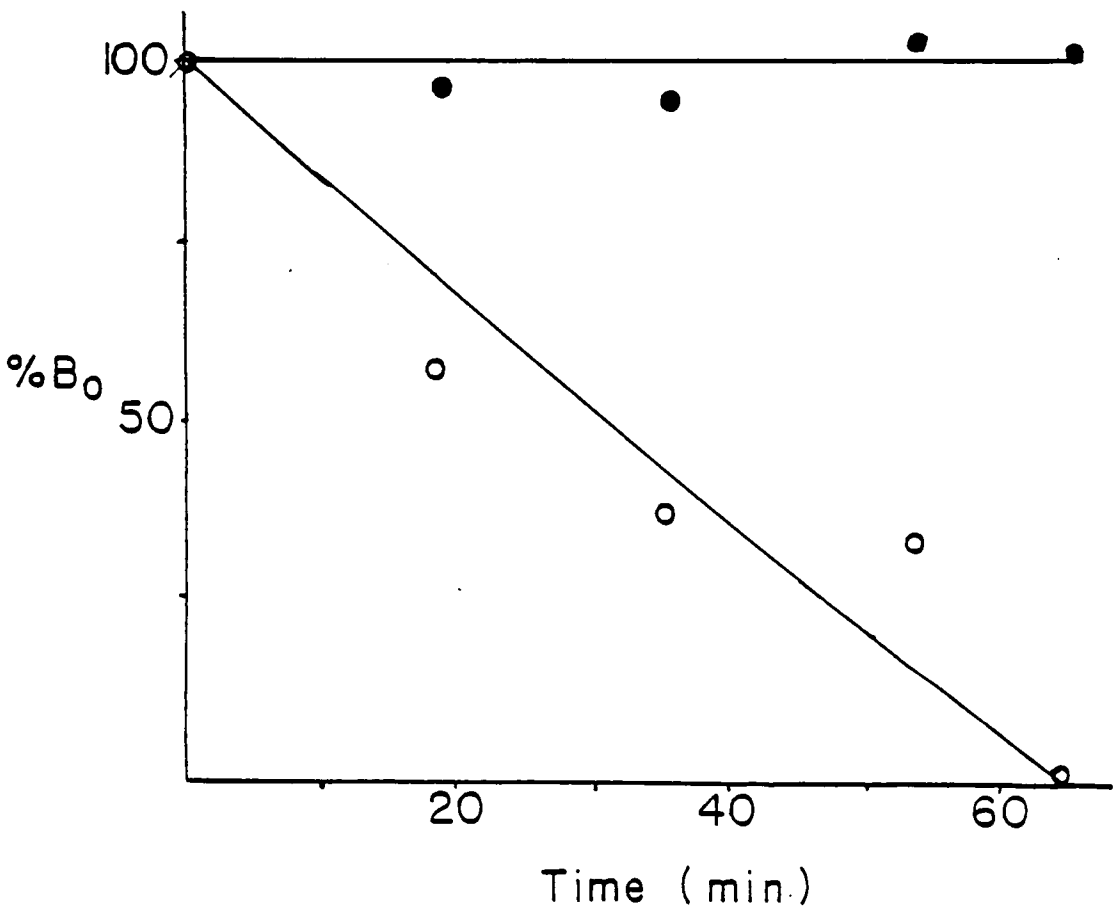

Figure 10.

GlcNAc did not bind to bacteria which had been homogenized or protease K treated. Additionally, all cell associated radioactivity on isolates 110H and 87, to which [^{3}H]-GlcNAc had been bound, was eliminated by homogenization.

CHAPTER IV

DISCUSSION

These investigations suggest that there are at least two adherence phenomena among B. bronchiseptica strains: one in which the monosaccharide GlcNAc is an essential component of the binding site on the surface of HLF to which B. bronchiseptica smooth phase isolates attach; and the other in which the adherence of B. bronchiseptica rough and intermediate phase isolates is facilitated by the divalent cations, Ca^{+2}, Mn^{+2}, Cd^{+2}, or Sr^{+2}.

