A STUDY OF INTERACTION BETWEEN *KLEBSIELLA PNEUMONIAE* STRAINS OF CLINICAL ORIGIN AND HEMOGLOBIN

BY

INDU TOMAR

A thesis submitted to the
Faculty of Graduate Studies, University of Manitoba
in partial fulfillment of the requirements for the degree
Master of Science

ISBN 0-315-33938-1

A STUDY OF INTERACTION BETWEEN KLEBSIELLA PNEUMONIAE STRAINS OF CLINICAL ORIGIN AND HEMOGLOBIN

BY

INDU TOMAR

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

Several clinical isolates of Klebsiella pneumoniae, when grown on a chemically defined medium supplemented with human red blood cells, produced red colored colonies. The red pigmentation of cultures resulted from absorption of either hemoglobin (Hb) or heme moiety from the medium. A localization study showed that most of hemoglobin was loosely bound to the bacterial surface. Subsulturing of accumulating isolates in nutrient or T-soy broth gave heterogenous cultures, consisting of red and white colonies. Experimental work showed that hemoglobin accumulation (HA) was accelerated by higher temperature, corresponding with faster growth at higher temperature. Accumulation was greater at lower pH values, possibly due to the effect of low pH on the red blood cell membrane. Various carbon sources used in the medium had no effect on HA. Nitrogen sources such as tryptose, yeast-extract and casamino acids were found to be inhibitory. Ammonium was repressive when the concentrations exceeded 75mM. Combinations of two amino acids, aspartic and glutamic acids inhibited HA. The addition of ferrous sulphate suppressed HA and optimum HA was observed when the iron source was absent.

One dimensional SDS-electrophoresis of membranes showed a significant difference between non-accumulators (no Hb uptake) and accumulators. Accumulators proved

to be lacking several protein bands in the region of 67,000 to approximately 35,000 daltons, which were present in the non-accumulating isolates.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. R.M. Lyric, of the Department of Microbiology, University of Manitoba, for his help and encouragement throughout the course of this study. I would also like to thank Dr. P. Maeba, and Roger Glufka for their help. Many thanks to David Kristjanson and Rajendra Grandhi for their help. I also thank the Natural Sciences and Engineering Research Council of Canada, and the Department of Microbiology for the financial support.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

TRIZMA	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetraacetic acid
HEPES	(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
TEMED	N,N,N',N'-Tetramethylethylenediamine
SDS	Sodiumdodecylsulphate
BSA	Bovine serum albumin
MW	Molecular weight
K	Kilodalton
MB	Minimum blood plates
HA	Hemoglobin accumulation
Hb	Hemoglobin
HM	Hepes-Mg^{++} buffer
OMP	Outer membrane protein

INTRODUCTION

INTRODUCTION

In response to iron deprivation in the environment, many species of bateria produce low molecular weight iron chelating agents known as siderophores. Microbial sequestering of iron by siderophores has been well accepted and documented (56,57,59). Other means of obtaining iron from host tissue have also been investigated and reported (66). Hemoglobin, the most abundant component of red blood cells has been suggested as an important source for microbial iron. Its significance and mode of uptake, however, is not well examined. The acquisition of iron from hemoglobin or hemoglobin-containing complex is suggested to be mediated by the hemolytic activity of bacteria. A recent study of iron uptake from hemoglobin was done on two hemolytic micro-organisms, Streptococcus pyogenes and Staphylococcus aureus and showed that increased iron acquisition was exhibited by S. pyogenes, which also had the greater hemolytic activity(66).

Klebsiella pneumoniae, unlike S. pyogenes and S. aureus, is not hemolytic on standard blood plates, and has not previously been shown to use hemoglobin or the heme moiety as a source of iron or nutrient.

This investigation was undertaken to study the interaction of clinical K. pneumoniae isolates with hemoglobin or the heme moiety present in the blood concentrate, when it was discovered that K. pneumoniae

strains, grown on a defined medium containing red blood cells showed red colored colonies on plates.

LITERATURE REVIEW

LITERATURE REVIEW

Discovery and Classification

In 1882, Friedlander reported the discovery of a micro-organism which he thought to be the causative agent of lobar penumonia. Although he identified the bacteria as a "micrococcus", the later work of Frankel and Weichselbaum showed that Friedlander's "micrococcus" was a short capsulated bacillus.

A variety of names have been given to Friedlander's bacillus since its initial discovery, depending on the source, pathogenicity, metabolic activities and morphology. Castellani called the new group of bacilli the Encapsulatus. The name Klebsiella was proposed by Trevisan in 1885. Medical workers referred to the bacteria either as the Bacillus mucosus group or as Friedlander's group (1). The name Aerobacter has also been used to refer to the Klebsiella genus. In the past, Klebsiellae of water and feces origin were referred to as K. aerogenes. This K. aerogenes was thought to correspond to the nonmotile, gas producing, and capsulated organisms described by Escherich in 1885, as Baketerium lactis aerogenes and later referred to as Bact. aerogenes. The latter bacteria were transferred to the genus Aerobacter. Thus, the terms Bacterium, Aerobacter, and Klesbsiella were used indiscriminately by successive generations of water bacteriologists. Later, Aerobacter aerogenes was

defined to be a motile organism and so distinguished from Klebsiella, but is now placed in the genus Enterobacter (2).

Classification of the genus Klebsiella into species has also undergone several rearrangements since its discovery. Much of the older work tried to subdivide the group on the basis of differences in their biochemical properties. It turned out to be a difficult task to find any correlations between the source of the organism, pathogenicity, and fermentative capabilities. Based on the early work, these organisms were divided into four groups, however, the workers expressed doubts as to the use of fermentation to differentiate the bacilli. In 1960, Cowan et. al. (3), recognized five species: K. aerogenes, K. pneumoniae, K. ozaenae, K. rhinoscleromatis, and K. edwardsii with two varieties: K. edwardsii var. edwardsii and K. edwardsii var. atlantae. In 1967, Durlakowa et. al. (4), and Slopek and Durlakowa (5), divided Klebsiella into six taxa of Cowan et. al., the names and ranks however, were slightly changed. Bascomb et. al. (6), also defined several groups in the genus Klebsiella, based on numerical taxonomy, one of which was K. pneumoniae and another composed of K. aerogenes, K. edwardsii and indole forming strains. In a study done by Naemura et. al. (7), in which 180 clinical and non-clinical Klebsiella isolates, (mostly clinical) were used, it was suggested that K. pneumoniae should include

Klebsiella groups proposed by Cowan et. al., and Bascomb et. al., except for K. oxytoca, the indole forming group.

In the eighth edition of Bergey's manual (8), three species were described in the genus Klebsiella: K. pneumoniae, K. ozaenae, and K. rhinoscleromatis. However, because of the DNA-relatedness studies done by Brenner et. al. (9), which showed that the three species of Klebsiella listed in the eighth edition of Bergey's manual and K. edwardsii were highly related, the ninth edition of Bergey's manual (10), considered all the previously described species as subspecies of K. pneumoniae. Also, in the ninth edition, two new species have been added to the genus Klebsiella, K. planticola (11), and K. terrigena (12). These two new species have shown to be distinct from K. pneumoniae according to numerical taxonomy, DNA-homology and biochemical tests.

Presently, the ninth edition of Bergey's manual recognizes four species in the genus Klebsiella. These are K. pneumoniae, K. oxytoca, K. terrigena, and K. planticola. Table 1 shows the differences between the four species and Table 2 shows the differences between the subspecies of K. pneumoniae.

Organism

The genus Klebsiella, consists of gram-negative facultatively anaerobic straight rods, which are either

Table 1. Differential Characteristics of the genus *Klebsiella*

Characteristics	*K. pneumoniae*	*K. oxytoca*	*K. terrigena*	*K. planticola*
Indole production	-	+	-	d
Pectate degradation	-	+	-	-
Fecal coliform test (gas production from lactose at 44.5°C)	+	-	-	-
Growth at 10°C	-	+	+	+
Fermentation of:				
Inulin	-	+	d	d
D-Melibiose	-	d	+	-
L-Sorbose	d	+	+	+
Utilization of:				
Gentisate or *m*-hydroxybenzoate	-	+	+	-
Hydroxy-L-proline	d	d	d	+

d some strains positive, some strains negative

Table 2. Differential characteristics of the subspecies of *Klebsiella pneumoniae*

Characteristics	*pneumoniae*	*ozaenae*	*rhinoscleromatis*
Gas from glucose	+	d	+
Acid from:			
Lactose	+	(+)	-
Dulcitol	d	-	-
Methyl red test	-	+	+
Voges-Proskauer test	+	-	-
Utilization of:			
Citrate (Simmon's)	+	d	-
Malonate	+	-	-
Urease	+	d	-
Utilization of organic acids (Kauffmann-Petersen):			
Citrate	d	d	-
d-Tartrate	d	d	-
Mucate	+	d	-
Lysine decarboxylase (Moller)	+	d	-
Arginine dihydrolase (Moller)	-	d	-

d some strains positive, some strains negative

(+) slow fermentation

arranged singly in pairs or short chains. All species are characteristically nonmotile. Generally, the bacteria in this genus give negative Methyl-Red test and positive Voges-Proskauer test. Other discriminative biochemical properties include fermentation of inositol, hydrolysis of urea, and lack of ornithine decarboxylase and H_2S production (10). Members of this genus have been differentiated superficially on the basis of their source. Bacteria of respiratory origin, other than K. pneumoniae subspecies pneumoniae are somewhat less active biochemically. Two features which distinguish the members of the genus Klebsiella from other members of the family, Enterobacteriaceae, to which this genus belong, are the presence of a large polysaccharide capsule and the ability to fix nitrogen. The capsule causes the colonies of Klebsiella, growing on agar to appear large, moist and mucoid. There are no particular growth requirements for the bacteria, however, a carbohydrate-rich medium increases capsule production.

The antigenic structure of these bacteria is like other enterobacteria, possessing both O and K antigens. However, capsular antigens (K) have been studied more extensively. Klebsiella species can be divided into several types by serological methods using both types of antigens, as well as phage and bacteriocin typing (13, 14, 15). Although serological typing based on K-antigens has been widely accepted and used, it is

stressed that use of more than one typing methods allows better discrimination between strains and is useful in epidemiological studies (16).

The first serological differentiation of Klebsiella was done by Julianelle (17), based on a determination of the K-antigens. He divided Klebsiella strains (Friedlander's bacilli) isolated from patients with lobar pneumonia into types A,B,C, and a heterogeneous group called X. By means of agglutination, precipitation, absorption and mouse protection tests, these capsular antigens were demonstrated to be highly specific. Later, other workers established three more types D, E, and F. In 1949, Kauffmann (18), extended the K-antigens to 14 and redesignated previous types with numbers. At present, 82 different K-types and 12 O-types have been described (19).

In addition to capsules, many Klebsiella strains possess fimbriae. There are two kinds of fimbriae in bacteria, type 1 and type 3. Type 1 fimbriae are referred to as "MS adhesin", or mannose sensitive, which is susceptible to inhibition by D-mannose. The other type which is a "MR adhesin", is resistant to mannose and associated with thinner fimbriae. Fimbriated strains of Klebsiella can possess either type 1 and or type 3 fimbriae. Micro-organisms possessing these types of fimbriae do not agglutinate fresh red blood cells, unless the red cells have been treated with tannic acid (20).

Pathogenicity

Klebsiellae are opportunistic pathogens which can give rise to bacteremia, pneumonia, urinary tract infections, metritis in mares and several other infections. The most frequent isolate from these infections is K. pneumoniae, which was originally thought to be the causative agent of lobar pneumonia. The production of capsular polysaccharides by K. pneumoniae and the virulence of this bacterium has been well established (21,22). It has been suggested that the capsule of K. pneumoniae increases its pathogenicity by preventing phagocytosis and may help in protecting the bacteria from complement-mediated lysis in serum. The presence of fimbriae may also play a role in the establishment of Klebsiella in the host (20).

