NUTRITIONAL REQUIREMENTS AND TRANSDUCIBILITY OF SALMONELLA PULLORUM

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Bruce C. Kline

1966

ABSTRACT

NUTRITIONAL REQUIREMENTS AND TRANSDUCIBILITY OF *SALMONELLA PULLORUM*

by Bruce Kline

Forty-five strains of *Salmonella pullorum* were assayed for their amino acid, vitamin, and (a) purine requirements. They were found to require predominantly only cysteine and leucine for growth on synthetic minimal agar. Eleven per cent of the strains were found to require an additional supplement of niacin or thiamine. Two strains were found which could apparently utilize either thiamine or leucine to satisfy the same growth requirement. A significant number of the strains were found to have an apparent increased back mutation frequency to leucine independence in the presence of thiamine and pantothenate. A study of potential sulfur sources showed that 33% of the strains responded to either sulfite, sulfide, or cysteine as a sulfur source and 13% responded only to either sulfide or cysteine as a sulfur source. Significantly, cysteine sulfinic acid, an organic equivalent of inorganic sulfite ion, was not utilized. A genetic attempt was made to roughly correlate the larger of the two groups to either the cysA mutant, the sulfate-thiosulfate permeaseless mutant of *Salmonella typhimurium*; or its nutritional counterpart, the *cys*B mutant. By interspecies transduction of *S. pullorum* and *S. typhimurium* it was possible to map the larger group of *S. pullorum* in the region of the *S. typhimurium cys*A locus.

NUTRITIONAL REQUIREMENTS AND TRANSDUCIBILITY OF *SALMONELLA PULLORUM*

By

Bruce C. Kline

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1966

This thesis is dedicated to all my
family, but especially to Mary Ann and Bruce Jr.

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation to Dr. D. E. Schoenhard for his very warm and knowledgeable guidance throughout the course of this study.

During this study, I was supported in part by a departmental research assistantship and my wife, Mary Ann.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Salmonella pullorum, the causative agent of bacillary white diarrhea in chickens, is a natural auxotrophic organism. A determination of the nutritional requirements of this organism would be of advantage in establishing a genetic system to analyse the virulent state of this organism.

At present the molecular basis of virulence of this pathogen is not completely understood. An endotoxin has been detected and partially characterized by Dooley et al (1958). Nonetheless, it is very difficult experimentally, if not impossible, to work backward from the presence of endotoxin to a complete molecular model of virulence. However, if one could establish that a region or certain regions of the chromosome control the heredity of virulence, then one would have taken the first step toward the intended goal; i.e., a basis would be established for believing one or several cellular constituents were responsible for virulence. Moreover, it might even fortuitously happen that the virulence determinant(s) would map in the same location as a known marker or markers.

To begin a genetic analysis one must first satisfy certain prerequisites, i.e., the bacteria must be genetically distinct and a competent transfer system must exist. The objects of this work are to determine what naturally occurring genetic distinctions can be inferred by examining the

nutritional requirements of auxotrophic strains of S. pullorum and which strains are competent recipients in transductional work.

The biochemical characterization of the strains, which represent samplings from 10 different states of the United States, also has a noteworthy measure of evolutionary and epidemiological significance.

LITERATURE REVIEW

Nutritional Requirements

Most species of Salmonellae grow well in a simple medium consisting of glucose, inorganic nitroger, and mineral salts (Lederberg, 1947). However, naturally occurring auxotrophic organisms of this genus appear and may be detected on ordinary nutrient agar as dwarf colonies or slow growers (Stokes and Bayne, 1957 and 1958a).

With respect to those species which are usually isolated from nature as natural auxotrophs, the nutritional requirement may be the same for all members of the species. An example of this is the omnipresent requirement for tryptophan in Salmonella typhosa (Fildes et al, 1933). Conversely, although the data are sparse, there may not be a general requirement for a particular nutrient in other naturally occurring auxotrophic species of Salmonella (Stokes and Bayne, 1958b).

The basis for natural auxotrophy is assumed by this author to be mutational in character. The precise way or ways in which a natural alteration of the genetic information encoded in DNA is altered is presently unknown. Possibly, an unstable tautomeric shift in the position of the hydrogen atoms on the purine and pyrimidine bases of the replicating DNA could cause, for example, a pairing of adenine with cytosine subsequently changing a codon's meaning (Watson

and Crick, 1953). Further, it has been observed in some strains of Salmonella typhimurium, for example LT-7, that natural mutants consistantly occur at a rate greater than that in other strains of this species. Demerec et al (1957) postulated the existence of a mutator gene to explain this phenomenon. Kirchner (1960) noted the ability of histidine requiring LT-7 strains, which possessed the postulated mutator gene, to revert to histidine prototrophy when treated with the base analogues 5-bromodeoxyuridine or 2-aminopurine in 95% of the mutants tested. Apparently, the mutator gene facilitates an event leading to a transition. The fact that a species, such as S. typhosa, is so stable in its requirement for tryptophan indicates that a naturally occurring deletion-type mutational event may have occurred. Thus, a third possible way of incurring a natural mutation may exist.

Salmonella gallinarum, and its variant in the older literature, S. pullorum, are natural auxotrophs. Today, both species are classified as S. gallinarum in the seventh edition of Bergey's Manual of Determinative Bacteriology. However, in the literature to be cited in this discussion they are treated as separate species. Further, the 45 strains used in this investigation were originally classified as S. pullorum on the basis of the distinguishing biochemical reactions reported in the older literature and the gross pathology of the host in response to infection.