Adherence of the smooth phase isolates was completely blocked and reversed by GlcNAc. Further, N-acetylmuramic acid a compound which chemically resembles GlcNAc but is not found in eucaryotic cells, inhibited 98% of the smooth phase adherence. GlcNAc appears to be the most important amino sugar residue in the binding of smooth phase isolates to HLF, but other N-acetylated amino sugars showed partial inhibition of adherence suggesting that the N-acetyl group may be important for binding. Sugars which lacked an N-acetyl group, with the exception of glucosamine, had no effect on adherence. In addition, the third carbon of the glucose moiety of GlcNAc does not appear to be involved in the binding since N-acetylmuramic acid has a substitution at this position. Further, GlcNAc appears to be acting by binding to the bacterial adhesin(s) since bacteria when incubated with GlcNAc then washed free of unbound GlcNAc were nonadherent whereas fibroblasts similarly treated still bound bacteria to their cell surface.

Other studies involving interactions between bacterial cells and mammalian cells indicate that specific carbohydrates may bind to the

bacterial cell surface and inhibit or reverse attachment. Incubation of Escherichlia coli with mannose or Vibrc cholera with fucose inhibits their subsequent adherence to epithelial cells (28,41). The neuramidiase dependent hemagglutination by human strains of Actinomyces naeslundii is inhibited or reversed by the addition of lactose (47). Galactose and N-acetylgalactosamine inhibit hemagglutination by Fusobacterium nucleatum (36), and a hemagglutinin of Pseudomonas aeruginosa has also been shown to bind galactose (20).

In all of the above cases, a compound capable of inhibiting and reversing bacterial adherence was generally considered to be an essential component of the mammalian cell receptor to which the bacterial adhesin binds. However, there is little information on the interaction between bacteria and the compounds postulated to be receptor components (22,40). The results of this study indicate that B. bronchiseptica smooth phase isolates can specifically bind GlcNAc, a non-utilizable carbon source (Part I). The binding of radiolabeled [^{3}H]-GlcNAc to the smooth phase isolate was saturable and reversible supports the hypothesis that GlcNAc inhibits bacterial attachment by binding to the bacteria. The intermediate phase isolate was also able to bind [^{3}H]-GlcNAc, but to a lesser extent than that displayed by the smooth phase isolate. The association between [^{3}H]-GlcNAc and B. bronchiseptica did not appear to be simply a result of transport of the amino sugar into the cell. Evidence that the relationship was truly that of surface binding was provided by the following observations: (1) GlcNAc was not utilized as a carbon source, (2) the level of and/or rate of [^{3}H]-GlcNAc binding was the same at both 4°C and 37°C,

(3) homogenization or treatment of the bacteria with protease K prevented the binding of radiolabel, and (4) all bacterial cell associated radioactivity was eliminated by homogenization of the bacteria.

It was observed that homogenization and protease K treatment not only resulted in the elimination of both adherence and GlcNAc binding but also were associated with the removal of pili from the cell surface. However, as homogenization or protease K treatment could undoubtedly cause the removal of bacterial cell surface components other than pili, the identify of the bacterial adhesin(s) is still uncertain. The specific binding of GlcNAc to B. bronchiseptica smooth phase isolate may be useful for the further isolation and characterization of the smooth phase adhesin(s).

Adherence of the rough and intermediate phase isolates was inhibited by various cationic chelators. The relative ability of these compounds to block adherence appeared to be correlated with their ability to bind divalent cations. EGTA, a chelating agent with a high selectivity for calcium, was the most effective inhibitor of adherence. Calcium, cadmium, strontium, or manganese are the divalent cations most likely to be involved in adherence since they were able to overcome the EGTA inhibition of adherence to HLF. One mechanism by which these cations could affect adherence may be by neutralization of surface charge. The influence of surface charge on the interaction of bacteria with mammalian cells and glass has been demonstrated by several investigators (21,42,49,58). The probable importance of this mechanism in adherence was shown by Heckels (25) who reported that pili reduce the negative charge on the gonococcal surface. He further showed that

neutralization of the negatively charged surface of non-piliated gonococci by chemical modification of the amino or carboxyl groups on the bacterial cell surface increased adherence of the non-piliated organisms to the same level as that observed for piliated gonococci. There is now additional information that pili may not be the sole adhensins which mediate gonococcal adherence (personal communication J. Swanson and J. Heckels, Adhesion Conference, ASM). If involved, the pili of the rough and intermediate phase isolates of B. bronchiseptica may also reduce surface charge, perhaps in this case by binding divalent cations.

