PIGMENT PRECURSORS IN WHITE CLINICAL *SERRATIA MARCESCENS* STRAINS

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Growth curves were determined for both pigmented and nonpigmented Serratia marcescens strains, and were determined to be identical for both types. Prodigiosin and MBC production were assayed in Nima, 9-3-3 and 9-3-3/WF cultures. This data was used to illustrate that production of the precursors is not on a stoichiometric and utilization basis. Nonpigmented strains were observed to produce faint syntrophic pigments when grown next to strain 9-3-3, and a technique was developed to better visualize this pigment. MBC, supplied in great enough quantities, allowed the formation of a variety of colors on MMH agar, as well as on TS agar. The range of temperature and the amounts of oxygen necessary for production of these pigments were explored and compared with that of the mutant WF. Antibiotics were also employed. It was determined that, while the production of the probable precursor produced by the clinical strains was less sensitive to temperature than WF's MAP, it was equally sensitive to oxygen concentrations and more sensitive to streptomycin. A model of pigment and precursor formation was developed.

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D.S.K.

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INTRODUCTION

History

Bizio first isolated Serratia marcescens polenta (from cornmeal mush) and named it in 1823 (7, 29). The name was later forgotten, and was not reassigned to the bacterium until later in this century. In between, 21 different names were given to the red bacterium, such as Micrococcus prodigiosus, Bacillus prodigiosus, Salmonella marcescens, and Chromobacterium prodigiosum (29).

The historical reports of red organisms growing on various items date back to the Middle Ages, and possibly even back to Biblical times. Its bright red pigment, which is outstanding even today, has often been mistaken for fresh blood. Many times throughout history, the appearance of "miraculous blood" has been reported. Since the "miraculous blood" has only recently been explained as being the pigment produced by Serratia, superstition often led to violence to defend the "blood," especially when it appeared on Holy Communion wafers.

Description

Serratia are Gram negative motile rods, and among other characteristics, are easily separated from closely related bacteria by being unable to ferment arabinose, having peritrichious flagella, and producing extracellular DNase (98). Serratia marcescens' pigment was long ago accepted as the characteristic nature of the bacterium. However, biochem-

ical tests have shown that 70% or more of the strains isolated today do not produce the pigment (24, 39, 82, 96). In addition biochemical tests are being developed to specifically identify Serratia which is being increasingly found as a human pathogen. This view of Serratia as an etiological agent is in conflict with the past conception of Serratia as a harmless saprophytic nonpathogen (25, 53, 81, 110).

Infection by Serratia

Serratia has not always been categorized as a pathogen in its interactions with man (8, 13, 56), and is considered to be a member of the normal microbial flora. Serratia has also been known to infect humans without causing any disease symptoms. The "red diaper syndrome" (87) refers to colonization of the gastrointestinal tract in infants by S.marcescens which became evident only when the soiled diapers were incubated at room temperature long enough for the bacteria to increase their numbers and produce the pigment. This "syndrome" apparently caused no harm to some of the infants who harbored Serratia in their gastrointestinal tracts (54, 87).

Serratia's pathogenicity was first recognized in 1913 (2), but was not fully accepted until 1963, when it was reported as a pathogen in paraplegic patients (8). However, most Serratia infections are nosocomial or opportunistic, usually infecting compromised or predisposed patients (1, 8, 57, 58), although bacteriological warfare tests by the U.S. Army were blamed for the death of one individual in 1950 in San Francisco (27, 62). Infection occurs in the newborn, children (3, 54) and in adults, with organisms found in the urine, sputum, wounds and

and blood. The majority of Serratia isolates are from nosocomial infections either of the urinary tract, particularly in catheterized patients (82, 86), or in the lungs and bronchioles (15). It can be controlled in the hospital environment by strict aseptic procedures and by hand washing (19).

Genetics and Drug Resistance

In 1956 Belser and Bunting (5) proved that conjugation existed in the genus when they found genetic markers transferred in sequence between cells. Thomas et al. (86) found, in a hospital epidemic, that multiple drug resistance had apparently been transferred to members of the genus Klebsiella in the same hospital. This finding was confirmed in vitro when plasmids conferring drug resistance were shown to pass from Serratia to both Klebsiella and Escherichia coli. Transmission of resistance plasmids in urine between Serratia and E. coli was demonstrated by Schaberg et al. (78). This was an important step in providing proof of plasmid transmissibility in the hospital environment. Because of this promiscuity of genetic exchange among the Gram negative rods, treatment of infections caused by Serratia and other Gram negative rods has become more complicated to handle than previously.

Patterns of Serratia's antibiotic resistances seem to be ecologically widespread. For example, an antibiogram-carrying strain of Serratia was found in four hospitals, probably carried on the hands of hospital personnel between the hospitals (77). Other isolates of multiply resistant Serratia are known (60, 82, 96), including instances of the development of resistance after usage of the drug for several months (46).

Identification and Speciation

As more and more reports on the pathogenicity of Serratia are published, hospital laboratories have begun to use all available techniques to identify isolates. As the state of the art of Serratia taxonomy has grown, so has the body of knowledge about Serratia, with the result of greater numbers of Serratia being isolated than in the past. Because of the greater accumulation of information, the definition of Serratia has changed through the years. This definition has changed to accommodate the growing number of Serratia isolates, as well as the developing biochemical differentiation tests, and the reassigning of species from one tribe to another. Thus, Serratiae has been identified as containing from one to seven species. A review of the history of the species assigned to Sarratiae is necessary.

Bergey's Manual of Determinative Bacteriology, 7th edition (7) recognized only five Serratia species when published in 1957, but in that same year, Davis, Ewing and Reavis (20) recommended that only one species, Serratia marcescens, be recognized because of homogeneity of biochemical reactions of some of the species of Bergey's. The 8th edition of Bergey's (9) lists only Serratia marcescens. However, Edwards and Ewing (24) later determined that Enterobacter liquefaciens should be transferred to the genus, Serratia, and be assigned the specific epithet Serratia liquefaciens. In 1974, Lennette et al. (48) recognized three species, Serratia marcescens, Serratia liquefaciens, and Serratia rubidaea.

In this thesis the three species of Lennette et al. are recognized. Serratia marcescens is identified as being unable to ferment arabinose,

while Serratia rubidaea and Serratia liquefaciens are able to ferment arabinose. Serratia rubidaea lacks ornithine decarboxylase, and produces red pigment, while Serratia liquefaciens produces ornithine decarboxylase (Table I).

In another study, Grimont and Dulong de Rosnay (38) recognized at least three taxa in a numerical taxonomic study of Serratia, and identified these as Serratia marcescens, Serratia marinorubra, and Serratia plymuthica. In their taxonomic scheme, S. marinorubra is identical to S. rubidaea but Serratia plymuthica possesses characteristics of Serratia killiensis, which had earlier been accepted as a subspecies of marcescens by Davis et al. (20).

Most recently Grimont et al. (1977) recognized four separate Serratia species, three of which are able to produce pigment: Serratia marcescens, S. plymuthica, and S. marinorubra (39). The fourth species is colorless, Serratia liquefaciens. In addition, their work was unable to taxonomically place several isolated variant strains.

Pigment Structure

Because of the visual and chemical resemblance to blood hemoglobin by the red pigment of both Serratia marcescens and S. rubidaea, early work investigated the porphyrin molecules present in both blood and prodigiosin, in order to compare the similarities and differences in their metabolic pathways. However, Serratia marcescens was found to be unable to use δ-aminolevulinic acid as a precursor to make prodigiosin, yet the acid is used in the synthesis of hemoglobin. Although prodigiosin is known to be a pyrryldipyrrylemethene structure, confusion existed as to

TABLE I. Characteristics of Lennette *et al.*'s *Serratia* Taxonomy.

Species	Pigment Produced	Arabinose Fermentation	DNase Production	Ornithine Decarboxylase Production
S. marcescens	+	-	+	+
S. liquefaciens	-	+	+	+
S. rubidaea	+	+	+	-

+ indicates the species possesses the characteristic or an ability to exhibit a positive biochemical test.

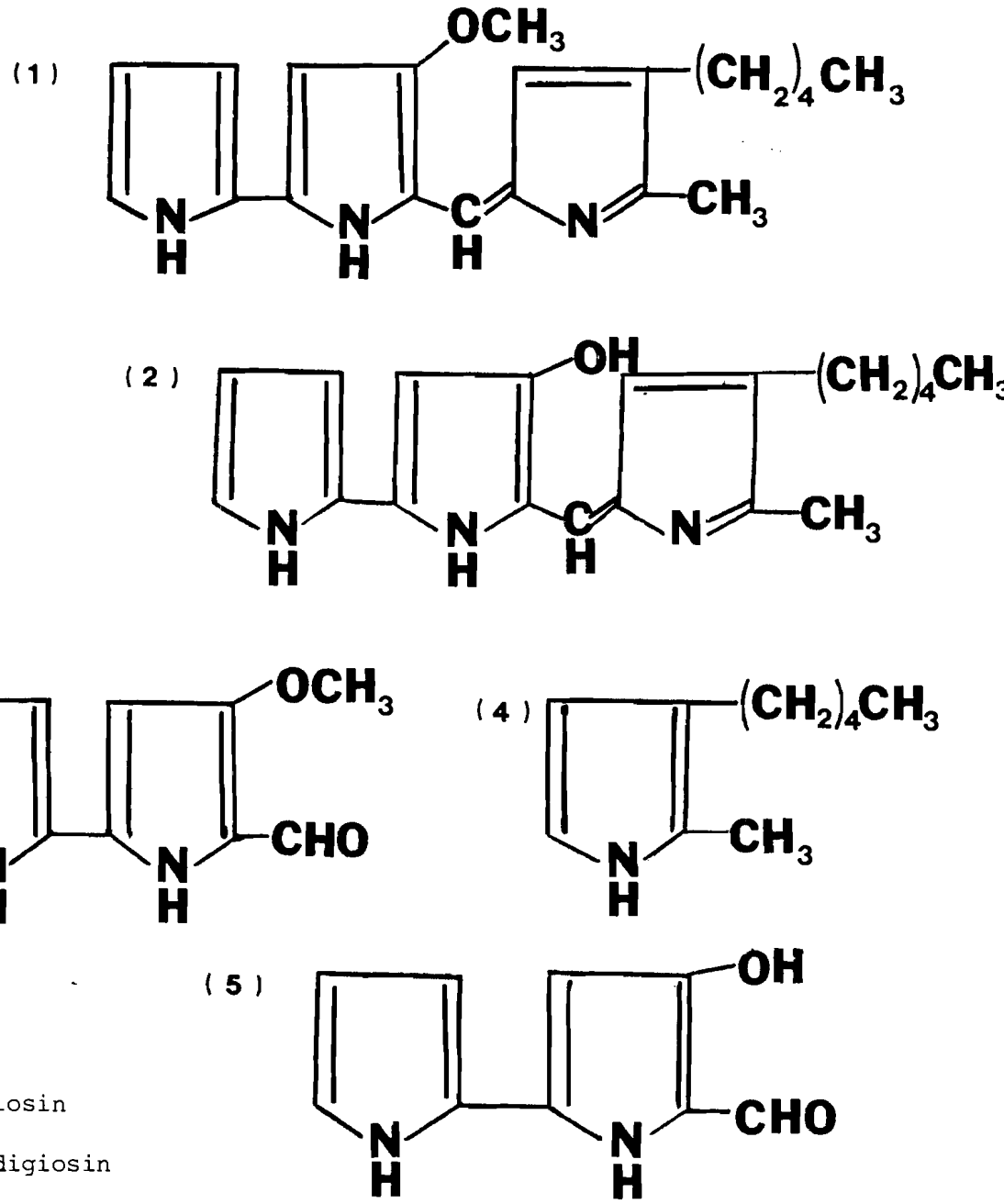
its exact structure. In 1960, Wasserman et al. (89) and Rapoport and Holden (70) both published work disproving a proposed nonlinear tripyrrole structure and offering proof that prodigiosin was linear in structure. Even before 1960, however, it was believed that prodigiosin was made up of two components, a bipyrrole and a monopyrrole, that were combined to form the molecule, prodigiosin (Fig. 1). Confirmation of the structure of this bipyrrole precursor came from Wasserman et al. (90). The mutants and techniques they used will be discussed in the next sections.

Other Serratia Pigments

Serratia is not the only microorganism capable of producing a red pigment. Gerber isolated a prodiginine (prodigiosin-like) pigment from Streptomyces and from other aerobic Actinomycetes (30, 31), and a prodiginine has been found that causes a pink stain on polyvinyl chloride (32). Hearn et al. have suggested, in view of the other prodigiosin-like compounds mentioned, that new nomenclature be adopted (40). The basic pyrryldipyrrylemethene structure, Gerber's prodiginine, was renamed prodigiosene.

Prodigiosin is not the only pigment produced by Serratia. Taylor and Williams (85) were unable to identify a particulate bound pigment of Serratia as prodigiosin. Prodigiosin itself is considered to be a comgination of several pigments. Green et al. (36) isolated four pigments chromatographically, one blue and the other three, red (101), and an orange component has also been visualized (105). The blue fraction had a greater molecular weight than the combined red fractions, but the

Figure 1. Structure of Prodigiosin and Its Known Precursors.



(1) Prodigiosin

(2) Norprodigiosin

(3) MBC

(4) MAP

(5) HBC

absorbence spectrum was similar to the red fractions. The colors of the wild-type and mutant strains can also vary quite widely, from pinks to blue or purple, and also to deep reds and oranges. Wasserman et al. (88) discovered a blue prodigiosin analog, a dipyrryldipyrromethene, from a mutant Serratia strain. Strain 9-3-3, which will be discussed in the next section, is able to produce a purple pigment which has been thought to contain bipyrrole (45). A second mutant, OF, is able to produce an orange pigment (42, 103) and will also be discussed in the next section.

