JOURNAL OF BACTERIOLOGY, June 1973, p. 999-1006
Vol. 114, No. 3

Printed in U.S.A.

Effect of Iron and Salt on Prodigiosin Synthesis in *Serratia marcescens*

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Received for publication 26 February 1973

Serratia marcescens wild-types ATCC 264 and Nima grew but did not synthesize prodigiosin in a glycerol-alanine medium containing 10 ng of Fe per ml. Wild-type 264 required the addition of 0.2 μg of Fe per ml for maximal growth and prodigiosin synthesis; Nima required 0.5 μg of Fe per ml. Three percent, but not 0.1%, sea salts inhibited prodigiosin synthesis in a complex medium containing up to 10 μg of Fe per ml. NaCl was the inhibitory sea salt component. The inhibition was not specific for NaCl; equimolar concentrations of Na_2SO_4, KCl, and K_2SO_4 also inhibited prodigiosin synthesis. Experiments with strains 264 and Nima and with mutant WF which cannot synthesize 4-methoxy-2-2'-bipyrrole-5-carboxaldehyde (MBC), the bipyrrole moiety of prodigiosin, and with mutant 9-3-3 which cannot synthesize the monopyrrole moiety 2-methyl-3-amylpyrrole (MAP) showed that both MBC synthesis and the reaction condensing MAP and MBC to form prodigiosin were relatively more sensitive to NaCl inhibition than the MAP-synthesizing step. The capacity of whole cells to condense MAP and MBC was present, but inactive, in cells grown in NaCl; removal of the NaCl from non-proliferating salt-grown cells restored the activity. Other evidence suggests the existence of a common precursor to the MAP- and MBC-synthesizing pathways.

Apollo 11 lunar material in a medium containing 0.1% (wt/vol) sea salts had no detectable effect on the growth of a number of terrestrial microorganisms (12). However, fluorescent pigment formation by *Pseudomonas aeruginosa* ATCC 15422 was inhibited, whereas synthesis of the red pigment prodigiosin by *Serratia marcescens* ATCC 264 was stimulated. These effects were traced to iron leached from the lunar material. It was also observed that increasing the concentration of sea salts in the growth medium from 0.1 to 3% prevented prodigiosin synthesis in the presence of lunar material. Although iron is required for prodigiosin synthesis (1, 2, 5), only Waring and Werkman (13) have reported quantitative data. In addition, the literature appears devoid of any reports on salt inhibition of prodigiosin biosynthesis.

This paper reports the results of further studies on the iron requirements for growth and prodigiosin synthesis in *S. marcescens*, identification of NaCl as the component in sea salt responsible for inhibition of prodigiosin synthesis, and the location and apparent mechanism of action of the NaCl block in the terminal biosynthetic pathway of the pigment.

MATERIALS AND METHODS

Organisms. The wild-type strains of *S. marcescens* used in this study were ATCC 264 (obtained from H. S. Ginoza) and Nima (kindly supplied by R. P. Williams). Mutants 9-3-3 and WF were also supplied by R. P. Williams. Mutant 9-3-3 is blocked in the synthesis of the volatile monopyrrole moiety of prodigiosin, 2-methyl-3-amylpyrrole (MAP), but produces the stable bipyrrole moiety, 4-methoxy-2-2'-bipyrrole-5-carboxaldehyde (MBC). Mutant WF is blocked in the synthesis of MBC, but produces MAP. Both mutants and the two wild types can couple MBC and MAP to form the linear tripyrrole prodigiosin. Although these reactions are presumed to be catalyzed by enzymes, only the enzymatic nature of the terminal condensation step has been established (8). These relationships were reviewed by Williams and Hearn (17) and are illustrated in Fig. 1.

Media. The organisms were maintained in stock culture on TS slants (1.0% ion agar no. 2 [Colab], 3.0% Trypticase soy broth [BBL]) or on slants of Brain Heart Infusion agar (Difco).

Medium ML was modified from a medium used previously in studies with lunar samples (9, 10, 12) by omitting the vitamins. The following stocks were prepared. (i) AA-NAB was a dry mixture of 18 g each of β-alanine; glycine; hydroxy-L-proline; and the DL amino acids: alanine, arginine, aspartic acid, aspara-