As with the smooth phase isolate, the adhesin(s) that mediates rough and intermediate phase adherence can be removed by homogenization of the bacteria, or protease K treatment. Both these treatments also denude the cell of pili. In addition, if these treatments are done in the absence of chloramphenical, within the same time frame (30 min.), the bacteria regain both their pili and the ability to adhere. From the above results one would predict that isolated pili should block attachment in a similar manner as was observed with E. coli (46). However, treatment of fibroblasts with pili isolated from each phase of B. bronchiseptica instead of inhibiting adherence as anticipated resulted in the enhancement of attachment. This enhancement may be related to the organisms' ability to autoagglutinate since clumps of bacteria as well as single cells were observed to be bound to the HLF. The hydrophobic properties of pili (8,9) which cause them to aggregate may have resulted in the observed enhancement of adherence. Alternatively, it is possible that the isolated pili preparations caused hidden

receptors/adhesins to be expressed. A third possibility is that the binding of pili or another bacterial adhesin in these preparations resulted in a localized reduction of negative charge on the HLF surface thus allowing for better interaction between the bacteria and mammalian cell. Clearly this phenomenon will require further examination.

This study indicates that B. bronchiseptica can interact with hamster lung fibroblasts in one of at least two ways: either by specifically binding to GlcNAc or a closely related cell surface component, or, via a divalent cation mediated event. The effect of GlcNAc or cationic chelators on B. bronchiseptica adherence appears to be a general phenomenon, not limited to HLF, since the ability of these compounds to inhibit the adherence of isolates to human buccal cells was comparable to that observed with HLF monolayers. Although the identity of the adhesin(s) is still in question, affinity chromatography using the postulated receptor as the ligand may provide the means for their purification. In addition, this technique could be used to determine whether an individual bacterium possesses more than one adhesin. The presence of multiple adhesins may explain the observation that although intermediate phase adherence was inhibitable by cationic chelators, it specifically bound GlcNAc. The capability of an organism to express multiple adhesins has been discussed in the literature and demonstrated for E. coli (2) where both mannose resistant and mannose sensitive adhesins are expressed on the same organism. Additional studies directed toward purification of the adhesin(s) of B. bronchiseptica and their corresponding mammalian cell receptor(s) are needed.

LITERATURE CITED

LITERATURE CITED

1. Arai, H., Munoz, J. (1979) The effect of stationary vs. shake cultures on the production of Bordetella pertussis fimbrial hemagglutinin. Infect Immun 25: 458-460.

2. Avots-Avotins, A., Fader, R. C., David, C. P. (1981) Environmental alteration and two distinct mechanisms of E. coli adherence to bladder epithelial cells. Invest Urology 18: 364-370.

3. Beachey, E. H. (1981) Bacterial adherence: Adhesion-receptor mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 143; 325-345.

4. Bemis, D., Greisen, H., Appel, M. (1977) Bacteriological variation among Bordetella bronchiseptica isolates from dogs and other species. J Clin Microbiol 5; 471-480.

5. Bemis, D., Greisen, H., Appel, M. (1977) Pathogenisis of canine bordetellosis. J Infect Dis 135; 753-761.

6. Bemis, D., Kennedy, J. (1981) An improved system for studying the effect of Bordetella bronchiseptica on the ciliary activity of canine trachael epithelial cells. J Infect Dis 144: 349-354.

7. Bergy, D., Harrison, F., Breed, R., Hammer, B., and Huntoon, F. (1923) Bergey's Manual of Determinative Bacteriology, 1st ed. The Williams and Wilkins Co., Baltimore.

8. Brinton, C., Jr. (1977) The piliation phase syndrome and the uses of purified pili in disease control, p. 33-70. In Proceedings of the 13th Joint U.S.-Japan Conference on Cholera, Atlanta, GA, September, 1977. Department of Health Education and Welfare Publication No. 78-1590. National Institutes of Health, Bethesda, MD.

9. Brinton, C. (1967) Contributions of pili to the specificity of the bacterial surface, p. 37-70. In B. D. David and L. Warren (ed.), The Specificity of Cell Surfaces, Prentice Hall, NJ.

10. Collier, A., Clyde, W. (1971) Relationships between Mycoplasma pneumoniae and human respiratory epithelium. Infect Immun 3; 694-701.