Syntrophic Pigmentation

Rizki (72) first noticed syntrophic pigmentation, calling it chromogenic induction, in 1954. The phenomenon was discovered when several stable white mutants, growing close to each other on an agar plate, developed color at the periphery of some of the colonies. Rizki called those strains which induced pigment in other strains, "inductors," and those strains which reacted, "reactors." He found that the intensity of the color formation differed, and also, that his normal HY strain, which was red, did not induct. This did not hold true for other wild type strains studied. Subsequently, he found that the two types, inductor strains and reactor strains, had different ultraviolet (UV) and visible spectra when assayed separately, but had a spectrum similar to that of prodigiosin when the chromogenic pigment was tested (73).

Williams and Green proposed the term syntrophic pigmentation in lieu of chromogenic induction (103). Davis (18) had described a phenomenon of syntrophism in which different auxotrophic mutants were able

to grow on medium which was deficient in the specific growth factors needed. Since each strain had a different metabolic block, their accumulated products were able to "cross feed" each other, thus permitting growth. This clearly appeared to be similar to chromogenic induction and was therefore adopted as the term having precedence.

The pigment was identified as being prodigiosin by Hearn et al. in 1972 (41), using mutants of two different wild type strains, HY and Nima. The pigment formed by the mutants in syntrophic pigmentation was analyzed using thin layer chromatography and by spectrophotometric means. Green and Williams (37) determined that the syntrophically derived pigment in a "white" mutant strain was identical to the wild type pigment in the Nima mutants.

Strain 9-3-3

Santer (75) confirmed that the phenomenon was a correlation of Davis' syntrophic feeding. She found that the donation of a noncolored precursor from one strain to another was coupled with a second noncolored precursor produced in the second strain, the acceptor. Santer found that 9-3-3, a mutant derived from an earlier mutant, 9-3, which itself was derived from the wild type red strain 274, produced a nonvolatile precursor. She developed a technique to extract this precursor which was later identified as the bipyrrole MBC shown in Fig. 1 (76).

The 9-3-3 mutant will produce a purple pigment under certain growth conditions. It is believed that this purple pigment contains some bipyrrole (45). It is not synthesized at 38° C, which implies a temperature-sensitive enzyme or intermediate is involved in its formation. Because the purple pigment and prodigiosin have similar ranges of produc-

tion in temperature and oxygen demand, as well as in media constituents, it has been suggested that the mechanism for prodigiosin production and purple pigment production are somewhat similar. This strain, 9-3-3, is also able to produce the monopyrrole, and hence, the pigment prodigiosin itself, under certain culture conditions.

The ability to produce the pigment molecule is induced by thiamine, but is inhibited by phosphate salts (44), and by casein peptone (33). This pigment, unlike the purple pigment and the normal, nonpigmented bipyrrole in 9-3-3, is identical to the red pigment of Nima, with the same visible absorption spectra and paper, thin layer, and column chromatographic characteristics. Thus, the addition of thiamine and the absence of phosphate salts in a medium can induce the formation of the second pigment precursor in strain 9-3-3 which is able to interact with MBC to form the red pigment prodigiosin.

Strain WF

Santer also found that WF, a mutant of the wild type Nima strain, was able to produce a volatile precursor (MAP) but she was unable to assay or analyze it. Deol et al. (21) isolated and characterized it, as a monopyrrole. In Figure 1, the structure of MAP is illustrated. WF cannot, therefore, produce the second precursor, MBC, and any color produced by this strain is due to syntrophic feeding of MBC and not to its inherent production.

Both strains WF and 9-3-3 are able to donate precursors to each other, enabling them, under ordinary culture conditions, to produce prodigiosin identical with the pigment produced by the wild type strain, Nima.

Strain OF

A third mutant, OF, which is a Nima derivative, produces an orange pigment, norprodigiosin. This pigment is identical to prodigiosin, except for the absence of a methoxyl group at the number 4 carbon in the bipyrrole precursor. The mutant makes normal MAP, but cannot exchange the hydroxyl group at the number 4 carbon to the methoxyl of MBC. Instead, it takes the intermediate bipyrrole precursor HBC (also illustrated in Figure 1) and attaches it directly to MAP. OF is able to act as a syntrophic donor and acceptor. In acting as an acceptor, receiving MBC from 9-3-3, OF is able to produce prodigiosin, and also feeds MAP to 9-3-3, enabling it to form prodigiosin. When OF acts as a donor, feeding WF HBC, WF is able to methylate HBC to MBC and form normal prodigiosin. Table II summarizes these syntrophic reactions.

Very little is known about the prodigiosin formation in the steps between raw amino acids and the known precursors. It has been suggested, because of the structural similarities between the bipyrrole and the monopyrrole, that they have common precursors. It has also been suggested that the pathway separates at some early point, remaining bifurcated until MBC and MAP are combined (91). However, no mutant has been found which is able to donate to both pyrrole-forming strains, except for OF and OF's block is known to be located at the end of the biosynthetic pathway. Because of this failure, there is no proof for the theory of a bifurcated precursor pathway. However, such an absence of common precursors does not rule out the possibility for cross-regulation between the separate routes for monopyrrole formation and bipyrrole production.

TABLE II. Donor-Acceptor Reactions.

Donor		Acceptor	
MBC		MAP--WF	MBC--9-3-3
	Nima	+	-
	WF	+	-
HBC	OF	+	-
MAP			
	Nima	-	+
	OF	-	+
	9-3-3	-	+

+ indicates prodigiosin able to form.
- indicates no pigment found.

Cell Physiology and Pigment Production

Prodigiosin has been identified as a secondary metabolite (97) because maximal production of the pigment occurs after cellular multiplication has ceased. It is evident, therefore, that the pigment is not essential for cellular growth and multiplication. A second proof of this fact is the evidence that the vast majority of strains are nonpigmented, and that the growth rates of pigmented and nonpigmented strains are identical (see RESULTS Section). Prodigiosin also fits the definition of a secondary metabolite from other findings. It is easily affected by physiochemical factors such as temperature, oxygen, and concentrations of phosphate and of metallic ions. One widely held hypothesis for the possible uses of such secondary metabolites suggests that it is not so much the molecule itself which is needed by the cell, as it is the process of creation of the molecule, in that these secondary metabolites are accumulating toxic, nonexcretable metabolic wastes.

In order to study the formation of pigment while the cells were not actively growing, Qadri and Williams devised a technique called nonproliferating cell culture, to enable the induction of pigment after cell growth had ceased (66). They incubated the cells in minimal medium for 48 hours at 38° C, a temperature which does not allow formation of pigment or precursors, and after centrifuging and washing, resuspended the cells in saline and incubated them at 25° C. Pigment was not formed by the downshift in temperature because the cells were in the stationary phase of their growth cycle. The addition of selected amino acids was found to cause formation of pigment in these cells (50, 66, 67, 69, 101). Among the individual amino acids added that were capable of inducing

pigment formation were histidine, proline, hydroxyproline, alanine, aspartic acid, serine, ornithine, and glutamic acid (67). Further work with labeled amino acids showed that the pyrrole groups of prodigiosin might be synthesized from 5-carbon skeletons of amino acids or the degradation of amino acids to a 5-carbon moiety, like proline, ornithine, and glutamic acid (101), but the biosynthetic pathways for this have not yet been determined (69). It was also found in these cell suspensions, that the kinetics for incorporation of amino acids paralleled the kinetics for prodigiosin formation (69) and that new protein had to be synthesized for the production of prodigiosin (49, 107).

Most of the work with the induction system has been done with the amino acid proline (79). Some aspects of its incorporation into the pigment are known; for example carbon number 2 of proline is incorporated into the MBC rings (80). With additions of proline to the nonproliferating cell cultures mentioned above, protein synthesis in the cells increases (107). Evidence that the entire proline molecule entered the pigment came from the incorporation of labeled proline into pigment by proline noncatabolizing mutants (49, 66). In normal cells, it appears that proline can either be incorporated intact (84) or be used as a nitrogen and carbon source for the pigment (107). No specific metabolic pathways for proline are yet known, and labeled intermediates have not yet been isolated.

Further data supporting separate mechanisms for the metabolism of MBC and MAP was derived from studies on glycine utilization. Glycine was incorporated into both pigment and bipyrrole, and was found in both the MAP and MBC and in the complete prodigiosin molecule (59, 80). It

was concluded that the rings of the bipyrrole were formed by a different mechanism than that forming the monopyrrole. Methionine was found to be involved in the methylation of the pigment (68). Were prodigiosin not a secondary metabolite, the amino acids would have been used for cellular metabolism in proliferating cells as a part of the general pool of nitrogen and carbon, and their role in pigmentation could not have been discovered.

Environmental Influence on Pigment Production

Radiation

Labrum and Bunting found that use of ultraviolet irradiation provided a higher percentage of stable color mutants than did natural selection (47). The effect of gamma irradiation was studied by Bartlett, O'Donovan, Neff and Webb (4, 9). Bartlett et al. (4) found that Nima, the red wild type strain, was more sensitive to gamma radiation than the three nonpigmented mutants they used. When strain 9-3-3 was used, the cells with syntrophic pigment were more sensitive to the radiation than cells without the syntrophic pigment. 9-3-3 became as sensitive as Nima, when allowed to form syntrophic pigment. Their conclusions were that one intermediate precursor might act as a protective agent, but that the pigment itself had a definite radiosensitive effect. Webb et al. (91) found that the sensitivity of UV and gamma radiation paralleled each other in these strains. Since 9-3-3 became as sensitive as Nima when allowed to form prodigiosin, and since WF was more sensitive than 9-3-3, it was concluded that the pigment produced no important radiosensitizing effect, but that some metabolites in the biosynthesis of MAP might be

radiosensitizers.

Temperature

The "red diaper syndrome" is an obvious effect of temperature sensitivity in Serratia, showing the inability of the bacterium to produce the pigment at 37°C, but still able to exhibit pigment production at room temperature. The effect of temperature on pigment, condensation enzyme, and precursor formation has been studied (99). Strain 9-3-3, an MBC producer, has maximum bipyrrole production at 33° C, and at 40° C, produces none. The monopyrrole produced by the mutant WF has its maximum production at 27° C, ceasing production at 37° C. The coupling enzyme, putting together MBC and MAP, in either WF or 9-3-3, ceases functioning at 40° C, and has the maximum activity at 33° C. Williams et al. (102) determined that no pigment is formed at 38° C.

Media Constituents

Phosphate effects on pigment metabolism were also mentioned earlier, when discussing 9-3-3's ability to produce a purple pigment, and also prodigiosin. It is known that inorganic phosphate can inhibit pigment production (109). Phosphate is also known to affect capsule production of Serratia marcescens (12). As the phosphate concentration is lowered, the accumulation of pigment paralleled the elaboration of capsule production. Poe and Hawkins (63) devised a rule of thumb to determine whether or not a strain will produce pigment. They found that, after 48 hours of growth, a strain which changed the pH of the media to the basic side generally produced pigment, while those that changed the media to the acid side generally did not produce pigment. The pH change

was at a maximum after 120 hours of growth, with up to a 2.5 pH unit change by some strains. They found no enhancement or inhibition by several carbohydrates and higher alcohols.

Linnane and Still (51) found that some unsaturated fatty acids allowed pigmentation at temperatures up to 42° C. They also found that oleic, linoleic, ricinoleic and erucic acids permitted pigment to form in a parrafin covered medium. The fact that pigment was formed anaerobically when the bacteria were supplied with these acids is particularly informative since prodigiosin formation requires oxygen in normal media (43).

Antibiotics

Antibiotics have been used to study pigment production as well as production of the precursors. Monopyrrole production is permitted at any level of antibiotics which allow cell growth, according to Gott and Williams (34). There was partial inhibition of monopyrrole synthesis by streptomycin (98). The synthesis of bipyrrole was inhibited by streptomycin (98), but the effects of other tested antibiotics were variable and depended upon the composition of the medium (34). The coupling enzyme was inhibited by cephalosporin and kanamycin (34) and not inhibited by other antibiotics, including streptomycin (98). Antibiotics, such as chloromycetin, aureomycin and terramycin did inhibit pigmentation, which was the only parameter measured (92). It is also known that streptomycin inhibits pigment production since precursor and coupling enzyme are both affected. Among other substances tested were several extracts of blue-green algae (197) which were able to inhibit pigment, but not growth, and were termed antipigmentation factors.

Proposed Pigment Functions

Gaps in our knowledge of *Serratia* and its pigment include the function of the pigment. Several Russian workers noted that the pigmentless variants of normally pigmented strains increased in extracellular nuclease activity. In addition, they found a decrease in the rate of growth and reproduction, but with an accelerated uptake of energy source, as well as an increase in respiration rate and a decrease in thermogenesis (64). These findings are in conflict with other work which noted no such decreases in growth and reproduction or increases in respiration rates (97) and with Yusupova *et al.*'s observations (111). Bunting (11) found that toxic concentrations of surface active agents were more lethal to white than red cells, but that when low concentrations were used, did permit the white variants in the aging cultures to continue growth after the red type began to decline. Bartlett *et al.* (4) found the wild type red strain Nima to be more sensitive to UV irradiation than the nonpigmented variants. However, all these studies utilized the normal red strains and white variant strains and not normally white strains. Overall conclusions have supported the contention that pigment does not appear to have any effect on the ability of the cell to survive. Since the majority of *Serratia* isolates, both clinical and nonclinical, are nonpigmented, investigations utilizing white strains may yield significant information about the function of the pigment or its precursors in the normal cell.

Statement of Problem

A number of researchers have considered pigment formation in Serratia marcescens. This Introduction has by no means covered every article written or all the work done on Serratia. In reviewing this literature, it was noted that most of the data was collected under a variety of physiological conditions, on many different media, and using many diverse strains. These reports unfortunately do not allow the transfer of information from one experimental system to another.

For this thesis, work in several areas was done to attempt to provide more information about pigmentation in Serratia. Some of the questions the research approached are presented here, in order to gain a perspective of this thesis and to point out potential areas of prodigiosin research.

For example, are the two precursors, MBC and MAP, produced on a stoichiometric basis? Are the metabolic pathways for the precursors completely separate, or do they bifurcate, and utilize common intermediates? Why is Nima able to feed both MBC and MAP, and is this a leakage from whole, viable cells, or is it from disrupted cells? Is the production of the precursors immediately followed by the complete uptake into pigment, or do the precursors have a period of time during which they can leave the cell? Does a blocked mutant like 9-3-3 produce more or less precursor than Nima, and why? Will there be any extra bipyrrole in a mixture of 9-3-3 and WF, a combination which will produce syntrophic pigment?