Historically, difficulties were encountered in classification because of the weak biochemical differences between the two organism. For this reason, and because of the

significant economic losses incurred with infection by the more virulent strains of S. pullorum, and because of the basic scientific interest in the "in vitro" phenomenon of slow growth exhibited by both of these organisms, early investigational efforts were directed to a study of nutritional requirements. The results, while showing definite patterns, were more or less easily interpretable depending on which one of the three aforementioned problems was considered.

Let us consider the first problem mentioned, classification, using nutritional requirements to distinguish between S. gallinarum and S. pullorum. In a total of 33 strains of S. pullorum tested by several workers, polyauxotrophy for some combination of cysteine, leucine, proline, and aspartic acid is the rule; whereas an infrequent requirement is found for the B vitamins--thiamine, niacin, pantothenate, and riboflavin; and no requirement for other vitamins or nucleic acid precursors is found (Johnson and Rettger, 1943; Lederberg, 1947; Kuwahara et al, 1958; Stokes and Bayne, 1958b). In contrast, in a total of 46 strains of S. gallinarum tested, there appeared an infrequent requirement for amino acids but a very frequent requirement for thiamine (Johnson and Rettger, 1943; Lederberg, 1947; and Stokes and Bayne, 1958b). In contrast to this last statement, of 11 additional strains of S. gallinarum tested for a thiamine requirement by Kuwahara et al (1958) only one strain required thiamine. Also, Stokes and Bayne (1958b) found in the 12 strains of S. gallinarum which they determined as only requiring thiamine that a supplementary mixture of

cysteine, leucine, and aspartic acid markedly stimulated growth in 10 of the strains. Moreover, Schoenhard and Stafseth (1953), as well as Gilfillian *et al* (1955), have shown that a mixture of vitamins stimulates the growth rate of a variety of *S. pullorum* strains. Thus, there seems to be an apparent overlap in the nutritional requirements of these species.

To what extent this overlap is real or simply due to the test procedure is difficult to assess. In view of the facts, however, that *S. pullorum* does grow very slowly on synthetic media, it does mutate quite frequently in at least the leucine locus (D. E. Schoenhard, personal communication), and that Lederberg (1950) has reported the presence of detectable amounts of sulfide ions in amino acid preparations, it seemed very desirable to find an assay procedure which would take these facts into account. This desire was intensified since it was suspected that many of the strains to be tested in this work would be cysteine or leucine auxotrophs. The addition of just the suspected requirements, utilizing the soft agar auxanographic method of Beijerinck as reported by Lederberg (1950), admirably resolved the complications of interpretation in the classical ommission technique performed in synthetic broth.

The results show that all 45 *S. pullorum* strains tested required at least cysteine and leucine; the leucine requirement has a high reversion frequency with both large and small colonial forms arising within the same strain; and that

thiamine and pantothenate may affect the requirement for leucine in some, but not all, strains. Having once established the nutritional requirements of the strains under consideration, the next step is to determine which of the strains are amenable to a transductional type of analysis.

Transduction

Transduction, the bacterial virus mediated transfer of bacterial genetic material from a donor organism to a recipient organisms, was first observed between auxotrophic mutants of S. typhimurium (Zinder and Lederberg, 1952).

It was subsequently shown that 3 types of transduction are possible, vis., generalized, specialized and abortive transduction. In generalized transduction any marker may be transferred from the donor to the recipient cell (Zinder and Lederberg, 1952). In contrast, in specialized transduction only a given region of the donor chromosome is transferred (Morse, 1954). This region is contiguous to the prophage region on the host chromosome. In abortive transduction any marker may be transferred, but the donor marker is not integrated into the genome of the recipient. It is transferred unilinearly within a clone (Ozeki, 1956, 1959).

A successful generalized transduction process is a function of several variables. Given that a particular recipient is permissive, it is a preliminary necessity that the recipient contains the proper surface receptor for donor virus attachment. S. typhimurium and S. pullorum share the common O antigen, 12. This antigen has been shown to be the phage receptor site of S. typhimurium for phage PLT22.

Fortuitously, S. pullorum phage P38 will transduce S. typhimurium recipient cells (Snyder and Schoenhard, 1960). This observation was employed as the basis for using a prototrophic S. typhimurium strain, LT2, as the donor in establishing the transducibility of S. pullorum auxotrophs.

The results of such transductions show that most S. pullorum are transducible to cysteine prototrophy by S. pullorum phage P38 grown on wild type S. typhimurium.

MATERIALS AND METHODS

Bacterial Strains

S. pullorum: 45 strains, as listed in table 2, were employed. They are from the collection of D. E. Schoenhard. They were originally isolated from a variety of sources in 10 different states of this country.

S. typhimurium: strain LT-2, a prototroph; strain LT-2 mutants: *cysA*-20, a cryptic mutant for sulfate and thiosulfate ions, which is deleted in all of the cistrons-a, b, and c, of the A locus (Dreyfuss, 1963); *cysJ*-538, a deletion mutant of the cysteine pathway which cannot reduce sulfite to sulfide, were used. *CysA*-20 and *cysJ*-538 are separated in their chromosomal location by approximately eleven minutes in experiments which determine time of entry during conjugation (Sanderson and Demerec, 1965). The involvement of the *cysJ* mutant in cysteine synthesis may be ascertained from the schematic pathway shown on page 15. These mutants are originally from the collection of M. Demerec and were kindly supplied by D. E. Schoenhard.

Phage

Phage P38, a generalized transducing phage of *S. pullorum*, was employed in this study. It has been described by Vaughan (1962). *S. typhimurium* phage P22 and Zinder's nonlysogenizing variant, H-4, were also employed in this study. They have been described previously by Zinder and

Lederberg (1952), Zinder (1958), and Adye (1962).

