STRAIN VARIATION AND GENETIC CHANGES

IN *PASTEURELLA MULTOCIDA*

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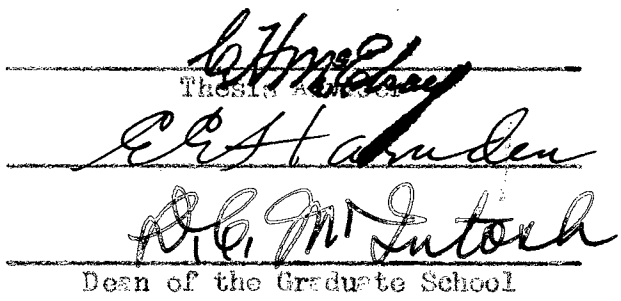

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PREFACE

I wish to express my sincere appreciation to Dr. A. Eisenstark for his cooperation and assistance and also for his opening to me a vast new phase of bacteriology which has provided for me a greater insight of the field. To Drs. P.C. White and L.T. Giltner I am grateful for the cultures they so kindly supplied for this study. I am indebted to Dr. A. C. Barr for the demonstration of invaluable technical and laboratory procedures.

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I. INTRODUCTION AND HISTORICAL REVIEW

Only recently has bacterial genetics become recognized as a substantial phase of the science of bacteriology. In considering the historical development of this field the bacteriologist has, by necessity, always made genetic assumptions, generally by implication. The existence of genetic continuity, of the presistence of characteristics from generation to generation, is conclusively an immediate prerequisite for taxonomic description and ultimately for any experimental work on bacteria. It is then demonstrated that, in bacteriological experiments the use of populations consisting of a mixture of generations, more material is needed than is contained in a bacterium, the indicator characteristics are thereby retained. Thus, is established an order of biological transmission, heredity.

The progress of bacterial genetics during the "Golden" or Cohn-Koch era and for some periods thereafter was limited by application of the principles and theories of "monomorphism." The acceptance of this dogma of non-variability did impede for some time any advances in the study of morphology, inheritance, and variation in bacteria. These theories are no longer accepted as the phenomenon can not now be accepted as errors in procedure.

It is now understood that bacteria are subjected to an infinite profusion of lineal variations that are of prime importance in all divisions of bacteriological research. The recent culminative integration of experimental data of bacterial genetics into a unified theory that includes all living forms has rapidly approached consummation.

Those trends of experimentation which have led to the establishment of bacterial genetics have been based on physiological and morphological effects mediated by genetic changes in bacteria. Specifically these fall into two groups of mutants--mutations for resistance to growth inhibitors, phages, and the like, or mutations affecting the biochemistry of the organism in a direct, obvious manner, detectable as nutritional, fermentative, or similar enzymatic changes. Other mutations amenable to genetic analysis include effects on pigment formation, colonial appearance, antigenic structure, virulence, motility, antibiotic production, and vitamin excretion.

At present there exists two methods by which the mechanisms of genetic interaction between clones may be classified. Lederberg (1951) defines these as (a) "infective transmissions, in which the interaction is mediated by extracts of filtrates which can be separated from the bacterial cells; and (b) sexual phenomenons which appear to require the integrity of the interacting clones." It is the first of these, "infective transmission," more commonly referred to as type transformation that concerns the experimental section of this thesis.

The phenomenon of transformation of pneumococcal types discovered by Griffith in 1928, was the first example of infective hereditary transmission. Subsequently the conditions necessary for transformations <u>in vitro</u> were demonstrated (Dawson and Sia, 1931). These researches culminated with the extraction and partial purification of the transforming principle by Avery <u>et al</u>. (1944).

Basically the phenomenon of transformation may be defined as: "An hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than sexual means, of a

genetically active unit directing the inheritable change" (Austrian, 1952). An additional feature of such reactions is that the genetically active material should be demonstrable in the progeny of the transformed cell and recoverable from such populations in quantities in excess of the amount requisite to induce the initial alteration.

Pneumococci are recognized in two phases, S and R (also called M and S, respectively) depending on the presence or absence or absence of a polysaccharide capsule. R Bacteria are not readily distinguished by serological methods, but the S types are characterized by the serologically specific reactions of the capsular material. The S types are designated with a Roman numeral, for example, S-III is one of the more prevalent types. R Mutants can be obtained from any of the S types particularly with the help of selection with anti-S serums. Such R mutants are generally avirulent for mice, but many are unstable, and will occasionally revert to S, though only to the same S type from which they arose. Other R mutants are stated to be completely stable, as corroborated by their avirulence in large doses. Griffith found that certain R cultures, by themselves avirulent, killed mice when inoculated with heat killed S cells of various types. Viable S cells of the same type as the heat-killed vaccine were recovered from the blood of the infected mice.