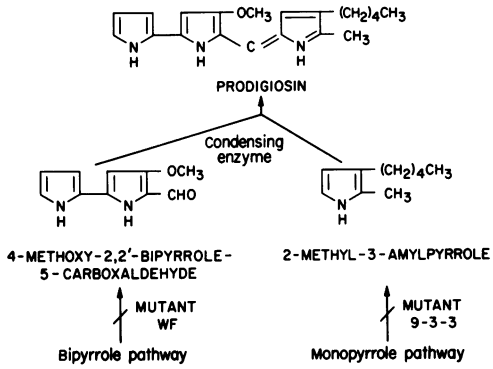

FIG. 1. *Terminal biosynthetic pathway for prodigiosin.*

gine, cysteine-hydrochloride, cystine, glutamic acid-hydrochloride, glutamine, histidine, isoleucine, leucine, lysine-hydrochloride, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine; and 0.98 g each of thymine, adenine, cytosine, guanine, hypoxanthine, orotic acid, xanthine, and uracil. (ii) LEM was a mixture of sodium lactate (60%), 2.5 vol; ethanol, 1.5 vol; and methanol, 1.5 vol. Medium ML contained (per liter of distilled water): AA-NAB, 4.68 g; LEM, 3.6 ml; sodium pyruvate, 1.0 g; sodium acetate, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; K_2HPO_4, 1.0 g; KNO_3, 1.0 g; glycerol, 1 ml; adjusted to pH 7.0 with NaOH.

Quantitative studies on the requirement of iron for growth and prodigiosin synthesis required the development of a chemically defined medium low in iron but capable of supporting good growth and pigment formation when iron was added. Medium GA was developed as a result of experiments with modifications of Bunting's medium (2) while taking into account observations on the induction of pigmentation by single amino acids (16) and the inhibition of pigment synthesis by high phosphate concentrations (3, 4, 15). Medium GA contained (per liter of distilled water): glycerol (Mallinkrodt, AR), 5.0 ml; L-alanine (Ajinimoto Co., Tokyo), 5.0 g; K_2HPO_4 (Baker, AR), 0.1 g; and $MgSO_4$ (Mann-Schwarz enzyme grade), 0.1 g. The pH was 7.2 to 7.3 after autoclaving. Iron was added to all media as required, either as $FeCl_3 \cdot 6H_2O$ or as sodium ferric diethylenetriamine pentaacetate (Sequestrene 330, Geigy Agricultural Chemicals, Ardsley, N.Y.). Both forms of iron seemed equally effective. Medium GA supplemented with 1 μg of Fe per ml is referred to as medium GAI.

Incubation. All cultures were incubated aerobically at 27 C for 72 h unless otherwise noted. Liquid cultures were grown in 300-ml Erlenmeyer flasks containing 50 ml of medium and were shaken on a New Brunswick model G26 rotary shaker (2.5-cm excursion diameter, 250 rpm).

Pigment intermediates and chemicals. DEP (2,4-dimethyl-3-ethylpyrrole), an analogue of the natural monopyrrole MAP, was purchased from Aldrich Chemical Co., Milwaukee, Wis. MBC was extracted from cultures of mutant 9-3-3 grown aerobically at 27 C for 4 days in 10 liters of 0.5% peptone (Difco)-1% glycerol medium by the procedures of Wasserman et al. (14). The yield was 229 mg of crude MBC, which was used without further recrystallization.

Instant Ocean sea salts, purchased from Aquarium Systems, Inc., Wickliffe, Ohio, was used without the trace elements. Synthetic 3% sea salts (SS) duplicated the major cations and anions in a 3% solution of Instant Ocean sea salts. It was prepared with reagent-grade chemicals and contained (mmoles per liter) $NaHCO_3$, 1.6; NaCl, 325; KCl, 6.9; $CaCl_2$, 6.7; $MgSO_4$, 18.7; $MgCl_2 \cdot 6H_2O$, 17.1; and H_3BO_3, 0.2. SS (0.1%) salts was prepared by appropriate dilution of 3% SS. Ultrapure NaCl was purchased from Alfa Inorganics, Inc., Beverly, Mass.; morpholinopropane sulfonic acid (MOPS) was from Calbiochem, Los Angeles, Calif.; and chloramphenicol was from the Sigma Chemical Co., St. Louis, Mo.

Analytical procedures. Medium GA and its individual components were analyzed for iron by using a Perkin-Elmer model 303 atomic absorption spectrophotometer. The individual components were also assayed for iron by the spectrophotometric pyridine-2-aldehydeoxime method of Poyer and McCay (11) with a Zeiss PMQ II spectrophotometer and 5-cm pathlength cells.

DEP, the analogue of MAP, can condense with MBC to form an analogue of prodigiosin. The visible absorption spectra of prodigiosin and prodigiosin analogues are virtually identical (R. H. Williams, Ph.D. thesis, Iowa State University, Ames, 1965). Therefore, the amounts prodigiosin and prodigiosin analogue per milliliter of culture were assayed spectrophotometrically by the following procedure. Suitable samples of culture (0.1–0.5 ml) were added to 5-ml volumetric flasks, brought to volume with hydrochloric acid-methanol (4 vol of 1 N HCl- 96 vol of methanol) (4) and extracted for 20 min to 2 h. The extracts were clarified by centrifugation at 5,500 $\times$ *g* for 15 min, and the amount of prodigiosin or its analogue, or both, in the extracts was determined as the difference in absorbance at 535 and 650 nm by using 1-cm pathlength cells in a Zeiss PMQ II spectrophotometer. The data are expressed as prodigiosin ($A_{535-650}$) per ml or pigment ($A_{535-650}$) per ml.