Media

The basal medium employed in this study was the minimal E medium of Vogel and Bonner (1956).

$MgSO_4 \cdot 7H_2O$	0.2 g
Citric acid$\cdot H_2O$	2.0 g
K_2HPO_4 (anhydrous)	10.0 g
$NaHNH_4PO_4 \cdot 4H_2O$	3.5 g
Glucose	5.0 g
Distilled water	1000 ml

Minimal E salts at single strength (E1X) is equivalent to the minimal E medium minus the carbohydrate.

Minimal E agar (E) was made by mixing a double strength, sterile, E salts solution at 45 C with an equal volume of sterilized, double strength (3% w/v) agar. The agar was supplemented with 0.5 g Na citrate before autoclaving. An aliquot of autoclaved 40% glucose solution (w/v) was added aseptically to make the final concentration of glucose 0.5% (w/v).

E soft agar was prepared the same as E agar, except the final concentration of agar was 0.7% (w/v).

Supplementation of E agar with amino acids was at a final concentration of 20 ug/ml of each amino acid. All organic supplements, except glucose, were sterilized by filtration through a millipore membrane, pore size, 0.45 μ. The final concentration of vitamins added was:

Thiamine$\cdot$HCl	1 μg/ml

Niacin	1 µg/ml
Ca pantothenate	2 µg/ml

Xanthine was also added to a final concentration of 5 µg/ml.

To indicate what supplementation was accomplished, the following abbreviations and their significance are employed in this communication: Ec, El, and Ecl indicate E agar with cysteine, leucine, and cysteine and leucine respectively. S agar is E agar supplemented with thiamine·HCl, niacin, calcium pantothenate, and xanthine to the final concentrations previously mentioned. The interpretation of the symbols Sc, Sl, and Scl is analogous to Ec, etc.

M-9 broth was employed in phage propagation. It was prepared according to Vaughn (1962).

2X M-9 Salts -- Each salt is added singly in the order listed until dissolved.

KH_2PO_4	6.0 g
Na_2HPO_4	12.0 g
NH_4Cl	2.0 g
Distilled water	1000 ml

Casamino acids (C.A.) -- 2X

Difco C.A. (lot 0230-01)	30.0 g
Distilled water	1000 ml
Norite A charcoal (activated)	2.0 teaspoons

This solution was mixed thoroughly and stored overnight at 4 C; filtered; and stored over chloroform until used.

M-9 Broth

2X M-9 salts	500.0 ml
2X Casamino acids	500.0 ml
1 M $MgSO_4 \cdot 7H_2O$	2.5 ml
25% NaCl	2.0 ml
20% Glucose	5.0 ml

The T2 buffer of Hershey and Chase (1952), employed as indicated, was prepared as follows:

Na_2HPO_4	3.0 g
KH_2PO_4	1.5 g
NaCl	4.0 g
K_2SO_4	5.0 g
$MgSO_4$ (0.1 M)	10 ml
$CaCl_2$ (0.01 M)	10 ml
Gelatin (1%)	1 ml
Distilled water	979 ml

Final pH, about 7.0

Autoclave at 10 lbs. pressure for ten minutes.

The indicator agars used for the detection of cysteine positive transductants were Si or El agar supplemented with 1.25% (v/v) reconstituted Difco nutrient broth. The Si enriched agar (Slem) was used for S. pullorum recipients. Slem or E enriched agar (Elem) was used for S. typhimurium recipients. According to Demerec and Hartman (1956), enrichment with nutrient broth increases the efficiency of transduction.

The complex media employed in this work were nutrient agar (NA), penassay agar (PA)--both purchased from the Difco Co., Detroit, Michigan--and tryptone agar. The tryptone agar was prepared as follows:

Tryptone, Difco		10.0 g
NaCl		8.0 g
Glucose		8.0 g
$CaCl_2$ (1 N)		0.1 ml
Agar	hard:	10.0 g
	soft:	7.0 g
Distilled water		1000 ml

Adjust pH to 7.0 and autoclave at 121 C for 15 minutes.

Nutritional Requirements

A. General

The auxanographic method of Beijernick, as described by Lederberg (1950), was employed in determining the nutritional requirements of the 45 strains of S. pullorum. Prior work by Robinson and Schoenhard (unpublished results) on a few of these strains had indicated a frequent requirement for cysteine and leucine. Earlier work by Schoenhard and Stafseth (1953) and Gilfillian et al (1955) had also indicated that a mixture of vitamins and xanthine was necessary for a rapid growth response in some of these same strains. Using these findings, the following test procedure was devised to pinpoint the requirements of the strains for cysteine, leucine, thiamine·HCl, niacin, Ca pantothenate and xanthine. The bacterial strain to be tested was grown 24 hours in 10 ml of penassay broth supplemented with