Dawson and Sia (1931) were able to demonstrate an in vitro transformation reaction while Griffith had been unable to obtain positive results. Transformation of type, as induced by growing small inocula of R forms in media containing vaccines (heat killed) prepared from heterologous S cultures, was most readily effected by employing anti-R serum in the culture medium.

The subsequent work (Avery et al., 1944 and McCarty, 1946) demonstrated that the active principle consists principally of a highly polymerized desoxyribonucleic acid, which does not require serologically detectable amounts of the capsular polysaccharide for its transforming action.

Transformation has been reported in several other genera of bacteria, these are the Staphylococci (Ryss, 1947), Bacillus (Hemminger and Hegredi, 1948), Salmonella (Mackenzie and Brewster, 1947), Shigella (Neil and Binder, 1947), Phytomonas (Rybak, 1949), Pseudomonas (Legroux and Genevray, 1933), Proteus (Dianzani, 1950), Hemophilus (Alexander and Leidy, 1950) and Escherichia (Youreka, 1948). These reports of directed genetic mutations have been accomplished chiefly by growing the organism to be changed in sterile cultural filtrates of a selected transforming strain and by using desoxyribonucleic acid extracts as outlined by Boivin (1947). To facilitate this method of inducing heritable genetic modifications it has been necessary to use distinct genetic markers. These have fallen into three general groups: (1) virulence, antigenicity or the R — S dissociation; (2) acquired drug resistance; and (3) fermentation reactions. Using the nuclear contracts prepared according to the method of Boivin (1947), Neil and Binder (1947) were able to change Shigella species from one antigenic type to another. Although they have indicated that their experiments are not yet sufficiently reproducible to permit the purification and characterization of the active agent.

Dianzani (1950) was able to induce lactose fermentation in Proteus strains OX2 and OX19 using desoxyribonucleic acid extracts from OX2 and OX19 strains that had been "adapted" to lactose fermentation.

There have been other reports of transformations in other bacteria, without enough details to permit an analysis. Lyss (1947) and Voureka (1948) have reported that staphylococci and E. coli grown in the presence of extracts of drug resistant bacteria acquired a heritable resistance to the drugs concerned. In this instance one must not overlook selection as a factor.

Reports of directed mutations using an undefined cultural filtrate of the prototype as the transforming agent have been cited by several researchers using various genera with the acquisition of virulence by avirulent strains as the marker. Legroux and Genevray (1933) observed that colorless, nonproteolytic, avirulent strains of Pseudomonas pyocyaneus could be transformed into pigmented, strongly proteolytic, pathogenic strains using this method.

Neuninger and Nogradi (1948) have shown an analogus situation in the conversion of motile Bacillus mesentericus to non-motile Bacillus anthracis. Similarly, Rybak (1949) was able to reverse the normal dissociation pattern of Phytomonas tumefaciens, S — mucoid, by using a special water soluble polysaccharide, isolated by alcohol precipitation, incorporated into the medium.

Another report of the induction of heritable virulence is seen in the reports of Mackenzie and Brewster (1947) in serological studies using Salmonella typhimurium. This was achieved by growing the avirulent strain in the cultural filtrates of the virulent strain.

One of the most recent comprehensive reports on the control of the genetic constitution of microorganisms serves to demonstrate the diversity of organisms that this phenomenon may effect. Alexander and Leidy (1950), using Hemophilus influenzae, were able to duplicate the pneumococcus

work of McCarty and Avery (1946). The transformation was accomplished by using an unpurified transforming principle which was proven to be deoxyribonucleic acid.

In the two decades that have elapsed since the research of Dawson and Sia (1933), there has been an unprecedented rise in the study of nucleic acid chemistry closely associated with the principles of transformation. This increase in knowledge has not arisen without criticism. The most evident flaw in the transformation principles, even with the pneumococcus and E. coli researches, has been the possibility that the so-called specific transforming principle may act as a selecting agent. Goodlow, Mika, and Braun (1950) in their work with Brucella abortus have found that the accumulation of the metabolite alanine controls population changes from smooth to nonsmooth types in growing cultures. This would seem to strengthen the arguments that deoxyribonucleic acid might be a selective agent, disregarding its loci in the nucleus.

With newer techniques and the application of statistical methods common to genetic studies, selection may then be eliminated.

Strong evidence for the transforming principle, as such, has been found by Zinder. He has been able, while using Salmonella sp., to correlate transformation with a cytological phenomenon, large body production. Also Lederberg's (1949) gene recombination experiments, by inference, give some genetic evidence which indirectly supports the transformation theory.

Lederberg (1951) stated, "Although the properties of actively lytic, parasitic bacterial viruses or bacteriophages are now widely appreciated, owing especially to a resurgence of interest in the growth and genetics of viruses, the significance of cryptic or lysogenic viruses has been

greatly underestimated."