In recent years, an increase in Klebsiella infections especially in hospitals has been noticed. Many of the isolated strains were shown to be resistant to multiple antibiotics, mainly because of the presence of R-factors (29).

There has been no definite correlation between a single serologic type of K. pneumoniae and virulence, nor is one serotype found to be more frequently associated with specific infections. However, some capsular types have been incriminated more than others as nosocomial types. The following types: 2,9,21, and 24 have been reported to be epidemic in hospitals (23, 24, 25, 26); and serotypes, 1-6 of Klebsiella strains have been most

frequently associated with upper respiratory tract infections. (27, 28).

In recent years, an association had developed between ankylosing spondylitis (a form of inflammatory arthritis mainly localized to the spine) and Klebsiella pneumoniae. Approximately 97% of patients who have ankylosing spondylitis (AS) have the histocompatibility antigen B-27 in their tissue type (30). This disease is extremely rare in individuals who are HLA-B27-. Ebringer et. al. (31) have shown a serological cross reactivity between HLA-B27+ lymphocytes and K. pneumoniae. Also, antibodies raised against K. pneumoniae in sera of rabbits when they were immunized with HLA-B27+ lymphocytes (32), further pointed to a relationship between the Klebsiella and histocompatibility antigen. Furthermore, Klebsiella organisms were found in the feces of patients with the active AS condition (33). Based on these observations, it was suggested that Klebsiella played an important role in the pathogenesis of ankylosing spondylitis (34). However, Geczy et. al. (35), suggested that the relationship between Klebsiella and AS was more complex than the simple cross reactions observed previously. It was shown that sera against certain Klebsiella isolates lysed the lymphocytes of HLA-B27+ patients with AS, but not of B27+ or B27- healthy individuals (36). In addition, it was found that culture filtrates of Klebsiella strains 'modified' lymphocytes from B27+

healthy individuals so that they were now lysed by antisera to the 'modifying' strains (37). The results obtained so far do not disprove nor support conclusively that there might be a causal relationship between Klebsiella and etiopathogenesis of AS.

Nitrogen Fixation

Besides being known as opportunistic pathogens, many strains of K. pneumoniae have the ability to fix molecular nitrogen. This organism has been used for extensive and detailed genetic analyses of the genes involved in nitrogen fixation. There are at least 17 nif genes involved in the reduction of dinitrogen, and the cluster of nif genes is organized into seven operons. The genes involved in nitrogen fixation occupy the region near the his operon (38).

Several physiological experiments done on K. pneumoniae have indicated that the expression of nif is very complex and responds to a variety of different environmental factors. Some of these factors include high levels of ammonium (39, 40, 41), certain amino acids (42, 43), temperature (44, 45) and oxygen (46, 47).

Nitrogenase, the enzyme which catalyzes the reduction of nitrogen is extremely oxygen labile. Generally anaerobic conditions are required for an organism to fix nitrogen. Organisms which fix nitrogen must have mechanisms by which to protect their nitrogenase

from being inactivated by oxygen. For strict anaerobes such as C. pasteurianum, there is no problem. Facultative anaerobes, like K. pneumoniae usually express their N_2 fixing ability only in the absence of oxygen. However, several reports have indicated that low concentrations (<10mm Hg) of dissolved oxygen are tolerated (47, 48) or possibly even used to sustain nitrogen fixation (49). Several reports (50, 51, 48) have indicated that production of slime or capsule by free living and facultative anaerobic diazotrophs may enable them to fix nitrogen by protecting the nitrogenase from excess oxygen. For example, Derxia gummosa, when grown on a nitrogen-free agar medium, gave two types of colonies (52). The colonies which were massive, yellow, and gummy occurred infrequently and were composed of nitrogen fixing organisms, while the thin white colonies did not fix nitrogen. This observation has also been supported by the work of Susan Hill (53). The colony dimorphism may have occurred when local oxygen concentrations on the agar surface were depressed and subsequently the growth of the "massive" colonies took place and allowed nitrogen-fixation.

Colony dimorphism has also been observed with K. pneumoniae (48). When K. pneumoniae were incubated in air in a medium supplemented with 200µg/ml casamino acid, large, yellow colonies were observed and they were shown to reduce acetylene in the assay method used for

nitrogen fixation.

The production of slime has been suggested to act as a diffusion barrier of oxygen. The slime may slow down the oxygen diffusion rate, and coupled with the high respiration rate of the micro-organisms (54), production of capsule may play a role in protecting nitrogenase (50).

For strict aerobes, such as Azotobacter vinelandii, two mechanisms are known by which they protect their nitrogenase from oxygen. One, known as conformational protection mechanism (54), increases enzyme stability to oxygen by allowing a complex formation between the two components of nitrogenase. This complex is unable to fix nitrogen until the intracellular oxygen levels are decreased to allow complex disassociation and restoration of nitrogenase activity. The second mechanism is known as respiratory protection (54). This mechanism involves increased utilization of substrates of reductants through high respiration rates which results in reduced internal oxygen concentrations, thereby protecting the nitrogenase.

Another way by which nitrogenase is protected from oxygen is observed in symbiotic diazotrophs, such as Rhizobium species. The presence of leghemoglobin (similar to the hemeprotein, myoglobin in human muscles), within the envelope membranes of plant root cells which surrounds the bacteroids, prevents free oxygen from

reaching the nitrogenase. Leghemoglobin acts by binding to free oxygen. This high affinity oxygen binding hemeprotein also permits the necessary high rate or bacteroid respiration at low pO_2, while simultaneously protecting the nitrogenase from free oxygen (38).

Iron Acquisition by Bacteria

Despite the natural abundance of iron in terrestrial habitats, earth's crust and in animal tissues, it is not readily available to bacteria for their assimilation. In neutral conditions, it predominates as highly insoluble ferric hydroxide polymers (55), which are inaccessible as a bacterial nutrient. During infections, hosts withold the free iron in their fluids, in the form of iron binding glycoproteins such as transferrins and lactoferrins (56, 57, 58). These glycoproteins have a very high affinity for iron and normally are partially saturated, with the result that the amount of free iron in body fluids is of the order of 10^{-8}M. This amount of iron is far too low to sustain bacterial growth. Thus, to be a successful pathogen, a bacterium must colonize and multiply in iron limited environments. The ability to obtain iron is therefore an important virulence factor for the bacteria.

It is well established that in response to iron deprivation, many species of bacteria produce low molecular weight iron chelating agents known as

siderophores. The siderophores studied to date belong to either of two chemical classes. One class of siderophores, namely phenolates, are derivatives of 2, 3-dihydroxygenzoic acid (eg. enterochelins). The second class of siderophores consists of hydroxamates, which are derivatives of hydroxamic acid (eg. ferrichrome) (56, 57, 59). Studies have also shown that under similar iron limiting conditions, several outer membrane proteins are expressed by micro-organisms (60, 61, 62, 63, 64). These outer membrane proteins have been suggested to facilitate iron uptake by acting as receptor sites for siderophores (65).

Microbial sequestering of iron by iron binding high affinity molecules has been well accepted and documented. However, another means by which some bacteria obtain iron from host tissue has also been reported, but its significance and mode of uptake is not well understood. Hemoglobin, the most abundant component of red blood cells, has been suggested as an important source for microbial iron (66). The idea that iron from hemoglobin (Hb) is used by bacteria is not a new one. Previous studies have shown that certain virulent bacteria grew well in the presence of Hb, although their growth was slowed down in its absence (67, 68, 69). Originally, it was thought that Hb served as the nutrient source for the bacteria (70). However, studies done by Davis and Yull (71), showed that Escherichia coli grew

in the presence of Hb but not with the globin moiety only. This observation was confirmed by several other workers studying hemolytic Staphylococci and Streptococci species (72, 73). These workers suggested that the heme moiety was the growth enhancing factor. Work of Bornside et. al. (67) suggested that the hemoglobin increased the virulence of E. coli in rats. When a simultaneous injection of hemoglobin and E. coli was given to rats, a lethal response was evoked, whereas, no lethal response was observed if E. coli was injected alone. The more recent work done by Maskell and Miles (74), also gave similar conclusions about the increase in virulence with the Hb administration. These workers found that Hb was a more potent enhancer of local infections in guinea pigs than ferric ammonium citrate, given in an equal or lesser iron content dose with respect to free iron. Among the bacterial species tested, Klebsiella pneumoniae and E. coli responded to both ferric citrate and Hb. As it is well understood that in certain bacteria virulence is dependent on higher levels of iron than required for normal metabolism, it seems apparent that hemolytic pathogens such as S. aureus and S. pyogenes, use hemoglobin to their advantage in acquiring iron. Francis et. al. (66) studied iron uptake from hemoglobin and haptoHb-hemoglobin complex, as the source of iron, using S. aureus and S. pyogenes. This study showed both of these bacteria were able to take up iron from both Hb and Hp-Hb

complex. However, greater iron acquisition was exhibited by S. pyogenes, which also had the greater hemolytic activity. Interestingly, S. pyogenes, showed red coloration in their pellets. It is possible that hemin is bound to the bacterial membranes in the free form and/or with a secreted heme binding protein.

A previous study done on Yersinia pestis (75), using concentrations of hemin in excess of the minimum requirement for growth revealed that many strains produced dark brown colonies when grown on a chemically defined medium, while other produced colourless colonies. The pigmentation resulted from absorption of hemin from the medium. Furthermore, after prolonged incubation, non-pigmented (NP) colonies were shown to develop from pigmented colonies. In a subsequent paper, the same authors (76), showed that NP mutant strains from virulent pigmented (P) strains had reduced virulence to mice, (while corresponding mutants derived from pigmented avirulent strains were avirulent). Moreover, the NP mutants from virulent P strains showed to increase their virulence when hemin or other iron compounds were added. However, no similar increased virulence was observed in NP mutants of avirulent P strains. It was suggested that the ability to produce pigmented colonies was related to their superior iron metabolism compared to NP strains. The requirement of red blood cells in the growth medium (X factor: growth promoting derivatives of blood pigments)

by some species of Haemophilus is a very well known phenomenon. This requirement has been regarded as the factor(s) necessary to permit growth aerobically by the organisms which are incapable of synthesizing porphyrins or haem from precursors of δ-aminolaevulinic acid (77).

Klebsiella pneumoniae, unlike S. aureus and S. pyogenes, is not hemolytic on standard blood agar, but our study showed that Klebsiellae are capable of picking up Hb, (similar to the Y. pestis), in a defined medium supplemented with red cells. Furthermore, under these conditions some of these strains were shown to be hemolytic as well. This thesis is concerned with physiology of K. pneumoniae strains of clinical origin, which were shown to accumulate hemoglobin from their growth medium.

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

Chemicals

Pyruvic acid disodium salt, Succinic acid disodium salt, D-L-Rhamnose and N'N'-BISmethylene acrylamide were obtained from Eastman Kodak Company. Matheson, Coleman and Bell supplied D-Fructose, Sodium citrate, Magnesium sulphate and Calcium chloride. Meso-Inositol was obtained from BDH Laboratories Chemical; Pyragallol, Ferrous sulphate and 2-Mercaptoethanol were from J. T. Baker Chemical Company. Sigma Chemical Company supplied L-Sorbose, Arabinose, Melibiose, Coomassie Brilliant Blue R-250, Trizma base, EDTA, Congo red dye, amino acids and Hepes. Ammonium persulfate, TEMED (N,N,N',N',-Tetramethylethylene-diamine), SDS (Sodium dodecyl sulfate), and Acrylmide were supplied by BIO-RAD Laboratories. Difco supplied vitamin-free casamino acids and Tryptose. Hemoglobin was a gift from Dr. R. Weselake of the Winnipeg Rh Institute. All other chemicals used were from Fisher Scientific Company. All chemicals were reagent grade or better.

