CHARACTERIZATION OF A 4.0 KILOBASE PLASMID FROM **PASTEURELLA MULTOCIDA**

by

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(ABSTRACT)

A 4.0 Kb (2.64 Mdal) plasmid was isolated from a fowl cholera strain of **Pasteurella multocida** (the Larsen strain) by alkaline lysis and cesium chloride purification. The plasmid, designated pLAR-1, was characterized in terms of its size and restriction sites. The restriction patterns produced by fourteen endonucleases were used to generate a restriction map. Five restriction enzymes cleaved the plasmid at multiple sites. Two enzymes, Bgl II and Sal I had unique sites on pLAR-1. Twelve of the fifty six strains of **P. multocida** surveyed contained plasmids of different sizes which hybridized to pLAR-1. Strains containing homologous plasmids were variable in serotype, dermonecrotoxin production, and origin (both in terms of the host and locale). pLAR-1 did not encode any of the enzymes necessary for the biochemical pathways contained

within the API-20E strip or siderophore production. pLAR-1 was cloned into the BamH I site of pBR322. Resultant clones were approximately 8.363 Kb in length, ampicillin resistant and tetracycline sensitive. The pLAR-1::pBR322 constructs were transformed into **Escherichia coli** DH5 alpha. Transcription of pLAR-1 was not detected using biotinylated pLAR-1 as a probe in a Northern analysis of mRNA extracted from the Larsen strain grown under nutrient rich conditions.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Pasteurella multocida is a small, gram - negative coccobaccillus approximately 0.3 x 0.4-0.8 um (width x length) belonging to the family **Pasteurelleacea** . In addition to **P. multocida** members of this family include **Pasteurella haemolytica, P. anatipestifer, P. ureae, P. canis, P. gallinarum, P. aerogenes,** and **P. piscidea** (Carter,1984; Olsen et. al., 1987). It has been proposed that the species **P. multocida** consists of three subspecies: **P. multocida** spp. **multocida, septica,** and **gallicida** (Mutters et. al.,1985) . Presently, **P. multocida** consists of five serogroups: A, B, D, E, and F (Roberts et. al.,1947; Carter,1955; and Rimler and Rhoades,1987). Each serogroup exhibits distinct characteristics. For example, strains belonging to serogroup A possess a capsule composed of hyalauronic acid while type D's are capable of producing dermonecrotoxin (DNT).

The hosts of **P. multocida** include bison, cattle, yak, rabbits, ducks, turkeys, chickens, sheep, swine, dogs, cats, and humans. All avian isolates from serogroup A are pathogenic, while mammalian isolates are either pathogenic or nonpathogenic (Timoney et. al.,1988). **P. multocida** is

the primary pathogen of diseases such as fowl cholera, hemorraghic septicemia, and snuffles. The severity of atrophic rhinitis in swine is dependent on prior exposure to **Bordetella brontiseptica** (Rutter et. al., 1984). **B. brontiseptica** may degrade the cilial lining thereby enabling the invasion and colonization by **P. multocida** (Jaques et. al., 1987). **P. multocida** is a secondary pathogen associated with complex diseases such as shipping fever, abortion, and pneumonia in cattle. Common serotypes associated with diseases in animals (pathogenesis) are shown in Table 1.

Recently, there has been an increase in the number of isolates capable of expressing antibiotic resistance. This could be due to the extensive use of antibiotics such as penicillins, triple sulfa, tetracycline, streptomycin, and/or chloramphenical to treat **P. multocida** infections . Similar observations have been documented in other gram-negative bacteria, e.g. **Vibrio anguillarum** (Torananzo et. al.,1983) and **Haemophilus influenzae** (Elwell et. al.,1975).

Antibiotic resistance can be either chromosomal or plasmid mediated. Plasmids are small, circular, autonomously replicating pieces of extrachromosomal DNA.

Table 1: Common serotype of **P. multocida** pathogenic in animals.

SEROTYPE	DISEASE CONDITION	HOST
A:12,A:3	SNUFFLES	RABBITS
A:1,A:3,A:5,A:8,A:9	FOWL CHOLERA	FOWL
B:2,E:2	HEMORRAGHIC SEPTICEMIA	CATTLE, BISON
D:1,D:3,D:4,D:10	ATROPHIC RHINITIS	SWINE
A:3,D:3	PNEUMONIA	SHEEP

Copy numbers of individual plasmids may vary from a single copy per cell to greater than 100 copies per cell . Some plasmids are transferable during cell to cell contact (conjugation) while others are known to be nonconjugal. In addition to antibiotic resistance, plasmids can encode virulence factors such as siderophores, siderophore regulating factors, bacteriocins and/or hemolysins (Elwell and Shipley,1980; Tolmasky et. al.,1985; Actis et. al., 1985, Tolmasky et. al., 1988). They are also known to encode restriction enzymes and conjugal factors (Elwell and Shipley.,1980; Hirsh et. al.,1981). In one study, greater than 90% of the **Pasteurella multocida** strains surveyed possessed plasmids (Hirsh et. al.,1985).

The objectives of this study were to : 1) characterize a plasmid from a fowl cholera isolate of **P. multocida** in terms of its size and restriction sites; 2) examine its phenotypic characteristics eg. antibiotic resistance, significance or prevalance both within and across serogroups and transcriptional activity; 3) clone the plasmid into an **E. coli** compatible vector in order to establish a potential shuttle cloning vehicle for further analysis of various genes from **P. multocida.**

LITERATURE REVIEW OF PASTEURELLA MULTOCIDA PLASMIDS

Extrachromosomal or plasmid DNA is common in **Pasteurella multocida** . Twenty-two to ninety percent of the strains surveyed contained plasmids variable in their; size, molecular weight, prevalance, ability to code for antibiotic resistance, incompatibility group, origin and mode of transmission.

P. multocida plasmids range from 1.3 to 27.5 Megadaltons (Mdal) (Berman and Hirsh,1978; Silver et. al.,1979; Hirsh et. al.,1981; Hirsh et. al.,1985; Haghour et. al.,1987; and Sriranganathan et. al., manuscript submitted). Within serogroup A, Sriranganathan et. al. (manuscript submitted) found plasmids to be relatively small, approximately 2 to 10 Mdal. Comparably sized plasmids were isolated by Haghour et. al. in 1987. However the serotypes of the strains bearing plasmids were not published. The serotype of **P. multocida** from which Hirsh (1981) isolated a 28.5 Mdal plasmid is also unknown. The strains in both of these studies were fowl cholera isolates so it is possible that they were type A's. Plasmids of types B and E plasmids range in size from 1.8 to 27.5 Mdal while strains belonging to serogroup D possess larger plasmids ranging in molecular weight from 2.3 to 20 Mdal

(Sriranganathan et. al., manuscript submitted). As of yet, serotype F has not been surveyed.

The prevalance of plasmids within **P. multocida** is well documented. 72 of the 75 strains surveyed by Hirsh (1981) contained plasmids. In their recent study they found plasmids in 41 of the 58 strains examined (Hirsh et. al.,1985). In a study by Sriringanathan et. al. (manuscript submitted), plasmids were isolated from fifty-seven percent of the type A's surveyed. Zero to four of the thirteen type B and E strains screened had plasmids depending on the method of DNA isolation used. Five of twenty-one type D stains contained plasmids.

Resistance plasmids (R-plasmids) account for only a small percentage of the antibiotic resistance found in field isolates of **P. multocida**. In contrast, Chang and Carter (1976) demonstrated antibiotic resistance in 81% of the 262 strains surveyed. Resistance to streptomycin alone or in conjunction with penicillin, and/or tetracycline was the most prevalant accounting for 82.1% of the total resistance observed. Haghour et. al. (1987) found similar patterns of antibiotic resistance in 75.3% of the 223 field strains surveyed. However, oxacillin resistance was included in their calculation. A subsequent study has

suggested that oxacillin resistance is characteristic of **P. multocida** in general and may be due to an inability of the drug to penetrate the cell wall (Sriranganathan et. al., manuscript submitted). The high percentage of resistance observed in Chang and Carter's study (1976) may have been due to bias in the study and does not represent conditions which may be found in the field. Therefore, the accurate level of antibiotic resistance observed in field isolates of **P. multocida** may be much lower than previously reported. Hirsh (1985) found that less than 16% of the forty three strains surveyed were resistant to streptomycin (Sm), tetracycline (Tet), and/or sulfonamide (Su). In their study, resistance to both triple sulfa and streptomycin was encoded by a single plasmid. The molecular weight of plasmids encoding for resistance to triple sulfa and streptomycin were 3.4 (Berman and Hirsh,1978), 6.0 (Hirsh et. al., 1985), and 7.2 Mdal (Hirsh et. al.,1981). Tetracycline resistance was encoded by a 3 Mdal plasmid (Silver et. al.,1979) or with triple sulfa and streptomycin resistance. Resistance to all three antibiotics was associated with a 4.4 Mdal plasmid (Berman and Hirsh,1978). As a result of restriction enzyme analysis, Berman and Hirsh suggest that this plasmid may be the result of a

tetracycline resistant transposon inserting into a plasmid carrying streptomycin and sulfonamide resistance (1978).

In terms of incompatibility (or Inc groups), those plasmids which have been tested belong to an unknown group. They can stably coexist with all 13 known Inc groups, however, $Sm^rSu^rTet^r$ resistant plasmids are not compatible with Sm^rSu^r plasmids (Berman,1978).

The origin of **P. multocida's** plasmids is unknown. Based on mole% guanine + cytosine content of DNA (mole% G+C) as a measure of relatedness, Berman and Hirsh (1978) suggest an enterobacterial origin for their plasmids similar to the R-plasmids of **Haemophilus influenzae** (Elwell et. al.,1975) and **Neisseria gonorrheae** (Elwell et. al., 1977). Of those plasmids surveyed, only one was within the mole% G + C of Pasteurella genomic DNA (40.8 to 43.9%) (Berman and Hirsh,1978; Silver et. al.,1979;and Hirsh et. al.,1981).