Bacteriophages and viruses are of exceptional biological importance in that they represent the simplest forms of living matter and are therefore of special interest in relation to the problems of the origin of life itself. In one respect, however, their properties seem to be paradoxical, for they can apparently flourish only within living cells--often specific cells--and it is therefore difficult to see how they could have come earlier in evolution.

Bacteriophages are particles which generally exhibit all the properties usually considered to be characteristic of ultraviruses; virulence, i.e. an infective and pathogenic power; a small size; and the property of being able to propagate only within a cell, often specific. In the so-called lysogenic bacteria, however, the bacteriophage occurs as a type of potential virus, a probacteriophage (Lwoff, 1952), which is neither infective nor pathogenic. In some cases, the probacteriophages may develop into bacteriophages which, if liberated by bacterial lysis, are able to infect and lyse sensitive bacteria belonging to the same related species.

Recently lysogenecity has been proven to be closely associated with infective transmissions. Zinder (1952) was able to isolate an infective, lysogenic agent capable of transferring hereditary traits from one strain to another. The term "transduction" has been applied to this phenomenon.

It is the purpose of this experiment to establish a simplified technique whereby this principle could be routinely demonstrated. Pasteurella multocida was chosen as the experimental organism as its position as the etiological agent of hemorrhagic septicemia, in domestic animals, is not thoroughly understood. Further this organism is most

constantly found in the normal flora of the mucous membranes of the respiratory tract of many domestic animals (Moore, 1895). Therefore the correlation of this system of nature and its genetic mechanisms with the modes and means of the pathogenesis of pasteurellosis would provide, directly or indirectly, an *in vitro* solution to the understanding of the pathogenic mechanisms involved.

II EXPERIMENTAL

A. Characterization of Strains

The Pasteurella strains used in these experiments were obtained from Dr. Philip G. White, American Scientific Laboratories, Madison, Wisconsin, and Dr. L. T. Giltner, Pathology, Division, Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C. The strains and available histories are listed in Table I. Stock cultures were maintained on Bacto-tryptose agar slants in screw-cap glass tubes and kept at room temperature. Because of the nature of this investigation transfers were made as infrequently as possible to avoid the higher rate of spontaneous mutations that occur in young cultures. A dual set of stock cultures were kept and one was used to check viability.

1. Materials and Methods

Biochemical and fermentation studies. To ascertain their general fermentative properties, these strains of Pasteurella were inoculated into lactose, glucose, and sucrose broth. The carbohydrate broth used was the standard Bacto-phenol red broth base. This medium had the following composition:

Bacto-beef extract	1 g.
Proteose peptone No. 3, Difco . .	10 g.
Sodium chloride	5 g.
Bacto-phenol red	0.018 g.
Water.	1 L.

Final pH 7.4

The carbohydrate sources were aqueous solutions of glucose, sucrose, and lactose. These were sterilized by filtration and added aseptically

to sterile broth base to a final concentration of one percent. The strains were inoculated and then incubated at 30° C. The reactions were observed 24 and 48 hours after inoculation.

The reactions of these strains on Bacto-Simmons citrate medium, Bacto-nutrient gelatin, and Bacto-litmus milk were used to determine their ability to utilize these compounds. The composition of these media are as follows:

Bacto-Simmons citrate agar

Magnesium sulfate	0.2	g.
Monoammonium phosphate	1	g.
Dipotassium phosphate	1	g.
Sodium chloride	5	g.
Sodium citrate	2	g.
Bacto-agar	15	g.
Water	1	L.

Final pH 6.8

Bacto-nutrient gelatin

Beef extract	3	g.
Bacto-peptone	5	g.
Bacto-gelatin	120	g.
Water	1	L.

Final pH 6.8

Litmus milk

Bacto-skim milk	100	g.
Bacto-litmus	5	g.
Water	1	L.

Final pH 6.8

The reactions were checked after 24, 36, and 48 hours of incubation at 30° C.

Colonial characterization. All strains were thinly seeded on Bacto-tryptose agar which has the following composition:

Bacto-tryptose	20	g.
Bacto-dextrose	1	g.
Sodium chloride	5	g.
Bacto-agar	15	g.
Water	1	L

The pH was adjusted to 6.9. For easier identification of colonial morphology, additional plates were prepared according to Levine and Gerber (1950) using 2, 3, 5, triphenyl tetrazolium chloride in the medium. A 1 percent sterile aqueous solution was added to the melted agar so that the final concentration of the dye was 0.005 percent. Approximately 20-ml. volumes of this medium were apportioned to petri plates, and then dried at 37° C. for 24 hours. These plates were thinly seeded with all strains, incubated at 30° C. The colony morphology was studied after four days incubation.