Organisms

Clinical isolates of Klebsiella pneumoniae were obtained from Dr. D. Hoban, of U. of Manitoba, and

selected isolates which were capable of accumulating hemoglobin on minimal blood plates were used throughout the study. These isolates were identified as PRIIR, PRIII, Kp5R, 46368 and 11057-1. The non-accumulators, used as controls (appeared white on blood plates), were designated PRI, PRIIW and Kp5W. (PRIIW and Kp5W were picked from PRIIR and Kp5R culture plates, growing on blood plates as white colonies).

Media

The medium used in this study was a chemically defined medium modified from Yu and Saddler (78), and contained per liter 8,700mg of K_2HPO_4. $3H_2O$, 1,500mg of KH_2PO_4, 3,000mg of $(NH_4)_2SO_4$, 400mg of EDTA, 240mg of $MgSO_4.7H_2O$, 100mg of NaCl, 14mg of $CaCl_2.2H_2O$. The initial pH of the medium was adjusted to 7.1 with 6N HCl. [Modifications of the Yu and Saddler medium included omission of 2-(N-Morpholino)ethanesulfonic acid, decreased $K_2HPO_4.3H_2O$ amount from 11,400mg to 8,700mg and the change in pH]. The solution was sterilized in the autoclave at 121°C for 15 min. The glucose used was prepared separately in 20% (w/v) solution and was sterilized by autoclaving. Aliquots of the sugar solution were then transferred to presterilized medium for a final concentration of 2%.

For final blood agar plates (MB), the salt solution was supplemented with 1.5% (w/v) agar and

autoclaved. After sterilization, the medium was allowed to cool before glucose and 4%(v/v) packed human red blood cells (obtained from Red Cross, Winnipeg) were added and poured in petri dishes. The agar plates were kept at room temperature overnight before utilization to ensure the sterility of the plates. When pure hemoglobin plates were required the packed red blood cells were substituted with 10-15% (v/v) of a sterile hemoglobin stock solution (obtained from Rh Lab, Winnipeg). Studies with nutrient supplements and different carbon sources were carried out on minimal blood agar plates. Supplementary components were either added to the medium before pH adjustment and sterilization or after sterilization, such as the amino acids, which were sterilized by passage through a 0.22 μm filter (Millipore Ltd., Mississauga, Ontario, Canada).

For nitrogen fixation studies, ammonium sulfate was omitted from the minimal medium which was supplemented with sodium molybdate (0.25mg/ml).

METHODS

Selection of Hemoglobin accumulating Strains

Upon receiving the clinical isolates of *Klebsiella*, the cultures were streaked on to the minimal blood plates. The plates were then incubated at 28°C for 2-4 days. Hemoglobin(Hb) accumulating cultures were chosen based

on their red colony color.

Identification of Klebsiella isolates

To identify and characterize the Klebsiella isolates (used in the study), to the species level, general biochemical tests and properties were used as described in the ninth edition of Bergey's manual.

Culture conditions

Working cultures of Klebsiella pneumoniae strains were maintained in minimal media broth at room temperature. These cultures were transferred weekly to fresh broth. The broths were used to inoculate the test medium and incubated appropriately as required.

Occurrence of Hb Accumulation phenomenon

Klebsiella cultures obtained from the hospital patients were checked for the production of red colonies on minimal blood plates. The cultures were streaked directly from the MacConkey plates on to the blood plates and incubated at 28°C. Bacteria other than Klebsiellae, obtained from the culture collection of University of Manitoba, Microbiology Department, were also checked for their ability to produce the red pigment from the minimal blood plates. The latter group was streaked directly to the minimal blood plates from their growth medium and also tested by growing in minimal broth

for 2 days before being streaked on to the MB plates, and incubated at 37° for several days.

Effect of various substrates and conditions on Hb accumulation

Wherever required, appropriate test conditions and substrates in MB plates were changed to see the effect they had on the Hb accumulation(HA) by the selected cultures. The plates were streaked from minimal broth and incubated for 2-4 days.

Localization of Hemoglobin

This experiment was designed to see whether Hb remained outside the cell, attached to the outer cell surface or was taken up inside the cells. The cultures were grown on MB plates until the growth and Hb accumulation was optimum (3 days), incubated at 28°C. The colonies were transferred by the innoculating loop in 10ml of saline (.85% w/v) and vortexed to disperse the cells. The cells were then centrifuged at 10K RPM for 5 minutes (RC-5 Superspeed Refrigerated Centrifuge, Sorvall). The pellet and supernatant were examined visually for the red coloration. The cells were washed and harvested as before. For the next two washes, 1M KCl solution was used. The suspensions of accumulators (PRIIR) and nonaccumulator (PRIIW) were examined by DU-8 Beckman Spectrophotometer for Hb presence, over the

absorbance 650-270nm range.

SDS-Electrophoresis

Strains were grown in 100ml of minimal broth in 250ml Erlemeyer flasks. The cultures were grown on a gyratory shaker at 150 RPM (G24 Environmental incubator Shaker, New Brunswick Scientific Co.) for 48h at 28°C. The cells were harvested by centrifuging at 10K RPM for 10 minutes. The pellets were suspended in 20ml of .01M hepes, pH 7.4, containing 10mM $MgCl_2$ buffer (HM) and homogenized once in a Sorvall Omni-Mixer set for the highest speed for 30 seconds, to remove the capsular material. Antifoam (Dow Corning Antifoam Spray) was used on the blade and container before each blending. Between each blending cycle, the mixer container and the blades were rinsed with distilled water. The cells were centrifuged again at 10K RPM for 20 minutes and resuspended in 15ml, (HM Buffer) 300μg of DNAase I was added to the suspension. The suspension was subjected to sonication until the suspension became clear. Unbroken cells were removed by centrifugation for 20 minutes at 10K RPM. The resultant supernatant was then centrifuged for 90 minutes at 40,000 RPM in a Spinco 60 Ti rotor (Beckman Instrument Company) at 5°C in an L2-65B Beckman ultracentrifuge to obtain a crude membrane extract. The pellet was suspended in 1% (w/v) Triton X-100 and HM buffer. The sample was then allowed to stand overnight in a -17°C

freezer. To clean the concentrate, 0.1ml of sample was added to 1 ml of 85% ethanol and left for 1h at 20°C. The sample was then centrifuged in the table top clinical centrifuge (GLC-2 General Laboratory Centrifuge, Sorvall) at 2,000 RPM for 10 minutes, and the alcohol was removed. Four fractions were obtained, S_1 (the slime layer obtained after the cells were blended in the mixer), S_2 (crude extract supernatant), S_3 (supernatant after ultracentrifuge), and S_4 (pellet from ultracentrifuge).

Protein Determination: Protein content of the four fractions was determined by the BCA Protein Assay method (Pierce Chemical Company). Instructions provided with the reagent were followed using the Micro Protocol and BSA as the standard. This protein assay is based on the principle of proteins reacting with alkaline copper II to produce copper I. The copper I then reacts with BCA protein assay reagent to form an intense purple at 562nm. The purple reaction product is water soluble, which enables the spectrophotometric measurement of an aqueous protein solution.

Electophoresis: The four fractions obtained were prepared in SDS-sample buffer [0.5M Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.1ml 2-mercaptoethanol and 0.05% (w/v) bromophenol blue] to give a final concentration of 2mg/ml protein. The mixtures were heated in boiling water for 2 min. Usually 10-20µl of the SDS-samples, which were equivalent to 10-20µg/ml protein,

were separated by one dimension SDS-electrophoresis (SDS-PAGE) in 9% polyacrylamide. The method of Laemmli (79) was followed using the Protean Slab Cell apparatus of BIO-RAD Laboratories. The gels were stained for 1h with 0.1% coomassie brilliant blue R in 85% ethanol and 10% acetic acid. The gels were then destaineb by successive immersion in 30% ethanol, 10% acetic acid for 1.5h, 25% methanol -10% acetic acid for 1h and 15% methanol -10% acetic acid overnight. The destaining was stopped by immersion in 7% acetic acid-2% glycerol for half an hour. The stained gels were dried on to filter paper in the BIO-RAD Slab dryer.

For molecular weight estimation, an electrophoresis calibration kit (Pharmacia Fine Chemicals) comprised of the Protein phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and β-lactalbumin was included in the electrophoretic procedure. Their molecular weights were 94,000, 67,000, 43,000, 30,000, 20,000 and 14,000 respectively.

Nitrogen fixation and Hb Accumulation

The ability to fix nitrogen by the K. pneumoniae strains was determined by using N-free minimal media plates, and acetylene reduction method described by Campbell and Evans (80). On the N-free plates, the cultures were streaked for colony isolation and incubated at 28°C, both aerobically and anaerobically

(in the glass jar). For the acetylene reduction method, cultures were grown aerobically in Pankhurst tubes containing N-free media for 48h. Then, Suba seals were placed on the tubes. The cultures were flushed with nitrogen gas for 2 min through a sterile 18 gauge hypodermic needle inserted into the Suba seal over the short arm of the Pankhurst tubes (P-tubes), and vented through a hypodermic needle inserted into the Suba seal over the long arm of the P-tubes. Sterile membrane filters were attached to each needle to ensure no contamination occurred. After N_2 flushing, Pyrogallol and NaOH, K_2CO_3 solutions were added as described by Campbell and Evans. 0.75ml of acetylene gas was injected into the long arm of the P-tubes, and mixed by moving the syringe plunger up and down several times. The tubes were incubated at 28°C and .25ml of gas samples were withdrawn at different time intervals and analyzed for ethylene production by (Perkin-Elmer Series 104 Chromatograph) gas chromatography. Pure ethylene was injected to determine the elution time for the ethylene peaks.

Detection of strains capable of absorbing Congo red dye

This experiment was based on the work done by Payne and Finklestein (81), who used an agar medium containing 0.01% Congo red dye to detect and differentiate virulent and avirulent colonies of several pathogenic bacteria. Their work showed that virulent

colonies which absorbed hemin from a synthetic medium and thus appeared pigmented also absorbed Congo red dye. In our experiment the minimal medium agar plates were supplemented with 0.01% Congo red dye before sterilization of the medium. Plates were then either streaked with the loop or diluted bacterial suspensions were plated on to the Congo red plates. The plates were incubated at 28°C for 2-3 days.

RESULTS

RESULTS

Characteristics of the Klebsiella isolates

The purpose of this project was to study the clinical Klebsiella isolates which on a chemically defined medium, supplemented with red blood cells, produced red colored colonies.

The general characteristics of Klebsiella strains used in this study are shown in Table 3. Based on the biochemical tests, all the Klebsiella isolates were identified to be K. pneumoniae. There was a major difference in the pigmented (red colony producers) and non-pigmented strains in their pattern of growth on agar plates and in broth. The pigmented isolates were mucoid and large colony producers on solid medium while the mon-pigmented isolates produced small colonies. In broth, the pigmented isolates formed a good pellicle on the surface as compared with the non-pigmented strains. When the cultures were examined under the microscope for differences in their ability to produce capsules, no differences were observed in their size.

Occurrence of Hemoglobin (Hb) accumulation phenomenon

To test the ability to accumulate Hb by Klebsiella strains of varied origin (clinical and culture collection strains), and other bacteria obtained from the Microbiology culture collection, the cultures were

Table 3. Characteristics of the Klebsiella isolates in the study.