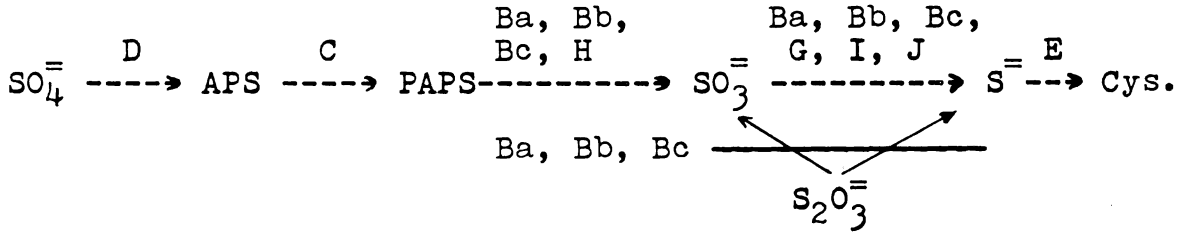
cysteine and leucine, 20 µg/ml of each. The cells were centrifuged; washed 2 times in equal volumes of E1X salt solution; and, finally, resuspended in an equal volume of minimal E broth. Next the minimal E broth suspension was incubated for 3 hours at 37 C in a water bath to starve the cells. To test the organisms, 0.3 ml of the starved cells were added to 3.0 ml of E soft agar (final concentration about 10^6 to 10^8/ml) in each of 8 test tubes. Then duplicate agar plates of E, Ec, El, and Ecl were overlayed with the soft agar-bacteria mixture. Each plate of a set, consisting of E, Ec, El, and Ecl, was then spotted with 4 disks, each disk containing 1 of the following 4 compounds in the amounts indicated: thiamine·HCl, 0.0025 µg; Ca pantothenate, 0.005 µg; niacin, 0.0125 µg, and xanthine, 0.0125 µg. The remaining set of plates was used to test the various combinations of cysteine and leucine. Although most positive results appeared within 15 to 24 hours at 37 C on the plates with adequate supplementation, incubation was prolonged to observe for back mutants or delayed growth responses. If no growth or poor growth resulted on the most highly supplemented plate, additional crystal tests with amino acids and/or vitamins were performed to determine the exact requirements. All tests were performed at least twice.

B. Specific--Cysteine Pathway

The nutritional classification of cysteine requiring mutants of S. typhimurium was originally performed by Clowes (1958). Since then it has been confirmed and extended by Dreyfuss and Monty (1963). The working assumption is made

that cysteine synthesis in S. typhimurium and S. pullorum is the same. To substantiate a nutritional finding in S. pullorum, deletion mapping utilizing phage propagated on known deletion mutants of S. typhimurium was performed.

Cysteine is synthesized from sulfide (reduced enzymatically from sulfate) and serine by the action of the enzyme serine transulfhydrase (Schlossman and Lynen, 1957). The pathway of sulfate reduction in S. typhimurium has been determined by Dreyfuss and Monty (1963) and is as follows:



(APS = adenosine-5'phosphosulfate; PAPS = 3'-phosphoadenine-5'phosphosulfate.) D, C, H, J, I, B, and E represent the corresponding mutant cysteine genes controlling the indicated step. Dreyfuss and Monty (1963) established the relationships between the sulfur sources utilized and the mutant gene as indicated in Table 1.

Therefore, to determine the nature of the cysteine deficiency, soft agar auxanography was performed with cysteine sulfinic acid (CSA), sulfite, and thiosulfate, 50 ug each per disk. Special sulfur-free E medium was used in which 0.2 g of $MgCl_2 \cdot 5H_2O$ was substituted for the $MgSO_4$ and Nobel (Difco) agar was substituted for regular agar. Sulfide utilization was tested in screw cap test tubes, utilizing sulfur free E1 broth and a filter-sterilized solution of Na_2S, final concentration of $S^=$ about 4×10^{-4} M.

Table 1. Growth responses of S. typhimurium cys mutants to various potential sulfur sources*

Sulfur Source	Mutant Classification					
	C,D,H	J,I,G	Ba, Bc	Bb	Ea,Eb	Aa,Ab,Ac
$SO_4^=$	-	-	-	-	-	-
CSA or $SO_3^=$	+	-	Slow	-	-	+
$S_2O_3^=$	+	+	-	-	-	-
$S^=$	+	+	+	+	-	+
Cysteine	+	+	+	+	+	+

*Dreyfuss and Monty, 1963.

Preparation of Transducing Lysates

The phage were cloned three times on donor cells, using the tryptone soft agar technique of Adam's (1959), prior to their usage in the production of transducing phage. One-tenth ml of an overnight M-9 broth culture of bacteria was transferred to 10 ml of fresh M-9 broth. The culture was aerated for three hours at 37 C. Phage particles were added to a final concentration of about 10^6/ml. The culture was then incubated at 37 C until cellular debris accumulating on the walls of the tube indicated extensive lysis had occurred. One ml of chloroform was then added and the incubation with aeration was continued for 3 to 5 minutes. Next the culture was centrifuged at 6,000 x g for 10 minutes to remove the cellular debris. The phage in the supernatant were assayed on the homologous cells. Phage stocks were

stored over chloroform at 4 C.

Transduction

Ten ml of an aerated, overnight PA culture were centrifuged at 6,000 x g for 10 minutes and resuspended in 1 ml of T2 buffer. All transducing phage lysates were diluted to 5×10^9/ml and irradiated, while shaken, with a UV light for 50 seconds at 41 cm. The lamp was a G. E. Germicidal, G_3OT8. Approximately 99.99% of the phage were not able to form plaques after the irradiation. The transduction was performed by adding 0.1 ml of the cell suspension to 0.9 ml of pre-warmed (37 C), irradiated phage (m.o.i. = 5). The mixture was incubated at 37 C for 10 minutes before plating 0.2 ml aliquots in triplicate on the indicator agar. Five times the number of cells per transductional plate were plated on control plates of the same agar to determine back mutation frequencies. Two-tenths ml of phage lysate without recipient cells were also plated on the indicator agar as a control of the sterility of the phage lysate. Furthermore, lysates were tested qualitatively for transductional potency before use, and recipient cells were tested concurrently for transducibility by phage lysates of "wild type" S. typhimurium or, if applicable, by nonallelic deletion mutants. Incubation was for 72 hr at 37 C. Representative transductants were identified on the basis of colonial morphology and somatic antigens D or B .