Determination of strain virulence for white Swiss mice. Subcultures were propogated in Bacto-tryptose broth at 30° C. until a slight turbidity was noted. These cultures were transferred to 250-ml. Erlenmeyer flasks containing approximately 100-ml. volumes of sterile Bacto-tryptose broth then incubated at 30° C. on a shaking machine, similarly re-subcultured until a heavy turbidity was produced in 2 hours while continuously being aerated by the action of the shaking machine. The cultures, multiplying under logarithmic conditions, were centrifuged until a clear supernatant broth was obtained. This supernatant was discarded and the packed cells were resuspended in 25-ml. volumes of 0.85% sodium chloride solution. This was then standardized to compare in optical density to a number 8 nephelometer standard, approximately 12 x 10^6 cells per ml. A young, white Swiss mouse was injected intraperitoneally with one-half-ml. volume of this cellular suspension, one mouse used per culture of each strain. Eight virulent Pasteurella strains were selected which had killed the mouse in less than 24 hours. These strains were re-subcultured as described previously and five mice per strain were inoculated intra-peritoneally to establish a comparative virulence standard.

2. Results

Fermentation and biochemical characteristics. The fermentation reactions, gelatin liquefication, citrate utilization and litmus milk reactions will be found in Table II.

Colonial cultural characteristics. Rough and smooth variants could be differentiated most readily after four days incubation on the TTC1 (2, 3, 5, triphenyl tetrazolium chloride) Bacto-tryptose agar. Typical smooth colonies were approximately 2 mm. in diameter and round with a sharply defined carmine red center. Rough forms were from 4 to 6 mm in diameter, irregular in shape and diffuse pink in color. Variants of some strains were also unable to reduce this dye. These colonies were rough in form, resembling the rough colony on Bacto-tryptose agar.

Besides the manifestations of R (rough), S (smooth), M (mucoid) and I (intermediate), other appearances such as peripheral papillae and fine granulations were noted. Elberg and Cheng-Lee Ho (1950) observed fluorescent, non-fluorescent, blue-fluorescent and various other fluorescent types. Fluorescence occurred only in young colonies and then only under certain conditions (e.g. after 16-24 hours at 37° C. on horse serum agar or longer on nutrient agar). The property disappeared after 72 hours at 37° C.

Three studies of colony morphology were completed. The assembled strains could be divided into two general groups according to the predominant colony form.

Group I contained strains producing large to small M type colonies having a very slimy tenacious consistency (methylene blue stains demonstrated capsular material surrounding the cell wall). Group I is composed of these strains:

O-1	509-L	B-4
O-2	AA	E-2
P-4	AB	E-3
P-6	AC	P-2
Bf-2	P-1	230

The colonies formed by this group are further characterized in that there was usually a well defined rim of clear material, the center of the colony being carmine red, large and rather flattened.

Group II contained strains in which the colonies were smooth to intermediate types. The strains chosen for study were selected from this colonial type. Two strains X-1 and Z-1 were found to have high dissociation rates, S ——— R, and were consequently selected. These strains would show fluorescence on Bacto-tryptose agar when grown at 37° C. The strains in this group are as follows:

T-2	E-1	Bf-5
BAL-P.c.	X-1	Bf-4
Z-1	Y-1	Bf-3
BAL-TTJ	1054	2050

Rough strains were selected at random from the parent S strains and sub-cultured until there were no reversions or back mutations to the smooth parent type.

Determination of strain virulence for white Swiss mice. Table III contains the results of the virulence survey of 27 strains. Table IV enumerates the comparative virulence of the strains found to be virulent in the survey, the results being recorded as the number of deaths in ratio to the total number of mice inoculated with each strain, and the time death occurred after inoculation.

3. Discussion

The purpose of characterization of these strains was to select genetically unstable, readily dissociable, or highly mutagenic strains which were to be used in the transformation experiments. The fermentation

reactions were disregarded for this purpose and used only as a limited tentative index of the associated strains. Colonial characteristics and virulence were used as the prime markers, the strains selected being highly variable and or highly pathogenic for white Swiss mice.

Strains Z-1 and X-1 were chosen for the investigation to determine the existence of demonstrable *in vitro* genetic phenomenon similar to type transformation using simplified laboratory procedures.

Strain X-1 proved to be the most readily discernible strain of this collection. While strain Z-1 was the most readily pathogenic for white Swiss mice, it also demonstrated a relatively high S ⟷ R dissociation rate.

A. Selection of Drug Resistant Strains and Establishment of Mutation Rates

1. Materials and Methods

Streptomycin resistance. Sub-cultures of smooth and rough variants of strains X-1 and Z-1 were grown in Bacto-tryptone broth. These strains were then transferred to broth containing sterile filtered streptomycin (Mixture-streptomycin sulfate, U. S. P. Eli Lilly and Company) in serial dilutions so that the final concentrations were 0.2 g. per ml., 0.02 g. per ml., 0.002 g. per ml., and 0.0002 g. per ml. Multiplying cells from the highest dilution in which growth appeared were transferred to the next lower dilutions. This procedure was continued until the cultures would grow in Bacto-tryptose broth containing 0.001 g. dihydro-streptomycin sulfate per ml.