A second approach to pigmentation in Serratia asks questions about pigment precursors in nonpigmented strains. Since the majority of

Serratia strains are nonpigmented, being nonpigmented must be the normal for Serratia, which is in direct opposition to the now-defunct but long held view that the pigment is essential to the cell, and that being pigmented is normal for Serratia marcescens.

Since the precursors themselves may be the essential products of the cell, a survey of nonpigmented strains would ascertain the presence of these. If precursors are present in the nonpigmented strains, questions such as: whether or not this precursor is the same as that produced by WF or 9-3-3; or do the cells produce one or both types of precursors; or are there any of the enzymes responsible for producing the pigment present in the normal cells; or, if present, is there any known function for them? The answers to these questions would throw some light onto the function of the pigment and the precursors.

In the work done for this thesis, all of the above questions were approached. Growth curves were determined for pigmented and nonpigmented strains, to confirm that there was no difference in growth rates. Pigment and MBC assays were used to construct a model of the rates of production and accumulation of these compounds. Nonpigmented strains, when tested for syntrophic ability, proved to behave similarly to the WF strain, accepting MBC from 9-3-3. Assays for MBC could therefore not be used for clinical strains, and the assay for prodigiosin destroyed the pigment produced by the clinical strains. Therefore, a technique of supplying MBC in large quantities on filter paper strips was developed. This technique allowed the visualization of the pigments produced by the clinical strains, and was used to determine some of the parameters of this precursor's production.

MATERIALS AND METHODS

Bacterial Strains

The four characterized Serratia marcescens strains, Nima, OF, WF and 9-3-3, were the gifts of Dr. R. P. Williams at the Baylor University College of Medicine, Houston, Texas. Strains which were not characterized in regard to their ability to produce, donate or accept pigment precursors were received from several sources. Strain ATCC 8100 was the gift of Dr. O. D. Smith, at Baptist Memorial Hospital in Kansas City, Missouri. One red strain was in the stock culture collection of the Division, and six strains, including one isolate identified as Serratia rubidaea (according to Bergey's Manual of Determinative Bacteriology, 8th edition, this strain is the species marcescens) were received from Dr. A. Ulrich at the Kansas State Health Laboratory in Topeka. Seventeen clinical culture isolates were the gift of Wesley Medical Center in Wichita, Kansas, and 79 other strains were received from the clinical laboratory at St. Francis Hospital, also in Wichita. Various other Gram negative and Gram positive organisms (Table III) were obtained from the Division's stock culture collection.

Media and Biochemical Tests

Cultures were grown and maintained on Trypticase Soy (TS) agar (BBL Division of Becton, Dickinson and Company). Following room temperature incubation for 24 hours, slants were refrigerated and then transferred

TABLE III. Gram Positive and Negative Bacteria.

Gram Negative
Arizona arizonae
Citrobacter sp.
Enterobacter aerogenes
Ent. cloacae
Escherichia coli
E. coli C-10
E. coli 11303
Proteus mirabilis
P. vulgaris
Pseudomonas aeruginosa
Ps. maltophilia
Gram Positive
Bacillus cereus
B. subtilis
Micrococcus lutea
Staphylococcus aureus

bimonthly. Experimental cultures were grown from stocks on any of several media. These other media included peptone-glycerol (P-G) agar and broth (47); a complex media devised by Bunting (10), which was used minus the phosphate salts; and a modified version of Mueller-Hinton broth and agar. The Modified Mueller-Hinton (MMH) media contained, per liter of distilled water, 3 g of beef extract, 5 g of tryptone, and 1.5 g of starch, with 17 g of agar added for a solidified medium.

Three biochemical tests, all purchased from Difco, were used to speciate the Serratia strains. These included sulfide-indole-motility medium (SIM), DNase test agar and phenol red broth base with 0.5% L-arabinose.

Speciation of Clinical Strains

The biochemical tests were used on all 150 clinical and 6 control strains of S. marcescens. Almost all Serratia marcescens were shown to be typical by means of these tests with few exceptions.

Although 5% of Serratia strains are considered to be nonmotile (39), only three of the isolates, or 1.9%, were nonmotile. Two of these were pigmented strains, and the third, a nonpigmented strain.

S. marcescens is typically arabinose negative. Two isolates produce acid and gas in the arabinose broth. Since S. liquefaciens is an arabinose fermentor, and nonpigmented, these two strains fit the criteria for S. liquefaciens. A third isolate, pigmented and arabinose positive, matched the criteria for S. rubidaea. One other strain also fermented arabinose, but was DNase negative and nonmotile. These results indicated that it was a Klebsiella species.

Growth Curves

Growth curves were established for Nima, the three mutants, and for 11 uncharacterized strains. Culture inocula were grown in TS broth at room temperature, without shaking for approximately 12-18 hours. The optical density at 375 nm was read in a Bausch and Lomb Spectronic 20 and the cells were pelleted in a Sorvall centrifuge at 12,500 gravities for 5 minutes. The cells were resuspended in sufficient medium to give an $A_{375nm} = 1.0$, and were added to the experimental growth flask to give an initial absorbency of 0.01. When strains were grown in mixed cultures to evaluate growth and production of pigment and MBC, the flasks received an equivalent inocula added at an initial optical density (O.D.) of 0.01 at 375 nm. The flasks were then placed on a wrist action shaker at ambient temperature and agitated during the course of the experiment.

All cultures were sampled at 45-minute intervals. The cultures were read without diluting, at 375 nm, until the O.D. was 0.25 or above. Samples above $A_{375nm} = 0.25$ were diluted 1:10 in TS broth for subsequent readings. Observations were terminated after 10.5 hours of sampling, or when the O.D. stabilized in stationary growth conditions.

Plate counts were made for Nima and the three mutants to correlate O.D. with cell numbers. Counts were made each time the O.D. was read, by the following technique: the cell suspension was diluted in 0.85% sterile saline, and 0.1 ml was plated, in duplicate, on TS agar plates; the sample was spread on the agar surface with an alcohol sterilized bent glass rod, and the plate was then incubated at ambient temperature for 24 hours.

Precursor and Pigment Assays

The assay for MBC was developed by Santer (75). Briefly, it consisted of shaking 1 ml of culture fluid with 6.0 ml of $CHCl_3$. Removal of the upper water phase was followed by a photometric reading of the $CHCl_3$'s O.D. at 363 nm and at 400 nm. The final relative amount of bipyrrole is determined by subtracting the reading at the nonspecific absorbence wavelength of 400 nm from that at the specific absorbence wavelength of 363 nm.

Williams and Gott (98) reported a simple technique for the assay of prodigiosin. The reading at the nonspecific absorbence wavelength of 655 nm is subtracted from the absorbency at the specific wavelength, 537 nm. A Hitachi-Perkin-Elmer 139 UV-Visible Spectrophotometer was used for both the bipyrrole and the prodigiosin assays. As a control for these assays, the norprodigiosin producing mutant OF was assayed. If these assay wavelengths were specific for MBC and prodigiosin, they would not show absorbency with OF's HBC and norprodigiosin, and this was indeed the case.

Statistical Analysis

Raw data from the growth curves, both the absorbency measurements and the viable cell counts were linearly regressed to correct for sampling errors and to provide statistical evaluation. Data from the pigment and MBC assays were also regressed. Correlations, to measure the agreement, were also determined for the data. A programable calculator was used for the calculations.

This information was then treated several ways to produce the final growth curve plots. For each strain tested, a growth curve was calculated for the O.D. readings versus time. For Nima and the other three well-characterized strains, a growth curve was calculated for cell numbers versus time. Also for these four strains, the correlation between cell numbers and absorbency was determined. The cell number growth curves were also correlated for these four characterized strains. Next, for all strains (including the 11 noncharacterized and the four well-characterized strains), the absorbency growth curves with respect to time were correlated for purposes of comparison. Overall, these correlations provided a general growth curve, for any strain of Serratia, using either O.D. or cell numbers.

The data from the bipyrrole and pigment production curves were then analyzed. In order to determine the total amount of MBC produced in the cultures, several assumptions about the pigment and the optical density were used in the analysis: (1) by assuming that the O.D. at λ_{max} measures the quantity of molecules in solution, the O.D. measured for pigment became equivalent to the O.D. for the combined precursor MBC, since one molecule of MBC is used per molecule prodigiosin; (2) since the MBC assay used a 6.0 ml volume, and the prodigiosin assay, a 4.3 ml quantity, a ratio was used to convert the O.D. for prodigiosin, representing the used MBC, to an O.D. to combine with the O.D. for free MBC, in order to determine how much MBC had been produced in total; and (3) the ratio used was:

$$\frac{\text{4.3 ml Prodig. assay volume}}{\text{measured O.D.}} = \frac{\text{6.0 ml MBC assay volume}}{\text{O.D. MBC (X)}} \qquad \text{Eq. 1}$$

Syntrophy in Mixed Cultures

Syntrophic pigment is the result of donation and acceptance of pigment precursors between different strains. When originally evaluating the ability of noncharacterized strains and of well-characterized Serratia strains to produce, donate or accept pigment precursors, a mixed culture was used. For this assay, two pure strains were grown separately in broth culture. Cells from each culture were then transferred with a cotton swab to an area on an agar plate, separated from the other by 5-7 mm. The technique used was similar to that of Santer (75). Although originally both strains were confined to growing in a single stripe on its half of the plate, best results were achieved when the entire half of the plate was inoculated. An uncharacterized strain was inoculated on one portion of the plate with one of the two well-characterized strains WF or 9-3-3, inoculated on the other half. Any color change in either strain was noted.

In some cases, the plates were sealed with Scotch tape to retain and concentrate the volatile MAP precursor in the plate, but this was later found to be unnecessary. In other instances, a different type of pigment detection technique was used. Williams and Gott (98) used a two-phase medium to confirm the donation of the volatile MAP to strain 9-3-3. The 9-3-3, grown on Bunting's complex media on a square of agar, was placed on the lid of the plate, formed pigment from the donor strain, grown in P-G agar in the bottom of the plate.

Paper Strips

Paper strips impregnated with MBC were prepared in the following manner. After bacterial growth, MBC was harvested by $CHCl_3$ extraction and added to the filter paper strips. The inoculum for the cultures was obtained by suspending 9-3-3 from a 48-hour TS culture in fresh P-G broth until barely turbid. This was then diluted $1:10^{-4}$, and 1 ml of the dilution was used to inoculate 50 ml of P-G broth. After growth for 24 hours at room temperature on a wrist action shaker, 5 ml of this was used to inoculate 200 ml of P-G broth.

The 200 ml of broth was shaken at 27° C, either on a platform shaker or in a water bath shaker, for three days. Once incubated, the broth culture was autoclaved and then cooled at 4-6° C for 12 hours or more. The contents of each flask of 200 ml was poured into a separatory funnel, and 100 ml of $CHCl_3$ was added to extract the MBC. After shaking, the layers were allowed to separate for up to one hour. The $CHCl_3$ and MBC layer was placed in a small flask, then the interface was removed from the separatory funnel and centrifuged for further phase separation. The interface-derived $CHCl_3$ layer was then added to the initial extract. The material obtained in this manner was added to 10 strips of Whatman #1 filter paper, having dimensions of 7.62 X 1.25 cm, and allowed to evaporate. The procedure was repeated until almost all was evaporated. Any scum, precipitation, or rusty color on the strips was rinsed off with fresh $CHCl_3$. The filter paper strips, now impregnated with MBC, were then wrapped in foil and autoclaved. Once the $CHCl_3$ evaporated, the MBC seemed to be stable for at least four months.

Syntrophy with Filter Paper Strips

A one strain test system using the filter paper strips was developed as a substitute for the mixed culture syntrophy described previously. Bacterial inocula were first grown in P-G or Modified Mueller-Hinton (MMH) broth. Inoculations were made with a cotton swab in a streak down the center of the MMH plate, which was not older than 48 hours. After the inoculation, the paper strip was transferred from its foil package to the lid of the plate, where it absorbed the condensed moisture there after which it was transferred to the surface of the agar, and placed perpendicular to the bacterial streak. The plate was then sealed with Scotch tape and incubated at room temperature. Observations were made until the plates were 5 days old, by which time no further color reactions occurred. Colors were identified by use of Ridgeway's Color Standards (71).

Antibiotic Tests

The influence of eight antibiotics on WF's MAP were determined via the Williams and Golt technique. Those antibiotics able to inhibit monopyrrole production were then evaluated as to their effect on WF's growth before MBC impregnated paper strips were used to further evaluate the antibiotic effect.

For tests on syntrophic pigment production, MMH agar plates were made with the antibiotic added to each plate before pouring in the melted agar. When used for these tests, these MMH agar plates were no more than 48 hours old. Bacterial inocula were grown up in either

P-G or MMH broth for 18-24 hours, centrifuged, and resuspended to a standard A_{375nm} = 1.0, and then diluted to yield 10 to 100 colonies per plate. To ensure adequate controls, inoculation of these drug containing plates was done in several ways. For each bacterial dilution, antibiotic-less control plates and MBC strip-less control plates were inoculated to determine how the presence of the strip changed the number and appearance of the colonies. Finally, when the plates with strips were inoculated, the inoculum was added, half to either side of the strip, and was then spread with a bent glass rod to prevent contact with the strip. Plates were then incubated at ambient temperature, and observations were made until the plates were 5 days old.

RESULTS

Optical Density Curves

Growth curve data were obtained for Nima, the three mutant *Serratia* strains (OF, 9-3-3, and WF) as well as for 11 other *Serratia* isolates. These 11 isolates represented organisms isolated from both clinical and nonclinical sources. Of these 11, five were pigmented while six were not.