Characteristic	PRIII	PRIIR	PRIIW	46368	11057-1	KW	KR
Citrate Utilization	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-
Methyl Red Test	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-
Encapsulation	+	+	+	+	+	+	+
Hb accumulation	+	+	-	+	+	-	+

streaked on to the MB plates directly from their growth medium. Figure 1 presents the varying shades of red observed in the clinical isolates. Table 4 shows the results of hemoglobin accumulation (HA) by clinical isolates of Klebsiella obtained from the hospital. Fifty-three isolates were tested and out of these bacteria, 16 of them showed no pigmentation. The rest of the cultures showed a mixture of red and white colonies, where red pigmentation ranged from slight hint of pink to darker red. The compiled data showed that approximately 70% of the isolates were capable of absorbing Hb or the heme moiety. Previous work (data not shown) showed 8 out of 19 clinical Klebsiella isolates were capable of producing red colonies. This data indicates that the phenomena of Hb absorption is not rare among Klebsiellae of clinical origin.

Bacteria, such as E. coli, Pseudomonas aeruginosa, Proteus vulgaris, S. aureus, Enterobacter aerogenes, Edwardsiella tarda, Enterobacter cloacae and other subspecies of K. pneumoniae, obtained from the microbiology culture collection, failed to show red pigment in their growth, with the exception of a K. pneumoniae subspecies pneumoniae strain, which showed faint pink colonies. When the same above bacteria were innoculated first in minimal broth before streaked on to MB plates, most of them failed to grow in the broth, and the ones that did (e.g. E. coli, E. aerogenes and K. pneumoniae) did not show any pigmentation on MB plates.

Table 4. Hemoglobin accumulation in the Klebsiella pneumoniae isolates obtained from the hospital.

No. of isolate	Hb Accumulation
42242	±
10128-2	+
42051-2	++
46857	-
46801	±
47164	±
42193	+
47117	++
11066-2	-
40575-4	+++
46388-2	±
42270-1	++
42258-3	Brown
42891-1	-
10170-3	++
10168-4	-
42987	-
42258-2	++
42258-1	Brown
42259-2	+
42260-1	Brown
40864-1	-
40862-1	Brown
40860-1	Brown
40863-3	Brown
38052-1	+
40177-3	Brown
42261-1	Brown
40177-6	-
38405-1	+
38409-1	+
39973-1	-

39202-2	++
37925-2	- sparse growth
40177-1	Brown
40237-2	++
42261-2	Brown
11057-1	++++
46018-1	-
45503-1	+++
45083-2	+++
44541-1	+++
44178-1	±
44027	+++
43448-1	-
10360-1	-
42948-3	++++
42248-1	-
*46546	++
*46109	-
*46125	-
*46307	-
*46368	++++

Number of isolates tested: 53
Positive(≥ ±)or Brown: 37
*Klebsiella isolates not identified to the species level
± Red at edges or slightly pink
Brown Colony color was brown rather than normal red

Effect of various substrates and conditions on HA

The following experiments were undertaken to study the effect of different substrates and conditions on HA. The variables studied were red blood cell concentration, incubation temperature, pH of medium, carbon sources, nitrogen compounds, iron and molybdenum. The HA was estimated according to the degree of red coloration shown by the different Klebsiella isolates in Figure 1.

Red Cell Concentration

An attempt to increase the Hb accumulation (HA) by increasing red blood cell concentration in medium, indicated that optimum HA was at 4% (v/v) red blood cell supplementation. Lower concentrations showed suboptimum HA, whereas higher than 4% (v/v) supplementation did not affect HA.

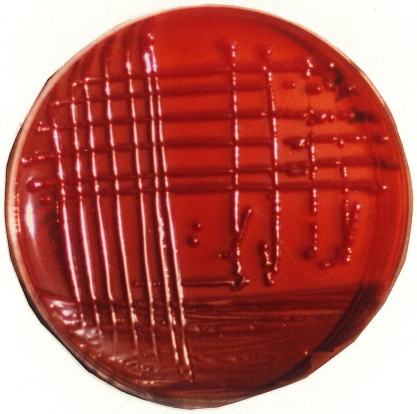
Temperature

Incubation temperature (data not shown) had the following effect on HA with respect to time. Incubation of plates at lower temperatures (18°C and 22°C) showed the same degree of red coloration as at 30°C or higher, however, it took a longer period of time (3-4 days) at lower temperatures to achieve the same degree of HA at 30°C or higher. At higher temperatures (30°C and 37°C), HA was observed within 24 hours. At 37°C, colonies

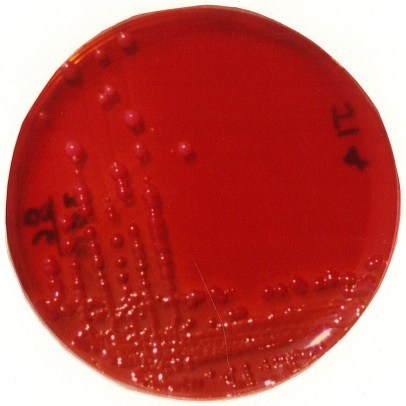
Figure 1. Variation of red color in Klebsiella isolates.

The isolates were streaked on to the defined medium supplemented with the red blood cell concentrate. The plus (+) signs represent the different degrees of red coloration.

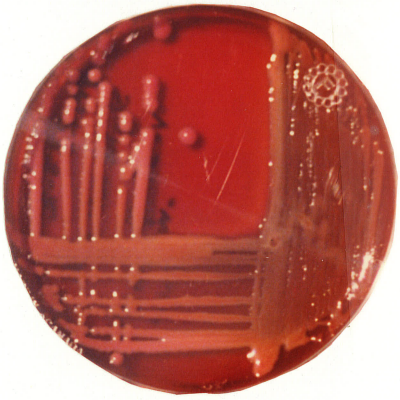
Photograph A. Depicts the pigmented and non-pigmented strains of PRII together (PRIIR and PRIIW).



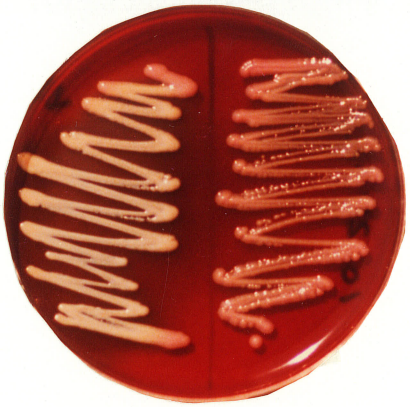
++++



+++



++



- +

A

became brown if kept longer than one day in incubation. The optimum temperature of HA was established at 28°C. It took 48 hours to see HA at this temperature.

pH

The results in Table 5 show that pH values on the acidic side support HA better than a pH of 7.4. Growth was found to be optimum at pH 7.1. Therefore, it was decided to use pH of 7.1 in media to obtain good growth and HA. When the growth medium were supplemented with tryptose (Table 6) the cultures showed only a trace of pigmentation in their growth.

Carbon Sources

The effect of varying substrates was investigated using concentrations of 2% (v/v). The change of carbon sources had no direct effect on pigmentation of the accumulating strains. However, varying the carbon sources had a great affect on K. pneumoniae strains with respect to slime production. The isolates which showed good growth (larger colonies) were slimy. From Tables 7A and 7B, it is apparent that the degree of HA is related to the growth of isolates, with better growth resulting in a greater degree of HA.

Nitrogen Sources

Preliminary studies showed that in the absence of

Table 5. Effect of pH on HA.

	pH							
	6.5		6.8		7.1		7.4	
Isolate	G	HA	G	HA	G	HA	G	HA
III	++++	+++	++++	+++	++++	+++	+++	+
IIW	+	-	++	-	++	-	++	-
IIR	+	±	++	++	++	-	++	-
46368	++	±	++	++	+++	++	+++	+
11057-1	+++	+++	+++	+++	++++	+++	+++	+
KR	+	++	++	+++	++	++	++	-
KW	+	-	++	-	++	-	++	-

± mixture of both white and red colonies

G Growth

HA Hemoglobin Accumulation

Table 6. Effect of pH on HA supplemented with 2% (w/v) tryptose.

	pH							
	6.5		6.8		7.1		7.4	
Isolate	G	HA	G	HA	G	HA	G	HA
III	+++	±	++++	-	++++	-	++++	-
IIW	++	-	++	-	+++	-	++	-
IIR	++	-	++	-	+++	-	++	-
46368	++	-	++++	-	++++	-	++++	-
11057-1	+++	-	++++	-	++++	-	++++	-
KR	++	±	+++	-	+++	-	+++	-
KW	++	-	++	-	+++	-	+++	-

± hint of red in some parts of the streaking growth

G Growth

HA Hemoglobin accumulation

Table 7A. Effect of various carbon sources on HA.

	ISOLATE					
	KW		KR		46368	
C-source	G	HA	G	HA	G	HA
Mannose	+	-	++	+	++	++
Glucose	+	-	++	+	+++	++
Sorbose	+	-	++	+	+	±
Melibiose	+	-	+	-	+	±
Sucrose	+	-	++	+	++	++
Pyruvate	+	-	++	+	++	+
Glycerol	+	-	++	+	+++	+++
Inositol	+	-	++	+	+++	++
Arabinose	+	-	++	+	++	++
Succinate	++	-	++	+	++	++
Rhamnose	+	-	++	+	++	++
NaCitrate	+	-	+	-	+	+
Fructose	+	-	++	+	++	++

	ISOLATE			
	III		11057-1	
C-source	G	HA	G	HA
Mannose	+++	+++	++	+
Glucose	+++	+++	++	++
Sorbose	+++	+++	+	±
Melibiose	++	++	+	-
Sucrose	++++	++++	+++	+++
Pyruvate	++	++	++	++
Glycerol	+++	+++	++	++
Inositol	+++	+++	+	±
Arabinose	+++	+++	+	+
Succinate	++++	++++	++	+
Rhamnose	+++	+++	++	+
NaCitrate	+	+	+	+
Fructose	+++	+++	++	++

Table 7B. Effect of various carbon sources on hemoglobin accumulation.

	ISOLATE			
	IIR		IIW	
C-source	Growth	HA	Growth	HA
Mannose	+	+	+	-
Glucose	+++	+++	+	-
Sorbose	++	++	+	-
Melibiose	++	+	+	-
Sucrose	+++	+++	+	-
Pyruvate	+	++	+	-
Glycerol	++	++	+	-
Inositol	++	++	+	-
Arabinose	++	++	+	-
Succinate	++	++	+	-
Rhamnose	++	++	+	-
Sodium Citrate	+	+	+	-
Fructose	+++	+++	+	-

± white mostly; few red colonies

HA Hemoglobin Accumulation

any nitrogen source in the medium, the clinical isolates growth on the plates consisted of irregular shaped colonies with a transparent appearance and watery consistency. This effect was more pronounced in the Hb accumulators. When the media was supplemented with various nitrogen containing compounds (Table 8), it was found that with the exception of ammonium, all of them suppressed HA. Furthermore, most of the isolates capable of accumulating Hb showed β-hemolysis. This was interesting since on the standard blood plates (5% v/v) no hemolysis has been reported by the members of the genus Klebsiella.

To further investigate the effect of ammonium on HA, varying concentrations of ammonium were added to the MB plates. Results of this experiment (Table 9) showed that ammonium concentrations of greater than 100mM inhibited HA in PRIIR and 46368, and PRIII, 11057-1 and KR were inhibited at less than 100mM. Growth at ammonium concentrations of 100mM and more was non-pigmented and showed large colony production.

Tables 10, 11 and 12 show the effect of amino acids on HA. The additions of glutamic acid or aspartic acid alone had no effect on HA. In the absence of ammonium in the medium, Hb accumulators showed augmented slime production. When the two amino acids were used in conjunction, as done by Shanmugam and Morandi (42), in a study on nitrogenase biosynthesis, [1mg/ml of

Table 8. Effect of various Nitrogen (N) sources on HA on minimal media blood plates.