Determination of streptomycin resistance mutation rate for culture X-1 rough variant. Forty-two 2-ml. volumes of 18 hour sub-cultures of

strain 2-1 rough variant in Bacto-tryptose broth were prepared. To each of forty sub-cultures 0.5-ml volume of dihydro-streptomycin sulfate containing 10,000 gamma was added. The contents of each culture was thoroughly mixed with 10-ml. volumes of 2% sterile Bacto-tryptose agar and plated.

One $1:10^{-6}$ dilutions were prepared from the remaining cultures in 0.85% sterile NaCl solution and 1-ml. volume of each diluted culture was mixed with 10-ml. volumes of sterile Bacto-tryptose agar and plated. These plates were incubated at 30^{0} C. and observed 72 hours later.

All colonies were counted and the total number of bacteria determined. The colonies on the plates to which dihydro-streptomycin sulfate had been added were checked for streptomycin resistance and for contamination by re-plating for typical colony appearance on 2, 3, 5, triphenyl tetrazolium chloride Bacto-tryptose agar containing 100 gamma dihydro-streptomycin sulfate per ml.

2. Results

Streptomycin resistance. When the cultures would grow in 0.001 g. dihydro-streptomycin sulfate per ml. or higher they were then transferred onto plain Bacto-tryptose agar slants, grown for three passages on Bacto-tryptose agar without streptomycin and rechecked for streptomycin resistance in Bacto-tryptone broth containing 0.001 g. dihydro-streptomycin sulfate per ml. A streptomycin resistant strain was selected and added to the stock collection and thereafter maintained on Bacto-tryptose agar slants in screw-cap tubes. Resistant cultures could be obtained by this method usually on the first transfer from one dilution to the next lower dilution, confirming a similarity of the one-step resistance in *Escherichia coli* and *Hemophilus parainfluenzae* var. *aureus* to streptomycin

(Demerec, 1948).

Determination of streptomycin resistance mutation rate for culture Z-1 rough variant. A total of 15 colonies from 10 of the 40 plates were streptomycin resistant and proved not to be contaminates by being sub-cultured on 2, 3, 5, triphenyl tetrazolium chloride Bacto-tryptose agar containing 100 gamma dihydro-streptomycin sulfate per ml. By using an average of the bacteria counted per ml. from the two blank plates, it was determined that 20×10^9 cells existed in the cultures used. The basic equation of Luria and Delbrück (1943),

$$M = \frac{2.3}{N} \log_{10} \frac{1}{P_o},$$

was used to calculate the mutations per cell division. "N" designates the total number of bacteria used in the determination, P_o the proportion of cultures without mutants, in this experiment 0.25, therefore:

$$M = \frac{2.3}{20 \times 10^9} \log_{10} \frac{1}{0.25} = 6.29 \times 10^{-11} \text{ mutations per cell division.}$$

3. Discussion

Evidence that bacterial resistance to penicillin and streptomycin is not induced by the composition of the medium but originates spontaneously through genetic changes comparable to gene mutation has been adequately demonstrated by Demerec (1948). This was substantiated as the numerous experiments, made in gathering data for his survival curves, showed clearly, that in large populations of bacteria there were always some individuals more resistant to antibiotics than others. In these experiments very small inocula (50-300 bacteria) were used. The proportion of resistant bacteria was too small to account for their presence by assuming that they came about through division of one or

more resistant individuals that may have been present in the inoculum. Therefore the resistant individuals must have originated in the experimental cultures. Two alternative possibilities were considered with respect to the mechanism of this origin: (1) that resistance was induced by some interaction between the antibiotics and the bacteria when they were together in the media; and (2) that it originated independently of the antibiotic, by mutation, the antibiotic acting only as a selective agent in the isolation of mutants by destruction of sensitive bacteria.

It was necessary to calculate the mutation rate since in the transformation experiments, streptomycin resistance was used as a transformation marker. The mutation rate so established provided a basis whereby a large increase in number of mutants served as evidence that induced genetic changes occurred in the cells subjected to experimental treatment.

C. Transformation Experiments

1. Materials and Methods

Cultures of a parent and daughter mutant were grown separately in 100-ml. volumes of Bacto-tryptone broth on a shaking machine in 250-ml. Erlenmeyer flasks. These were transferred frequently to assure a logarithmic multiplication growth phase, a decided visible turbidity being produced after two hours agitation at 20° C. One-half-ml. volume of a cellular suspension of each culture was then transferred to separate arms of a glass "U" tube constructed with an ultra fine Corning fritted glass filter disk fused between the arms (B. Davis, 1950) (See plate I). A 5 pound per square inch negative pressure was applied for 15 minutes to the arm containing the daughter culture. This process was repeated

two times. The "U" tube was then allowed to incubate for 4 hours at 30° C. A 1-ml. volume sample of each arm was then removed and plated on sterile 2, 3, 5, triphenyl tetrazolium chloride Bacto-tryptose agar to observe any population changes in colony morphology. At this time portions of the untreated samples were plated to serve as controls.