Cell mass was measured turbidimetrically at 650 nm by using a Bausch & Lomb Spectronic 20 spectrophotometer. Viable counts were determined by standard surface plate counts of decimal serial dilutions on TS agar.

Location of the salt block. The following procedures were used in experiments (see Table 3) aimed at identifying the metabolic step affected by salt in the terminal pathway of prodigiosin biosynthesis in the wild types and mutants WF and 9-3-3. MBC (1.25 mg) dissolved in ethanol was added to the main compartment of 500-ml Nephelo screw-capped flasks (Bellco Glass Co., Vineland, N.J.), and the solvent was removed by gentle heating in an airstream. Medium GAI (25 ml) containing various amounts of ultrapure NaCl was placed in the main compartment and sterilized by autoclaving. The volatile monopyrrole DEP (0.1 ml) was added to the side arm and allowed to diffuse into the main compartment be-

cause direct addition to 25 ml of GAI was inhibitory. After inoculation, all flasks were incubated aerobically at 27 C for 69 to 71 h on the rotary shaker. Prodigiosin or prodigiosin analogue, or both, per milliliter of culture was determined spectrophotometrically as described above. Viable counts per milliliter of culture were used as a measure of cell mass because a precipitate of unknown composition formed when cultures were diluted for turbidimetric measurements. The data obtained in this series of experiments are expressed as pigment ($A_{535-650}$) per 10^9 cells.

The following procedures were used in experiments (see Table 4) to determine whether salt blocked the monopyrrole pathway leading to MAP synthesis in strains Nima, 264, and WF. Since no direct assay for MAP has been developed, the syntrophic interaction of these strains (the donors of volatile MAP) with mutant strain 9-3-3 (the recipient capable of combining the MAP of the donors with its own bipyrrole MBC to form prodigiosin) was used to measure indirectly the MAP furnished by the donors (4). The donor strains were spread in triplicate on the surface of petri plates of GAI-1% Ionagar no. 2 supplemented with ultrapure NaCl as required. The recipient strain (9-3-3) was spread in triplicate on agar plates of the same medium without added NaCl. The inoculated halves of the donor and recipient plates were fastened together with adhesive tape, with the recipient uppermost, and incubated at 27 C in the dark for 5 days. The cells of mutant 9-3-3 were washed off the surface of the triplicate plates with 2.0 ml of water per plate, and pooled. Turbidimetric measurement of total cell mass and spectrophotometric measurement of the prodigiosin synthesized were determined as described above. The data obtained are expressed as prodigiosin ($A_{535-650}$) per unit cell mass.

Nature of salt inhibition of the condensing enzyme. The following procedure was used in experiments (see Table 5) to determine the mode of action of NaCl in salt-blocked cell suspensions. Two sterile buffer solutions were prepared, one composed of 0.05 M MOPS containing 1 μg of Fe per ml, the other of identical composition but containing 3% ultrapure NaCl (MOPS-NaCl). Both buffers were adjusted to pH 7.17 with KOH. The two wild types and mutants WF and 9-3-3 were grown in medium GAI containing 3% ultrapure NaCl and were harvested after 67 h. Each culture was then divided into two equal portions. One portion was washed three times in 0.05 M MOPS, the other in 0.05 M MOPS-NaCl, and both portions were resuspended in 25 ml of their respective buffers. MBC (1.25 mg) was added to the main compartment of 500-ml Nephelo flasks, followed by 14 ml of 0.05 M MOPS or 0.05 M MOPS-NaCl buffer, 1.0 ml of chloramphenicol solution, and 10 ml of cell suspension. The final concentration of chloramphenicol was 50 μg/ml in all flasks. Preliminary experiments established that this concentration completely prevented the growth of all the strains of *S. marcescens* used in these experiments. DEP (0.1 ml) was added to the side arm of all flasks. Incubation was at 27 C on the shaker for 44 h. Zero time and 44-h viable counts were made to confirm that no proliferation of cells had occurred. Prodigiosin and prodigiosin analogue per milliliter were determined at zero time and 44 h as previously described. The data, expressed as Δ pigment ($A_{535-650}$) per milliliter, represent the change in the quantity of pigment synthesized between zero time and 44 h.