The actual mode of transfer of these plasmids is unknown. Hirsh et. al. (1981) cited evidence for a conjugal plasmid that is responsible for the transfer of itself and a smaller, R-plasmid between strains (Hirsh et. al.,1981). Only the smaller 7.2 Mdal plasmid is capable of transferring across families (Hirsh et. al.,1981); this is

the only direct evidence for horizontal transfer within **P. multocida**. Most R-plasmids within **P. multocida** are thought to be nonconjugal even those that have very similar, if not identical restriction maps. Silver et. al. (1979) suggests as an explanation,that there has been a selection for R-plasmids from a common pool of genetic elements .

MATERIALS AND METHODS:

CULTURES AND STORAGE CONDITIONS:

P. multocida strains: Larsen, 3831, 2013, AND 3008B were from G. R. Carter's [Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechinic Institute and State University, (VMRCVM, V.P.I. and S.U.)] culture collection. Strains 1085, 1167, and 1845 were given to us by K. R. Rhoades (University of California at Davis). Strains 24R, 8, 470, were isolated in our laboratory from turkeys suffering from fowl cholera in Harrisonburg, Va. Strains 613 and 613/15B/417 were isolates from sheep on the same farm. **E. coli** DH5 alpha was from S. M. Boyle (V.M.R.C.V.M., V.P.I. and S.U.). All other cultures were from N. Sriranganathan's (V.M.R.C.V.M., V.P.I. and S.U) culture collection. For long term storage, cultures were grown in Trypticase Soy broth with 0.3% yeast extract (Difco) and stored in liquid nitrogen. Frequently used strains were grown overnight in Brain Heart Infusion Broth (BHIB, Difco), Yeast extract-Proteose peptone-Cystine Broth (YPC, Namoika and Murata,1961), or Luria-Bertani broth (LB, Maniatis, 1982).

Overnight cultures were supplemented with 15% glycerol and stored at -80°C. For short term storage, **P. multocida** cultures were plated onto either BHI plates or Blood Agar (Difco) supplemented with 5% horse or sheep's blood. **E.coli** American Type Culture Collection (ATCC) strain 25922 and **S. aureus** ATCC strain 25923 were streaked onto LB plates. All the culture plates were incubated 18 to 48 hours at 37°C to ensure adequate growth. **P. multocida** strains were subcultured every 2 to 3 weeks. Culture plates were stored at 4°C when not in use.

GROWTH CONDITIONS:

P. multocida cultures were grown in either YPC or BHIB at 37°C in a rotary incubator at 150-200 rpm. **E. coli** and **S. aureus** cultures were grown in LB, YPC, or BHIB under conditions similar to those used with **P. multocida**. For specific conditions see desired section in materials and methods.

ANTIBIOTIC SUSCEPTIBILITY:

The modified procedure of Bauer et. al. (1966) was used to measure antibiotic susceptibility. Overnight cultures were diluted with sterile saline to a turbidity approximating 0.5 MacFarland's Standard. The bacterial suspensions were streaked onto Mueller-Hinton Agar (BBL) supplemented with 5% sheep's blood. Discs (BBL) containing known quantities of the following antibiotics were dispensed onto the streaked plates: Ampicillin (10 mcg), Erythromycin (15 mcg), Gentamycin (10 mcg), Cephalothin (30 mcg), Penicillin (10 units), triple sulfa (1 mg), Carbenicillin (100 mcg), Neomycin (30 mcg), Tetracycline (30 ug), Nitrofurantoin (300 ug), Clindamycin (2 ug), and Streptomycin (10 mcg). Plates were incubated at $35^{o}C$ for 18 hours. The zones of inhibition were measured to the nearest millimeter using a sliding calliper (Manostat, Fischer Scientific). Strains were judged susceptible, resistant, or intermediate according to the performance standards for antimicrobial disc susceptibility tests proposed by the Clinical Laboratory National Committee for Clinical Laboratory Standards (1973).

PLASMID ISOLATION: LARGE SCALE:

Two methods were employed, a phenol:chloroform extraction (R. Moore, personal communication) and an alkaline lysis procedure (Birboim,1979).

I. PHENOL CHLOROFORM EXTRACTION: 1% of an overnight culture was used to inoculate BHIB which was then incubated for 18 hours under conditions previously mentioned. Cultures were centrifuged at 10,000 x g for 10 minutes. Cell pellets were then resuspended in 1/7 the original volume of STE buffer (10mM Tris, pH 8.0, 10 mM NaCl, 1 mM EDTA, pH 7.6). Equal volumes of Tris-buffered phenol-chloroform with a pH of less than 7.6 were added to the cell suspensions. This mixture was then rotated on a rotary shaker for 30 minutes to increase cell lysis. Lysed samples were centrifuged at 7000 x g at 14^{o}C for 45 minutes. The aqueous layer was removed and the remaining pellicle was re-extracted with an equal volume of STE. Samples were centrifuged as previously mentioned. The aqueous fractions were pooled and then extracted with an equal volume of chloroform until protein pellicles were no longer visible at the interface. Samples were then ethanol

precipitated by adding two and one-half volumes of 95% ethanol. Plasmid preparations were resuspended in 1/10 TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0) containing 20 ug/ml of RNase (Sigma Chemical Co.) and incubated for 15-30 minutes at 37^{o}C. Resulting plasmid DNA was electrophoresed through a 0.7% agarose (Sigma Chemical Co.) gel containing TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) and 10 mM ethidium bromide. The plasmid DNA concentration was approximated by comparison to lambda DNA digested with Hind III (Bethesda Research Laboratories) which was electrophoresed simultaneously as a molecular weight standard.

II. ALKALINE LYSIS: 1% of an overnight culture was used to inoculate 500 mls of BHIB and incubated as previously stated. Cells were pelleted by centrifugation at 10,000 x g for 10 minutes for nonencapsulated strains and up to 1 hour for encapsulated strains. Cell pellets were resuspended in 10 mls of solution I (50 mM Tris-HCl pH 8.0, 10mM trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), 100mM glucose, 5mg/ml lysozyme) and incubated for 30 minutes at room temperature. After incubation 20 mls of solution II were added (10mM NaOH, 1%

SDS) and incubated for 5 minutes. Finally, 15 mls of solution III (3M Sodium Acetate, pH 4.8) were added and the lysed samples were then incubated for 60 minutes. Unless stated otherwise , all incubations were on ice. The samples were centrifuged at 7000 x g for 45 minutes at 4^{o}C to pellet protein, chromosomal DNA and intracellular debris. The aqueous phase was removed and ethanol precipitated with 2 1/2 volumes of 95% ethanol overnight at -20 ^{o}C.

CESIUM CHLORIDE PURIFICATION:

The procedure outlined by Maniatis (1982) was used to purify large amount of plasmid DNA. Approximately 1.00 grams of cesium chloride were added for every milliliter of supernatant of cell lysate. After the cesium dissolved, ethidium bromide (EtBr, Sigma Chemical Co.) was added to a final concentration of 1 mg/ml. Precipitated protein was removed by centrifugation at 5000 x g for 10 minutes at 20^{o}C. The supernatants were centrifuged for 48 hours at 150,000 xg in an SW60 Ti rotor at 20^{o}C. Plasmid bands were extracted through side puncture with an 18 gauge needle. Excess EtBr was removed through a series of 1-butanol

extractions. Samples were then dialyzed against TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for approximately 12-18 hours at room temperature in an Amicon dialyzation unit with a YM10 membrane. Plasmid DNA concentrations were determined through gel electrophoresis as described under large scale plasmid extractions.

PLASMID ISOLATION:SMALL SCALE:

Three methods of small scale plasmid isolations were used: I. Alkaline -lysis method,(Birboim,1979) II. Ish-Horowitz alkaline lysis,(Maniatis,1982) and III. phenol-extraction (R. Moore, personal communication).

I. The alkaline lysis method mentioned above was scaled down for 5 mls. of culture. This method was used to isolate **P. multocida** plasmids.

II. The Ish-Horowitz alkaline lysis procedure was used to isolate plasmids from DH5 alpha transformants. Overnight cultures were grown in LB containing 100 ug/ml ampicillin and then lysed acccording to Maniatis (1982).

III. The phenol extraction was used to quick screen transformants. Colonies were removed from a plate and resuspended in 40 ul TE . An equal volume of phenol:chloroform was added and the samples were vortexed. After a five minute incubation at room temperature, the samples were centrifuged for 10 minutes at 12,000 x g at $4^{o}C$. The aqueous phase was removed and 10 ul of each sample was electrophoresed to screen for desired clones.

RNA ISOLATION:

RNA was isolated according to the method of Szumanski and Boyle (manuscript submitted). Overnight cultures were used to inoculate 10 ml of BHIB broth. Cultures were then incubated at $37^{o}C$ and harvested at 80-120 Klett units. This represents a culture density of approximately 4 to 7 x 10^{7} colony forming units (c.f.u.) per milliliter in mid-log phase. Cells were then lysed in the presence of phenol:chloroform with beta-mercaptoethanol. Cell lysates were centrifuged at 10,000 x g for 20 minutes at $4^{o}C$ and the aqueous phase removed. One-half volume of 7.5 M Ammonium acetate was added to the aqueous layer. RNA was further purified through a series of ethanol

precipitations. RNA samples were electrophoresed in 0.7% agarose gels in TBE containing 10 ug/ml EtBr to verify that they were not degraded prior to Northern analysis. Concentrations of RNA were determined by measuring the OD at 260 nm in a Response II UV-VIS spectrophotometer (Gilford) with quartz cuvettes. NOTE: ALL glassware used in this procedure was either ashed or brand new. All solutions were made with DEPC (Diethylpyrocarbonate, Sigma Chemical Co.) treated water to inactivate endogenous RNase.

RESTRICTION ENDONUCLEASE ANALYSIS:

Two hundred to five hundred nanograms of plasmid DNA from the Larsen strain of **P. multocida** (pLAR-1) was digested according to the manufacturer's recomendations. Unless otherwise noted the restriction enzymes were purchased from Bethesda Research Laboratories (BRL). The units of restriction enzyme per microgram of DNA neccessary for complete digestion are listed in Table 2. Digestions with multiple enzymes were done according to Maniatis (1982). The digested samples were electrophoresed through

Table 2: Amount of restriction endonuclease per microgram of DNA necessary for complete digestion of pLAR-1.