2. Results

The preliminary transformation attempts were conducted using rough ——— smooth reversion as the genetic markers. Two parent strains, Z-1 and X-1, and their respective rough variants were treated as outlined in the Materials and Methods Section. In these first preliminary tests, population samples of treated rough, untreated rough, treated smooth, and untreated smooth were streaked on 2, 3, 5, triphenyl tetrazolium chloride Bacto-tryptose agar and incubated for 72 hours at 30° C. The colonies on these plates were counted quantitatively and qualitatively. The data obtained are found in Table V.

The second group of transformation experiments consisted of exposing streptomycin susceptible rough and streptomycin susceptible smooth variants to cultural filtrates of streptomycin resistant smooth cultures. Four trials each of the two combinations were completed with no induced or increased rates of streptomycin resistance in the treated populations.

The third group of transformation experiments were patterned after Hotchkiss' (1952) experiments in which he induced transformation in pneumococci by penicillin lysates. Strain Z-1 smooth (parent) was transferred to one arm of the "U" tube and streptomycin resistant Z-1 rough variant to the other arm. Dihydro-streptomycin sulfate was added to the arm containing the parent culture so that the final concentration

was 0.01 g. per ml. This was allowed to incubate 2 hours at 30° C. before negative pressure was applied. During this time the smooth population was destroyed by antibiotic lysis. Plate counts demonstrated complete destruction of the population by the streptomycin. The rough cells being streptomycin resistant were unaffected by the streptomycin. The treated rough cells were plated and no significant smooth colonial changes were noted in 6 trials involving 21,725 rough colonies.

During the course of these transformation experiments it was noted in the case of both strains 2-1 and 2-1 that the smooth cultures were lysogenic for thier respective daughter rough strains. Lysis was noted usually six to eight hours after the two strains had been transferred to the arms of the "U" tube. Direct total plate counts of the rough strains before and after lysis demonstrated a population reduction from 56×10^7 cells per ml. to 10×10^6 cells per ml. Procedures for demonstration of plaques were attempted with no consistent results. Although plaques were demonstrated there was no consistency in numbers with the dilution of the filtrate.

3. Discussion

Theoretically the failure to secure significant transformation in the first two experiments is probably the result of; (1) absence of nuclear transforming agents, (2) incomplete transforming systems, as compared to pneumococcal transformations, (3) selection of strains failing to participate in the reaction under comparable conditions, and (4) insufficient sampling of the experimental populations. To alleviate the possibility of insufficient nuclear material in the cultural filtrates, the streptomycin smooth lysates were then instigated, although these experiments produced no results of significance. It is

also possible that Pasteurella cells contain a similar enzyme found in pneumococcal cells (Avery, MacLeod, McCarty, 1944). This enzyme is capable of destroying the activity of the transforming principle and was found to be highly active in the autolysates of a number of different pneumococcal strains. The fact that this cellular enzyme is released during autolysis may explain, in part, at least, the observations of Dawson and Sia (1931) that it is essential, in bringing about transformation in the test tube, to use a small inoculum of young and actively growing rough cells. The irregularity of the results and often the failure to induce transformation when large inocula are used may be attributable to the release from autolyzing cells an amount of this enzyme sufficient to destroy the transforming principle in the reaction system.

Zinder and Lederberg (1952) has shown that Salmonella typhimurium, when grown in the presence of weak phages, produces a filterable agent capable of transferring hereditory traits from one strain to another. Individual filtrates could transduce many different traits, but no more than one to a single bacterium. It is then pertinent to point out the importance of lysogenesis in the Pasteurella strains. With this similarity there possibly exists a transduction system which may be demonstrated with the selection of the proper strains.

III. SUMMARY AND CONCLUSIONS

Bacterial genetics has become established as a unified division in the field of bacteriology. All phases of bacteriology are intimately dependent upon the genetic factors involved and the physiological and morphological variations commonly seen in bacteria are the tools of the bacterial geneticist.

There are two basic mechanisms with which genetic interaction has been studied: (a) infective transmission or type transformation and (b) sexual phenomena or gene recombination.

This report is concerned with the establishment of simple laboratory procedures whereby type transformation could be induced using Pasteurella multocida as the experimental organism. Pasteurella multocida was selected as the experimental organism as its relationship as the ethiological agent of hemorrhagic septicemia, pasteurellosis, or the pneumo-enteritis complex is not thoroughly understood.