	Urea		Y.E.		C.A.		Tryp.		Ammonium	
Isolate	G	HA	G	HA	G	HA	G	HA	G	HA
III	++	-	++++	-	++++	-	++++	-	+++	+++
IIW	+	-	+++	-	+++	-	++	-	++	-
IIR	+	-	+++	-	+++	-	++	-	+++	++
46368	+	-	++++	-	+++	-	++++	-	+++	+++
11057-1	+	-	++++	-	++++	-	++++	-	+++	+++
KR	+	-	+++	-	+++	-	++	-	+++	+++
KW	+	-	+++	-	+++	-	++	-	++	-

2% w/v of each N-source added to the media

Y.E. yeast extract
C.A. vitamin free-casamino acid
Tryp. Tryptose
G Growth
HA Hemoglobin Accumulation

Table 9. Effect of varying concentration of ammonium on Hb accumulation.

	ISOLATE IIW		IIR		46368	
Ammonium (mM)	G	HA	G	HA	G	HA
0	+	-	+	-	+++	+++
25	++	-	+++	±	++++	++
50	+++	-	+++	±	++++	++
75	+++	-	+++	-	+++	++
100	+++	-	+++	-	+++	-
150	+++	-	+++	-	+++	-
200	+++	-	+++	-	+++	-
250	+++	-	+++	-	+++	-

	III		11057-1		KW		KR	
Ammonium (mM)	G	HA	G	HA	G	HA	G	HA
0	+++	+++	+++	++++	++	-	+++	+++
25	++++	++	++++	+++	+++	-	+++	±
50	++++	++	++++	+++	+++	-	+++	±
75	++++	++	+++	+++	+++	-	+++	±
100	++++	-	++++	-	+++	-	+++	-
150	++++	-	++++	-	+++	-	+++	-
200	++++	-	++++	-	+++	-	+++	-
250	++++	-	++++	-	+++	-	+++	-

± white mostly, few red colonies

G Growth

HA Hb Accumulation

mM Millimolar

Table 10. Effect of various concentrations of Glutamic acid on HA, with and without ammonium (3mg/ml).

	GLUTAMIC ACID							
	10µg/ml				100µg/ml			
	$-NH_4^+$		$+NH_4^+$		$-NH_4^+$		$+NH_4^+$	
Strains	G	HA	G	HA	G	HA	G	HA
III	++	BR	+++	++	+++	BR	+++	++
IIR	++	BR	+++	++	++	BR	+++	+++
IIW	+	-	++	-	+	-	++	-
11057-1	+	B	+++	+++	+	B	+++	+++
46368	++	BR	+++	++	++	B	+++	+++
KR	+	B	+++	+++	++	B	+++	+++
KW	+	-	++	-	+	-	++	-
	150µg/ml				200µg/ml			
III	+++	BRr	++++	BR	+++	BRr	+++	BR
IIR	+++	BRr	+++	++	+++	BRr	+++	+++
IIW	++	-	++	-	+	-	++	-
11057-1	+++	+++	++++	+++	++	B	+++	+++
46368	+++	BRr	++++	B	+++	BR	+++	+++
KR	++	BRr	++++	B	+++	BR	+++	+++
KW	+	-	++	-	+	-	++	-

BR Brown and runny
BRr Brown and red colonies, runny
B Brown
LB Light Brown
RT Runny Transparent

Table 11. Effect of various concentrations of Aspartic acid on HA, with and without ammonium (3mg/ml).

Concentration	ASPARTIC ACID 10µg/ml				100µg/ml			
	$-NH_4^+$		$+NH_4^+$		$-NH_4^+$		$+NH_4^+$	
Strains	G	HA	G	HA	G	HA	G	HA
III	++	BR	+++	BRr	++	BRr	+++	BRr
IIR	++	BR	+++	+	++	BR	+++	+++
IIW	+	-	++	-	+	-	++	-
11057-1	++	BR	+++	++	++	BR	+++	+++
46368	++	BR	+++	++	++	BR	+++	+++
KR	++	BR	+++	+++	+	B	+++	++++
KW	+	-	++	-	+	-	++	-
	150µg/ml				200µg/ml			
III	+++	BRr	+++	BRr	++++	++BR	++++	BRr
IIR	++	BR	+++	+++	+++	BR	+++	+++
IIW	+	-	++	-	++	-	++	-
11057-1	++	B	+++	+++	+	B	+++	+++
46368	+++	BR	+++	+++	++	BR	+++	+++
KR	++	B	+++	++++	++	BR	++	++++
KW	+	-	++	-	+	-	++	-

Table 12. Effect of glutamic acid and aspartic acid together on HA.

		G100μg/ml:A200μg/ml
Strains	Growth	HA
III	++++	BRr
IIR	+++	BRr
IIW	+	-
11057-1	+++	B
46368	+++	BR
KR	++	B
KW	+	-
		G1μg/ml:A200μg/ml
III	+++ very slimy	++
IIR	+++ slimy	-
IIW	+	-
11057-1	+++ very slimy	-
46368	+++	-
KR	+++	-
KW	++	-

glutamic acid with 200µg/ml of aspartic acid (Glmg/A200µg)], this combination of concentrations resulted in repression of HA (Table 12).

Iron supplementation

Ferrous sulphate supplementation was used to determine whether the K. pneumoniae isolates were sequestering iron from the medium. If so, then, they should stop accumulating the Hb if an excess of iron is provided in the medium. It is difficult to remove completely the contaminating metals in the macronutrients of the culture medium. Even in the minimal medium, there is usually enough Fe^{+3} contamination (0.03 to 0.2µg/ml), especially in the sugars and phosphates to support growth. For K. pneumoniae, maximal growth is achieved by adding 0.03µg/ml iron, which makes it apparent that K. pneumoniae can do well without adding iron to the medium.

Table 13 shows the effect of varying concentrations of ferrous sulphate on Hb accumulation. Increasing concentrations of ferrous sulphate resulted in decreased HA. The highest degree of HA was observed when no iron was added to the medium. Figure 2 shows the photographs of HA on MB plates with various iron concentrations.

Molybdenum supplementation

The addition of Molybdenum (Mo) to the MB plates had no effect on pigmentation. In the presence of

Table 13. Effect of varying concentration of ferrous sulphate on hemoglobin accumulation on minimal medium blood plates with and without ammonium supplementation (3mg/ml).

	0µg/ml				50µg/ml			
	$-NH_4^+$		$+NH_4^+$		$-NH_4^+$		$+NH_4^+$	
Strains	G	HA	G	HA	G	HA	G	HA
III	++	BR	+++	++	++	BR	+++	+
IIR	++	BR	+++	+++	+	BR	+++	+
IIW	+	-	++	-	+	-	++	-
11057-1	+	B	+++	+++	+	B	+++	+
46368	++	B	+++	++	+	B	+++	+
KR	+	B	+++	++	+	B	+++	+
KW	+	-	++	-	+	-	++	-
	100µg/ml				500µg/ml			
III	++	RT	+++	+	++	BR	+++	LB
IIR	+	RT	+++	+	+	BR	+++	LB
IIW	+	RT	++	-	+	-	+++	LB
11057-1	+	B	+++	B	+	-	+++	LB
46368	+	B	+++	B	+	-	+++	LB
KR	+	-	+++	B	+	-	+++	-
KW	+	-	++	-	+	-	++	-

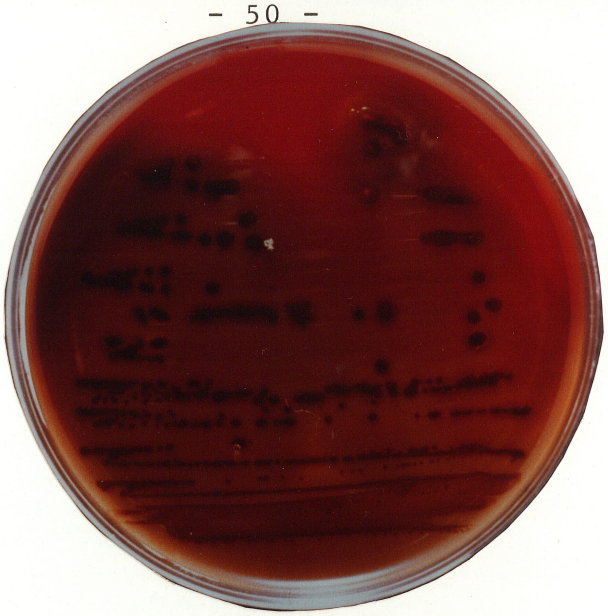
HA Hb accumulation
RT Runny and Transparent
B Brown
BR Brown and Runny
LB Light Brown

Figure 2. Effect of ferrous sulphate on PRIIR

Photograph 1. Without ammonium, 0ug/ml ferrous sulphate.

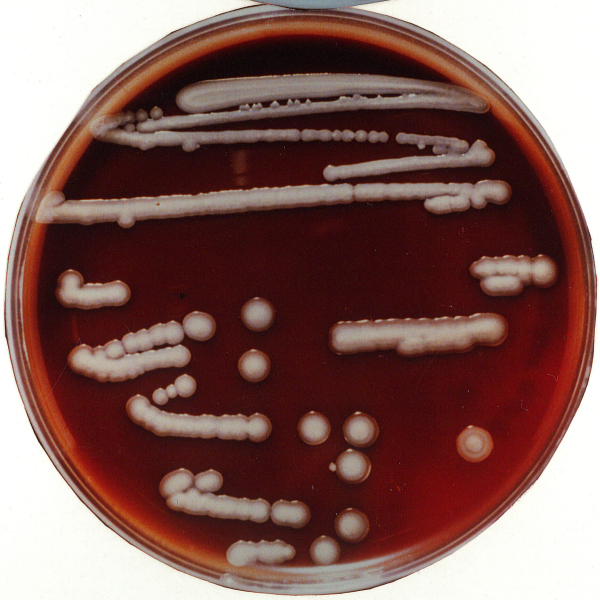
Photograph 2. 100ug/ml of ferrous sulphate

Photograph 3. 500ug/ml of ferrous sulphate



1

2



3

ammonium (Table 14), the pigment was evenly spread in the growth ie. the growth of the cultures was evenly colored. In the absence of ammonium, the cultures showed irregular growth and brown coloration.

Hb localization

The purpose of the localization study was to find out the nature of the association between the bacterium and the pigment. It is difficult to know whether the Hb or hemin is bound to the bacterial surface or is absorbed loosely. However, it was demonstrated that the most of Hb or hemin was recoverable from the cultures that were pigmented. When the scraped colonies were suspended, by vortexing, in PBS, the solution became red. When the suspension was centrifuged, the resultant pellet appeared slightly pinkish with the majority of the red color remaining in the supernatant. The third wash supernatant was colorless. No change was observed in the resulting pellets after each wash with PBS. The pinkish hue in the pellets remained after three washes with the KCl salt solution.

Figure 3 depicts the absorption spectrum of red blood cells (RBC) in reduced and oxidized state and Figure 4 illustrates the absorption spectrum of pure Hb, in reduced and oxidized forms. Both of these figures show reduced maxima at approximately 555nm and 430nm wavelength; and peaks of 415 nm, 576nm and 540nm are

Table 14. Effect of supplementing molybdenum (.25mg/ml) to the minimal medium blood plates on HA.

	$-NH_4^+$		$+NH_4^+$	
Strains	G	HA	G	HA
III	+++	BR	++++	BRr
IIR	+++	BR	+++	+++
IIW	+	-	++	-
11057-1	++	BR	++++	++++
46368	++	BR	++++	+++
KR	++	B	+++	+++
KW	+	-	++	-

BR Brown and Runny

B Brown

BRr Brown, runny and red

HA Hb accumulation

G Growth

Figure 3. Absorption spectra of red blood cells. Oxidized red blood cells (HbO_2) were obtained by exposing the blood sample to oxygen.