RESULTS

The overall motivation of the work, to be reported in this section, is the development of a deeper understanding of the biochemical and genetic nature of S. pullorum. Data of this nature are being gathered in conjunction with the results of other studies so that a systematic investigation of virulence may be conducted at the molecular level. This particular work falls into 3 main categories: nutritional studies, genetic studies, and preliminary molecular studies of one of the nutritional requirements, the cysteine deficiency.

A brief comment on the significance of the incubation period employed in the first of these studies is in order. Lederberg (1950) reported that Escherichia coli showed positive growth responses to supplementation in 6 to 10 hrs if the soft agar overlay technique was used. A longer period of incubation has been found necessary with S. pullorum to observe positive results. This observation is not at all surprising in view of the smaller size and slower growth rate of the latter. In most of the strains used in these tests, positive results occurred in 15 to 24 hrs. A strong response at 48 hrs was also taken as evidence of completed supplementation. A weakly positive response after 48 hrs was taken as evidence of an additional leaky requirement. The responses of the S. pullorum strains to the various combinations of

amino acids and vitamins are listed in Table 2.

Zinder and Lederberg (1952) and Zinder (1953) reported that transduction, not transformation, occurred with S. typhimurium phage lysates. Likewise, Snyder and Schoenhard (1960) reported transduction with S. pullorum phage lysates. Furthermore, Schoenhard (1963) reported on the transduction of S. pullorum by phage lysates of S. typhimurium and visa versa. Thus, it is the object of the second of these studies not to show the means of genetic transfer, but the fact of genetic transfer. The results of the crosses between phage lysates prepared on prototrophic S. typhimurium and the various S. pullorum recipient strains showed that all the strains listed in Table 2, except strain 11, were transducible to cysteine prototrophy. Strains 13, 16, 26, 50, and 54 were indeterminable due to high reversion frequencies on the indicator agar. The highest transductional frequency observed was 5.5×10^{-8}, representing approximately 50 colonies per plate.

In view of the frequency of both the cysteine and leucine requirements of these strains, it is of fundamental interest to ask if these mutants are all deficient in a common step in their respective synthetic pathways or in different steps. Thus, the object of the third part of the study is defined.

Preliminary characterization of a cys mutant was accomplished in S. typhimurium using the crystal tests described by Dreyfuss and Monty (1963). The pattern of responses they found are listed in Table 1. The same tests were applied to

the S. pullorum strains. The primary result (Table 3) was that 84% of the 45 strains tested gave a qualitative response indicative of either a cysA or a cysBa,c mutant. However, in these tests the growth responses of the strains to CSA were very slow and very weak, but the responses to sulfite, while not as strong as the responses to cysteine in comparable tests, were rapid.

With S. typhimurium, cysA mutants can be distinguished from cys Ba,c mutants by the generation time. Dreyfuss and Monty (1963) reported that cysA mutants with CSA as a sulful source, have a generation time equivalent to that of the cys^+ genotype; whereas cysBa,c mutants with CSA as the sulfur source have a generation time approximately 3 times longer. Because of the instability and toxicity of sulfite, and the extremely weak response of S. pullorum to CSA, this method of resolution is eliminated. However, a cysA deletion mutant of S. typhimurium was available to use for deletion mapping. All the strains that were transducible with phage propagated on cys^+ S. typhimurium, were tested for transducibility with phage lysates from strain cysA-20. Only strain 14 was found to be transducible. Strain 14 was found to be a cysBb mutant by the crystal tests, and thus this transduction result was expected. Strains 33, 43, and 45 also were found to be cysBb by the crystal tests, but they were not transducible in any of several attempts. These latter results rendered all the negative results of the S. pullorum X cysA-20 transductions suspect. Therefore, a very carefully controlled transductional analysis of one of

the strains, 35, was performed to ascertain if the negative results with cysA-20 donor in this strain were due to the nature of the transductional manipulations or materials. The results shown in Table 4 indicate that the negative results are not due to non-transducing phage lysates, non-transducible recipients, or the conditions employed.

The data recorded in Table 2 indicate that both strains 50 and 54 can be grown on Ec medium plus thiamine or leucine at 37 C. It was accidentally discovered that strain 54 grows at room temperature on E medium plus thiamine. Tests at 25 C of strain 54 in minimal broth resulted in just visible growth (1-5 x 10^7 cells/ml) in 15 days; whereas a similar growth response using the same quantity (1% v/v) of inoculum in minimal broth plus thiamine·HCl (0.05 ug/ml) required only 6 days. In another type experiment 5-10 x 10^7 cells were spread on minimal agar and minimal agar plus thiamine to observe the nature of the responses and the frequency of back mutations. A heavy, confluent lawn of growth on minimal agar plus thiamine was observed in 10 days; whereas approximately 500-1000 distinct colonial shapes against a light background of growth were observed after 16 days incubation of the minimal agar plates. In both of the aforementioned experiments the culture used for the inoculum was washed 3X with EIX salts solution prior to usage.