11. Duguid, F. (1968) The function of bacterial fimbriae. Arch Immunol Therap Exptl 16; 173-188.

12. Dugid, J. P., Clegg, P., Wilson, M. (1979) The fimbrial and non-fimbrial haemagglutinins of Escherichia coli. J Med Microbiol 12; 213-227.

13. Evan, A. (1918) Further studies on Bacterium abortus and related bacteria. II. A comparison of Bacterium abortus with Bacterium bronchisepticus and with the organism which causes Malta fever. J Infect Dis 22; 580-593.

14. Ferry, N. (1910) A preliminary report on the bacterial findings in canine distemper. Amer Vet Rev 37; 499-504.

15. Fukimi, H., Sayami, E., Tomizawa, J., Uchida, T. (1953) Nutritional requirements and respiratory patterns of pertussis-parapertussis-bronchiseptica group of microorganisms. Jap J Med Sci Bio 16; 587-601.

16. Gabridge, M., Taylor-Robinson, D., Davies, J., Dourmashkin, R. (1979) Interaction of Mycoplasma pneumoniae with human lung fibroblasts: Characterization of the In Vitro model. Infect Immun 25; 446-454.

17. Galli-Valerio (1896) Cited by Smith, T. (1913).

18. Gibbons, R., van Haute, J. (1975) Bacterial adherence in oral microbial ecology. Ann Rev Microbiol 25: 19-44.

19. Gibbons, R. J., van Haute, J. (1974) Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect Immun 3: 567-573.

20. Gilboa-Garber, N., Mizraki, L., Garber, N. (1972) Purification of the galactose binding hemagglutinin of Pseudomonas aeruginosa by affinity chromatography using sepharose. EBS Lett 28; 93-95.

21. Gingell, D., Garrod, D., Palmer, J. (1970) Divalent cations and cell adhesion. p. 59-64. In Cuthbert, A. (ed.). Calcium and Cellular Function, Macmillian, London.

22. Glasgow, L., Hill, R. (1980) Interaction of Mycoplasma gallisepticum with sialoglycoproteins. Infect Immun 30; 353-361.

23. Goldhammer, A., Cook, G., Wolff, J., Berkowitz, S., Klee, C., Manclark, C., Hewlett, E. (1981) Spurious protein activators of Bordetella pertussis adenylate cyclase. Em J Biochem 115; 605-609.

24. Goodnow, R. (1981) Biology of Bordetella bronchiseptica. Microbiol Rev 45; 1-30.

25. Heckels, J., Blackett, B., Everson, J., Ward, M. (1976) The influence of surface charge on attachment of Neisseria gonorrhoeae to human cells. Gen Microbiol 96; 359-364.

26. Holt, L. (1972) The pathology and immunology of Bordetella pertussis infection. Med Microbiol 5: 407-424.

27. Jones, G. (1977) The attachment of bacteria to surfaces of animal cells. In Microbial Interactions. Reissig, J. (ed.). Chapman and Hall, London.

28. Jones, G. W., Abrams, G. D., Freter, R. (1976). Adherence properties of Vibrio cholerae; Adhesion to isolated rabbit brush border membranes and human erythrocytes. Infect Immunity 14; 240-245.

29. Keusch, G. (1979) Specific membrane receptors: Pathogenic and therapeutic implications in infectious diseases. Rev Infect Dis 1: S17-S29.

30. Korhonon, T., Svanborg-Eden, C. (1980) Binding of purified Escherichia coli pili to human urinary tract epithelial cells. FEMS Microbiol Lett 7: 237-240.

31. Kellog, D., Peacock, W., Deacon, W., Brown, L., Pickel, C. (1963) Neisseria gonorrhoeae I. Virulence genetically linked to colonial variation. J Bacteriol 85; 1274-1279.

32. Linnemann, C., Jr. (1978) Host-parasite interaction in Pertussis, p. 3-18. In International Symposium on Pertussis. Dept. Health, Education, and Welfare Publication No. 79-1830. NIH, Bethesda, MD.

33. Mardh, P., Westrom, L. (1976) Adherence of bacteria to vaginal epithelial cells. Infect Immun 13; 661-666.

34. McGee, Z., Dourmashkin, R., Gross, J., Clark, J., Taylor-Robinson, D. (1977) Relationship of pili to colonial morphology among pathogenic and non-pathogenic species of Neisseria. Infect Immun 15; 594-599.