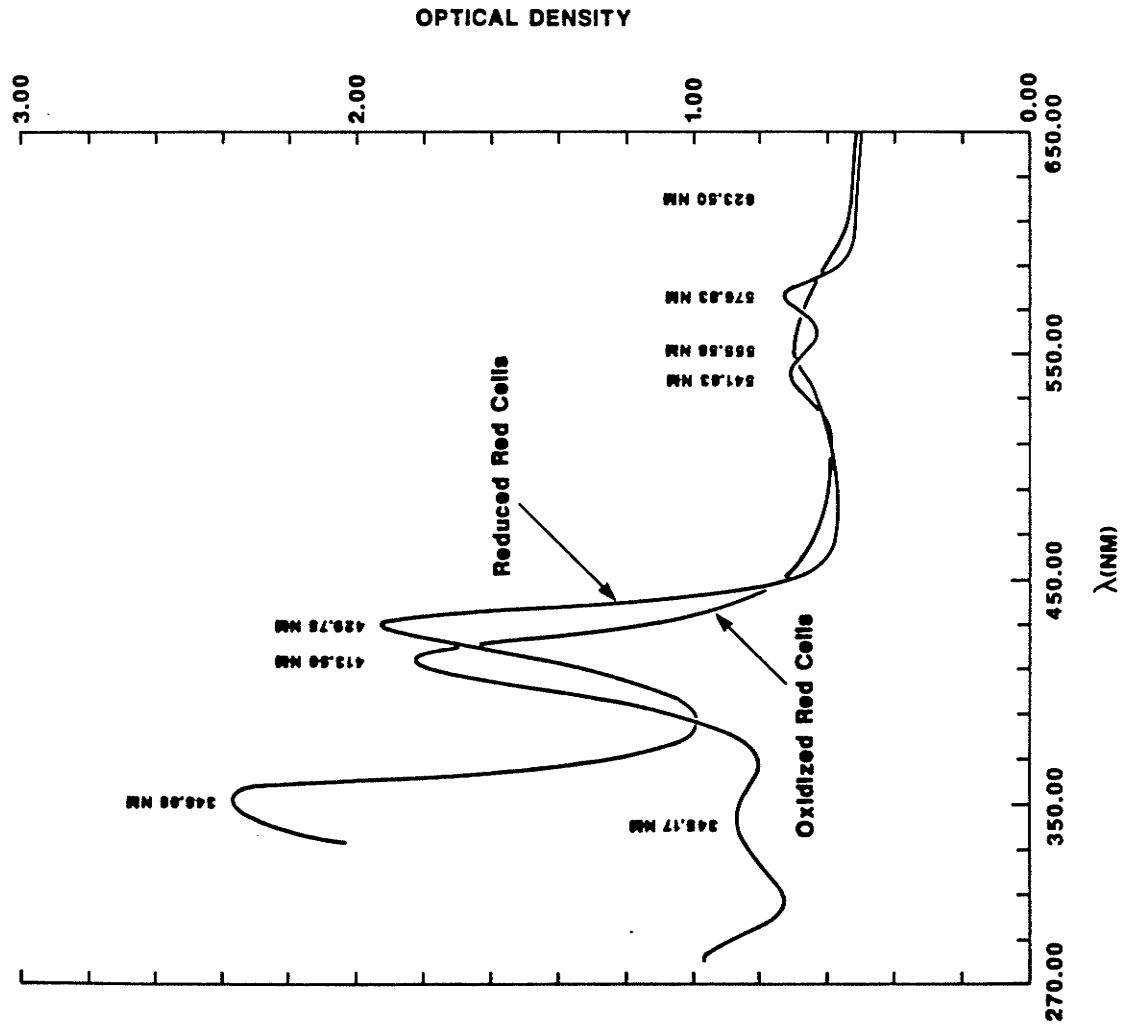
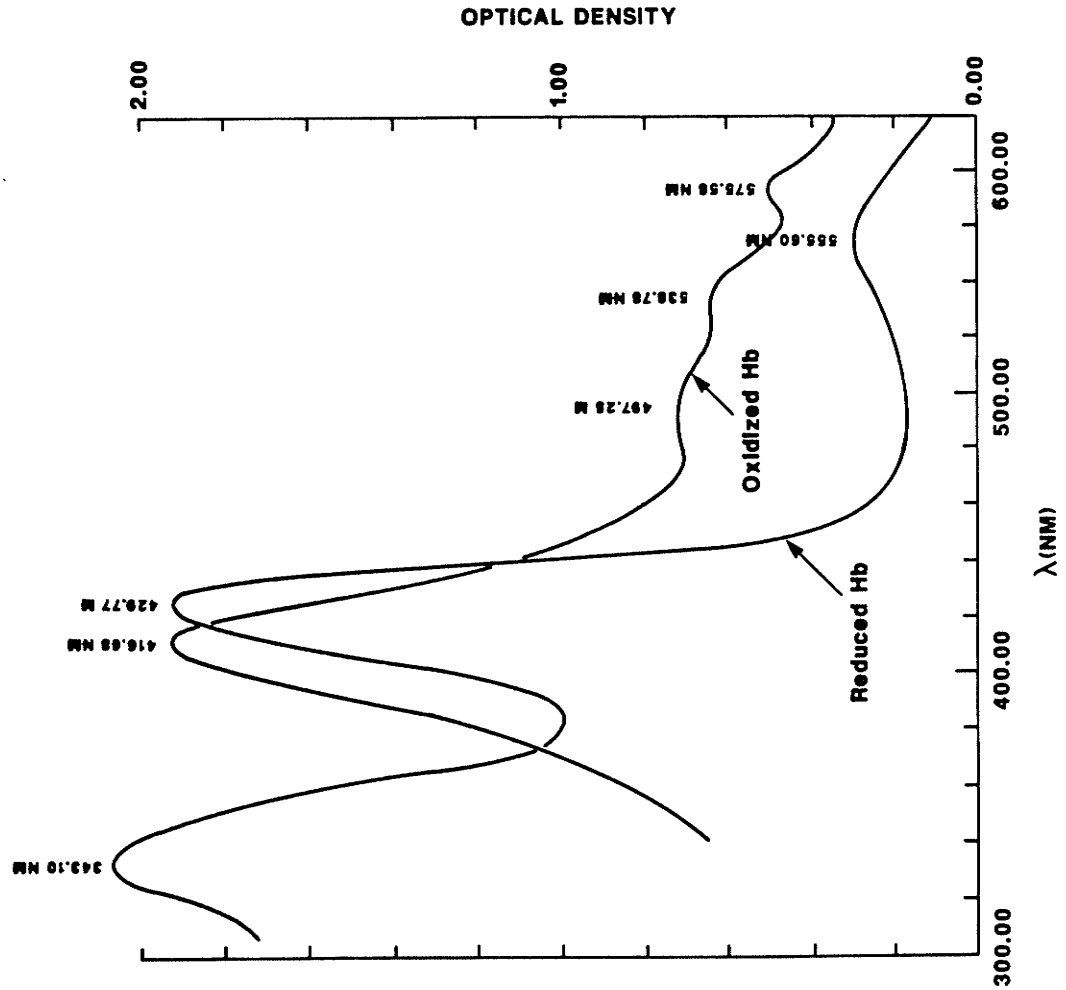

OPTICAL DENSITY
3.00
2.00
1.00
0.00
650.00
550.00
450.00
350.00
270.00
λ(NM)
623.50 NM
576.83 NM
555.58 NM
541.83 NM
Reduced Red Cells
428.75 NM
413.58 NM
346.88 NM
348.17 NM
Oxidized Red Cells

Figure 4. Absorption spectra of Hb in oxidized and reduced states.

Hb was reduced by Sodium dithionite. Oxidized hemoglobin was obtained by exposing pure hemoglobin solution to oxygen.


OPTICAL DENSITY
2.00
1.00
0.00
600.00
500.00
400.00
300.00
λ(NM)
576.56 NM
555.60 NM
538.78 NM
497.28 M
Oxidized Hb
Reduced Hb
428.77 M
416.68 NM
343.10 NM

predominant in the oxidized form. A spectrophotometric examination of pigmented isolates revealed similar absorption maxima (Figure 5) to pure Hb or to RBC in oxidized form. Nonpigmented isolates treated in the same manner showed no absorption band. It is clear from these results that red colored colonies were due to adsorption and retention of Hb or heme moiety. Additionally, it seems that, at least some of Hb or hemin was tightly bound to the cell surface.

SDS-Electrophoresis

Three isolates of K. pneumoniae, PRIII, PRIIW and 46368 were grown and four fractions (S1, S2, S3, and S4) were prepared and then analysed by one-dimensional electrophoresis as described in Materials and Methods.

Coomassie blue stained gels (Figure 6) revealed many bands, with the major differences being in the S4 fractions of three isolates. Protein bands between the molecular weights of approximately 35K and 67K (Lane S4: columns a and b) were only present in PRIIW, a non-accumulator. There are a few very faint bands present in PRIII and 46368 isolates in the region (columns a and b), however, it is apparent that there are significant differences between the accumulating and non-accumulating isolates.

Figure 5. Absorption spectra of Hb accumulating and non-accumulating isolates.

The organisms were scraped off a plate and suspended in the PBS buffer, pH7.4.

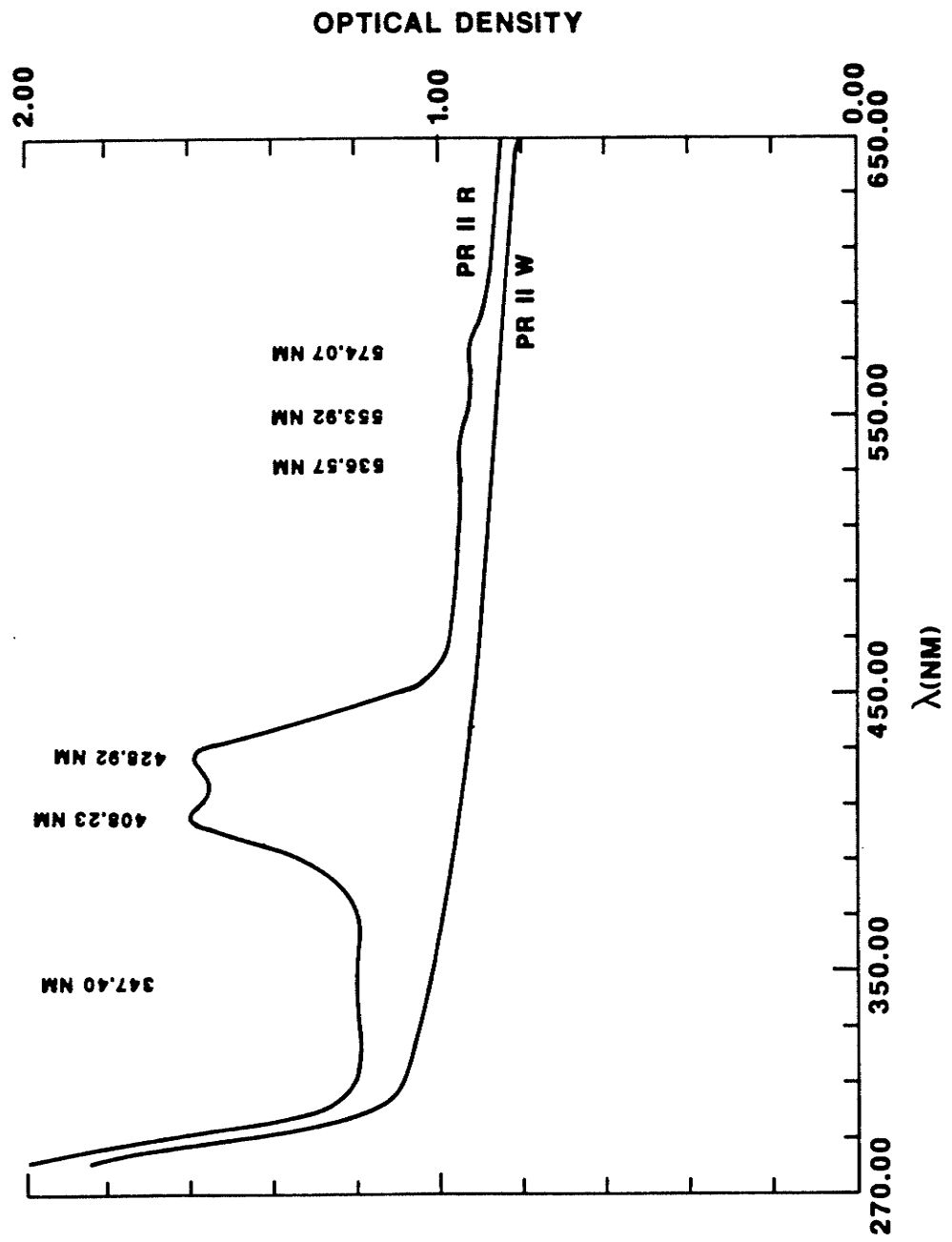

OPTICAL DENSITY
2.00
1.00
0.00
650.00
550.00
450.00
350.00
270.00
λ(NM)
PR II R
PR II W
574.07 NM
553.92 NM
536.57 NM
428.92 NM
408.23 NM
347.40 NM