Table 2. Growth at 37 C of *S. pullorum* strains related to amino acids, vitamins and a purine

Strain of *S. pullorum*	Set I: E medium supplemented with amino acids				Set II: Same as Set I, plus each plate contains 4 disks impregnated respectively with thiamine, niacin, pantothenate and xanthine			
	–	Cys	Leu	Cys, Leu	–	Cys	Leu	Cys, Leu
3				+		tp		w
4				+		tp		w
5				+		tp		w
6				pro				
9				+ –		weak nia		nia
10				+		tp		w
11				+				w
12				+				w
13			bm	+				w
14		bm		+		tp		w
15				+				w

Table 2 (Continued)

Strain of *S. pullorum*	Set I				Set II			
	–	Cys	Leu	Cys,Leu	–	Cys	Leu	Cys,Leu
16			bm	±		tp		nia
17				+				w
18				+				w
19		bm		+		tp		w
20				+		tp		w
21				+				w
22				+				w
23		bm		+				nia(w)
24		bm		+				w
25		bm		+		tp		w
26		bm	bm	+		tp		w
27		bm		+		tp		w
28				+				w
29		bm		+		tp		w
30		bm		+				w

Table 2 (Continued)

Strain of *S. pullorum*	Set I				Set II			
	–	Cys	Leu	Cys,Leu	–	Cys	Leu	Cys,Leu
31		bm		+		t		w
32				+				w
33		bm		+				w
34				+				w
35		bm		+		t		w
36				+				w
37		bm		+		tp		w
38				+				w
40		bm		+		t		w
43				+				w
45				+				w
46		bm		+		t		w
47				+				nia(w)
48				+		t		w
49		bm		+		tp		w

Table 2 (Continued)

Strain of *S. pullorum*	Set I				Set II			
	–	Cys	Leu	Cys, Leu	–	Cys	Leu	Cys, Leu
50		bm	bm	+		thia		w
51				+		tp		w
53				+				w
54			bm	±		weak thia		weak thia*

Set I

\+ = confluent, opaque growth over whole plate within 48 hours.
± = confluent, translucent growth over whole plate within 48 hours.
pro = confluent opaque growth circumscribing point on agar surface seeded with proline crystals.
bm = random, isolated colonies of *S. pullorum*; apparently back mutants.

Set II

thia, nia, xan, panto } confluent, opaque growth circumscribing the disk impregnated with the indicated chemical.

w = confluent, opaque growth over whole plate.
nia(w) = confluent, translucent growth over whole plate, greater opacity circumscribing nia disk.
weak nia, weak thia } confluent translucent growth circumscribing niacin or thiamine disks.
t = discrete colonies around thiamine disk.
tp = discrete colonies around both thiamine and pantothenate disks.
* = greater opacity than supplemention without leucine.

Table 3. Growth responses of S. pullorum and S. typhimurium strains to potential sulfur sources

Cultures	Agar plate tests				Broth	Agar and/or broth
	CSA 5 days	$SO_3^=$ 2-4 days	$S_2O_3^=$ 5 days	$SO_4^=$ 5 days	$S^=$ 1 day	No S 5 days
S. pullorum:						
3, 4, 5, 6, 9, 10 11, 12, 17, 18, 19 20, 23, 24, 25, 27 28, 30, 31, 32, 34, 35 36, 37, 38, 40, 46, 48, 51, 53	weak	+	-	-	+	-
16, 21	weak	-	-	-	+	-
15, 22, 26, 29	-	+	-	-	+	-
13, 14, 33, 43, 45 47, 50, 54	-	-	-	-	+	-
S. typhimurium *						
cysA-20	+	+	-	-	+	-
cysBac	+	+	-	-	+	-
cysBb	-	-	-	-	+	-
cysJ-538	-	-	+	-	+	-

Agar plates were made with sulfur-free E medium as described in the Materials and Methods section. Likewise, the minimal broth was sulfur free. The agar and broth were supplemented with the appropriate amino acids and niacin indicated in Table 2.

*Positive response within 24 hours.

Table 4. Summary of results of deletion mapping of *cys* marker in *S. pullorum* 35.

Lysates of donor bacteria	Recipient Bacteria*				
	S. typhimurium			*S. pullorum*	
	cysA-20	*cysJ*-538	*tryA*-4	*cys*	*his*
S. typhimurium					
cys^{+}LT2	75.0	75.0	75.0	2.0	1.7
cysA-20	0.0	100.0		0.0	1.7
cysJ-538	4.0	0.0		0.14	
S. pullorum					
35	0.0	0.0	0.2	0.0	

*Listed according to species and marker transduced.

Numerical values represent frequency of transduction x 10^{-8} and are calculated as follows:

$$\frac{\text{No. recombinants/ml}}{\text{No. of plaque forming units/ml}}$$

Procedures are described in the Materials and Methods section.

DISCUSSION

The results of the nutritional studies at 37 C show that of the 45 strains of S. pullorum tested all require at least cysteine and leucine. Moreover, in addition to these amino acids, 1 strain requires proline. Niacin and thiamine stimulate growth resulting in a larger population. This stimulation is true for 4 strains and 1 strain respectively. Furthermore, in 1 strain, which neither requires nor is stimulated by thiamine, thiamine replaces leucine. In the strain stimulated by thiamine, thiamine also replaces leucine, but growth is heaviest when thiamine and leucine are combined. It also appears that thiamine and pantothenate stimulate to varying degrees the apparent leu ---> leu^+ reversion in a large number of the strains.

The majority of the strains respond to various inorganic sulfur ions corresponding to the cysA or cysBa,c type of mutants defined in S. typhimurium. Genetic evidence indicates in one of these strains that it is the cysA mutation which is present. Recombination at the cys locus of 40 of 41 strains of S. pullorum occurs, since they were transduced to cysteine independence by lysates of prototrophic S. typhimurium, LT-2.

The findings of a general nutritional requirement for cysteine and leucine are in general agreement with those of Lederberg (1947) and Stokes and Bayne (1958b). Johnson and Rettger (1943) did not include information on the cysteine requirement in 10 strains of S. pullorum, which they assayed.