Twenty-seven strains of Pasteurella multocida were obtained. Fermentation studies were executed as a limited taxonomical index of the assembled strains. Comparative virulence of strains was evaluated using white Swiss mice and colonial dissociation rates were studied by incorporating 2, 3, 5, tryphenyl tetrazolium chloride into the medium. This dye readily differentiates rough, smooth, and other colonial variants.

Two strains of Pasteurella multocida were selected for relatively high colonial variation mutation rates and virulence. An additional genetic marker was established by selectively isolating streptomycin

resistant strains. Mutation rates were established for streptomycin resistance for one of the selected rough strains.

Transformation attempts were conducted using three groups of markers; (a) rough ——— smooth, (b) streptomycin susceptible ——— streptomycin resistant, and (c) rough ——— smooth using streptomycin as a lytic agent to liberate the cellular transforming principle.

A glass "U" tube with an ultra-fine fritted glass disk (Corning) separating the arms was used to bring cultural filtrates into contact with susceptible cells.

Transformation from rough to smooth was observed in a very low frequency. It is thought by the writer that this could possibly have been the result of a back mutation as this had been observed during the course of the experiments. In these experiments two lysogenic strains were noted. These may serve to establish transduction, a related form of type transformation.

It is concluded that this method will have to be further investigated. It is indicated that more virulent strains should be used. In the transformation of R to S, the R cells must be made to grow in aggregates, this can be done either by agglutination, with anti-R serum, a medium containing sodium chloride, or by the use of semisolid agar.

TABLE I

Pasteurella multocida Strains

Code	Source	History
X-1	Amer. Sci. Lab.	P. multocida, Type I, ATCC 7707.
Y-1	" " "	P. multocida, Type II, ATCC 6530, (AJVR 4:110, 1943).
Z-1	" " "	P. multocida, Type III, ATCC 6535.
AA	" " "	P. avicida, (Bergey's 5th): history unknown: type undetermined.
AB	" " "	(Same as above)
AC	" " "	(Same as above)
B-4	" " "	Pasteurella sp., isolated from bovine hemorrhagic septicemia 1946.
Bf-2	" " "	Pasteurella sp., isolated from buffalo hemorrhagic septicemia 1940, (Globe #100).
Bf-3	" " "	P. bubaliseptica, Globe #101, buffalo hemorrhagic septicemia, 1940.
Bf-4	" " "	P. bubaliseptica, (no record of isolation).
Bf-5	" " "	P. bubaliseptica, (no record of isolation).
E-1	" " "	P. equiseptica, ATCC #4957, 1937.
E-2	" " "	P. equiseptica, Globe #200, equine hemorrhagic septicemia 1940, (isolated 1931).
E-3	" " "	P. equiseptica, Globe #202, equine hemorrhagic septicemia (isolated 1929).
O-1	" " "	P. oviseptica, Globe #503, ovine hemorrhagic septicemia.
O-2	" " "	P. oviseptica, from clinical case of hemolytic septicemia in sheep.
P-1	" " "	P. suiseptica, U.S.P.H. #D2 (via Abbott Laboratories).
P-2	" " "	P. suiseptica, U.S.P.H. #B-1 (via Abbott Laboratories).
P-4	" " "	P. suiseptica, from lung of hog with hemolytic septicemia; Globe #300, Kinsley 1924).
P-6	" " "	P. suiseptica, from heart of hog with hemolytic septicemia; Globe #302.
T-2	" " "	P. avicida, from turkey heart blood, Globe #630.
2050	B.A.I.	P. avicida, Type I, Drs. Lyons and Little.
1054	"	P. bubaliseptica, Type II, Drs. Lyons and Little.
BAI-III	"	P. muricida, Type III, Drs. Lyons and Little.
230	"	P. equiseptica, no history.
590-L	"	P. boviseptica, no history.
BAI-P.o.	"	P. oviseptica, isolated from a goat.