Enzyme	Amount	Enzyme	Amount
Acc I	4 units	Rsa I	3-4 units
Bgl II	2 units	Sal I	10-12 units
Hind III	2 units	Taq I	3-4 units
Hinf I	2 units		

0.7% agarose gels (Sigma Chemical Co.) under conditions suggested by Maniatis (1982). Both TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE buffers were used. Approximate sizes of the restricted fragments were determined by comparison to the molecular weight standards of lambda DNA digested with Hind III, 1 kilobase and 123 basepair ladders (BRL).

LIGATION AND TRANSFORMATION:

The plasmids, pLAR-1 and pBR322 (BRL) were digested to completion with Bgl II and Bam HI respectively. Plasmids were then mixed to give a molar ratio of 2:1 (pLAR-1:pBR322) to which 1 unit of T4 DNA ligase (BRL), 2 ul of 10x ligase buffer, ATP (final concentration of 66uM) and sterile water was added to obtain a final volume of 20 ul. Ligation mixtures were incubated at room temperature overnight and then used to transform **E. coli** DH5 alpha using the Hanahan procedure (1983). For transformation, **E. coli** DH5 alpha was inoculated into SOB (Hanahan, 1983) broth and grown in a shaking incubator at $37^{o}C$ until a turbidity of 90-120 Klett units (approximately 4-7 x 10^{7} c.f.u./ml). Cells were then pelleted and transformed with

50 to 100 ng of ligated DNA. Initial selection was on LB plates containing 100 ug/ml ampicillin (LB-amp). Subsequent screening was done on LB-amp, LB-tet (50 ug/ml tetracycline), and LB plates to separate vector background from those transformants containing the insert.

SOUTHERN BLOTTING :

Plasmid samples were electrophoresed overnight through 0.7% agarose gels containing 10 ug/ml EtBr in TBE buffer . The plasmid profiles were documented with a Polaroid Land Camera using either Type 55 or 57 film. Prior to Southern hybridization, gels were treated with 0.2N HCl, denatured, and then neutralized according to the method described by Ausubel et. al. (1989). The gels were overlayered with MSI nitrocellulose membranes (.22 microns, Fisher) and allowed to transfer overnight. The following morning, membranes were dried for 90 to 120 minutes under vacuum at 80^{o}C. For long term storage, membranes were stored at room temperature in the dark.

NORTHERN BLOTS:

Prior to loading, samples were diluted and denatured according to Ausubel et. al. (1989). RNA was electrophoresed through a 1.2% agarose gel in 1x MOPS (3-N-morphilino propane-sulfonic acid) running buffer. The RNA remained denatured throughout electrophoresis due to the addition of 37% formamide. Gels were electrophoresed at 5V/cm for 3 hours until the bromophenol blue dye front had migrated approximately 1/2 way through the gel. Gel was then incubated in 10x SSC for 45 minutes and then transferred to MSI nitrocellulose membranes (.22 microns, Fischer) according to the methods of Ausubel et. al. (1989). The following day blots were dried 1.5 to 2.0 hours at 80^{o}C under vacuum and stored as previously mentioned.

DOT BLOTS:

Dot blots were done according to Davis et. al. (1986). A Bio-Dot apparatus (Bio-Rad) was used to transfer samples to a MSI nitrocellulose membrane (.22 microns,

Fischer). One to two ug of DNA was diluted in 10 ul TE and denatured by incubation at 95°C for 10 minutes. Forty ul of 20x SSC was added and the denatured DNA samples were applied to the nitrocellulose under vacuum. RNA samples were suspended in 40 ul of 20x SSC and transfered directly to the nitrocellulose membrane under similar conditions. Membranes were dried under vacuum for 1.5 to 2.0 hours at 80°C.

BIOTINYLATION OF PROBE:

Both chromosomal and plasmid DNA from the Larsen strain were biotin-labeled with biotin-7-dATP according to the methodology suggested by BRL. The BlueGENE nonradioactive nucleic acid detection system was used. One to two micrograms of DNA were nick-translated using 2 units DNA polymerase I (BRL) in the presence of 200 picograms of DNA Pol I/DNAse I (nick translation grade, BRL). The probe was separated from unincorporated nucleotides by passing it over a Sephadex G-50 column equilibrated with 1x SSC. Collected fractions were then resuspended in 10 ml of hybridization fluid. See section on hybridization and

color detection for components of hybridization fluid.

HYBRIDIZATION AND COLOR DETECTION (DNA):

Dried membranes were placed in a Hybridease (Hoeffer) chamber for both prehybridization and hybridization. Blots were incubated in 10 ml of prehybridization fluid for four hours at 42°C. Prehybridization fluid consisted of 50% formamide, 5x SSC, 5x Denhardt's solution, 25 mM sodium phosphate, pH 6.5, and 0.5 mg/ml herring sperm DNA. Hybridization was done according to the manufacturer's suggestions (BRL) [1]. The probe was denatured at 95°C for 10 minutes. Hybridization buffer contained 45% formamide, 5x SSC, 1x Denhardt's solution, 20 mM sodium phosphate, pH 6.5, 0.2 mg/ml denatured herring sperm DNA, 5% dextran sulfate, and 0.1 - 0.5 ug/ml denatured probe. The hybridization buffer was added to the Hybridease chamber and incubated at 42°C overnight. The following day membranes were washed in successive dilutions of SSC and 5% SDS and then in a solution of 0.1 M Tris-HCl pH 8.0 and

[1]-Blu-GENE™ Nonradioactive Nucleic Acid Detection System. BRL. Cat. no.8279SA.

0.15 M sodium chloride (Tris/sodium chloride buffer) for 1 hour at 65^{0}C. Prior to color detection, membranes were incubated with Strepavidin-Alkaline Phosphatase (1 ug/ml), washed in Tris/sodium chloride, and finally in Tris/sodium chloride buffer with bovine serum albumin (3% final concentration, Sigma Chemical Co.). To visualize the biotin-strepavidin complex, Tris/sodium chloride buffer containing nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP) was added. Reactions proceeded for 15 to 90 minutes at which point they were stopped by the addition of TE (10 mM Tris-HCl, 1 mM EDTA).

HYBRIDIZATION AND COLOR DETECTION (RNA):

The prehybridization and hybridization conditions for membranes containing RNA were similar to those for DNA. 5% SDS (sodium dodecyl sulfate) was added to both prehybridization and hybridization fluid to inactivate any contaminating RNase. Membranes were prehybridized for four hours to overnight followed by an overnight hybridization with the biotinylated probe. Conditions for detection of the probe have been described elsewhere in the materials and methods section.

SIDEROPHORE ACTIVITY

The method described by Snipes et. al. (1989) was used to induce siderophore activity. Overnight cultures of the Larsen strain of **P. multocida** were used to inoculate 10 mls. of BHIB, BHIB supplemented with the iron chelators ethylene diamine-di(O-hydroxyphenyl) acetic acid (EDDA, 1 mM final concentration) and alpha-alpha dypyridyl (20 uM final concentration), and BHIB containing the iron chelators and 200 mM $FeCl_3$. Cultures were incubated until a Klett reading of 120 to 150 units. Cells were then lysed and total RNA was isolated according to the methods of Szumanski and Boyle (manuscript submitted). See RNA isolation previously mentioned under materials and methods.

BIOCHEMICAL TESTS:

Biochemical activities were measured using an API-20E strip specific for **Enterobacteriaceae** and other gram negative organisms (Analytab. Inc., Sherwood Medical). Tests were done according to manufacturer's instructions.

All chemicals and solutions were supplied by the manufacturer. Overnight cultures grown in either BHIB [for **P. multocida** strains or on selective plates (pLRBR clones and **E. coli** DH5 alpha)]. The turbidity of the resuspended samples was comparable to a MacFarland's standard of 0.5. Strips were incubated for 18 hours at 37°C, read, and then reincubated for another 24 hours. Tryptophane deaminase, (TDA), Indole, (IND), Voges-Proskauer, (VP), and Nitrate reduction tests were performed after 48 hours.

TRANSFORMATION OF P. MULTOCIDA:

A modified Hanahan procedure (1983) was used to transform four plasmidless strains of **P. multocida** with the constructs pLRBR-21 and pLRBR-67. These strains were 67/70, 3875, 1053, and P86-347 (see results section for serotypes). Overnight cultures grown in YPC were used to inoculate 10 mls. of YPC containing 20 mM magnesium. (The magnesium stock is 1 M $MgCl_2$ and 1M $MgSO_4$). These cultures were incubated in a rotary shaker at 37°C until an absorbance of 90 to 110 Klett units. This represents a turbidity of approximately 4 x 10^7 c.f.u. per ml. Cells were then pelleted and transformed according to the methods

proposed by Hanahan (1983). After the heat shock, transformed cells were incubated in YPC containing 20 mM glucose and 20 mM magnesium in a shaking water bath at 37°C for two to four hours. Initial selection was on BHI-amp plates containing 100 ug/ml ampicillin. Subsequent selection was on BHI, BHI-amp, and BHI-tet plates (25 ug/ml tetracycline). An R-plasmid isolated from **P. multocida** strain 1085 transferring streptomycin resistance was used as a control to ensure that the cells were competent. **E. coli** DH5 alpha was used a control for the Hanahan procedure.