Growth curves using optical density (O.D.) versus time were regressed and treated statistically for Nima, the three mutant strains and the 11 isolates. The data points derived from the regression equations were plotted and used as the growth curves for each of the 15 individual strains. Only results for Nima and the mutants are listed in Table IV. Since correlations among the growth curves were +0.99, the information for Nima and the three mutants was then combined to present a generalized growth curve. This generalized growth curve's regression formula is:

$$\log (O.D.) = 2.27 \log (t) - 1.45. \qquad \text{Eq. 2}$$

Growth curves of the other strains were compared and correlated to this combined O.D. versus time curve and all had correlations of +0.98. This meant that none of the 15 strains differed in any radical manner from each other in regard to their growth rates in the test system.

TABLE IV. Statistical Regression Formulas for Growth Curves

Culture	Statistical Formula for Live Cells	Statistical O.D.A. = 375 nm Formula
Nima	log (cell count) = 6.48 + 2.89 log (t)	log (O.D.) = -1.34 + 2.17 log (t)
OF	log (cell count) = 6.97 + 2.58 log (t)	log (O.D.) = -1.46 + 2.33 log (t)
9-3-3	log (cell count) = 6.59 + 2.76 log (t)	log (O.D.) = -1.51 + 2.21 log (t)
WF	log (cell count) = 6.82 + 2.87 log (t)	log (O.D.) = -1.52 + 2.36 log (t)

TABLE V. Statistical Regression Formulas for Pigment and Bipyrrole Assays

Culture	Pigment	r*	Bipyrrole	r
Nima	Pigment O.D. = -.16 + 0.017t	+0.89	MBC O.D. = -0.007 + 0.0009t	+0.79
9-3-3	No pigment in 9-3-3		MBC O.D. = -0.07 + 0.1000t	+0.83
9-3-3/WF	Pigment O.D. = -.02 + 0.002t	+0.88	MBC O.D. = -0.0007 + 0.0002t	+0.71

r* = Coefficient or correlation

Live Cell Number Growth Curves

Nima, OF, WF, and 9-3-3 were also used to determine a growth curve using live organism cell counts in order to derive a population curve based on viable cell numbers. The regressions and correlations for these are also listed in Table IV. The generalized formula for live cell numbers versus time is:

$$\log (\text{cell \#'s}) = 3.55 \log(t) + 6.19. \qquad \text{Eq. 3.}$$

This equation had a correlation of +0.84 between the cell number and the time data for all four organisms.

Live Cell Number versus O.D.

The third step, that of correlating live cell number to O.D., allowed the use of O.D. to estimate the live cell numbers from the O.D. measurement. The general correlation formula for Nima and the three mutants was:

$$\log (\text{cell \#'s}) = 8.59 + 1.23 \log (\text{O.D.}) \qquad \text{Eq. 4.}$$

This equation had a correlation of +0.97 for all data on Nima, OF, WF and 9-3-3. Since the growth curves of the other 11 isolates were essentially identical to the generalized O.D. growth curve formula, this equation was held to be valid for any *Serratia* strain, in any of the tested media. In addition, this equation could only be used to represent the bacteria while they are in the logarithmic or early stationary phase of growth, because as cell cultures grow older, the presence of dying or nonviable cells creates a discrepancy between the O.D. measurement and the numbers of viable cells.

Pigment and Bipyrrole Assay

The raw data collected during these experiments was adjusted to a normalized base using the wild type Nima strain as the comparison. The O.D. data for Nima in peptone-glycerol (P-G) broth was linearly regressed to provide an estimate for these conditions (Table V). Statistically derived data points, representing sampling times, were calculated from this equation and were then converted to statistically derived cell numbers using Equation 4 above.

Data for the 9-3-3 and 9-3-3/WF cultures were treated the same way as those for Nima's (Table V). Although the growth curves were all essentially identical, there were small differences in cell numbers at different test times. Because of these differences from Nima, a technique to directly compare the amounts of bipyrrole and pigment produced by WF and 9-3-3 with the amounts produced by Nima was needed. In order to do this, the cell numbers present in the other cultures were compared to the numbers present in Nima, and the number in Nima's culture was considered to be 100%. The formula used was:

$$\frac{\text{cell \#'s mutant}}{\text{cell \#'s Nima}} \times 100 = \% \text{ of Nima cells} \qquad \text{Eq. 5.}$$

Likewise, the amount of pigment in the cultures was compared to that in Nima, using the formula:

$$\frac{\text{Pig. (MBC) O.D. mutant}}{\% \text{ of Nima cells (Eq. 5)}} \times 100 = \text{adjusted Pig. (MBC) O.D.} \qquad \text{Eq. 6}$$

The O.D. data for both the pigment and the bipyrrole assays were linearly regressed, and the statistical O.D. for each sampling time was obtained (Table V). One difference between the regressions done on the assays and those done on growth curves was that the best correlations

were obtained for the assays by using a plain linear regression formula, time versus O.D. Equation 5 was used for 9-3-3 and 9-3-3/WF to determine the % of cells compared to Nima. Equation 6 was then used, adjusting the pigment or bipyrrole O.D. for each sample to be directly comparable to that of Nima, as if there were actually the identical number of cells present in both cultures.

With these adjusted data, Figures 2 and 3 were constructed, showing the relative statistically derived amounts of pigment and precursor produced by Nima and the other cultures.

Further analysis of these assays, using information drawn from Figures 2 and 3, was done according to the methods described in the Materials and Methods. Table VI lists this information. By these assays, the wild type strain Nima produces MBC in amounts comparable to that produced by 9-3-3. Also, the 9-3-3/WF culture produced much less pigment and MBC than either of the other two cultures.

Clinical Syntrophic Pigmentation in Agar Cultures

Peptone-Glycerol Agar

The 118 clinical strains received as uncharacterized in regard to precursor production were plated opposite both strain WF and 9-3-3 on peptone-glycerol (P-G) agar. Several types of results were seen in the clinical strains (Table VII). In each case, 9-3-3 turned a purple-bluish color, and WF, a slight pink. However, both of these colors were normal for these strains on P-G agar. The white noncharacterized strains did show color reactions, acting as acceptors of MBC from 9-3-3 in a definite pattern, although in no case was the color change in these

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Figure 2. Production of Prodigiosin.

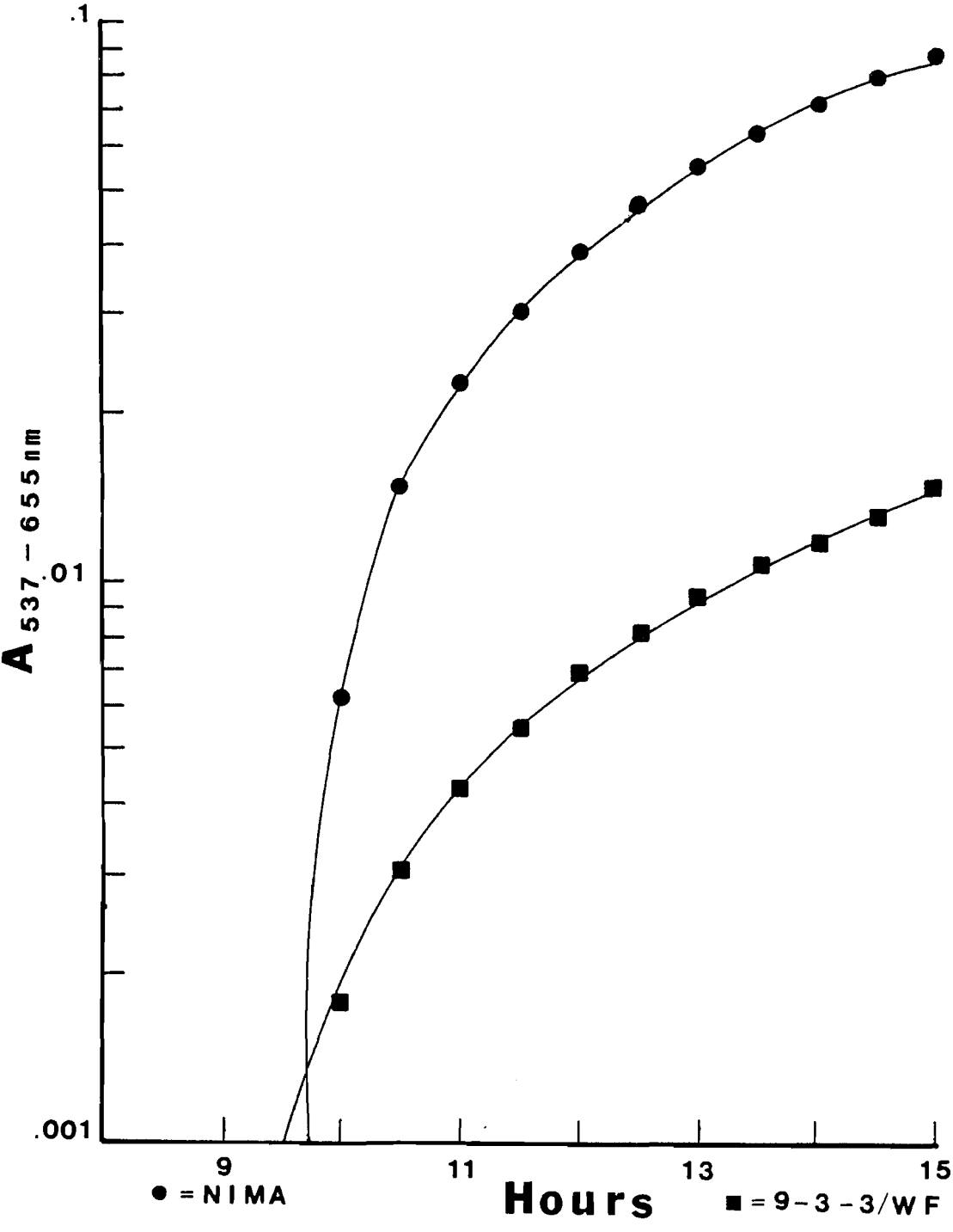


Figure 3. Production of MBC.

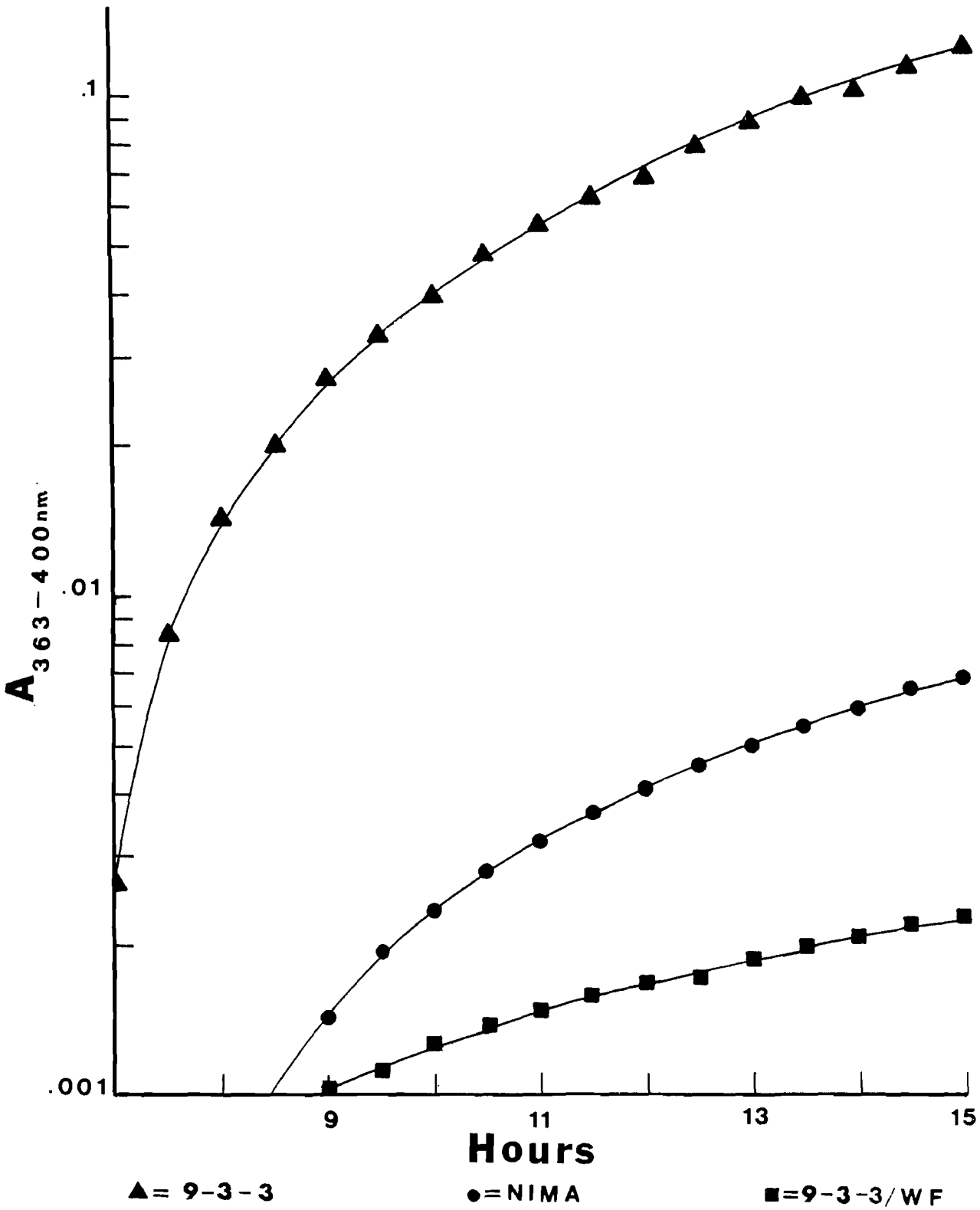


TABLE VI. O.D. Results of Assays

Culture	O.D. Free MBC	O.D. Total MBC	O.D. Pigment
Nima	0.007	0.137	0.09
9-3-3	0.124	0.124	---*
9-3-3/WF	0.002	0.023	0.015

*No pigment in 9-3-3.

TABLE VII. Reactions of Clinical Strains on P-G Agar.