35. McKenzie, R., Wood, A., Blackall, P. (1979) Pneumonia associated with Bordetella bronchiseptica in captive Koalas. Aust Vet J 55; 427-430.

36. Mongiello, J. R., and Falkier, W. (1979) Sugar inhibition of oral Fusobacterium nucleatum haemagglutination and cell binding. Arch Oral 24; 539-545.

37. Moreno-Lopez, M. (1952) El genero Bordetella. Microbiol Espanol 5; 177-181.

38. Nakase, Y. (1957) Studies on Hemophilus bronchiseptica. II. Phase variation of H. bronchiseptica. Kitasato Arch Expl Med 30; 73-77.

39. Ofek, L., and Beachey, E. (1980) Bacterial adherence. Adv Intern Med 25; 503-532.

40. Ofek, I., Beachey, E., Sharon, N. (1978) Surface sugars of animal cells as determinants of recognition in bacterial adherence. TIBS 7; 159-160.

41. Ofek, I., Mirelman, D., Sharon, N. (1977) Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors. Nature (London) 265S; 623-625.

42. Osada, Y., Une, T., Ikeuchi, T., Ogawa, H. (1974) Effect of calcium on the cell infectivity of virulent Shigella flexneri 2a. Jap J Microbiol 18; 321-316.

43. Pittman, M. (1974) Genus Bordetella Morono-Lopez 1952. p. 282-283. In Buchanan, R. E., and Gibbons, N. E. (eds.), Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

44. Proom, H. (1955) The minimal nutritional requirements of organisms of the genus Bordetella (Lopez). J Gen Microbiol 12; 63-75.

45. Punsalang, A., Sawyer, W. (1973) Role of pili in the virulence of Neisseria gonorrhoeae. Infect Immunity 8; 255-263.

46. Salit, I., Gotschlich, E. (1977) Type 1 Escherichia coli pili: Characterization of binding to monkey kidney cells. J Exper Med 146; 1182-1194.

47. Saunders, J., Miller, C. (1980) Attachment of Actinomyces naeslundii to human buccal epithelial cells. Infect Immunity 29: 981-989.

48. Schaeffer, A., Amundsen, S., Schmidt, L. (1979) Adherence of Escherichia coli to human urinary tract epithelial cells. Infect Immun 24; 753-759.

49. Seglen, P. (1973) Preparation of rat liver cells II. Effect of ions and chelators on tissue dispersion. Exp Cell Res 76; 25-30.

50. Smith, J. (1977) Microbial surfaces in relation to pathogenicity. Bacteriol Rev 41; 475-500.

51. Smith, H. (1974) Microbial interference with host defense mechanisms. Monogr Allergy 9; 13-38.

52. Smith, J., Pearce, J. (1972) Microbial pathogenicity in man and animals. 22nd Symposium on the Society for General Microbiology. Cambridge University Press, Cambridge.

53. Smith, T. (1913) Some bacterialogical and environmental factors in the pneumonias of lower animals with special reference to the guinea pig. J Med Res 24; 291-324.

54. Snell, J., Lapage, S. (1973) Carbon source utilization tests as an aid to classification of non-fermentating gram negative bacteria. J. Gen Microbiol 74; 9-20.

55. Biometry (1960) Sokal, R., and Rohlf, F., W. H. Freeman and Co., San Francisco, CA.

56. Svenborg-Eden, C., Hansson, H. (1978) Escherichia coli pili as possible mediators of attachment to human urinary tract epithelial cells. Infect Immun 21; 229-237.

57. Swanson, J. (1973) Studies on gonococcus infection. IV. Pili: Their role in attachment of gonococci to tissue culture cells. J Exp Med 137; 571-582.

58. Taylor-Robinson, D., Manchu, R. (1967) Adherence of mycoplasma to glass and plastic. J Bacteriol 94; 1781-1782.

59. Wolff, J., Cook, G., Goldhammer, A., Berkowitz, S. (1980) Calmodulin activates prokaryotic adenylate cyclase. Proc Natl Acad Sci 77; 3841-3844.

60. Yokomizo, Y., Shimezer, T. (1979) Adherence of Bordetella bronchiseptica to swine nasal epithelial cells and its possible role in virulence. Res Vet Sci 27: 15-21.