RESULTS:

ANTIBIOTIC SUSCEPTIBILITY:

The Larsen strain of **P. multocida** was susceptible to twelve of the thirteen antibiotics tested. To see if this profile was common to the species in general or specific to the Larsen strain, antibiotic susceptibility tests were performed on strains containing known R-plasmids, strains containing plasmids of unknown function, and plasmidless strains. The results are shown in Table 3. All thirteen strains were susceptible to ampicillin (AM), gentamycin (GM), cephalothin (CF), penicillin (P), chloramphenical (C), and carbenicillin(CB). All strains except for 3008B and 1167 were sensitive to trimethoprim (SXT). 3008B was the only strain resistant to tetracycline (TE). 8 of the 11 strains tested were susceptible to clindamycin (CC) whereas 1167, 470, and 3831 were resistant. Resistance to triple sulfa (SSS) was found in 5 of the strains; p1085, p1167, p 1845, 24R, and 8. Results from strains 2013 and 3008B were variable with respect to triple sulfa. Erythromycin (E) was the most variable with six strains being sensitive, 4

Table 3: Results from Antibotic Susceptibility Test

		ANTIBIOTICS TESTED												
		AM	E	GM	CF	P	OX	SSS	SXT	C	TE	N	CB	CC	PLASMID
	Larsen	s	i	s	s	s	r	s	s	s	s	i	s	r	pLAR-1
S	P1085	s	s	s	s	s	r	r	s	s	s	i	s	r	pVM 109
T	P1167	s	s	s	s	?	r	r	r	s	s	s	s	r	pVM 109
R	P1845	s	s	s	s	?	r	r	s	s	s	i	s	?	pVM 110
A	3831	s	s	s	s	s	r	s	s	s	s	s	s	r	yes
I	2013	s	s	s	s	s	r	i/s	s	s	s	i	s	r	yes
N	3008B	s	i	s	s	s	r	i	s	s	s	s	s	r	no
	24R	s	?	s	s	s	r	r	s	s	s	s	s	r	no
	8	s	r	s	s	s	r	r	s	s	s	s	s	r	yes
	470	s	?	s	s	s	r	s	s	s	s	i	s	r	?
	613	s	s	s	s	s	r	s	s	s	s	i	s	r	no

Strains were judged susceptible (s), intermediate (i) or resistant (r) according to the Clinical Laboratory National Committee for Clinical Laboratory Standards (1973).

? denotes variable response

intermediate, and 4 resistant. Oxacillin (OX) resistance was found in all strains tested.

RESTRICTION MAPPING:

A 4.0 kilobase (kb) plasmid was isolated by cesium chloride banding or alkaline lysis from the Larsen strain of **P. multocida**. This plasmid was designated pLAR-1. Restriction endonuclease analysis revealed two unique sites : Bgl II and Sal I (Table 4). These sites are separated from one another by approximately 1.0 kilobase (kb). Digestion with the restriction enzyme Acc I produced two fragments, 3.1 and 0.48 kb in length. Hind III cleaved pLAR-1 into two fragments 1.85 kb in length. Hinc I, Rsa I, Sau 3A, and Taq I restricted the plasmid into three or more fragments. Figure 1 is a representative of the gels used to estimate DNA fragments separated by agarose gel electrophoresis. A series of double, triple, and quadruple digests were used to map these sites relative to one another. This data was used to generate the restriction map shown in Figure 2. Seven of the fifteen restriction endonucleases surveyed were unable to digest the plasmid (Table 5). Isoschizomers of these enzymes have been designated with an asterisk. Restriction enzymes which do

not have sites within pLAR-1 include Bal I, BamH I, Cla I, EcoR I, Hae III, Hpa II, Msp I, Pvu II, Pst I, Sal II, Sma I, Xba I, and Xma I.

SOUTHERN BLOT ANALYSIS:

pLAR-1 hybridized to 12 of the 56 strains of **P. multocida** surveyed across serogroups A,B,D, and E. Figures 3 and 4 represent an agarose gel and corresponding Southern blot from this survey. Ten of these strains were of serogroup type A, while two were of serogroup type D. All 12 strains contained at least one plasmid per strain, however the sizes of these plasmids ranged from 2.5 to 4.2 kb. Six of the strains were serotype A:3 while the remaining were A:1, A:4, A:5, A:6, and A:9. The serotypes of the type D strains were not recorded. Strains found to hybridize with pLAR-1 were isolated from various hosts including seven avian and two mammalian sources from the United States and Brazil respectively (Table 6).

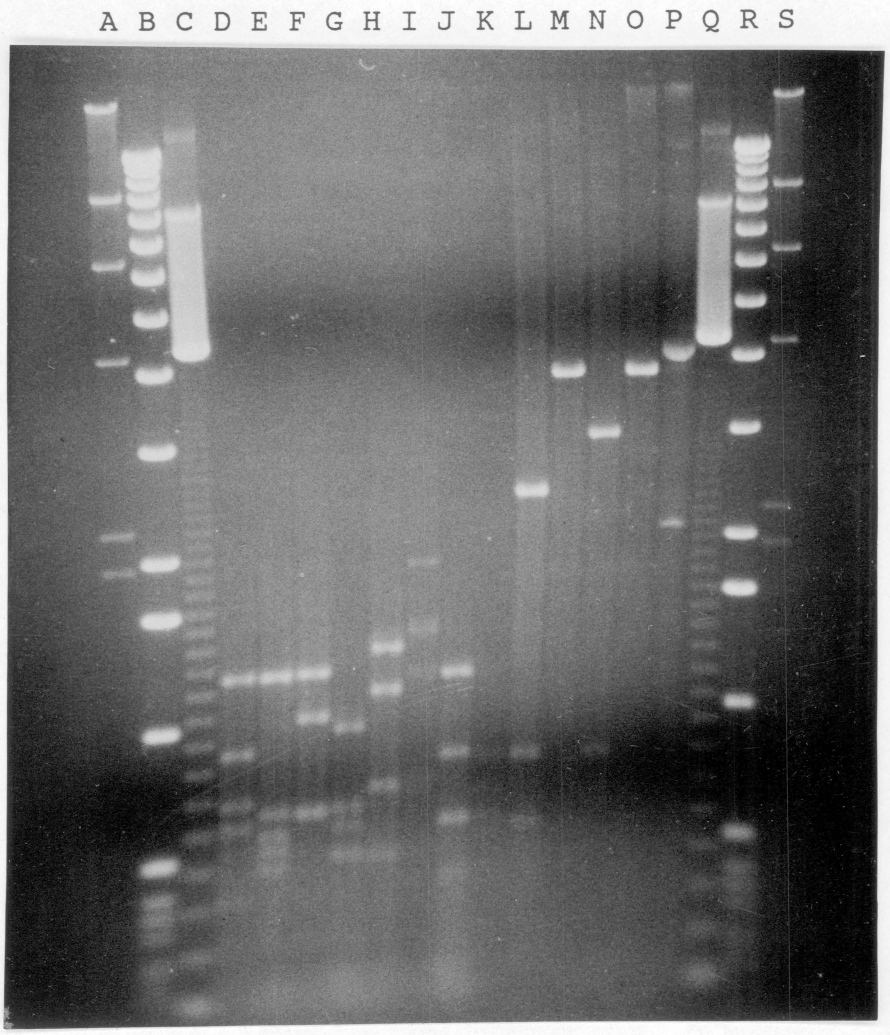

Figure 1: A representative gel of the restriction endonuclease digestions used to generate the restriction map of pLAR-1. Lanes A and S are lambda/Hind III, lanes B and R: 1 kilobase ladder, lanes C and Q: 123 basepair ladders, lanes D through P are pLAR-1 digests: lane D: Rsa I/Hind III, lane E: Hind III/Hinf I, lane F: Hinf I, lane G: Hinf I/ Taq I, lane H: Taq I, lane I: Hind III/ Taq I, lane J: Sau 3A, lane K: Sau 3A/Acc I, lane L: Acc I/ Bgl II, lane M: Bgl II, lane N: Bgl II/Sal I, lane O: Sal I, lane P: undigested pLAR-1.

Table 4: Restriction enzyme analysis of pLAR-1

NO. OF SITES	RESTRICTION ENZYME	LENGTH (IN BASEPAIRS)	TOTALS
1	Bgl II	3958	3958
1	Sal I	3975	3975
2	Acc I	3100, 480	3580
2	Hind III	1850	3700
4	Hinf I	1350,1150,740,250	3490
4	Taq I	1360, 1100, 680, 480	3620
5	Rsa I	1150,850,640,550,375	3565
2	Bgl II/Sal 1	2900,970	3870
2	Hind III/Sal I	1850,1750	3600
3	Acc I/Bgl II	2500,1000,700	4200
3	Bgl II/Hind III	1800,1000,900	3700
4	Acc I/Taq I	1350,1150,490,280	3320
5	Acc I/Rsa I	1130,800,600,340,280	3150
5	Hinf I/Taq I	1100,850,640,550,357	3610
5	Hinf I/Hind III	1350,730,630,560,520	3790
5	Hind III/Rsa I	1350,950,720,580,300	3900
5	Taq I/Hinf I	1100,800,700,660,540	3610
5	Taq I/Sal I	1100,790,650,590,440	3570
6	Hind III/Taq I	1350,790,720,500,380, 270	3610
6	Hinf I/Rsa I	680,560,500,420,390,300	2850
3	Bgl II/Hind III/ Sal I	1800,1100,900	3800
5	Bgl II/Hind III/ Sal I/Taq I	900,780, 640,520,380	3220

$\bar{X}$ = 3622 +/- 292.3

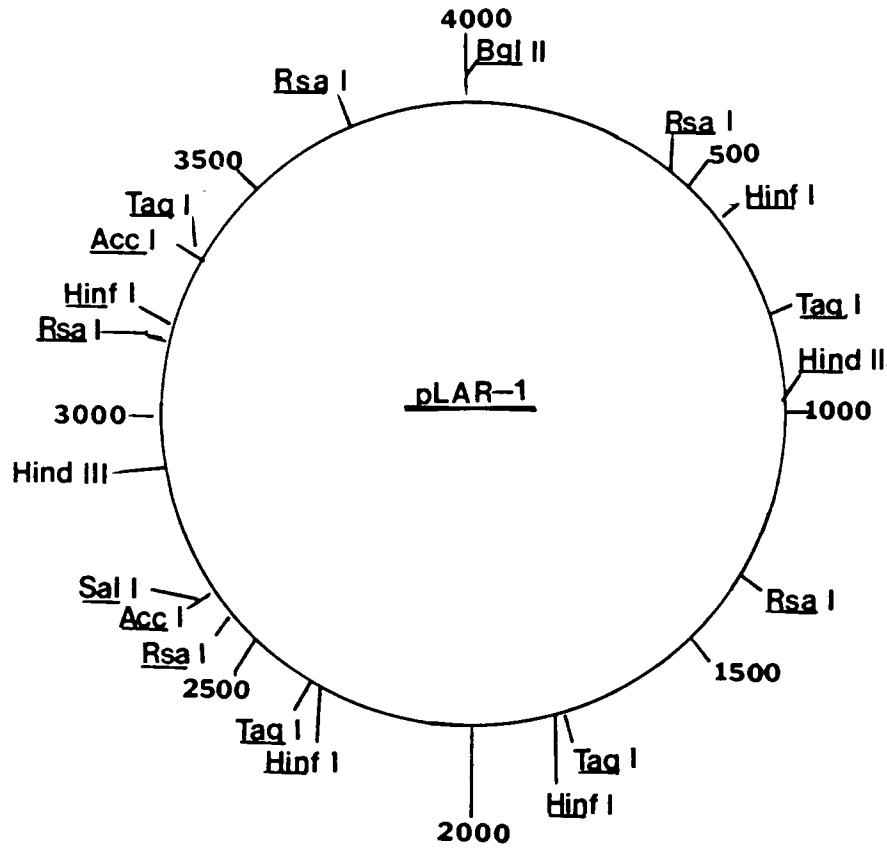

Figure 2: A restriction map of pLAR-1 using the following enzymes: AccI, BglII, Hind III, Hinf I, Rsa I, Sal I and Taq I.