Clinical Strains		versus WF	versus 9-3-3		
No.	Percent	(+ or -)	Pink	Purple	Pink/Purple
118	100.0	-			
52	44.1		+	-	-
17	14.4		-	+	-
30	25.4		-	-	+
19	16.1		-	-	-

+ indicates a color change as described in the headings.
- indicates that the color change was not achieved.

acceptor strains as intense as the color change in the 9-3-3 and WF syntrophic pigment.

Results listed in Table VII show that, of the 118 noncharacterized white strains, 44.1% changed to a slight pink color, while 14.1% changed from a white to a slight purple color, and 25.4% became pinkish with a slight purple border. Only 16.1% showed no ability to change in color at all. None of the clinical strains showed any ability to be syntrophic donors to 9-3-3 or to donate or accept from WF, the monopyrrole donor.

Trypticase Soy Agar

TS agar was the nutrient medium originally used in the syntrophic pigmentation experiments. Results from 49 clinical isolates grown on TS indicated that there were two types of responses to 9-3-3 (Table VIII). The first response was a positive, pink response by the clinicals. Twenty-six out of the 49 strains, or 53%, showed the pink positive response on TS agar. When plated on P-G agar, 13 of these 26 showed a positive response to 9-3-3. The other two did not produce color when grown versus 9-3-3 on P-G agar.

The second response seen on TS agar was a negative, or no color change, when plated versus 9-3-3. Of the 49 clinical strains, 47% or 23 showed this negative response. However, when these strains were grown versus 9-3-3 on P-G agar, 13 showed color reactions, while 10 did not. Those 13 clinical strains displayed all three types of positive responses on P-G agar: namely, pink, 4 strains, purple, 4 strains; and pink with purple edge, 5 strains (Table IX). Overall, 75.5% of the 49 clinical strains showed a positive response when grown on P-G agar, while only 53% showed positive responses on TS agar, making P-G the

TABLE VIII. Syntrophic Pigmentation of 49 Clinical Strains on TS and P-G Agars.

Number of Strains	Reaction on TS Agar	Reaction on P-G Agar
24	+	+
2	+	-
13	-	+
10	-	-

+ indicates color formed.
- indicates no color formed.

TABLE IX. P-G Agar Color Reactions of Isolates Producing No Color on TS Agar.

Number of Strains in Group	P-G Reaction
4	Pink
4	Purple
5	Pink with Purple Edge
Total 13	

more sensitive test system.

Syntrophic Pigment in Broth Cultures

When flasks were grown with 9-3-3 as the donor and one of 9 selected clinical strains as the acceptor in P-G broth, there was some evidence of color formation in only 1 out of 9 test flasks. Color appeared in this flask by 11.75 hours of growth, but was destroyed by the assay procedure. Therefore, although a pigment appeared at the normal time for pigment to become visible in a flask, the pigment that appeared was not stable and did not react like prodigiosin. In this instance, as reported earlier by Santer (75) there appeared to be no correlation to the production of syntrophic pigment in agar or in broth; while it may appear on the agar plate, it may not in a broth culture.

Paper Strip Assay and Clinical Strains

Preliminary Work

Once the basic technique of growing 9-3-3 and extracting the bipyrrole had been tried with success, the next trials involved determining the best technique for adding the bipyrrole impregnated strips to the media. Tests were run by embedding the strip in the melted agar, as well as by placing the strip on the surface of the agar. Those strips embedded in the agar showed no ability to donate bipyrrole to WF, which remained white. When the strip was laid on the surface, either over the bacteria or with the bacteria inoculated over it, the strip and the bacteria showed color. In addition, samples taken from these colored strips from the top and bottom, as well as areas well away

from the original sites of inoculation, showed the strips to be covered with bacteria. This covering of the strip by the bacteria allowed the bacteria access to the bipyrrole, and thus, an easy way of showing the pigment. The conclusion reached was that, upon embedding the paper strip, the bipyrrole must diffuse throughout the media to become available and cannot be concentrated in the specific area needed, that is, the area of bacterial growth. The technique of laying the strip on top of the agar was adopted, since these strips were able not only to induce pigment formation by the bacteria, but to show the pigment and provide a permanent record of syntrophic pigmenting ability.

Paper Strip Preparation Variables

A variety of conditions were used to determine the best technique for culturing 9-3-3 and extracting the bipyrrole to impregnate the paper strips. These tests not only used the mutant strain WF, but also used four clinical strains, E220, E222, E223, and 2698B, which were shown to give equally strong color reactions, albeit weaker colors than WF. These clinical strains are referred to as the control clinical strains. Results of some tests led to the following conclusions. It was found that proteose peptone-glycerol broth inhibited bipyrrole production but that Bacto peptone-glycerol broth did not. The age of the cultures affected the amount of bipyrrole present in that a 3-day-old culture was able to supply more usable bipyrrole than a 5-day or older culture. Autoclaving did provide somewhat stronger colored strips and was adopted to eliminate the use of living cultures, with the attendant possibilities of supplying live inoculum to the test cultures. For the same reason, autoclaving was used for the impregnated strips. Cooling of the

autoclaved broth before addition of chloroform allowed quick layer separation.

Once these conditions for providing bipyrrole were established, other experiments were done to maximize the amounts of bipyrrole extracted and usable to both WF and the clinical strains. One experiment was done to determine the effect on the amount of usable MBC extracted under different conditions, used acid (HCl to make a pH of 3.0) or base (NaOH to make a pH of 10.0) added to the P-G broth after autoclaving. Strips made from the flasks treated in this manner did show pink when strain WF was used, but none of the control clinical strains showed any color formation. In the same experiment, sodium lauryl sulfate was added (0.2 g/100 ml) to the broth before the $CHCl_3$ was added. Again, strips made from this extract showed a pink color when WF was used, but none of the control clinical strains showed any ability to form a pigment. In the same experiment, a flask's contents were extracted with $CHCl_3$ and then immediately placed on the strips and evaporated. Color was seen on these strips, not only when WF was used, but with one of three control clinical strains as well. This indicated that the freshly extracted MBC had more usable MBC in it than the extracts with acid, base or sodium lauryl sulfate.

Other experiments showed that strips made from 3-4 day old cultures, inoculated with 9-3-3, which had formed some of the purple pigment, were used, gave the strongest color reactions with the clinical and control mutant strains. Again, the strongest colors were formed in strips made immediately after the $CHCl_3$ extraction. Those experiments which led to strips providing enough bipyrrole for both WF and control

clinicals to show color were adopted for use in growing 9-3-3 and impregnating the paper strips.

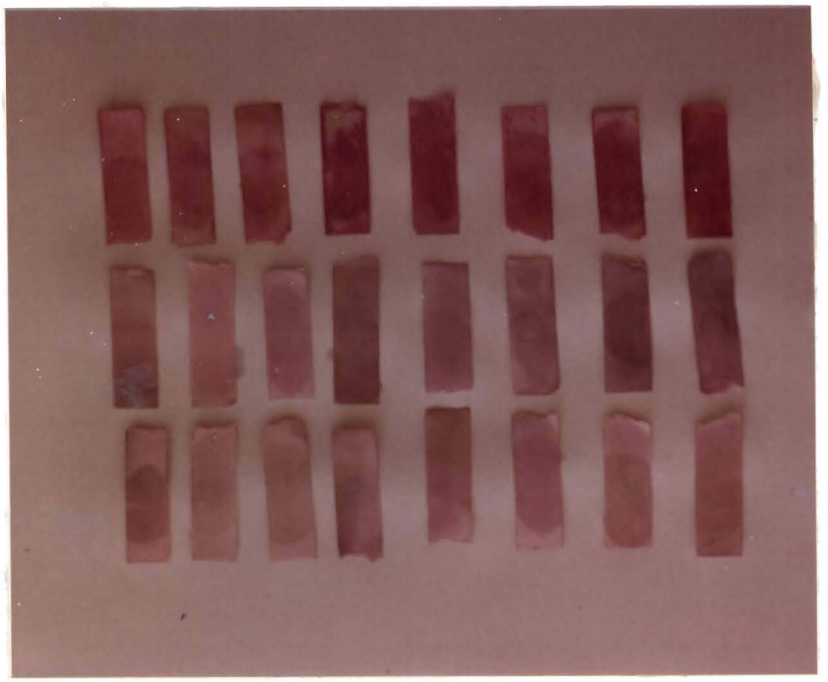
Paper Strip Controls

In order to use the assay to test for the clinical strains' ability to pigment, many flasks of 9-3-3 had to be grown and extracted. The first, and continuing, problem therefore faced was how to ensure that the effect of the variability in the MBC on the strips was minimized as much as possible. This variability is demonstrated by Figure 4.

Several steps were followed in order to determine whether or not the strips from each flask would give reproduceable results when used with the clinical strains. The time required to pool the $CHCl_3$ extracts to provide all strips with the same amount of MBC allowed the development of a blue-colored compound in the $CHCl_3$. Therefore, representative strips made from each batch of flasks were chosen and used with the mutant control strain WF as well as with any one of the four clinical control strains. If the colors were weak or nonexistent on any of these clinical strains, strips from these flasks were not used in an experiment.

Overall, although P-G agar syntrophic pigmentation provided clear color distinctions, like pinks and purples, the filter paper strips gave no such clear colors. Colors on the strips were not all the same pink or purple, and often were combinations of these basic colors. Such colors on the strips ranged from very light pinks to deep lavendars, to grays and to intense pinkish purples. (Classification of the colors on the strips was done by using Ridgeway's Color Standards.) Several colors appearing on the strips were not represented in Ridgeway's Standards

Figure 4. Variability in Color Produced by WF, E223, and ATCC 8100 on Strips from Different Batches of Flasks.



and are so marked.

Control organisms listed in Table III of Materials and Methods were tested with impregnated strips to determine the specificity of the assay system and none were able to produce pigment on the strips. The growth of the 11 Gram negative organisms was not inhibited. However, growth of all four Gram positive control organisms was inhibited, in one form or another. For example, growth of the two Bacillus species and Staphylococcus aureus was inhibited from 10 to 14 mm out from these strips at 24 hours, but after 3 days of growth, the growth came to within 1 mm from the edge of the strips. M. luteus was prevented from growing even after incubating for 5 days after inoculation.

In addition, a control on the broth was run by extracting a sterile flask of broth with $CHCl_3$ and impregnating paper strips with this extract. None of the strips showed color changes.

Experimental

Each of the 118 noncharacterized strains was tested with filter paper strips obtained from two different flasks to evaluate the flask differences. Most strains gave similar colors when the strips from different flask extracts were used, although the colors were usually lighter or darker, or of a different shade. For example, strain 13417A gave one strip colored vinaceous lavendar and the second strip colored deep dull lavendar. Several strains did not give uniform results from the duplicate platings, giving grays and pinks or purples. An example of this type of reaction would be that of strain 12633, which had one strip colored livid pink and the second strip colored pale vinaceous lilac.

Results of the MBC-impregnated filter paper strip tests showed that 116 out of 118 nonpigmented Serratia marcescens strains were able to produce color on these strips. A comparison of the results for syntrophic pigment using 9-3-3 as a live donor on P-G agar yielded only 99 strains showing color, or 83.9% of the strains. In all, only two strains gave negatives, showing no color change on the paper strips at all. The distribution of the color results for the 116 positive isolates are illustrated in Table X. Table XI lists all colors observed in the paper strips. Many of those colors mentioned in Table XI are illustrated by the photographs in Figure 5.

Among those 40 strains which either did not react, or are not otherwise mentioned, in these results included the two negative Serratia marescens strains, Nima and the three mutants OF, WF and 9-3-3, two S. liquefaciens, one S. rubidaea, and one Klebsiella species. The rest, some 30 strains, were all pigmented Serratia, and thus had the ability to produce both precursors.

Of all Serratia marcescens isolates, only two showed no ability to form pigment with the paper strip assay. These two were among those which were negative on the early syntrophic pigment assay on P-G agar. When supplied with both MBC (paper strip) and MAP (WF culture in lid), neither strain was able to form pigment. These results indicate that the two strains, 1-2232 and 2-2232, which were both isolated from the same patient, do not possess the coupling enzyme, and without this enzyme, there is no information on their ability to form the precursor. These results also indicated that, while production of the monopyrrole precursor and utilization of the monopyrrole and fed bipyrrole are not

TABLE X. Results of Strip Colors From Different Batches of Bipyrrole.

Strip A Color	No. Strains A Color	Strip B Results: Purples/ Violets	Lavendars	Lilacs	Pinks	Grays
Purples/ Violets	38	18	3	10	5	2
Lavendars	30		13	10	6	2
Lilacs	12			4	7	1
Pinks	34				32	2
Grays	2					2
Total	116					

TABLE XI. Colors Observed on MBC-Impregnated Paper Strips with Clinical Strains.