TABLE II

Biochemical and Fermentation Reactions

Strain	Lactose	Glucose	Sucrose	Citrate	Gelatin	Litmus Milk
AA	≠	≠	≠	-	NL	Litmus reduced
AB	≠	≠	≠	-	NL	Litmus reduced
AC	≠	≠	≠	-	NL	Litmus reduced
B-4	≠	≠	≠	-	NL	Litmus reduced
Z-1	≠	≠	≠	-	NL	Litmus reduced partially
T-2	≠	≠	≠	-	NL	Litmus reduced partially
230	≠	≠	≠	-	L	no change
2050	≠	≠	≠	-	NL	no change
509-L	≠	≠	≠	-	NL	Litmus reduced
BAI-III	≠	≠	≠	-	L	Litmus reduced
BAI-P.o.	≠	≠	≠	-	L	Litmus reduced
1054	≠	≠	≠	-	NL	Litmus reduced
P-1	b	b	b	-	L	no change
P-2	b	b	b	-	L	Litmus reduced
P-4	b	b	b	-	NL	Litmus reduced
P-6	b	b	b	-	NL	Litmus reduced
O-1	≠ slow	≠ slow	≠ slow	-	L	no change
O-2	≠ slow	≠ slow	≠ slow	-	L	Litmus reduced
E-1	b	b	b	-	NL	no change
E-2	b	b	b	-	NL	Litmus slightly reduced
E-3	b	b	b	-	NL	no change
Bf-2	≠ slight	≠	≠	-	L	Litmus reduced
Bf-3	b	b	b	-	NL	Litmus reduced
Bf-4	b	b	b	-	NL	Litmus reduced
Bf-5	b	b	b	-	NL	no change
Y-1	≠	≠	≠	-	NL	Litmus reduced
X-1	≠	≠	≠	-	L	Litmus reduced

(≠ acid b basic - no growth NL not liquified L liquified)

TABLE III

Preliminary Virulence Survey of Assembled Strains

Strain	Time of Death After Inoculation
2050	Death in 16 hours.
1054	Death in 24 hours.
BAI-III	Death in 16 hours.
Y-1	Death in 24 hours.
Y-1	Death in 16 hours.
Z-1	Death in 15 hours.
O-1	Not virulent.
O-2	Not virulent.
P-4	Not virulent.
P-6	Not virulent.
230	Death in 16 hours.
509-L'	Not virulent.
BAI-P.o.	Death in 16 hours.
AA	Not virulent.
AB	Not virulent.
AC	Not virulent.
B-4	Not virulent.
Bf-2	Not virulent.
Bf-3	Not virulent.
Bf-4	Death in 16 hours.
Bf-5	Death in 24 hours.
E-1	Death in 24 hours.
E-2	Not virulent.
E-3	Not virulent.
P-1	Not virulent.
P-2	Not virulent.
T-2	Not virulent.

TABLE IV

Comparative Virulence of Selected Strains

Strain	Ratio	Time of death after inoculation				
		Mouse #1	Mouse #2	Mouse #3	Mouse #4	Mouse #5
X-1	0/5	-	-	-	-	-
Y-1	5/5	16 hours	16 hours	18 hours	18 hours	24 hours
Z-1	5/5	6 hours	8 hours	8 hours	8 hours	8 hours
2050	5/5	18 hours	24 hours	24 hours	26 hours	92 hours
230	0/5	-	-	-	-	-
1054	0/5	-	-	-	-	-
Bf-4	2/5	24 hours	36 hours	-	-	-
Bf-5	1/5	18 hours	-	-	-	-
B. I-III	5/5	5 hours	6 hours	6 hours	8 hours	8 hours

TABLE V

Preliminary Transformation Data

Cultures	Number of Colonies							
	Treated Populations				Untreated Control Population			
	S		R		S		R	
	S	R	R	S	S	R	R	S
Z-1 S and Z-1 R	708	1	488	0	894	0	901	0
X-1 S and X-1 R	1090	3	860	1	964	3	932	1
Z-1 S and Z-1 R	1113	2	643	2	890	2	1194	3
X-1 S and X-1 R	710	0	542	0	802	1	1012	1
Z-1 S and Z-1 R	955	4	628	3	697	0	1083	0
X-1 S and Z-1 R	998	0	539	1	1317	1	1499	2

PLATE I

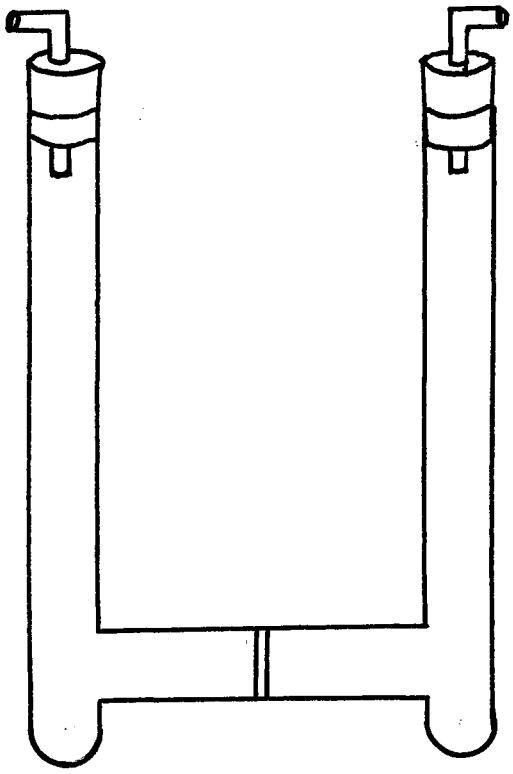

Diagram of glass "U" tube with Corning Fritted glass U. F. filter disk.

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