Table 5: Restriction endonucleases which do not cleave pLAR-1.

Bal I *	Pvu II
Bam HI	Pst I
Cla I +	Sal II *
Eco RI +	Sma I
Hae III	Xba I
Hpa I	Xma I *
Msp I *	

* denotes isoschizomers (See text for details)
+ Sigma Chemical Co., remaining enzymes were purchased from Bethesda Research Laboratories.

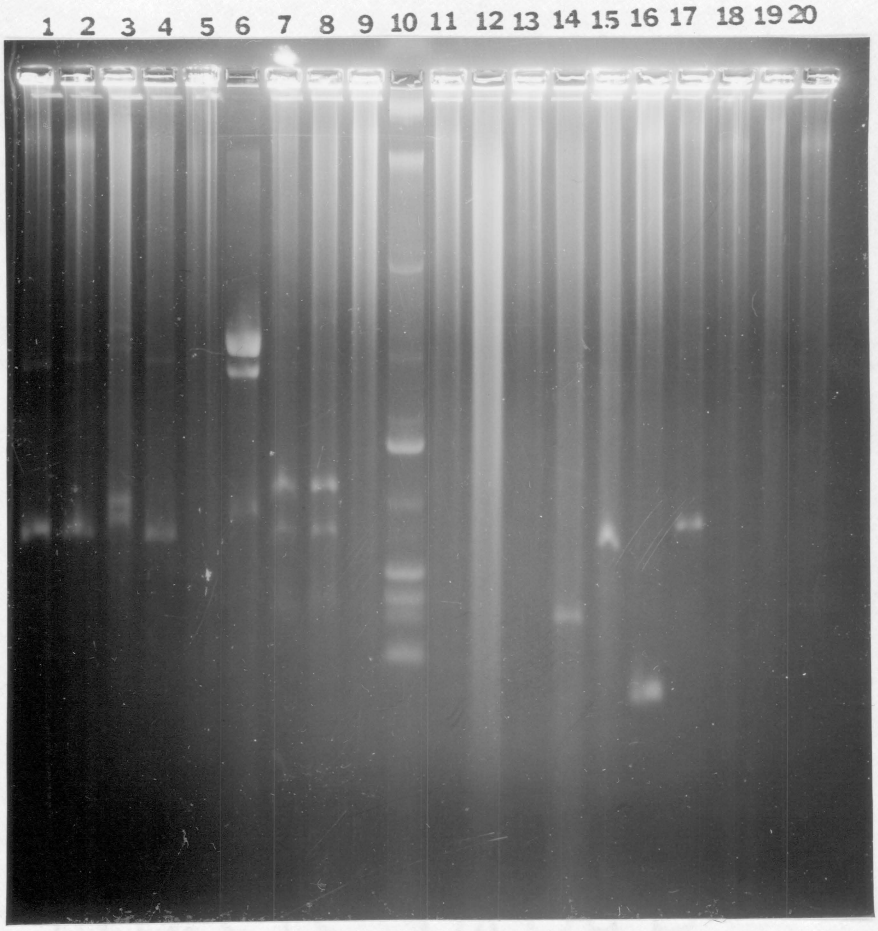

Figure 3: A representative gel used for Southern blot analysis. Lane 1: X-73, lane 2: 1059, lane 3: Liver, lane 4: P86-270, lane 5: P86-338, lane 6: Larsen, lane 7: P86-349, lane 8: P86-350, lane 9: P86-304, lane 10: **E. coli** V517, lane 11: P86-308, lane 12: P86-332, lane 13: P1662, lane 14: P1702, lane 15: P-2192, lane 16:P-1581, lane 17: 2095, lane 18:2100, lane 19: 2237, lane 20: 2723.

Figure 4: Southern Blot resulting from the transfer of the agarose gel shown in figure 3 . Lane 1: 2723, lane 2: 2237, lane 3: 2100, lane 4: 2095, lane 5: 1581, lane 6: P2192, lane 7: P1702, lane 8:1662 lane 9: P86-332, lane 10: P86-308, lane 11: **E. coli** V517, lane 12: P86-304, lane 13: P86-350, lane 14: P86-349, lane 15: Larsen, lane 16:P86-338, lane 17: P86-270, lane 18: Liver, lane 19: 1059, lane 20: X-73

TABLE 6: Strains of **Pasteurella multocida** which hybridized to pLAR-1 using Southern Blot analysis.

STRAINS	SERO-TYPE	DNT	ANIMAL ORIGIN	PATHOGENESIS	SIZES (KB)	LOCALE OF ISOLATE
LARSEN	A:3,4	-	TURKEY	FOWL CHOLERA	4.0	VIRGINIA
X-73	A:1	-		FOWL CHOLERA	3.8	CALIFORNIA
1059	A:3	-	TURKEY	FOWL CHOLERA	3.8	CALIFORNIA
LIVER	A:3	-	AVIAN	FOWL CHOLERA	4.0,4.2	VIRGINIA
P86-270	A:3		CHICKEN	FOWL CHOLERA	3.8	MARYLAND
P86-349	A:3		TURKEY	FOWL CHOLERA	3.8	PENNSYLVANIA
N.C.	A:3				3.8	N. CAROLINA
I.O.	A:3				3.9	IOWA
1702	A:5		TURKEY	FOWL CHOLERA	2.5	CALIFORNIA
2095	A:9		TURKEY	FOWL CHOLERA	4.0	CALIFORNIA
3866	D	+	SWINE	A. RHINITIS		BRAZIL
3869	D	+	SWINE	A. RHINITIS		BRAZIL

CLONING:

pLAR-1 was cloned into the BamH I site of the tetracycline (tet) gene in pBR322. Clones containing the insert were selected by screening for colonies which were ampicillin resistant and tetracycline sensitive. This phenotype was due to insertional inactivation of the tet gene by pLAR-1. Six clones resulted which were tetracycline sensitive and ampicillin resistant. These were designated pLRBR-21, pLRBR-26, pLRBR-28, pLRBR-53, pLRBR-56, and pLRBR-67. (The numbers correspond to the clone tested, eg. pLRBR-21 was the 21^{st} clone tested.) Restriction analysis with the enzymes Cla I and Hind III confirmed the insert was pLAR-1 and the vector was pBR322 (Table 7). Southern blot analysis using pLAR-1 as the probe also confirmed these results (figures 5 and 6). Clones pLRBR21,pLRBR-26, pLRBR-28, and pLRBR-56 gave the same restriction patterns therefor pLRBR-21 was chosen as a representative clone of this group. The restriction pattern of pLRBR-53 very similar to pLRBR-21 however one band was consistantly smaller in both the Sal I and Hind III digests. This could have been due to a deletion in pLAR-1. Digestion of the clones with the restriction

enzyme Sal I verified the pLAR-1 was cloned in both orientations (Table 7). pLRBR-21 respresents one orientation of pLAR-1 into pBR322 and pLRBR-67 represents the second orientation. Restriction maps of pLRBR-21 and pLRBR-67 generated from this data are shown in Figures 7 and 8.

SIDEROPHORE AND GENERAL TRANSCRIPTIONAL ACTIVITY OF pLAR-1:

To determine whether pLAR-1 was cryptic, total RNA was isolated from the Larsen strain cultured under nutrient rich conditions eg. in BHI broth. No mRNA was detected by Northern analysis using biotin labeled pLAR-1 as a probe. In an attempt to induce transcription of pLAR-1, the Larsen strain was grown in BHI broth in the presence of the iron chelators ethylene diamine-di(O-hydroxyphenyl) acetic acid (EDDA) or alpha-alpha dypyridyl. Again, no mRNA bands were detected upon hybridization using biotin-labeled pLAR-1 as the probe. Data from these experiments is shown in Figure 9. Chromosomal DNA isolated from the Larsen strain was nick-translated and used as a control for hybridization conditions. pLAR-1 or chromosomal DNA were used on each

Table 7: Restriction enzyme analysis of pLRBR clones.