APPENDIX

TABLE A-1

COMPOSITION OF BRUCELLA BROTH[a]

	g/l
Bacto-Tryptone	10
Bacto-Peptamin	10
Bacto-Dextrose	1
Bacto-Yeast Extract	2
Sodium Chloride	5
Sodium Bisulfite	0.1

[a]Difco Laboratories, Detroit, MI.

TABLE A-2

COMPOUNDS TESTED FOR THEIR ABILITY TO SUPPORT THE GROWTH OF, OR INHIBIT THE ADHERENCE OF *B. BRONCHISEPTICA* ISOLATES

Carbohydrates		
Arabinose	Cellobiose	Glycerol
Xylose	Maltose	Inositol
Lyxose	Sucrose	Sorbitol
	Lactose	Mannitol
Galactose	Trehalose	Dulcitol
Sorbose	Mellobiose	
Glucose		Starch
Rhamnose	Raffinose	
Mannose		
Fucose		
Amino Acids		
Lysine	Cysteine	Glycine
Arginine	Methionine	Alanine
Histidine		Valine
	Phenylalanine	Leucine
Asparatic Acid	Tyrosine	Isoleucine
Asparagine	Tryptophane	Serine
Glutamine		Threonine
Glutamate	Proline	
Amino Sugars		
N-acetylglucosamine	N-acetylmannosamine	Galactosamine
N-acetylgalactosamine	Glucosamine	Mannosamine
Organic Acids		
Citrate	Succinate	Malate
Pyruvate	Lactate	Acetate
α-Ketoglutarate	Oxaloacetate	Fumarate

TABLE A-3

LOGARITHMS OF BINDING CONSTANTS FOR SOME METAL COMPLEXES

Ligand	Ca^{+2}	Mn^{+2}	Mg^{+2}
Citrate	4.8	3.5	3.2
Succinate	1.2	NA	1.2
Acetate	0.5	1.0	0.65
Pyruvate	0.2	1.1	0.78
EDTA	10.6	13.8	8.8
EGTA	10.9	12.2	5.3

Figure A-1. Growth curve of B. bronchiseptica smooth (□), intermediate (○), and rough (▲) phase isolates. All were grown in Brucella Broth at 37°C.