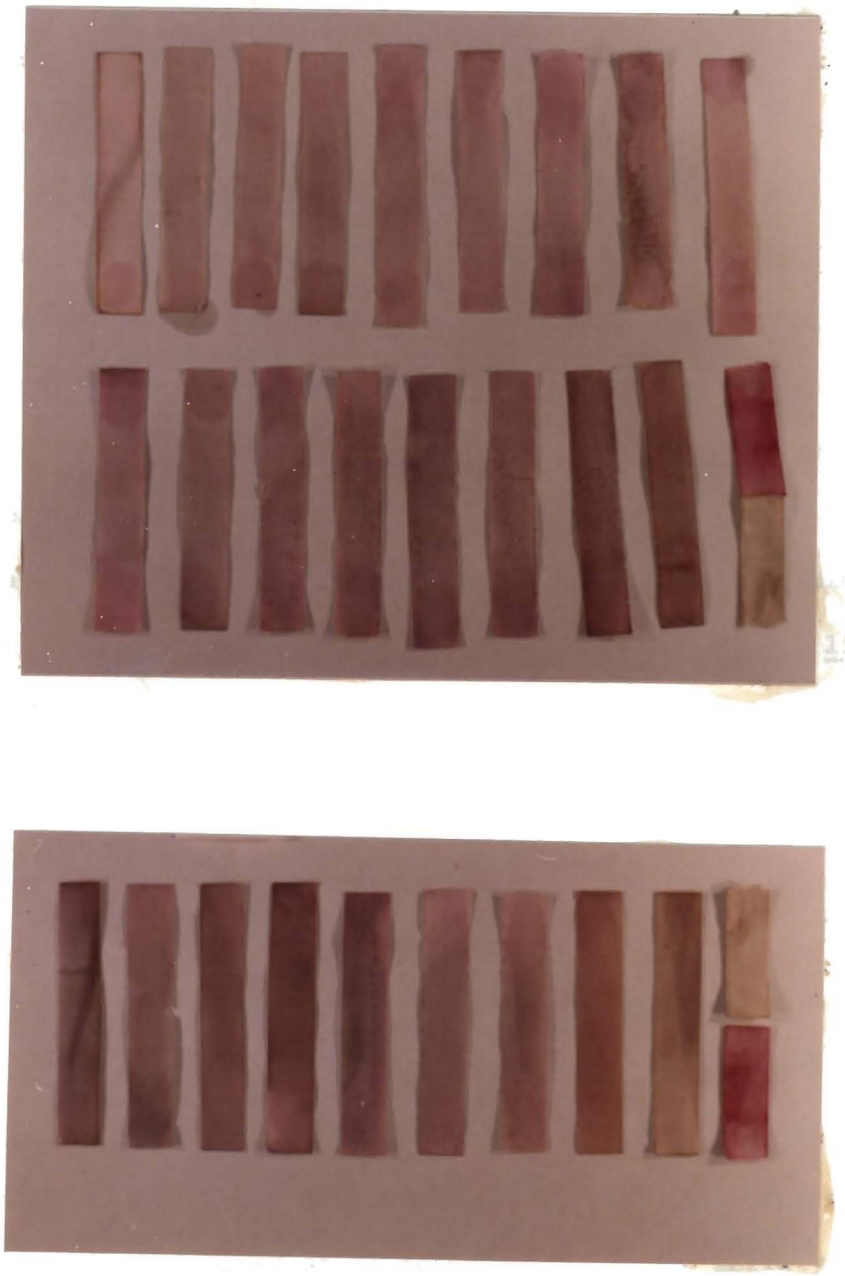
Purples/Violets	Lavendars
Pale Lobiela Violet	Vinaceous Lavendar
Light Vinaceous Purple	Dull Lavendar
Pale Amparo Purple	Deep Vinaceous Lavendar
Pale Rose Purple	Deep Dull Lavendar
Rose Purple	
Dull Lavendar Purple	

Lilacs
Light Pink Lilac
Light Pale Lilac*
Light Vinaceous Lilac
Pale Vinaceous Lilac
Pale Lilac
Vinaceous Lilac

Pinks	Grays
Light Pink*	Light Plumbago Gray
Light Livid Pink*	Light Purple Gray
Livid Pink	Pale Varley's Gray
Very Light Purple-Pink*	Heliotrope Gray
Mallow Pink	Pale Gray Vinaceous
Rosalane Pink	Pallid Purple Drab
Cameo Pink	Pink Purple Drab
Pale Amaranth Pink	Vinaceous Gray
Pale Vinaceous	Light Quaker Drab
Mauvette	

* signifies a color not illustrated in Ridgway's Color Standards.

Figure 5. Colors Produced by Clinical Strains on MBC-impregnated Paper Strips.



universal among Serratia marcescens, over 98.3% of the nonpigmented isolates have this characteristic.

It must also be noted in this discussion that, although negative paper strip and syntrophic reactions are recorded for the two S. liquefaciens strains, this was not strictly the case. No pink or purple colors were noted on any paper strips when the two S. liquefaciens strains were grown. However, the colors of the strips did change from a white to a dark grayish color. The reason for this is unknown. However, S. liquefaciens has been placed as a Serratia species on the basis of morphological, physiological, biochemical and C source utilization tests by Grimont et al. (39). The ability to change the color of the MBC-impregnated paper strip was observed to be a characteristic of the Serratia species alone. These reactions illustrate that S. liquefaciens is more closely related to S. marcescens than earlier workers (9, 20) had assumed.

The Effects of Growth Conditions on Pigmentation

In these experiments, clinical red and white strains were used, as well as WF, 9-3-3, Nima and the ATCC white S. marcescens strain. Petri dish cultures were either sealed with Scotch tape, grown in a candle jar, or in an anaerobic Gas-pak jar. White strains were supplied with an MBC-impregnated paper strip to determine their ability to produce monopyrrole and to use the MBC precursor to make pigment under the test conditions.

Scotch tape plate results (Table XII) were identical to the results which were obtained from plates which were not taped. The only excep-

TABLE XII. Effect of Growth Conditions on Pigmentation.

Serratia Strain	Colors Observed on Paper or in Bacteria	
	Tape Sealed	Candle Jar
Red Strains		
S. rubidaea	Red	Little Red
Nima	Red	Deep Red
13338	Red	Deep Red
11885	Red	Deep Red
2938A	Red	Deep Red
9-3-3	White	Deep Purple
White Strains		
WF	Rose Red	Rose Pink
E223	Cameo Pink	Pale Amaranth Pink
ATCC 8100	Rosalane Pink	Pale Vinaceous
D157	Rose Purple	Vinaceous Lavendar
2978B	Dull Lavendar	Plumbago Blue
1502A	Dull Vinaceous Lilac	"Light Pink"
5384	Dull Lavendar	Dull Lavendar
15823C	Pale Lobiela Violet	Lobiela Violet
9-2 1986 *Serratia*	Light Vinaceous Purple	Pale Lilac

tion was 9-3-3, which required room atmosphere or candle jar conditions to produce the purple pigment. These tests indicated that the optimal conditions for producing the syntrophic pigment are room atmosphere, which provided the darkest colors on the strips. Under anaerobic conditions, in the Gas-pak jar, little growth was produced, and there were no colors on any strip or in any colonies.

Another parameter of normal and syntrophic pigment production surveyed was the effect of temperature on red Serratia strains, and on white and red Serratia strains supplied with the bipyrrole-impregnated paper strips. The test was designed to see the effect of high and low growth temperatures on the production and utilization of MAP.

Table XIII lists the effect of different incubation temperatures on red strains. It was found that red Serratia are uniformly inhibited from producing pigment at 40° C, but that only a few are inhibited at 35° C. As reported in the Introduction, studies done on the mutants WF and 9-3-3 have shown that the production of the bipyrrole ceases at 40° C, the production of the monopyrrole ceases at 37° C, and the coupling enzyme ceases to function at 40° C. If this is so, then in red strains, the production of the pigment must cease at 37° C, when the monopyrrole can no longer be produced. While these data do not contradict this upper limit, they show that there is variability in the upper limit, and for several of the red strains, the upper limit for pigment is 35° C.

The effects of different incubation temperatures on the ability of the control and clinical strains to produce their pigment when supplied with the MBC-impregnated paper strip are listed in Table XIV. These

TABLE XIII. Effect of Temperature on Naturally Red Strains of *Serratia*.

Strain	20° C	25° C	30° C	35° C	40° C
Nima	orange-red	red	red	red	white
2938A	orange-red	red	red	white	white
11885	orange-red	red	red	red	white
13338	red	red	red	red	white
S. rubidaea	red	red	red	lt. red	white
9-3-3*	purple	purple	purple	white	white

*9-3-3 is normally purple on this medium.

TABLE XIV. Temperature Effects in Filter Paper Tests by White *Serratia* Strains.

Strain	20° C	25° C	30° C	35° C	40° C
WF	red	red	red	light red	white
ATCC 8100	pink	dark pink	dark pink	pink	white
E223	pink-purple	pink	pink	dark pink-purple	dark pink
D157	light pink	dark pink	dark pink	white	white
2978B	dark pink-purple	pink-purple	pink-purple	pink	pink
1502A	pink	pink	pink	pink-purple	light pink
5384	pink	pink	pink	pink	pink
15823C	pink	pink	pink	pink	white
9-2 1986 S.	dark pink	pink	pink-purple	pink-purple	pink

strains showed a non-uniform response to different incubation temperatures. Some strains showed inhibition at 35° C, others at 40° C, and a few, no inhibition at 40° C. These results indicate that, in the clinical strains, the monopyrrole can be produced at temperatures above the inhibitory temperature in WF, and that the coupling enzyme is also active at these temperatures. Again, these results show that there are varied responses to incubation temperature, and that the upper limit of pigmentation or enzyme activity and monopyrrole production, is not 37° C, but is a range of temperatures, and varies with the strain studied. In addition, these clinical nonpigmented strains can respond at 40° C, while the mutant strain WF, and the normal and clinical red strains cannot.

The next experiment explored the use of MBC on paper strips to visualize the production of MAP and coupling in red Serratia grown at higher temperatures. The results are listed in Table XV. Seven out of 10 red strains showed the ability to produce MAP and couple it to MBC at 40° C, a higher active temperature than reported elsewhere for the enzyme. These strains, grown at that temperature, without the MBC precursor, were all white, but when MBC was supplied, they formed pigment. This indicates that the bipyrrole precursor was unable to be produced at this temperature, but that the monopyrrole was produced. This is opposite the conclusions reached by Williams et al. (99) when they used the mutants 9-3-3 and WF.

The results of these three temperature experiments indicate that the bipyrrole is more susceptible to temperature than either the monopyrrole or the coupling enzyme. These results also indicate that there

TABLE XV. Effect with MBC of Temperature on Red *Serratia* Strains.

Serratia Strain	Color	Room Temperature No Strip	40° C With Strip
Nima	red	white	pink
13338	red	white	red
5920B	red	white	pink
10152A	red	white	pink
28984	red	white	pink
E19	red	white	pink
482 Ul.	red	white	pink
S. m. John	red	white	white
OF*	orange	white	white
S. rubidaea	red	white	white

*OF is a normally orange Nima mutant.

is a range of upper pigmenting temperature limits that are effective for different strains, rather than a single absolute upper temperature limit as shown by Williams (120).

Effect of Antibiotics on Monopyrrole Production

This investigation also compared the effects of antibiotics on monopyrrole production in clinical isolates, with their effects on WF (34, 98). Eight different antibiotics were used. The first step was to determine whether a sublethal concentration of antibiotics for WF could be found which would inhibit 9-3-3 from forming syntrophic pigment when plated by the Williams and Gott technique. The second step took those antibiotics for which there were sub-lethal concentrations and obtained growth curve information for strain WF. The last step involved known numbers of bacteria, both WF and clinicals, which were added to the MMH agar plate which had both the proper concentration of antibiotic and an MBC-impregnated paper strip, and observation of the formation of the pigment by the bacteria.

The determination of the sub-lethal, monopyrrole-inhibiting concentrations of antibiotic was originally done by the Williams and Gott technique, using 9-3-3's ability to combine the volatile monopyrrole to produce pigment. The eight antibiotics, carbenicillin, kanamycin, novobiocin, polymyxin B, dihydro-streptomycin, rifampin, puromycin, and tetracycline were tested at various concentrations. The results are listed in Table XVI. For carbenicillin, kanamycin, polymyxin B, and tetracycline, no concentration of antibiotic was found which inhibited the production of the monopyrrole. Rifampin, at 5.0 µg/ml, was able to

show inhibition of monopyrrole, but the growth was greatly affected. In contrast to these five antibiotics, streptomycin, novobiocin and puromycin were all able to show inhibition of monopyrrole production by this plating technique. These three antibiotics were chosen to use in the next experiments.

In the second step, growth curves for WF in novobiocin, puromycin and streptomycin were done. The growth curves of WF in puromycin, at 100 µg/ml, and streptomycin, at 0.4 and 0.6 µg/ml, correlated with the generalized growth curve, Eq. 2. Therefore, these two antibiotics, at these concentrations, showed no inhibitory growth effects on WF. However, novobiocin broth, at either 80 or 100 µg/ml, did not allow WF to grow normally. Instead of a growth curve as done for puromycin and streptomycin, it was determined that novobiocin had a definite killing effect on the majority of WF cells. MMH agar plates were prepared with 100 µg/ml of added novobiocin. Dilutions of WF were added to determine the percentage of cells killed by the antibiotic. Novobiocin killed WF completely when WF concentrations of less than O.D. 1 were used. Between concentrations of O.D. 1 (4×10^8 cells/ml) and O.D. 2 (9×10^8 cell/ml) WF could grow, and the novobiocin-resistant colonies grew at what appeared to be the same rate as normal WF on non-antibiotic MMH agar plates.

For the third step, WF was added to the MMH antibiotic agar plates, along with the bipyrrole-impregnated paper strips. Bipyrrole was allowed to diffuse from the strip through the agar, since no bacteria were inoculated on top of the strip. This resulted in red bacteria, and white paper strips. Neither novobiocin nor puromycin were able to

TABLE XVI. Effect of Selected Antibiotics on WF

Antibiotic	Concentration Permitting Growth and Pigment (µg/ml)	Concentration Inhibiting Pigment/Not Growth	Concentration Inhibiting Growth
Carbenicillin	50.0	none	100.0
Kanamycin	1.75	none	2.0
Novobiocin	50.0	80,100	120.0
Polymyxin B	100.0	unknown	unknown
Puromycin	50.0	100	unknown
Rifampin	1.0	5.0	10.0
Dihydro-streptomycin	0.2	0.4, 0.6	1.0
Tetracycline	150.0	none	unknown

inhibit monopyrrole production in WF or in the clinical strains. Streptomycin, as well, was unable to inhibit syntrophic pigment by WF, but was able to inhibit syntrophic pigment in the only clinical strain tested. This implies that the ability of the clinical strain to syntrophically pigment was more sensitive to streptomycin than the ability of the known mutant strain WF. Thus, as reported by Gott and Williams (34), WF showed some sensitivity to monopyrrole production in the presence of streptomycin, but not a complete inhibition of production. However, the one clinical strain tested, E223, was inhibited by streptomycin. Likewise, the finding that puromycin and novobiocin do not adversely affect either strain points to an approximately equivalent response by secondary metabolites to those drugs.

DISCUSSION

This Discussion will focus on four areas. The first deals with the work on MBC and prodigiosin assays. The second area concerns strain 9-3-3 and the production of MBC in different media, while the third area is that of the paper strip assays with WF and the clinical strains. The last area of this Discussion will present a model for synthesis and location of precursor and pigment in both pigmented and nonpigmented strains, as well as provide an explanation for the various affectors of pigmentation as outlined in the Review of the Literature and Discussion sections.