Figure 6. Electropherogram of K. pneumoniae isolates. One dimensional SDS-PAGE and staining was carried out as described in Materials and Methods. Column a: PRIII; Column b: 46368;

Lane C: PRIIW

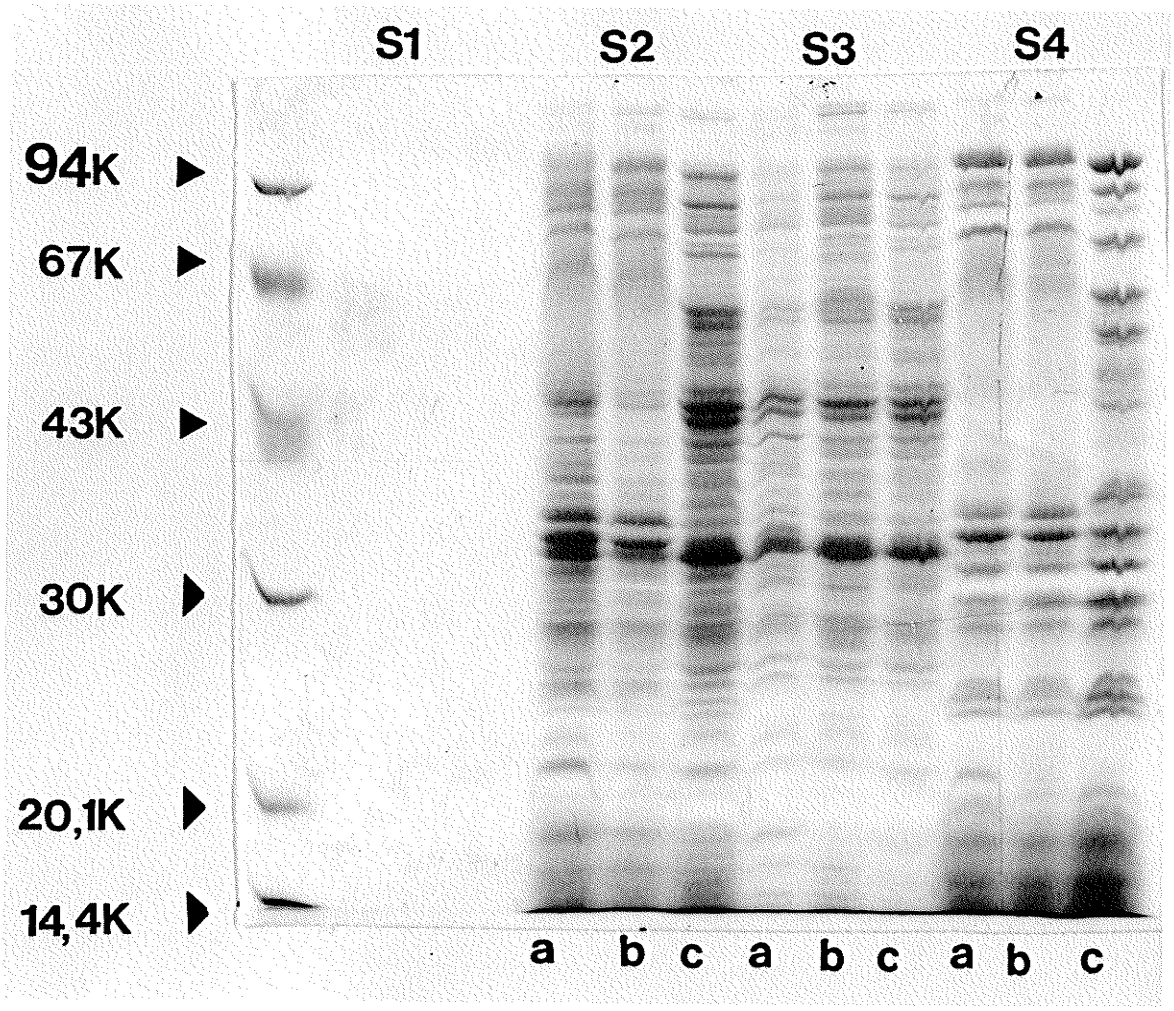
Lane S1: Slime layer

Lane S2: Crude membrane extracts

Lane S3: Soluble Triton X-100 extracts

Lane S4: Insoluble Triton X-100 extracts

Numbers indicate the molecular weights of the marker proteins in kilodaltons(K).


S1
S2
S3
S4
94K
67K
43K
30K
20,1K
14,4K
a b c a b c a b c

Nitrogen fixation and HA

Although nitrogen fixation was detected by the clinical isolates studied, it proved to be poorly reproducible so not date are shown.

Congo red dye absorption

All of the isolates were capable of absorbing the red dye from the medium.

DISCUSSION

DISCUSSION

This work was initiated following the observation that certain clinical isolates of K. pneumoniae, when grown on a minimal medium supplemented with red blood cell concentrate produced red pigmented colonies. Spectrographic study of pigmented cells showed that red pigmentation was due to the accumulation of hemoglobin by the bacteria. This phenomenon was apparent only in some clinical isolates and was not present in any of the other bacterial species tested.

The first series of experiments were performed to attempt to define the optimal conditions for demonstration of hemoglobin accumulation by K. pneumoniae. The organism has not been reported to be hemolytic on standard blood agar plates, and it will not accumulate hemoglobin on standard blood plates. In our study it was found that HA was favored at pH values close to seven, and a red blood cell concentration of 4%. Using only red blood cells, a minimal salts medium, and a carbohydrate, normally glucose, those cultures which could accumulate hemoglobin would also hemolyse the red cells. Both the HA and hemolysis are prevented when blood serum is present in the medium.

The HA strains could also accumulate on a medium containing sterile hemoglobin instead of red cells, but cells were normally used because of their accessibility

and cost. Cultures were able to accumulate hemoglobin on a wide variety of carbohydrate substrates, with the greatest amount of accumulation corresponding to the growth obtained. On substrates which supported growth poorly (eg. citrate) it was difficult to discern whether there had been accumulation or not. The best accumulation was observed on those substrates which resulted in the production of large quantities of extra-capsular material or slime. Since the majority of the red color could be removed from cells by two or three washings in PBS, it would appear that the majority of the Hb is retained in the slime layer.

The Hb localization study indicated that Hb or hemin was bound loosely to the bacterial surface. It is possible that the Hb or hemin bound to the outer membranes of bacterium was either in free form and/or with a secreted-binding protein. The fact that not all of the red color in the pellets came off after several washes, suggests that the part of the total Hb or hemin bound to the cells was bound to the cell surface, or probably with a receptor molecule on cell surface.

One of the major findings was the observation that nitrogen containing compound supplementation inhibited HA. Initially, this led us to speculate that perhaps Hb accumulation was involved in nitrogen fixation, possibly having a similar role as leghemoglobin in Rhizobium-legume System. It was possible that Hb

molecules or hemin, by surrounding the bacterial colony on the surface protected the nitrogenase from oxygen. This idea was also supported by the slimy growth of accumulating isolates on nitrogen free medium. Furthermore, the inhibition by serum, tryptose, high concentrations of ammonium, and the combination of aspartic acid and glutamic acid further helped promote the idea that nitrogen fixation and HA somehow were related since these compounds are known to repress the expression of nitrogenase genes. Unfortunately, except for an initial positive result, no nitrogen fixation could be detected in any of the clinical isolates. Lack of consistent results may have been due to the difficult methodology, however, this feature requires further investigation.

The results of the excess iron supplementation experiment showed increased suppression of HA with increased content of iron in the medium. This indicated that HA was involved in mediating iron sequestering for K. pneumoniae, ie. excess iron in the growth medium would stop the bacteria from using Hb as the iron source. If iron was the reason to pick Hb or hemin from the growth medium, then why was the HA suppressed in the presence of nitrogen compounds and specifically in the presence of high concentrations of ammonium? Since ammonium and other macronutrients used in minimal media, such as sugars and phosphate could be contaminated with iron, it is possible that increasing the ammonium

concentrations increased the iron content of growth medium, thus resulting in HA repression. Another possibility is that HA is coordinately controlled by both Fe and N compounds.

One of the most interesting results came from the SDS-PAGE study. The coomassie blue R-250 stained gels of non-accumulators and accumulators showed major differences between them. Several protein bands between the molecular weights of 67,000 and 35,000 are missing in accumulating strains (Figure 6, lane S4; a and b columns). Work of Lodge et. al. (82) and others have shown that outer membrane proteins of K. pneumoniae (studied by SDS-PAGE) expressed in iron depleted conditions were missing in cultures in which iron was sufficient. Six outer membrane (OM) proteins have been reported to be expressed in iron depleted medium which vary from 66 to 83 kilodaltons (Kdal). The 83Kdal OM protein has been proposed to be an enterobacteria receptor protein and 35.5 and 39 Kdal proteins to be related to the OmpF and OmpC Porin proteins of E. coli.

In this study, the protein bands in the non-accumulating strain were not observed in the region where polypeptides occur due to iron deprivation, rather in the region where some nif genes products are found. Experimental work has shown that polypeptides of nif genes are found between 22,000 to 120,000 daltons, and most of them occupying the region between 22Kdal to 66Kdal

(83). This suggests that non-pigmented colonies could be expressing some nif genes products.

Work of Sterkenberg et. al. (84), which examined the effect of nutrient limitation on outer membrane proteins (OMPS) of K. pneumoniae, revealed four major proteins, when cultured at a high specific growth rate, but at a lower growth rate, showed that OMP expression was dependent on imposed nutrient limitation. Several additional OMPs were expressed under various nutrient limiting conditions. In the present study, the coomasie blue gels of non-accumulators showed similarity with OMPs expressed in the region of 45Kdal to 66,200 daltons in Sterkenberg et. al. work. Their protein bands were present most clearly in ammonium limitation gels, and expressed in other nutrient limiting conditions in lesser amounts. This suggests that protein expressed by non-accumulators are part of normal OMPs present in K. pneumoniae, and that Hb accumulating strains are mutants lacking them. It is plausible that accumulators are lacking some OMPs which are associated with uptake of limiting nutrients. Further work is required to explain the results from the SDS-PAGE study of accumulators and non-accumulators.