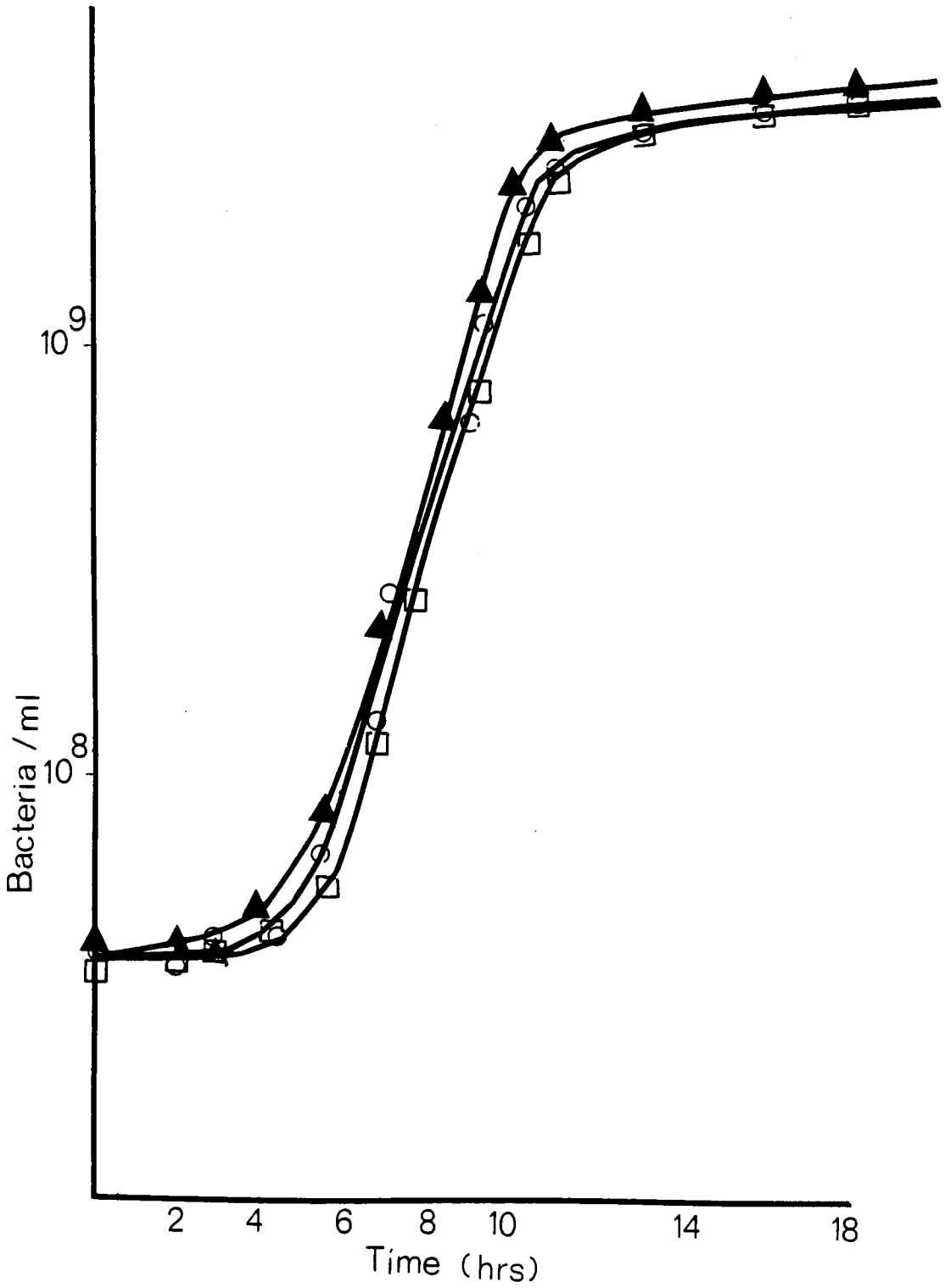

Figure A-1.

Figure A-2. Transmission electron micrograph showing late log growth phase B. bronchiseptica smooth phase isolate (X 60,000). Note the hair-like structures (pili) surrounding the cell.