Assays

Information drawn from Figures 2 and 3 was compiled in Table VI and provided the opportunity to explore some of the questions raised in the Introduction. They include: (1) why is Nima able to feed both MBC and MAP, and is this a leakage from whole viable cells, or from disrupted cells; (2) is the production of the precursors immediately followed by a complete uptake into pigment; and (3) will there be any extra MBC in a 9-3-3/WF culture, a combination which can produce syntrophic pigment?

That the manufacture of pigment precursors is not immediately followed by the incorporation of the precursors into pigment is illustrated by the assay results which measured excess MBC in Nima. The assays also measured excess MBC in the 9-3-3/WF culture, which could mean that 9-3-3 produced more MBC than WF produced MAP. The samples for the assays were

taken from cultures which were either in the logarithmic or early stationary phase of growth. Cell death is rare in logarithmic phase, but there is some cell death in the early stationary phase of growth. Further, according to Davis (18), syntrophic growth is assumed to be the result of the excretion and incorporation of precursors from viable cells to viable cells. Not only was MBC produced in Nima that was not incorporated immediately into pigment, but Nima is known to be able to feed both 9-3-3 and WF by excreting precursors. Since excess MBC was measured in cells which were in logarithmic or early stationary phase, the MBC is presumed to be the result of excretion from viable cells and not the result of cellular lysis. Whether MAP leakage is the result of cellular excretion or lysis is unknown, since there is no assay for MAP.

One explanation for the ability of MBC and MAP to be excreted by Nima suggests that these precursors may not be produced by the same enzyme system. Since pigment precursors are produced in pigmented cells which are not pigment incorporated, information concerning the stoichiometry of precursor production as well as the control of precursor production could help define the role of pigment precursors in the cell. Questions about the rate and amount of production of MBC in 9-3-3, compared to that in Nima, as well as about whether or not the pathways for MBC and MAP production have common intermediates or are co-regulated, have become important to answer.

Table VI listed the amount of MBC produced by 9-3-3 at $O.D._{363-400nm}$ as 0.124, and the total amount of MBC produced by Nima as 0.137 at $O.D._{363-400nm}$. Nima produced 10% more precursor than 9-3-3. The fact that 9-3-3 did not produce more precursor than Nima demonstrates that

there is no enhancement of MBC production in 9-3-3 when production of MAP is blocked. No enhancement of MBC production also leads to the inference that there is no point of bifurcation between MAP and MBC pathways. Williams and Hearn (106) reported that several mutant strains, blocked at "point 7" in both the MBC and MAP pathways, were able to produce pigment if they were supplied with syntrophically fed precursors from mutants blocked at higher points on either pathway. Their suggestion was that this represented a common intermediate in precursor synthesis. However, they did not consider the equally likely proposal that these strains had two defective enzymes, or one defective enzyme which was nonspecific in function. Since MBC production was not enhanced by the inability to produce MAP as a controlled bifurcated pathway would be, a common intermediate precursor does not seem likely. In addition, Shrimpton, Marks and Bogorad (80) found that proline was incorporated into different positions on the MBC and MAP pyrrole rings, indicating that they were formed by different mechanisms.

If the precursors are produced separately, by different mechanisms, as suggested, the regulation of production must be considered. The fact that excess nonpigment-bound MBC precursor is produced by Nima, and that MBC is produced at all by 9-3-3 without the production of MAP indicates a lack of cross regulation of precursor synthesis. Work showing cellular regulation of precursor synthesis was done by Gott and Williams (34). They determined that, while all antibiotics used blocked MBC production in minimal medium, only cephalosporin and polymyxin B inhibited MBC production in complete medium. Among the antibiotics used were streptomycin, penicillin and tetracycline analogues, as well as the

detergent-like polymyxin B. Tetracyclines and streptomycins were able to affect MBC while the mutant was growing on minimal, but not on complete medium. Since tetracycline affects the 50 S ribosome moiety and interferes with the transcription of mRNA, and streptomycin interferes with the 30 S ribosome moiety to prevent initiation of mRNA transcription, MBC inhibition by them suggests that either the ribosome-mRNA complete transcribing MBC enzymes were affected, or that under these conditions, cellular production of MBC was inhibited. Since these antibiotics had no effect on MAP production, MAP enzymes and synthesis were differently affected again indicating a lack of co-regulation.

Overall, information provided by the assays answered questions about precursor and pigment production by illustrating that excess pigment precursors produced by Nima is not immediately incorporated into pigment, and that 9-3-3 showed no enhancement of MBC production. Further, the idea of separate pathways, without common precursors and no co-regulation seems to have some support from this data, in that there was no 9-3-3 MBC enhancement. This hypothesis is supported by other evidence, particularly work done by Gott and Williams on antibiotic inhibition (34), and by Shrimpton et al. (80) on proline incorporation into MBC and MAP. This hypothesis will be incorporated into the last section of this Discussion, and form the foundation for the model of pigment and precursor production offered at that time.

Strain 9-3-3

Five media were used to grow strain 9-3-3. These were Bunting's modified complete medium minus the phosphate salts, P-G broth, MMH agar,

proteose peptone-glycerol broth, and Trypticase Soy (TS) agar. As mentioned in the Introduction, Serratia pigment production is sensitive to such media components as phosphate salts and thiamine. Hence, media considerations are relevant.

9-3-3, in addition to producing the bipyrrole MBC, produces a purple pigment. When producing MBC in any quantity, the purple pigment also appears in the 9-3-3 culture. It may contain bipyrrole (45) but its specific structure is unknown. Because of its appearance whenever there is a sufficient quantity of MBC to feed the control strains, the presence of the purple pigment in 9-3-3 flasks became somewhat of a guide for this work. Those growth flasks showing some purple pigment production provided impregnated paper strips with enough MBC to be colored by clinical strains.

Data on different agar syntrophic pigmentation results show that formation of purple pigment is a sign of sufficient MBC accumulation to permit clinical strains to show color. TS agar prevented formation of purple pigment by 9-3-3. Out of the 49 strains tested on TS and P-G agar (Table VIII), 23 strains were unable to show any syntrophic pigment on TS agar. Of these 23 negative strains, 13 were able to show syntrophic pigment when plated versus 9-3-3 on P-G agar, which allows formation of larger amounts of MBC and purple pigment. Later tests with MBC-impregnated filter paper strips showed that all but two of the 49 strains were able to pigment on MMH agar. In addition, several clinical strains were used on later tests versus MBC-impregnated paper strips on TS agar. All of these strains were able to show pigment formation. The conclusions drawn from these results are that, when unable to form

purple pigment, not enough MBC is produced by 9-3-3 to ensure formation of color on impregnated paper strips.

Strain 9-3-3 required either an unsealed Petri dish, or candle jar conditions to form purple pigment (Table XII). When grown in Scotch taped Petri dishes, and in anaerobic Gas-pak jars, no purple pigment was formed. And, additional observation has concluded that, in cultures in identical P-G media, the amount and vigor of the shaking controlled the amount of purple pigment, and therefore, the amount of MBC produced. 9-3-3 cultures which are shaken very vigorously, or which have larger surface-to-air ratios, produced more purple pigment. The production of prodigiosin is known to be oxygen dependent (43), and the need for shaking 9-3-3's in order to form purple pigment illustrates the purple pigment's oxygen dependency as well. Temperature control of purple pigment is similar to that seen in prodigiosin (Table XIII), which also illustrates the interrelatedness of these two pigments.

Jackson et al. (44), and Witney et al. (109) offer ideas on how media contents affect the production of MBC in 9-3-3; those effects noted in this work confirm their observations. These media allowed good formation of MBC and purple pigment: Bunting's complete medium, P-G broth and MMH agar. The two media which inhibited purple pigment production and allowed minimal MBC production were proteose peptone-glycerol broth and TS agar. A detailed comparison of media composition is impossible, since the Difco Manual (23) and the BBL Manual (74) list different components, some of which are undefined, such as primary proteose N and peptone N. However, the effect of media on 9-3-3's MBC was important in controlling the use of the paper strip assay, as well as

in supplying information to be considered in the last section of this Discussion.

Paper Strip Assay, WF and Clinical Strains

When first plated versus 9-3-3 on P-G agar, the clinical strains were divided into several groups, both by color and by their earlier ability to pigment with 9-3-3 on TS agar. Those colors which developed when plated versus 9-3-3 on P-G agar were classified as pink, purple, and pink with purple edge. A possible explanation for these colors would be the assumption that: those strains which produced pink colors used the MBC precursor from 9-3-3 to conjugate to a native made precursor; the purple color in the clinical strains were the result of alterations in 9-3-3's purple pigment; and the pink with purple border a combination of the two.

However, these color division observations were not confirmed by other tests, when these clinical strains were supplied with MBC-impregnated paper strips. Those strains producing pink on P-G agar colored the strips in a wide range of pinks and purples. This same type of result was seen for strains included in all color divisions on P-G agar. In all, pigment colors on the paper strips represented 35 different colors. All the colors observed had some element of pink in them, although none were as red as the control WF strips. However, the fact that there was such a variety of colors suggested that, if the nonpigmented strains were producing pigment precursors, perhaps not all the precursors were identical.

In fact, there are two different possible explanations for the

production of the pigments by these nonpigmented strains. The first explanation is the hypothesis that these strains do produce pigment precursors, which act similarly to MAP, and that these strains also produce the conjugating enzyme. The second hypothesis states that there is an almost universal ability in the clinical nonpigmented strains to chemically alter the noncolored MBC into a vividly colored pigment without the addition of any native precursor.

The question about the ability of normally nonpigmented strains to utilize pigment precursors has not been approached before. However, since the phenomenon of syntrophic pigmentation by the clinical strains seemed to be similar to that pigment produced syntrophically to WF, and both hypotheses seemed to be equally likely, culture and growth conditions were used to determine the limits of the clinical strains' abilities and hence, whether similar enzymes governed the two precursor systems.

When growth conditions were changed, and the cultures supplied with MBC, clinical strains reacted as WF did (Table XV). WF and the clinicals, grown in an anaerobic Gas-Pak jar, produced very slight growth without pigment. When grown in a candle jar, WF's color became lighter than normal, as did the colors of the clinical strains' strips. Scotch taping of plates had no effect on either WF or clinical strains. Therefore, for the various atmospheric conditions used, these nonpigmented strains' abilities to produce pigment are affected by the same conditions as WF's. Production of the pigment prodigiosin is only possible in a well-aerated culture (43), but the red strains used showed similar reactions (Table XII).

Incubation temperatures were able to differentially affect pigment by WF and clinical strains (Table XIV). WF was very red at 20°, 25° and 30° C, but less red at 35° C, and white at 40° C. Clinical strains' abilities, however, were not inhibited at 40° C, with five out of eight clinical strains able to form pigment. These results indicated that, if these nonpigmented strains were producing a precursor, this precursor's synthesis was less affected by temperature than WF's.

These results tend to support the hypothesis that the clinicals are creating a precursor to couple to MBC and not just converting MBC. However, there is no solid evidence to prove it. In working with the antibiotics and their effect on monopyrrole production, it was hoped that an antibiotic could be found which was able to suppress WF's and the clinicals' ability to form pigment.

The antibiotics used in work reported in this thesis represented a variety of mechanisms of action and these are listed in Table XVII. For carbenicillin, kanamycin, polymyxin B and tetracycline, no ability to inhibit production of the monopyrrole was seen. These results agree with those reported by Gott and Williams (34) and by Wiel (92). However, rifampin, not tested elsewhere, did show an ability to inhibit monopyrrole production in WF, although only at the limit of growth, and was not tested further. None of the three antibiotics which were tested with paper strips, puromycin, novobiocin and dihydrostreptomycin, showed an ability to inhibit WF's monopyrrole production. Streptomycin was seen to cause a decrease in the amount of monopyrrole produced by WF, again agreeing with the literature (34), although streptomycin and not dihydrostreptomycin was the specific antibiotic used. But, unlike WF,

TABLE XVII. Antibiotics, Mechanisms of Action.

Antibiotic	Site of Action	Mode of Action
Dihydro-streptomycin Kanamycin	Ribosome, 30S	Aminoglysides, enzyme unable to recognize and cause release of protein from ribosome, causes misreading of mRNA.
Puromycin	Ribosome, 50S	Premature release of peptide chain from ribosome by binding to 50S moiety and replacing aa-tRNA.
Tetracycline	Ribosome, 50S	Prevents binding of aa-tRNA to A site.
Rifampin	DNA	Blocks initiation of transcription.
Novobiocin	DNA and RNA	Inhibits synthesis, cytoplasmic membrane disrupted.
Carbenicillin	Cell wall	Inhibits synthesis.
Polymyxin B	Cell wall	Detergent action, renders cell envelope nonfunctional.

the one clinical strain tested did have its pigmenting ability inhibited by streptomycin.

Those antibiotics used by other researchers on S. marcescens to determine their effect on pigment and pigment precursors have come from several antibiotic families. These have included streptomycin, tetracycline, penicillin and their derivatives, as well as polymyxin B, bacitracin and chloramphenicol (Table XVIII).

Studies using each antibiotic to examine its effect on MBC, MAP and coupling enzyme have not been done since some of the reported work was only used to see if pigment was inhibited, and did not specify which part of the pigment reaction was affected. Overall, work has shown that the coupling enzyme was affected only by high concentrations of kanamycin and cephalosporin. Cephalosporin acts like penicillin, and kanamycin blocks the initiation of translation. However, every antibiotic tested was able to affect MBC production. This suggests that MBC is very sensitive to changes in the medium, as well as changes to the cytoplasmic membrane and cell wall.

MAP production, both by work in this thesis and by others' results (34), is sensitive only to streptomycin. But even though less MAP is produced when grown with streptomycin, there is no inhibition. Three clinical strains, grown with both novobiocin and puromycin, showed no inhibition. One strain was grown in the presence of streptomycin and production of the pigment was inhibited. This would suggest that, perhaps, these clinical strains are not producing MAP, but an analogue of it, to be used within the cell. Since 9-3-3 was unable to show any color change when "fed" from the clinical strains, and thus, illustrated

TABLE XVIII. Reported Antibiotic Effects on Pigment and Precursors.