RESTRICTION ANALYSIS OF pLRBR-21

NO. OF SITES	RESTRICTION ENZYME	LENGTH OF FRAGMENTS (IN BASEPAIRS)
1	Cla I	8900
2	Sal I	5000,3450
3	Hind III	5050,2200,1476

RESTRICTION ANALYSIS OF pLRBR-67

NO OF SITES	RESTRICTION ENZYME	LENGTH OF FRAGMENTS (IN BASEPAIRS)
1	Cla I	8900
2	Sal I	7800,1300
3	Hind III	5050,2200,1600

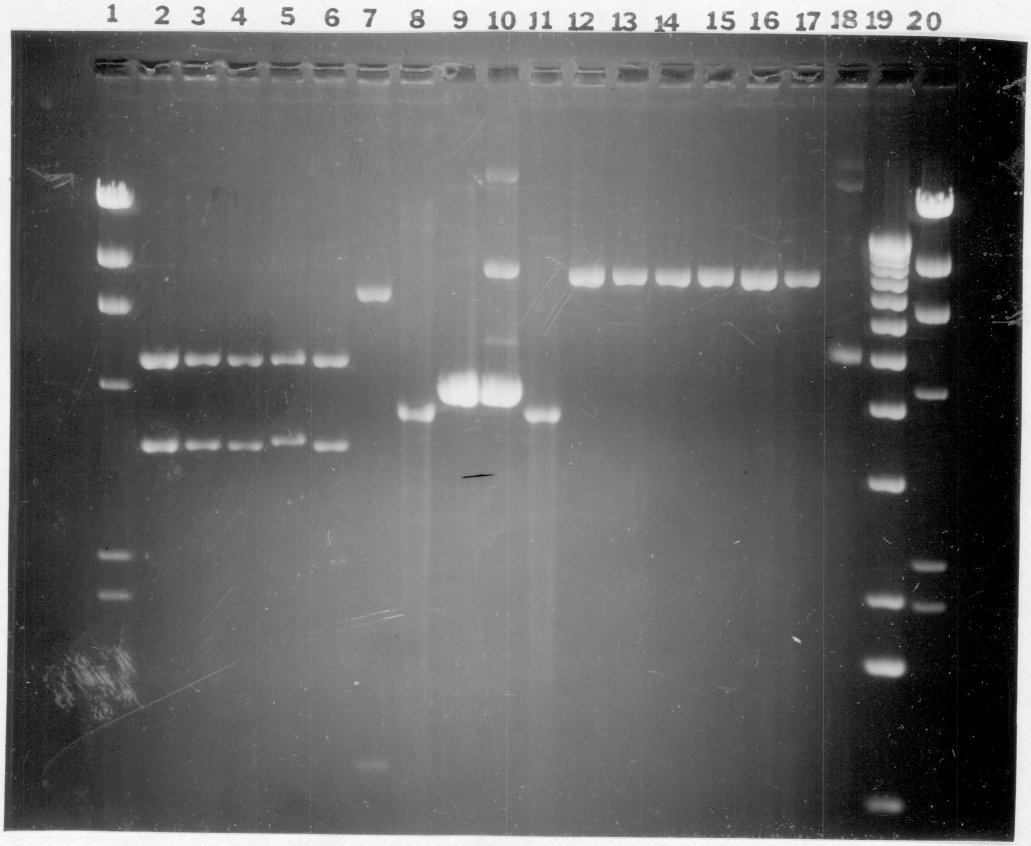

Figure 5: The agarose gel used used for identification of the clones; pLRBR-21 and pLRBR-67. Lane 1:Lambda/Hind III, lanes 2 thru 9: pLRBR-21, 26, 28, 53, 56, 67, pLAR-1, and pBR322 digested with Sal I, lane 10:pBR322 digested with Cla I, lane 11: pLAR-1 digested with Bgl II, lanes 12 thru 17: clones pLRBR-21, 26, 28, 53, 56, and 67 digested with Cla I, lane 18: pLRBR-26 undigested, lane 19: 1 kilobase ladder, lane 20: lambda digested with Hind III.

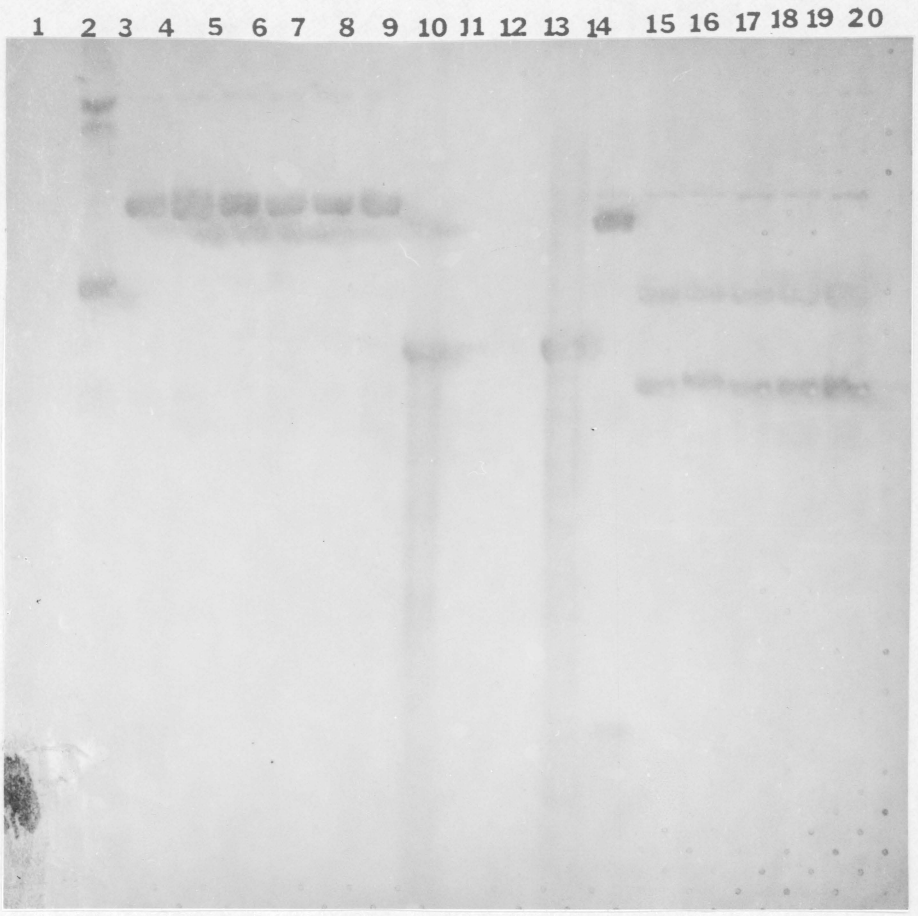

Figure 6: The Southern blot resulting from the transfer of the agarose gel shown in figure 5. Lane 1:Lambda/Hind III, lane 2: 1 kilobase ladder, lanes 3:pLRBR-26 undigested, lanes 4 through 9: pLRBR-67, 56,53 ,28, 26, 21 digested with Cla I,lane 10: pLAR-1 digested with Bgl II, lane 11:pBR322 digested with Cla I, lanes 12 through 19: pBR322, pLAR-1, pLRBR-67, 56, 53, 28, 26, 21 digested with Sal I, lane 20: lambda digested with Hind III.

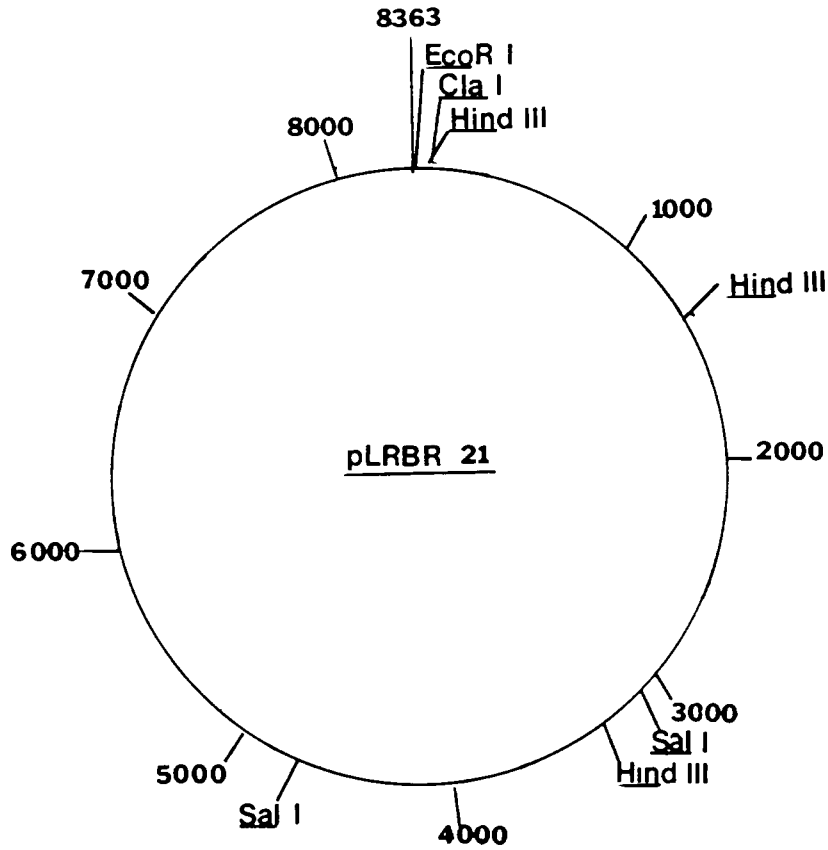

Figure 7: A restriction map of pLRBR-21. The G in the Eco RI site is designated as one of both pBR322 and pLRBR-21. The Bgl II and BamH I sites of plAR-1 and pBR322 (respectively) were lost upon ligation. The remnants of these sites are at bases 56 through 58 and 4056 through 4058 of pLRBR-21.