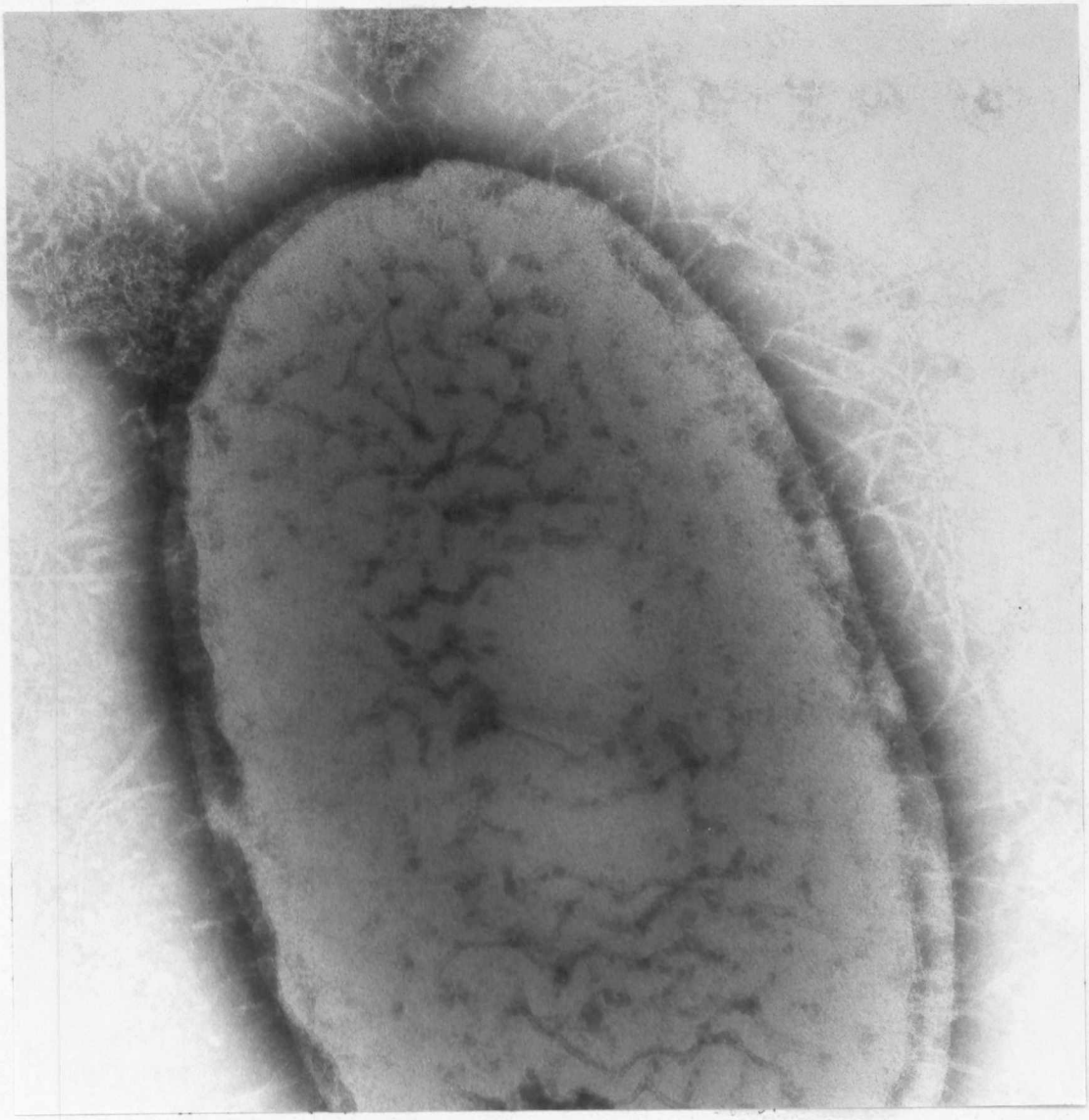

Figure A-2.

Figure A-3. Transmission electron micrograph showing late log growth phase B. bronchiseptica intermediate phase isolate (X 60,000).

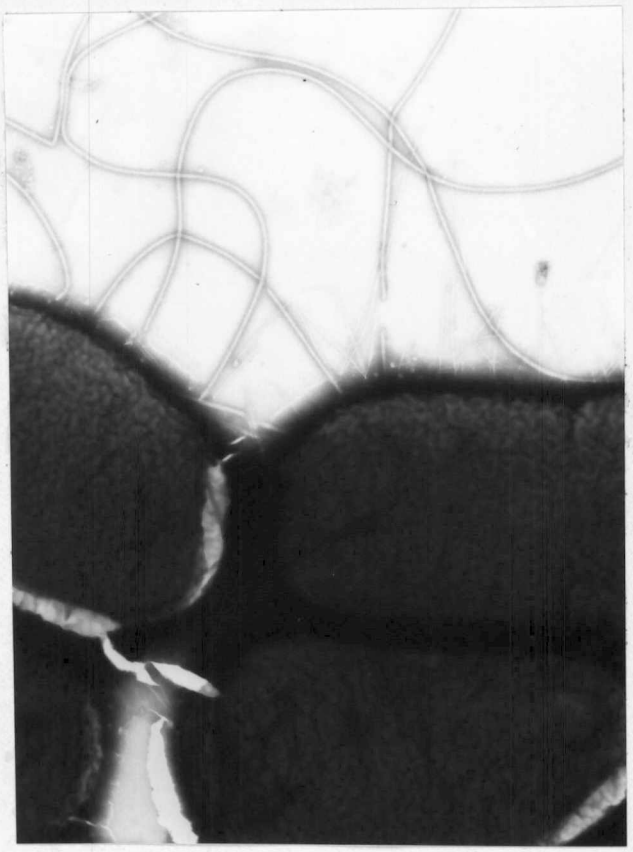

Figure A-3.

Figure A-4. Transmission electron micrograph showing late log growth phase B. bronchiseptica rough phase isolate (X 60,000).

Figure A-4.

VITA

Balbina Jean Plotkin was born in South Bend, Indiana on June 22, 1955. She attended elementary schools in that city and received her secondary education in Richmond, Indiana. She was graduated from Richmond Senior High School in June, 1973. The following September she entered Indiana University and in May, 1977, she received a Bachelor of Science degree in Microbiology. In the fall of 1977, she accepted a teaching assistantship at The University of Tennessee. She was awarded the Doctor of Philosophy degree in Microbiology in December, 1982.

She is a member of the American Society for Microbiology.