Antibiotic	Antibiotic Family	Site of Action	Pigment/Precursor Effects MAP	MBC	Pig.	CE**
Streptomycin	Aminoglyside	(Cell membrane) P10 protein, 30S ribosome moiety, misreading	+ -	+	-	-
Neomycin	Aminoglyside	"	-	+	NR*	-
Kanamycin	Aminoglyside	"; other 30S sites as well	-	+	NR	+
Tetracycline	Tetracycline	Prevents recognition 30S ribosome moiety	-	NR	NR	NR
Aureomycin	Tetracycline	"	-	+	+	-
Terramycin	Tetracycline	"	-	+	+	-
Chloromycetin	Chloramphenicol	50S ribosome fragmentation rxn inhibited	-	+	+	-
Polymyxin B	Polymyxins	Detergent action cell envelope	-	+	NR	NR
Bacitracin	Bacitracin	Cell wall building inhibited	NR	NR	-	NR
Penicillin	Penicillin	"	NR	NR	-	NR
Carbenicillin		"	-	NR	NR	NR
Cephalosporin	Cephalosporin	"	-	+	NR	+

+ indicates inhibition.
- indicates no inhibition.
* NR--not recorded in paper.
**CE--coupling enzyme.

no leakage of precursor from clinical strains, this could be the case.

The specificity of streptomycin's effect on monopyrrole production has some reaching effects on the system of precursor production. Bermingham, Deol and Still (6) observed that, while low concentrations of streptomycin did not affect pigment formation, changes were observed in the cytoplasmic membrane. Streptomycin permitted the accumulation of phosphatidylethanolamine, and a decrease in acetone-soluble dipsipeptides. Cyclic dipsipeptides are suspected to be ion carriers across the cell membrane, and the decrease could explain the efflux of ions through the cell membrane that is seen in higher concentrations of streptomycin. Other effects by higher concentrations of streptomycin (28) which are seen in the bacterial cell are: (1) an inhibition of protein synthesis; (2) stimulation of RNA synthesis; (3) inhibition of cellular respiration; and (4) loss of low molecular weight materials from the intracellular pool. Streptomycin's ability to affect MBC production, at such low amounts (6) could stem directly from membrane effects, while those actions of streptomycin responsible for inhibition of translation could be the cause of monopyrrole decrease or inhibition.

However, kanamycin is also an aminoglyside antibiotic, with much the same range of activity as streptomycin. Streptomycin is thought to be specifically bound to the P10 protein of the 30S ribosome moiety (83). Kanamycin, however, not only affects this area, but has other attachment sites as well. As an example of the different actions of the two antibiotics, streptomycin is able to block steps in virus coat proteins that kanamycin is unable to block (83). The inability of kanamycin and the ability of streptomycin to affect monopyrrole production provides

a second difference in action mechanisms.

The difference in the sensitivity of precursor in clinical strains and the WF illustrates that there are differences between the MAP produced by WF and the probable precursor produced by the clinical strains. Overall, the clinical strains seem to produce smaller amounts of precursor, but are able to maintain its production at higher temperatures than WF. WF and clinical pigmentation act the same under different atmospheric conditions and both are affected by streptomycin, although the clinical strain was affected to a greater degree.

There are several other ideas which can be considered about the differences and similarities between clinical and control pigment. Prodigiosin is not a single pigment, and appears to come in a wide range of shades naturally. The variety of colors observed produced on paper strips by clinical strains are thus in keeping with known information on prodigiosin's color ranges. Another factor is that the color produced on these strips by some of the clinical strains fades away after several weeks. Prodigiosin is not light stable (106). A further report of instability in color produced by a pigmenting strain is from Weinberg (93). HY, a strain often used in pigment research, produces a pigment that fades away when grown on complex media. Thus, the phenomena noted in this work are not unknown to occur in red strains.

Model for Precursor and Pigment

A large amount of uncoordinated information is available concerning pigmentation. Such information includes the data recorded here and elsewhere on antibiotics, which has been listed in Tables XVI and XVIII.

Additional work on phosphate inhibition (12, 44, 109) and on the requirements for pigment synthesis (22) is also important.

The early portions of this Discussion determined that MBC and MAP precursor productions are separate biochemical pathways. The two precursors do not have any known common intermediates and are probably not formed by the same mechanisms. Ability of nonpigmented cells to form pigment when fed MBC indicated the presence of a probable pigment precursor within these cells. It also indicated that these strains possess a coupling enzyme which functions similarly to the known MBC and MAP coupling enzyme.

New information on pigment has come from several authors who have suggested that the pigment is located in the cell envelope or associated with the cell wall (61, 65, 85). Loriya et al. (52) correlated extracellular protease production, which is located on the cell membrane (35, 55) and pigment production. They saw that, when protease production was inhibited by antibiotics, pigment synthesis was also inhibited. They also recorded that, in pigmentless mutants, extracellular protease concentrations were lowered. It is their suggestion that if both enzyme systems are located on the cell membrane, any change affecting one would affect the other. Others' results presented in the antibiotic section suggest that this not fully the case.

Using the information about antibiotic effects, protease, as well as phosphate inhibition, along with the other cited work in the Introduction, a hypothesis about the location and function of the pigment and the probable precursor has been developed. Table XIX lists the hypothetical sites of each element in the pigment system.

TABLE XIX. Proposed Location of Precursors, Enzymes and Pigment.

Product or Enzyme	Enzyme System Location	Location of Product/Enzyme
Probable Precursor	Cytoplasm	Cytoplasm
MAP	Cytoplasm	Cytoplasm
MBC	Cytoplasmic Membrane	Cytoplasmic Membrane
Coupling Enzyme	Cytoplasm/ Cytoplasmic Membrane	Cytoplasm/ Cytoplasmic Membrane
Prodigiosin		Cytoplasmic Membrane

Probable Precursor

The probable precursor, discovered in the clinical nonpigmented strains, is produced in the cytoplasm. Strain E223 was affected by streptomycin, indicating that the presence of the precursor is dependent on an actively formed enzyme system. This precursor is a normal constitutive product of the cell.

MAP

MAP may, or may not, be a further metabolized form of the probable precursors. Its enzyme system is also located in the cellular cytoplasm. Since its production is also somewhat affected by streptomycin, but not by any other antibiotic tested, this may represent a new specific site of action for streptomycin and dihydrostreptomycin in Serratia marcescens. The MAP produced in large quantities in pigmented and mutant cells is due to an active enzyme system, and is excreted, while the probable precursor is not produced in large enough quantities in nonpigmented cells to be excreted.

MBC

The bipyrrole precursor, MBC, is present only in pigmented strains and their mutants. This precursor represents genetic information in the cell which is either absent from most Serratia, or whose enzyme system is never transcribed. The location of this precursor and its enzyme system is on the cytoplasmic membrane. Production of MBC is dependent on the normal functioning of the cytoplasmic membrane and any change in the normal structure and function of this membrane is responsible for inhibiting the MBC production. In Tables XVII and XVIII, those anti-

biotics responsible for membrane damage (streptomycin) and for cell wall synthesis disruption (polymyxin B), along with any of several ribosome and DNA-active antibiotics were responsible for either inhibiting MBC production specifically, or inhibiting pigment at a nonspecific point. Since only one precursor seems to be the affected precursor, this is the only precursor whose enzyme production system is present on the cytoplasmic membrane.

Coupling Enzyme

In all but two S. marcescens strains used in this thesis, a functioning coupling enzyme was present. The location of this enzyme is uncertain. Although it is not affected by many of the situations which affect MBC production, it is slightly affected by high concentration of cephalosporin, which is a cell wall synthesis inhibitor. Whether this antibiotic is also able to affect coupling enzyme as a part of this function, or the effect is because of a different site of action, is unknown.

It is known that coupling enzyme's activity is dependent on Mg^{++} and ATP, that it is not phosphate suppressed, it has an active site sulfhydryl group, and that it is not inhibited by 40° C temperature. Because of the ineffectualness of phosphate and the antibiotics to affect its activity, the most logical location for the coupling enzyme would be in the cytoplasm.

However, Fiil and Branton (26) noted structural changes in the cytoplasmic membrane of E. coli caused by Mg^{++} starvation, and indicated that the starvation had affected the membranes indirectly through ribosome breakdown. Thus, proper maintenance of the membrane and of ribosome

function involves Mg^{++}, which could account for the dependency of coupling enzyme on Mg^{++}. Therefore, the possibility exists that this coupling enzyme attaches the pigment to the cytoplasmic membrane, and in doing so, alternates between the interior of the cell and the membrane. Since kanamycin is able to inhibit coupling enzyme activity, the enzyme is probably continually manufactured in the cell.

Coupling enzyme is a nonspecific enzyme which is able to function by condensing MAP with MBC, HBC to MAP, MBC to MBC (to form purple pigment), and the probable MAP-like precursor to MBC. Its nonspecificity has been demonstrated by the ability of Serratia marcescens to make analogues of prodigiosin (40, 42, 45), and by the variety of natural structures classified as prodigiosenes (30, 31, 32).

Prodigiosin

The pigment prodigiosin is located on the cytoplasmic membrane. It is known to be membrane bound (61, 85), and is probably located on the cytoplasmic membrane (65). The pigment is not excreted from the cell, but is retained by the cell. Williams (97) suggested that, since the pigment is a secondary metabolite, its formation comes from the accumulated, nonexcretable waste products. However, this is nonacceptable since there have been no differences in life span or viability recorded between pigmented and nonpigmented cells and living cells of Nima are known to be able to excrete both precursors, thus demonstrating their ability to be excreted separately from the cells.

Function of Prodigiosin

There have been several suggested functions for pigment. These

include a UV and gamma radiation sensitizer (4, 91), precursor of catalase (16), and an ion carrier (104). Pigment was found not to be the sensitizing agent, but a precursor of MAP was suggested to be responsible for the sensitivity. Any relation of pigmenting ability to catalase production has not been verified (108), but some correlation was noted, although prodigiosin is not a metabolite in the catalase pathway. However, the location of catalase in the cytoplasm of Staphylococcus lactis (55) would eliminate the association between loss of pigment and catalase as being an effect of the membrane-bound elements, and leaves open to question the real association between the two.

The hypothesis of pigment acting as an ion carrier does fit with known data. Firstly, pigment, particularly the blue fraction of chromatographed prodigiosin, is known to concentrate Ca^{++} and Fe^{+++}. Secondly, a class of Fe^{+++} carrier molecules, siderophores, is known to exist (94, 95). If both the pigment and the probable precursor were able to function as siderophores, the persistence of a minority of pigmented strains would be explained, particularly if prodigiosin were able to function somewhat more economically than the bound probable precursor.

Relation of Proposed Model to Known Effects

This proposed theory would explain a number of results, such as oxygen, temperature, antibiotics and media composition, which have not been interrelated before. These are listed in Table XX.

TABLE XX. Model-Based Pigment Affector Explanations.

Affector	Effect	Explanation
Higher alcohols and CHOs (63)	Either inhibit or enhance pigment	Ability to be incorporated into cytoplasmic membrane, determine effect on MBC and pigment production.
PO_4^{3-} (44, 109)	Pigment inhibited	Internal phosphate concentration constant, membrane transport affected, therefore effect on MBC enzyme system.
PO_4^{3-} or peptide concentration decreased (12)	Capsule and pigment production parallel increase	Each repeating subunit of capsule is built upon a membrane lipid which is then transported across cytoplasmic membrane (19), capsule production and MBC both dependent on membrane, no direct connection.
Unsaturated F.A.'s, in high temp. and low oxygen (51)	Pigment permitted	Ability to be incorporated into the phospholipid membrane layer as free fatty acids to prevent the confirmational changes due to higher temperature and lower oxygen.
Low oxygen (43)	Inhibit pigment and MBC	MBC inhibited by membrane activity and enzyme activity.
Cytoplasmic membrane and envelope-affecting compounds and antibiotics (11, 17, 34, 99)	Inhibit pigment and MBC	Disruption of membrane structure and alterations in function inhibit MBC synthesis.
Unknown (52)	Pigment and protease inhibited	Both present on cytoplasmic membrane, when one site disrupted, other is.
Unknown (64)	Pigment inhibited thermogenesis, growth, reproduction decrease, energy source uptake nuclease, respiration rate increased	Pigment greater efficiency than probable precursor, cell steps metabolic functions down, loses enzymes, less heat produced, reproduction slowed, nuclease and energy source uptake give cell needed new sources, respiration increases.

Suggestions

Although the specific function for prodigiosin and precursors is still unknown, the proposed separate production of precursors and production model has opened new avenues for exploration. Use of other mutants, blocked at different points other than 9-3-3 would allow the range of the coupling enzyme and probable precursor to be investigated. Isolation of the proposed pigment precursor as a radioactively tagged substance would allow the direct visualization and investigation of its synthesis and possible connections to catalase. In addition, the use of nonpigmented cells to represent the normal Serratia marcescens would allow the further exploration of the function of the pigment precursors in the normal cell.

Summary

Results of work done for this thesis have confirmed that the growth curves of different Serratia strains, both pigmented and nonpigmented, are identical. The development of a sensitive indicator of syntrophic pigment allowed the visualization of pigment in clinical, nonpigmented strains. The presence of a MAP-like precursor, and of a coupling enzyme was seen to be almost universal in these strains, with 116 out of 118 white Serratia marcescens strains showing an ability to produce the pigment. This ability was absent in all other Gram negative and Gram positive organisms tested. Compilation of data for different growth conditions, and utilization of antibiotics to attempt to selectively inhibit the pigment from forming in WF and nonpigmented strains allowed the development of a new model for pigment and precursor production. The model proposed that production of the two precursors was separate, and that there is production of a MAP-like early precursor in all strains. In addition, the MBC bipyrrole precursor was seen to act as an antibiotic, with a bacterial activity range similar to that of prodigiosin.

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