Five of these strains required leucine. They did find, however, that in 2 of 45 strains tested for vitamin requirements, 2 strains required niacin. A comparison of the foregoing studies and my study indicate the amino acid and vitamin requirements are substantially similar.

With respect to the transducibility of S. pullorum by phage lysates of S. typhimurium, the observation of Schoenhard (1963) has been confirmed and extended to include 38 more strains of S. pullorum.

No studies have been reported about the utilization of various sulfur ions in cysteine requiring S. pullorum strains. The findings of this work show that the mutants fall into primarily 2 groups, those that utilize either sulfide or cysteine as a sulfur source, and those that utilize sulfite, sulfide, or cysteine as a sulfur source. In Neurospora (Horowitz, 1955) and S. typhimurium (Clowes, 1958) CSA has been found to be nutritionally equivalent to sulfite. Lineweaver and Monty (1961) have demonstrated with cell free preparations of S. typhimurium that CSA is desulfinated to sulfite. Therefore, one could readily imagine 3 possible situations whereby CSA is weakly or not at all utilized: 1) cells are impermeable to CSA, 2) cells lack or have an altered enzyme responsible for desulfination of CSA to yield sulfite, ammonia, and pyruvate, or 3) there is a branched pathway in which CSA is a precursor to cysteine, but it is genetically blocked. Since at the present moment the number of steps involved in sulfate reduction by S. pullorum and the number of genes controlling each step are unknown, it

is impossible to choose which one, if any, of the above 3 situations is correct.

The genetic data gathered to resolve whether or not the S. pullorum deficiency is analogous to the cysA or cysBa,c, lesion, as defined in S. typhimurium, is at best weak. This follows from the following reasons: 1) the reciprocal crosses of S. pullorum donor and S. typhimurium recipients always gave negative results, even though the phage lysate was known to be a good transducing lysate, as judged by its capacity to transduce other markers from S. pullorum to S. typhimurium. (Schoenhard, 1963), and 2) whereas the cysteine loci in S. typhimurium are in 5 different regions on the chromosome (Mizobuchi et al, 1962), this situation may not prevail in S. pullorum. Thus, interspecies transductions in either direction may be hindered by nonhomology (Zinder, 1960) if the cotransduction of nonallelic outside markers occurs. To visualize this last statement more clearly, imagine that integration may proceed by way of breakage and reunion (Fox and Allen, 1964) according to a model analogous to the triple stranded DNA-messenger RNA complex suggested by Zubay (1962), and discussed recently by Schaeffer (1964). Thus, only stretches of donor cys^+ determining DNA, unlinked to nonhomologous, nonallelic DNA, are potentially capable of integration. This hypothetical explanation only accounts for a reduced frequency of transduction. It still does not explain the unidirectional transduction observed in S. pullorum x S. typhimurium cysJ-538 crosses. To do this, it is necessary to define speculatively more a priori states of the donor and recipient genomes.

This is really not warranted by the data.

The finding that thiamine substitutes for leucine in fulfilling one of the nutritional requirements of both strains 50 and 54 is an unexpected observation. Also related to this observation is the apparent increased frequency of *leu* --> *leu*$^+$ back mutations in the presence of thiamine and pantothenate. These observations may not be the first of this type. Stokes and Bayne (1958b) observed that 10 *S. gallinarum* strains, which took seven days for appreciable growth in minimal broth supplemented with thiamine, produced full growth in 1 day if the supplementation was casein hydrolysate or a mixture of cysteine, leucine and aspartic acid. These findings were not further pursued in any depth.

SUMMARY

The predominant nutritional requirements of the 45 strains of S. pullorum tested in this study were found to be cysteine and leucine. Five of the 45 strains have an additional leaky requirement for a vitamin, 4 requiring niacin and 1 requiring thiamine.

Nutritional studies of the cysteine requirement indicated that there are primarily 2 groups of organisms. The largest group, comprising approximately 80% of the strains, grew on either sulfite, or sulfide, or cysteine as a sulfur source. The remaining group, comprising 13% of the strains, grew on either sulfide or cysteine as a sulfur source.

Interspecies transductional tests lent support to the inference that one strain, 35, which belongs to the larger of the above 2 groups, corresponds to the cysA type mutant of S. typhimurium. This mutant is impermeable to sulfate and thiosulfate ions. Other interspecies transductional tests indicate that 40 of 41 strains of S. pullorum are transducible by lysates of "wild type" S. typhimurium.

The leucine deficiency was found to have an undetermined association with the vitamins thiamine and pantothenate as evidenced by an apparently increased back mutation rate to leucine prototrophy.

BIBLIOGRAPHY

Adams, M. H. 1959. Assay of phage by agar layer method, pp. 1450-451. In M. H. Adams, Bacteriophages. Intersciences Publishers, Inc., New York.

Adye, J. 1962. Effects of nitrous acid on transduction by Salmonella phage P22. Virology 18:627-632.

Clowes, R. C. 1958. Nutritional studies on cysteineless mutants of Salmonella typhimurium. J. Gen. Microbiol., 18:140-153.

Demerec, M. and Z. Hartman. 1956. Tryptophan mutants in Salmonella typhimurium. Genetic studies with bacteria, Carneg. Inst. Wash. Pub. 612:5-17.

Demerec, M., E. L. Lahr, T. Miyake, I. Goldman, E.Balbinder, S. Banic, K. Hashimoto, E. V. Glanville, and J. P. Gross. 1957. Bacterial genetics. Carneg. Inst. of Wash. Yearbook 57:390-406.