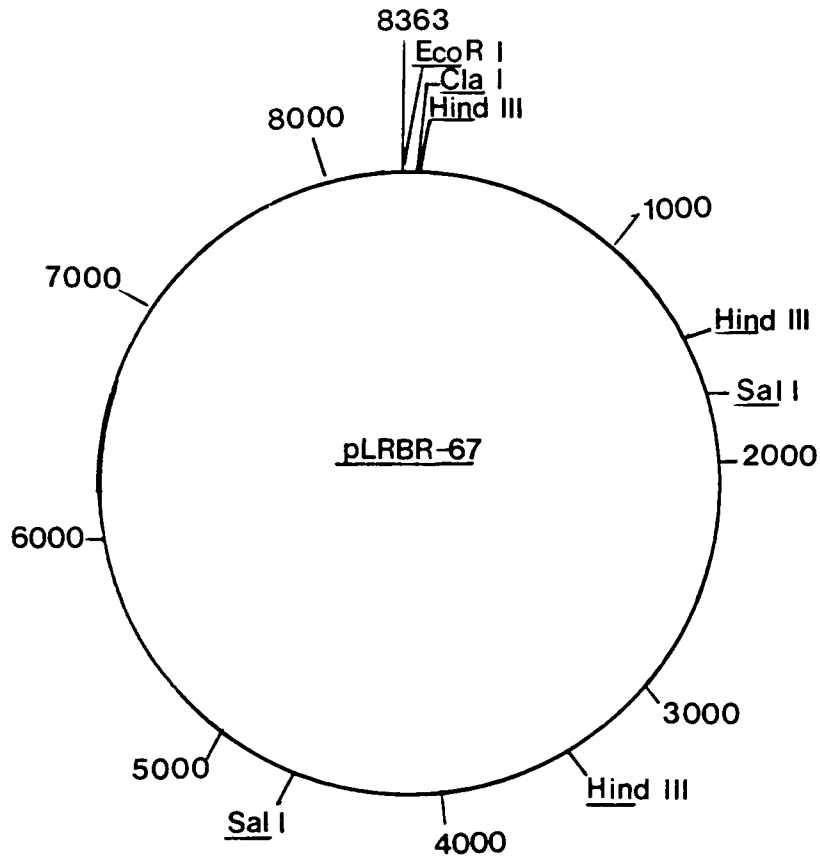

Figure 8: Restriction map of pLRBR-67. The G in the Eco RI site is designated as one of both pBR322 and pLRBR-67. The Bgl II and BamH I sites of plAR-1 and pBR322 (respectively) were lost upon ligation. The remnants of these sites are at bases 56 through 58 and 4056 through 4058 of pLRBR-67.

blot as controls for the procedure. Results from the controls validated that the probes were able to detect both DNA and RNA, and the conditions were appropriate for Northern analysis.

BIOCHEMICAL ACTIVITY OF pLAR-1:

There were no differences in biochemical activities between **E. coli** DH5 alpha and DH5 alpha containing the clones pLRBR-21 and pLRBR-67. Clones were assayed for activities of beta-galactosidase (ONPG), arginine dehydrolase (ADH), lysine decarboxylase (LDC), ornithine decaroboxylase (ODC), citrate utilization (CIT), hydrogen sulfide production (H_2S), urease (URE), tryptophane deaminase (TDA), indole production (IND),VP, gelatine utilization (GEL), sugar utilization such as glucose (GLU) , mannose (MAN), sorbitol (SOR), rhammose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY), arabinose, and for oxidase and nitrate production.

TRANSFORMATION INTO P. MULTOCIDA:

Unsuccessful attempts have been made to transform four strains of **P. multocida**; Larsen, 67/70, 3875, and P86-347. These strains were plasmidless with the exception of the Larsen strain and representated four of the five serogroups. Larsen is serotype A:3,4, P86-347 is A:3, 67/70 is B:2, 1053 is E:2, and 3875 is from serogroup D. A tetracycline resistance plasmid isolated from **P. multocida** strain 1085 was used as a transformation control (eg. to verify that the **Pasteurella** strains were competent). **E. coli** DH5 alpha was used as a control for the Hanahan procedure. The strains of **P. multocida** surveyed remained viable throughout the protocol, however they were unable to survive on BHI plates containing streptomycin.

DISCUSSION:

GENERAL CHARACTERISTICS OF pLAR-1

The plasmid isolated in this study has a molecular weight of 2.64 Mdal (approximately 4 Kb) and is similar to those reported in the literature (Berman and Silver,1978, Haghour et. al.,1987, Hirsh et. al.,1981, Hirsh et. al.,1985, Silver et. al.,1979,). Many of the low molecular weight **Pasteurella** plasmids, eg. 3.0, 3.2, 6.0 and 7.4 Mdal, are nonconjugal R-plasmids (Hirsh et. al.,1985, Hirsh et. al.,1981, Silver et. al.,1979, Berman and Hirsh,1978). It has not been determined if pLAR-1 can be tranferred during conjugation; however, given the data presented here, it can be concluded that pLAR-1 does not confer antibiotic resistance against any of the thirteen antibiotics tested. Similarly, other studies have found little or no correlation between the presence of plasmids and antibiotic resistance in **P. multocida** (Hirsh et. al.,1981, Hirsh et. al.,1985, Silver et. al.,1979). Resistance to oxacillin by other **Pasteurella** strains has been demonstrated elsewhere (Haghour et. al., 1987; Sriranganathan et. al., manuscript submitted). Oxacillin resistance could be due to an

inability of the drug to penetrate the cell wall (Sriranganathan et. al., manuscript submitted). Though carbenicillin resistance was found across all strains of **P. multocida** tested, it was actually an artifact of the media (Fales et. al., 1986). The strains became susceptible to carbenicillen when the antibiotic susceptibilty tests were repeated using laked horse blood.

There is no evidence to suggest that pLAR-1 encodes for capsular or O-antigens (used in serotyping **P. multocida**), dermonecrotoxin (DNT) production, or host specificity . Serotypes A:1, A:3, A:4, A:5, A:9, and serogroup D all had strains that contained plasmids which hybridized to pLAR-1. Conversely, five out of nine type A:3 strains did not hybridize with pLAR-1. Some strains containing homologous plasmids did produce DNT, however there was no correlation between toxin production and the presence of plasmids within or across serogroups. Plasmids isolated from both avian and mammalian **P. multocida** were homologous with pLAR-1 thereby suggesting that pLAR-1 does not have a role in host specificity. Finally, the distribution of strains showing homology with pLAR-1 is worldwide, eg. the Larsen strain was isolated in Virginia,

X-73 from California, I.O. from Iowa, N.C. from North Carolina, 3869 and 3866 are from Brazil . This evidence seems to support 1) Silver et. al.'s (1979) theory that **Pasteurella** plasmid DNA was selected from a common pool of genetic elements and/or 2) the plasmids originated from a common ancestral strain.

The actual function of pLAR-1 is unknown. Northern analysis of total RNA isolated from cells grown under nutrient rich, iron limiting, or iron rich conditions demonstrates that pLAR-1 is not transcribed during normal cell growth or under iron limiting conditions. This suggests that pLAR-1 is not responsible for sequestering iron from the media through siderophores and siderophore regulating factors. These factors are known to be encoded by plasmids in other gram-negative organisms eg. **Vibrio anguillarum** (Crosa et. al., 1977; Crosa et. al., 1980). In addition, expression of 96,000, 84,000, and 80,000 daltons (molecular weight) outer membrane proteins have been induced under iron limiting conditions in **P. multocida** strain P1085 (Snipes et.al.,1989). Whether these proteins are encoded on the chromosome or plasmid mediated has not been illucidated. pLAR-1 is much smaller than plasmids isolated

from gram negative bacteria which have been reported to encode siderophore and/or siderophore regulating factors. For example, Tolmosky et. al. (1987) isolated a 65 kilobase plasmid from **V. anguillarum** which codes for an outer membrane protein (OM2) and the siderophore anquibactin.

pLAR-1 does not encode enzymes responsible for the biochemical activities tested by the API 20-E strip. Neither of the pLAR-1 derived clones, pLRBR-21 or pLRBR-67 tested differently when compared to the parental strain of **E. coli** DH5 alpha . However, **E. coli** DH5 alpha may have some inherent problems for expression of **Pasteurella** DNA, eg. codon usage, differences in the intracellular concentrations of individual amino acid , or mRNA stability.

pLAR-1 appears to be a cryptic plasmid based on Northern analysis. The inability to detect transcription may be due to a number of factors. First, the Larsen strain may not have been incubated under conditions which would induce pLAR-1's transcription. Secondly, if pLAR-1 is a low copy number plasmid then transcripts may be present in extremely low quantities. The biotinylated probe may not have been sensitive enough to detect these transcripts.

pLAR-1 AS A CLONING VEHICLE

pLAR-1 was cloned into the BamH I site of pBR322 and expressed in **E. coli** DH5 alpha. In order to be used as a cloning vehicle or shuttle vector, pLRBR constructs must be able to transform **P. multocida**. A transformation system and potential cloning vehicles such as pLRBR-21 and pLRBR-67 established in **P. multocida** would be very valuable to study genes of interest. Such a system could be used not only within the species **P. multocida** but also to study closely related organisms such as **P. hemolytica, P. piscidea** and possibly members of the genera **Actinobaccillus** and **Haemophilus** as well.

The advantages of establishing a system within **Pasteurella** over **E. coli** are based on plasmid stability and gene expression. There are numerous examples within the literature of heterologous genes and/or plasmid instability within **E. coli** (Silver et. al., 1979; Rimler and Rhoades, 1989). Though **E. coli** DH5 alpha is a recombination ($recA^-$), restriction minus (hsdR) host, there may be some difficulty with pLAR-1 in **E. coli** in general due to codon usage, promoter recognition , promoter strength, errors in transcriptional and/ or translational processing. A 3.0

Mdal plasmid encoding penicillin resistance has been isolated from **P. hemolytica**. This plasmid is capable of conferring antibiotic resistance to both **P. hemolytica** and **E. coli**. However it was found to be unstable in **E. coli** K12 (Silver et. al.,1979). To circumvent a similar situation pLRBR clones were used to transform four plasmidless strains of **P. multocida**. The hypotheis was: if the plasmid constructs were derived from a strain of **P. multocida** and the hosts into which the constructs are transformed share the same restriction and methylation systems as the Larsen strain, then the plasmids, i.e. pLRBR-67 should not be restricted upon transformation. However these were not sucessful in transforming **P. multocida** strains: Larsen, P86-347, 67/70, or 3875.

The lack of successful transformation using the pLRBR clones may have been attributed to a number of causes. First, pBR322 is an **E. coli** derived plasmid. This portion of the construct may not be methylated appropriately to avoid recognition by **Pasteurella**'s restriction system therefore the pBR322 portion of the construct may have been degraded upon transformation into **P. multocida**. To address this problem and bypass the problems associated with working with an ampicillin resistant **Pasteurella** plasmid

(ampicillin is one of the drugs of choice when treating **Pasteurella** infections), the kanamycin resistance gene (pUC4-K, BRL) encoding aminoglycoside resistance was ligated into the Bgl II and Sal I sites of pLAR-1. Expresssion of kanamycin resistance was not detected in either **E. coli** DH5 alpha or HB101. Since the actual location of genes on pLAR-1 are unknown, it is possible that the Bgl II and/or Sal I sites are within genes necessary for plasmid replication eg. origin of replication. Second, insertion of the kanamycin resistance gene may influence regions of DNA in areas other than the actual restriction sites eg. disrupt regulation and/or transcription of downstream portions of a cistron.