Previous work done on Y. pestis, which produced pigmented colonies, when growth on excess hemin, showed that pigmented colonies were considerably more virulent than non-pigmented colonies. They also found that

virulence of non-pigmented strains could be augmented to the level of pigmented colonies if sub-lethal amounts of iron compounds were injected along with the organisms. From the results, it was suggested that the ability to absorb hemin is a mechanism by which a virulent strain is able to derive adequate supplies of iron for growth. It is possible that in K. pneumoniae, HA is related to virulence, also enabling them to sequester iron in iron limited environments. Since the HA strains were larger and more slimy compared to the non-accumulators, they do in fact have some advantage under the growth conditions tested.

Upon prolonged incubation of pigmented cultures on the minimal medium or in nutrient or T-soy broths, non-pigmented variants were observed in the pigmented isolates. However, the reverse, non-pigmented to pigmented was never observed.

The results of the present study suggest that HA might be involved in iron metabolism and that nitrogen fixation and iron metabolism may be related. Moreover, the use of Hb by this opportunistic pathogen implies that the HA phenomenon might be important clinically.

Work of Payne and Finkelstein (82), who used absorption of congo red dye to differentiate several virulent and avirulent strains of some pathogenic bacteria, found that virulent strains absorb the red dye and produced red colonies. Similar experiments done

on K. pneumoniae showed that cells of all the isolates were capable of absorbing the red dye.

However, to prove definitively the role of HA in virulence, animal tests are required for Hb accumulating and non-accumulating isolates. If it is shown to be related, then, the ability to absorb Hb and use it to their benefit, may be recognized as one of the important virulence factors in K. pneumoniae.

REFERENCES

REFERENCES

1. Zinsser, H., and S. Bayne-Jones. 1939. A textbook of Bacteriology 8th ed., D. Appleton-Century Company Inc., New York. p482.

2. Holmes, B., and R.J. Gross. 1984.In: Topley and Wilson's Principles of Bacteriology, Virology and Immunity Vol 2. Edward Arnord. p292, 296.

3. Cowan, S.T., K.J. Steel, C. Shaw, and J.P. Duguid. 1960 J. Gen. Microbiol. 23: 601-612.

4. Durlakowa, I., Z. Lachowicz, and S. Slopek. 1967. Arch. Immunol. Ther. Exp. 15: 490-496.

5. Slopek. S., and I. Durlakowa. 1967. ARch. Immunol. Ther. Exp. 15: 481-487.

6. Bascomb, S., S.P. Lapage, W.R. Willcox, and M.A. Curtis. 1971. J. Gen. Microbiol. 66: 279-295.

7. Naemura, L.G., S.T. Bagley, R.J. Seidler, J.B. Kaper, and R.R. Colwell. 1979. Curr. microbiol. 2: 175-180.

8. Buchanan, R.E., and Gibbons, N.E. (Eds.) 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins, Baltimore, Maryland. pp290-340.

9. Brenner, D.J., A.G. Steigerwalt, and G.R. Fanning. 1972 Int. J. System. Bacteriol. 22: 193-200.

10. Bergey, D.H., N.R. Krieg, and J.G. Holt. (Eds). 1984. Bergey's Manual of Systematic Bacteriology. 9th ed. Vol 1 Williams and Wilkins. Baltimore, London. pp461-465.

11. Bagely, S.T., R.J. Seidler, and D.J. Brenner. 1981. Curr. Microbiol. 6: 105-109.

12. Izard, D., C. Ferragut, F. Gavini, K. Kersters, J. De Ley and H. Leclerc. 1981. Int. J. Syst. Bacteriol. 31: 116-127.

13. Slopek. S., 1978. In: Bergan and Norris (Eds). Methods in Microbiol. Vol 2. Academic Press, London. pp193-222.

14. Slopek. S., and J. Marexz-Babczyszyn. 1967. Arch. Immunol. Ther. Exp. 15: 525-529.

15. Edmondson, A.S., and E.M. Cooke. 1979. J. Hyg. 82: 207-223.

16. Cooke, E.M., T. Sazegar, A.S. Edmondson, J.C. Brayson, and D. Hall. 1980. J. Hyg. 84; 97-101.

17. Julianelle, L. A. 1926. J. Exp. Med. 44: 113-128.

18. Kauffmann, F. 1949. Acta Pathol. Micrbiol. Scand. 26: 381-406.

19. Orskov, I., and F. Orskov. 1984. Methods in Microbiology, Ed. T. Bergan. 14: 143.

20. Duguid, J.P. 1959. J. Gen. Microbiol. 21: 271-286.

21. Costerton, J.W., R.T. Irvin, and K.J. Cheng. 1981. CRC Critical Rev. Micrbiol. 8: 303-338.

22. Domenico, P., D.L. Diedrich, and D.C. Strauss. 1985. Can. J. Microbiol. 31: 472-478.

23. Orskov, I. 1952. Acta Pathol. Scand. Supp. 93: 259-269.

24. Richard, C. 1973. Ann. Biol. Chem. 31:295-303.

25. Riser, E., P. Noone, and R.E. Thompson. 1978. J. Hyg. 80: 43-56.

26. Casewell, M. and H.G. Talsania. 1979. J. Infect. 1:77-79.

27. Steinhauer, B.W., T.C. Eickhoff, J.W. Kislak, and M. Finland. 1966. Ann. Intern. Med. 65:1180-1194.

28. Eickhoff, T.C., B.W. Steinhauer, and M. Finland. 1966. Ann. Intern. Med. 65:1163-1179.

29. Montgomerie. J.Z. 1979. Rev. Infect. Dis. 1:736-753.

30. Brewerton, D.A., M. Caffrey, F.D. Hart, D.C.O. James, A. Nichools, and R.D. Sturrock. 1973. Lancet 1:904-907.

31. Ebringer, A., P. Cowling, N. Suh, D.C.O. James, and R.W. Ebringer. 1976. In: HLA and Disease. Eds. J. Dausset and A. Snejgaard. Inserm Publications, Paris. p27.

32. Welsh, J., H. Avakian, P. Cowling, A. Ebringer, P. Wooley, G. Panayi, and R. Ebringer. 1980. Br. J. Exp. Pathol. 61:85-91.

33. Ebringer, R.W., D.R. Cadwell, P. Cowling, and A. Ebringer. Ann. Rheum. Dis. 37:146-151.

34. Avakian, H., J. WElsh, A. Ebringer, and C.C. Entwhistle. 1980. Br. J. Exp. Pathol. 61:92-96.

35. Geczy, A.F., H.V. Bashir, K. Alexander, and J.P. Edmonds. 1980. Lancet 1:50.

36. Seager, K., H.V. Bashir, A.F. Geczy, J. Edmonds, and A. De Vere-Tyndall. 1979. Nature:277:68-70.

37. Geczy, A.F., K. Alexander, H.V. Bashir, and J. Edmonds. 1980. Nature 283: 782-784.

38. Brill, W.J. 1980. Microbiol. Rev. 44:449-467.

39. Streicher, S.L., K.T. Shanmugam, F. Ausubel, C. Morandi, and R.B. Goldberg. 1974. J. Bact.120: 815-821.

40. Tubb, R.S. 1974. Nature 251:481-485.

41. Tubb, R.S., and J.R. Postgate. 1973. J. Gen. Microbiol. 79: 103-117.

42. Shanmugam, K.T., and C. Morandi. 1976. Biochem. Biophys. Acta. 437:322-332.

43. Yoch, D.C., and R.M. Pengra. 1966. J. Bact. 92: 618-622.

44. Hennecke, H., and K.T. Shanmugam. 1979. Arch. Microbiol. 123:259-265.

45. Brooks, S.J., J.J. Collins, and W.J. Brill. 1984. J. Bact. 157:460-464.

46. St. John, R.T., V.K. Shah, and W.J. Brill. 1974. J. Bact. 119:266-269.

47. Klucas, R. 1972. Can. J. Microbiol. 18:1845-1850.

48. Hill, S. 1975. J. Gen. Microbiol. 91:207-209.

49. Hill, S. 1976. J. Gen. Microbiol. 93:335-345.

50. Wilcockson, J. 1977. J. Gen. Micrbiol. 101: 311-317.

51. Wilcockson, J., and D. Werner. 1978. J. Gen. Microbiol. 108:151-160.

52. Jensen, H.L., E.J. Peterson, P.K. De, and R. Bhattachary. 1960. Arch. Microbiol. 36:182-195.

53. Hill, S. 1971. J. Gen. Microbiol. 67:77-83.

54. Dalton, H., and J.R. Postgate. 1969. J. Gen. Microbiol. 56:307-319.

55. Bullen, J.J. 1981. Rev. Infect. Dis. 3:1127-1138.

56. Finkelstein, R.A., C.U. Sciortino, and M.A. McIntosh. 1983. Rev. Infect. Dis. 5:5759-5777.

57. Weinberg, E.D. 1978. Microbiol Rev. 42:45-66.

58. Weinberg, E.D. 1984. Physiol. Rev. 64:65-102.

59. Neilands, J.B. 1981. Ann. Rev. Biochem. 50: 715-731.

60. Lam, C., F. Turnowsky, E. Schwarzinger, and W. Neruda. FEMS Microbiol. Lett. 24:255-259.

61. McIntosh, M.A., and C.F. Earhart. 1977. J. Bact. 131:331-339.

62. Sciortino, C.V., and R.A. Finkelstein. 1983. Infect. Immun. 42:990-996.

63. Williams, P., M.R.W. Brown, and P.A. Lambert. 1984. J. Gen. Microbiol. 130:2347-2355.

64. Sterkenberg, A., E. Vlegels, and J.T.M. Wouters. 1984. J. Gen. Mcirobiol. 130-2347-2355.

65. Chart, H., and E. Griffiths. 1985. J. Gen. Microbiol. 131:1503-1509.

66. Francis, R.T., J.W. Booth, and R.R. Becker. 1985. Int. J. Biochem. 17:767-773.

67. Bornsise, G.H., P.J. Bouis Jr., I. Cohn Jr. 1968. J. Bact. 95:1567-1571.

68. Quie, P.G., and L. W. WAnnamaker. 1961. J. Bact. 82:770-783.

69. Whittenburg, R. 1978. In: Streptococci. Eds. Skinner, F. A., and L.B. Quesnel, Academic Press, New York. pp51-64.

70. Lee, T.J., D.H. Ahrenholz, R.D. Nelson, and R.L. Simmons. 1979. Surgery 86:41-48.

71. Davis, J.H., and A.B. Yull. 1964. J. Traum. 4: 84-89.

72. Bryan-Jones, D.G., and R. Wittenburg. 1969. J. Gen. Microbiol. 58:247-260.

73. Jensen, J. 1957. J. Bact. 73:324-333.

74. Maskell, J.P., and A.A. Miles. 1984. J. Med. Microbiol. 18: 377-384.

75. Jackson, S., and T.W. Burrows. 1956. Brit. J. Exp. Path. 37:57-576.

76. Ibid., pp577-583.

77. Smith, J.W.G. 1984. In: Topley and Wilson's Principles of Bacteriology, Virology and Immunity. Vol 2. Edward Arnord. pp379-386.

78. Yu, E.K.C., and J.N. Saddler. 1982. App. Env. Microbiol. 44:777-784.

79. Lameli, U.K. 1970. Nature 227:680-685.

80. Campbell, N.E.R. and H.J. Evans. 1969 Can. J. Microbiol. 15:1342-1343.

81. Payne, S.M., and R.A. Finkelstein. 1977. Inf. Imm. 18:94-98.

82. Lodge, J.M., P. Williams and M.R.W. Brown. 1986. J. Bact. 165:353-356.

83. Roberts, G.P., and W.J. Brill. 1980. J. Bact. 144:210-216.

84. Sterkenburg, A., E. Vlegels and J.T.M. Wouters. 1984. J. Gen. Microbiol. 130:2347-2355.