Dooley, E. S., D. F. Holtman, and C. D. Jeffries. 1958. Alterations in the blood chemistry of chicks treated with the endotoxin of Salmonella pullorum. J. Bacteriol. 75:719-729.

Dreyfuss, J. 1964. Characterization of a sulfate- and thio-sulfate-transportation system in Salmonella typhimurium. J. Biol. Chem. 239:2292-2297.

Dreyfuss, J. and K. J. Monty. 1963. The biochemical characterization of cysteine-requiring mutants of Salmonella typhimurium. J. Biol. Chem. 238:1019-1024.

Fildes, P., G. P. Gladstone, and B. C. J. G Knight. 1933. The nitrogen and vitamin requirements of B. typhosus. Brit. J. Exptl. Pathol., 14:189-196.

Fox, M. S. and M. K. Allen. 1964. On the mechanism of deoxyribonucleate integration in pneumococcal transformation. Proc. Natl. Acad. Sci. U. S. 52:412-420.

Gilfillan, R. F., D. F. Holtman, and R. T. Ross. 1955. A synthetic medium for propagation and maintenance of virulent strains of Salmonella pullorum. Poultry Sci. 34:1283-1288.

Hershey, A. D., and M. Chase. 1952. Independent function of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. 36:36-59.

Horowitz, N. H. 1955. p. 631-633. In W. D. McElroy and B. Glass (ed.), Amino acid metabolism, Johns Hopkins Press, Baltimore.

Johnson, E. A. and L. F. Rettger. 1943. A comparative study of the nutritional requirements of Salmonella typhosa, Salmonella pullorum, and Salmonella gallinarum. J. Bacteriol. 45:127-135.

Kirchner, C. E. J. 1960. The effects of the mutator gene on molecular changes and mutation in Salmonella typhimurium. J. Mol. Biol. 2:331-338.

Kuwahara, S., S. Yasushi, T. Owa, and M. Shinoda. 1958. Nutrition of Salmonella pullorum and Salmonella gallinarum. I. Growth factor and amino acid requirements. Japan. J. Microbiol. 2:343-353.

Lederberg, J. 1947. The nutrition of Salmonella. Arch. Biochem. 13:287-290.

Lederberg, J. 1950. Isolation and characterization of biochemical mutants of bacteria. p. 5-50. In R. W. Gerard (ed). Methods in Medical Research, vol. 3. Year Book Publishers, Chicago.

Leinweber, F. J., and K. J. Monty. 1961. The mode of utilization of cysteine sulfinic acid by bacteria. Biochem. and Biophys. Research Communs. 6:355-358.

Morse, M. L. 1954. Transduction of certain loci in Escherichia coli K-12. Genetics 39:984-985.

Mizobuchi, K., M. Demerec, and D. H. Gillespie. 1962. Cysteine mutants of Salmonella typhimurium. Genetics 47:1617-1627.

Ozeki, H. 1956. Abortive transduction in purine-requiring mutants of Salmonella typhimurium. Genetic studies with bacteria. Carneg. Inst. Wash. Pub. 612:97-106.

Ozeki, H. 1959. Chromosome fragments participating in transduction in Salmonella typhimurium. Genetics 44:457-470.

Sanderson, K. E., and M. Demerec. 1965. The linkage map of Salmonella typhimurium. Genetics. 51:879-913.

Schaeffer, P. 1964. Transformation, p. 87-153. In I. C. Gunsalaus and R. Y. Stanier (ed.), The bacteria, vol. 5. Academic Press, Inc., New York.

Schlossman, K. and F. Lynen. 1957. Biosynthese des cysteins aus serin und schwefelwasserstaff. Biochem. Z 328: 591-594.

Schoenhard, D. E. 1963. Transduction of Salmonella pullorum by PLT-22 phage. Bacteriol. Proc., p. 30.

Schoenhard, D. E., and H. Stafseth. 1953. Growth curves of Salmonella pullorum in different media. J. Bacteriol. 65:69-74.

Snyder, R. W. and D. E. Schoenhard. 1960. Isolation of Salmonella gallinarum mutants and their use in transduction experiments. Bacteriol. Proc., p. 79.

Stokes, J. L. and H. G. Bayne. 1957. Growth rates of Salmonella colonies. J. Bacteriol. 74:200-206.

Stokes, J. L. and H. G. Bayne. 1958a. Dwarf colony mutants of Salmonellae. J. Bacteriol., 76:136-141.

Stokes, J. L. and H. G. Bayne. 1958b. Growth-factor dependent stains of Salmonellae. J. Bacteriol. 76: 417-421.

Vaughan, R. W. 1962. Generalized transduction of the galactose marker in Salmonella pullorum. M. S. Thesis, Michigan State University, East Lansing, Michigan.

Vogel, H. J. and D. M. Bonner. 1956. Acetylornithase of Escherichia coli partial purification and some properties. J. Biol. Chem. 218:97-106.

Watson, J. D. and F. H. C. Crick. 1953. The structure of DNA. Cold Spr. Harb. Symp. quant. Biol. 18:123-131.

Zinder, N. D. 1953. Infective heredity in bacteria. Cold Spr. Harb. Symp. quant. Biol. 18:261-269.

Zinder, N. D. 1958. Lysogenization and superinfection immunity in Salmonella. Virology 5:291-326.

Zinder, N. D. 1960. Hybrids of Escherichia and Salmonella. Science 131:813-815.

Zinder, N. D. and J. Lederberg. 1952. Genetic exchange in Salmonella. J. Bacteriol. 64:679-699.

Zubay, G. 1962. A theory on the mechanism of messenger RNA synthesis. Proc. Natl. Acad. Sci. U. S. 48: 456-471.