Second, another problem presented itself which might have influenced expression of the pLAR-1/Kan resistant clones. Osgood and Heyn (1989) recently showed that **E. coli** DH5 alpha (which has a wild type ribosomal protein L20) had difficulty expressing aminoglycoside resistance. Kanamycin concentrations (50 mg/ml) used in the selection plates would have killed recipients before they were able to express the enzymes encoded in the kanamycin resistance gene.

SUMMARY:

The results of this thesis are:

1) A four kilobase plasmid has been isolated from the Larsen strain of **P. multocida** and designated pLAR-1. It has been characterized in terms of its restriction sites which were then used to generate a restriction map.

2) pLAR-1 does not code for antibiotic resistance, O-antigen production, dermonecrotoxin production, host specificity, siderophore production or any of the biochemical activities tested.

3) Plasmids which are homologous with pLAR-1 have a worldwide distribution.

4) pLAR-1 may be a cryptic plasmid due to the inability to detect a RNA transcript when using pLAR-1 as a probe.

5) pLAR-1 has been cloned into pBR322 in both orientations. These clones have been designated pLRBR-21 and pLRBR-67 and may have the potential to be used as cloning vehicles for further study of genes of **P.** multocida.

LITERATURE CITED

Actis, L.A., S.A. Potter, and J.H. Crosa. 1985. Iron-regulated outer membrane protein OM2 of **Vibrio anguillarum** is encoded by virulence plasmid pJM1. J. Bacteriol. **161**.736-742.

Ausubel,F.M., R.Brent, R. Kingston, D. Moore, J Seidman, J. Smith, and K. Struhl. Current Protocols in Molecular Biology. John Wiley & Sons. New York. 1989

Bauer, A.W., W.M. Kirby, J.C. Sherris, and M. Turck. 1966. Antibiotic susceptibiltiy testing by a standardized single disc method. Am. J. Clin. Pathol. **45**:493-496.

Berman, S. and D.C. Hirsh. 1978. Partial Characterization of R-Plamids from **Pasteurella multocida** Isolated from Turkeys. Antimicrob. Agents Chemother. **14**(3):348-352.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Research. **7**:1513:1523.

Carter, G.R. 1955. Studies on **Pasteurella multocida**. A hemagglutination test for the identification of serological types. **Am. J. Vet. Res. 16**:481-484.

Carter. G.R. 1984. **Pasteurella.** In J.G. Holt and N.R. Kreig, eds., Bergey's Manual of Systematic Bacteriology, vol. 1. Williams & Wilkins, Baltimore. pp. 552-557.

Carter, G.R. 1988. The genus **Pasteurella** in pp. 104-110.

Carter, G.R. and Chengappa, M.M. 1986. Identification of types B and E **Pasteurella multocida** by counterimmunoelectrophoresis. Vet. Rec. **108**:145-146.

Chang, W.H. and G.R. Carter. 1976. Multiple Drug Resistance in **Pasteurella multocida** and **Pasteurella haemolytica** from Cattle and Swine. JAVMA. **169**. 710-712.

Crosa, J.H., M.H. Schewe, and S. Falkow. 1977. Evidence for plasmid contribution to the virulence of the fish pathogen **Vibrio anguillarum**. Infect. Immun. **18**(2)509-513.

Crosa, J.H., L.L. Hodges, and M.H. Schiewe. 1980. Curing of a plasmid is correlated with an attenuation of the virulence in the marine fish pathosgen **Vibrio anguillarum**. Infect. Immun. **39**.509-513.

Davis,L.G., M.D. Dibner, and J.F. Battey Basic Methods in Molecular Biology. Elsevier Science Publishing. New York. 1986.

Elwell, L.P. and P.L. Shipley. 1980. Plasmid mediated factors associated with virulence of bacteria to animals. Annu. Rev. Microbiol. **34**:465-496.

Elwell, L.P., J. de Graaff, D. Seibert, and S. Falkow. 1975. Plasmid-linked ampicililin resistance in **Haemophilus influenzae** type b. Infec. Immun. **12**:404-410.

Elwell, L.P., M. Roberts, L.W. Mayer, and S. Falkow. 1977. Plasmid mediated beta lactamase production in **Neisseria gonorrhoeae**. Antimicrob. Agents Chemother. **11**:528-533.

Fales, W.H., J.N. Berg, and L. W. Morehouse. 1986. Use and comparison of minimal inhibitory concentration and disk diffusion antimbicrobial susceptibility testing with bovine isolates of **Pasteurella haemolytica** type 1 and **Pasteurella multocida** recovered from Missouri cattle with bovine respiratory disease complex. Amer. Assn. Veterinary Laboratory Diagnosticians. 29th Annual Proceedings. 1-8.

Flossman, K.D., G. Muller, P. Heilmann, and H. Rosner. 1984. Influence of iron on **Pasteruella multocida**. Zbl. Bakt. Hyg. A. **258**:80-93.

Haghour.R., E. Hellman, and J. Schmidt. 1987. Plasmids and resistance to 9 chemotherapeutic agents of **Pasteurella multocida** and **Pasteurella haemolytica**, epedemiological Aspects. J. Vet. Med. **34**:509-518.

Hanahan,D. 1983. Studies on transformation of **Eshericia coli** with plasmids. J. Mol. Biol. **166**:557-580.

Helsinki, D.R. 1973. Plasmid determined restance to antibiotics: molecular properties of R factors. Annu. Rev. Microbiol. **27**:437-470.

Hirsh, D.C., L.D. Martin, and K.R. Rhoades. 1981. Conjugal transfer of an R-Plasmid in **Pasteurella multocida**. Antimicrob. Agents Chemother. **20**(3):415-417.

Hirsh, D.C., L.D. Martin, and K.R. Rhoades. 1985. Resistance plasmids of **Pasteurella multocida** isolated from turkeys. Am. J. Vet. Res. **46** (7):1490-1493.

Jacques, M. 1987. Adherence of **Pasteurella multocida** to porcine upper respiratory tract cells. Current Microbiology. **15**:115-119.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Laboratory handbook in molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. Ed.

Mutters, R., P. Ihm, S. Pohl, W. Frederiksen, and W. Mannheim. 1985. Reclassification of the genus **Pasteurella** Trevisan 1887 on the basis of deoxyribonucleic acid homology, **Pasteurella canis, Pasteurella stomatis, Pasteurella anais,** and **Pasteurella langaa**. Int. J. Syst. Bacteriol. **35**(3):309-322.

Namoika and M. Murata. 1961. **Pasteurella multocida**. Cornell Vet. **51**:498-507.

Olsen,I., S.K. Rosseland, A.K. Thorsrud, and E. Jellum. 1987. Differentiation between **Haemophilus apraphrophilus, H. aphrophilus, H. influenzae, Actinobacillus actinomycetemcomitans, Pasteurella multocida, P. haemolytica,** and **P. ureae** by high resolution two dimensional protein electrophoresis. Electrophoresis. **8**: 532-535.

Osgood,C and D. Heyn. (1989) Enhancement of Geneticin resistance in **E. coli** DH5 strains. Focus. **11**(2):33.

Rimler, R.B., and K.R. Rhoades. 1989. **Pasteurella multocida**. in **Pasteurella** and **Pasteurellosis**. eds. Adlam and Rutter. Academic Press. New York. pp.37-73.

Rimler, R.B., and K.R. Rhoades. 1987. Serotype F, a new capsular serogroup of **Pasteurella multocida**. J. Cin. Microbiol. **25**:615-618.

Roberts, R.S. 1947. An Immunological Study of **Pasteurella septica**. J. Comp. Pathol. **57**:261-278.

Rutter, J.M., R.J. Taylor, W.G. Crighton, I.B. Robertson, and J.A. Benson. (1984). epidemiological study of **Pasteurella multocida** and **Bordetella bronchiseptica** in atrophic rhinitis. **115**:615-619.

Szumanski, M. and S.M. Boyle. manuscript submitted. Analysis and sequence of the speB gene encoding agmatine ureohydrolase, a putrescine biosynthetic enzyme in **Escherichia coli**. J. Bacteriol.

Silver, R.P., B. Leming, C.R. Garon, and C.A. Hjerpe. 1979. R-Plasmids in **Pasteurella multocida**. Plasmid. **2**:493-497.

Snipes,K.P., L.M. Hansen, and D.W. Hirsh. 1988. Plasma- and iron-regulated expression of high molecular weight outer membrane proteins by **Pasteurella multocida**. Am. J. Vet. Res. **49**(8):1336-1338.

Sriranganathan, N., S.M. Boyle, L. McGonagle, and G.R. Carter. Plamid Analysis of **Pasteurella multocida** of Animal Origin. Journ. of Clin. Micrio. manuscript submitted.

Timoney, J.F., J.H. Gillespie, F.W. Scott, and J.E. Barlough. The genus **Pasteurella** in Hagan and Bruner's Microbiology and Infectious Diseases of Domestic Animals. 8th ed. Comstock Publ. Assoc. pp104-116.

Tolmasky, A.E., L.A. Actis, and J.H. Crosa. 1988. Genetic analysis of the iron uptake region of the **Vibrio anguillarum** plasmid pJM1: molecular cloning of genetic determinants encoding a novel **trans** activator of siderophore biosynthesis. J. Bacteriol. **170**(4):1913-1919.

Tolmasky, M.E., L.A. Actis, A.E. Toranzo, J.L. Barja, and J.H. Crosa. 1985. Plasmids mediating iron uptake in **Vibrio anguillarum** strains isolated fro turbot in Spain. J. of Gen. Microbiol. **131**. 1989-1997.

Toranzo, A.E., J.L. Barja, R.R. Colwell, and F.M. Hetrick. 1983. Characterization of plasmids in bacterial fish pathogens. Infect. Immun. 39(1):184-192.

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