RESULTS

The inhibitory component in sea salt. We have confirmed previous observations (12) that *S. marcescens* ATCC 264 normally did not form prodigiosin in medium ML plus Instant Ocean sea salts, that supplemental iron ranging from 0.05 to 10 μg/ml elicited pigment production in 0.1% Instant Ocean sea salts, and that 3% Instant Ocean sea salts prevented prodigiosin synthesis even though iron over the same concentration range was added to the medium. Identical results were obtained when 0.1 and 3% SS were used in place of Instant Ocean sea salts.

The inhibitory component in 3% SS was determined by omitting the major salts individually from 3% SS in tubes of ML broth supplemented with 2 μg of iron per ml. The data in Table 1 show that NaCl at a concentration of 325 mM in 3% SS was responsible for pigment inhibition.

The question of whether the inhibition by 325 mM NaCl resided in the Na^+ or Cl^- moiety of the salt, or was indeed specific to NaCl, was approached by testing for prodigiosin inhibition by equimolar concentrations of NaCl, Na_2SO_4, KCl, and K_2SO_4 in 3% SS tubes of ML broth supplemented with 2 μg of iron per ml. The data in Table 2 show that 325 mM concentrations of all the salts tested proved equally inhibitory to prodigiosin synthesis. This suggests that the inhibition was not specific to Na^+ or Cl^- or to any of the individual salts tested but could be caused by nonspecific cation or anion effects or be a function of the total ionic strength.

Iron requirements for growth and pigmentation. Medium GA was used to test *S. marcescens* wild-types Nima and ATCC 264 for their iron requirements for growth and prodigi-

TABLE 1. *Effect of 3% synthetic sea salts on prodigiosin synthesis in S. marcescens ATCC 264*[a]

Component deleted	Prodigiosin synthesis
None	–
NaCl	+
$MgSO_4$ and $MgCl_2 \cdot 6H_2O$	–
KCl	–
$CaCl_2$	–

[a] Visual observation of prodigiosin production in ML broth tubes; +, prodigiosin production; –, no prodigiosin.

TABLE 2. *Effect of 3% synthetic sea salts and other salts on prodigiosin synthesis in S. marcescens ATCC 264*

Deletion[a]	Replacement[a]	Prodigiosin[b] synthesis
None	None	–
NaCl	None	+
NaCl	Na_2SO_4	–
NaCl	KCl	–
NaCl	K_2SO_4	–

[a] At a concentration of 325 mM.

[b] Visual observation of prodigiosin production in ML broth tubes; +, prodigiosin synthesis; –, no prodigiosin.

osin synthesis. Analysis revealed that medium GA contained approximately 6 ng of Fe per ml in the absence of added iron. The inocula (0.1 ml) for the experiment, taken from liquid cultures grown in medium GA supplemented with 2 μg of Fe per ml, contributed approximately 4 ng of Fe per ml of medium. Therefore, the inoculated medium without added iron contained approximately 10 ng of Fe per ml.

In the absence of added iron, both wild types synthesized negligible amounts of prodigiosin although growth was possible (Fig. 2). The two strains differed in their iron requirements for maximal growth and pigment synthesis. Strain Nima required about 0.5 μg of added iron per ml, whereas approximately 0.2 μg of added iron per ml was sufficient for maximal growth and pigment synthesis in strain 264. In other experiments with both strains, the addition of up to 10 μg of Fe per ml did not alter these maximal values. Therefore, medium GAI (containing 1 μg of added Fe per ml) was used in subsequent experiments.

Effect of NaCl on growth and pigmentation. *S. marcescens* wild-type 264 was much more sensitive to NaCl than wild-type Nima. A number of experiments showed that with 264 the amount of prodigiosin synthesized per unit cell mass began to decrease at 0.05 to 0.3% NaCl, whereas up to 1.0% NaCl consistently had no effect on maximal growth and prodigiosin synthesis in Nima. Both strains showed further decreases in the prodigiosin synthesized per unit of cell mass at higher NaCl concentrations, often reaching complete inhibition at salt concentrations which varied between 0.5 to 3.0% NaCl in strain 264 and 2.5 to 3.0% NaCl in strain Nima. Inhibition of pigment synthesis in salt-grown cultures was not the result of preferential selection of nonpigmented cells since surface plates of these cultures on TS agar gave uniformly pigmented clones. A representative experiment on the effect of NaCl on growth and pigment synthesis in Nima and 264 is illustrated in Fig. 3.

Location of the salt block. An attempt was made to determine which pathway in the terminal biosynthesis of prodigiosin was blocked by NaCl (Table 3). It is evident that *S. marcescens* wild-type 264, when grown in 0.5% NaCl, could not synthesize prodigiosin or prodigiosin analogue when given only the monopyrrole (DEP) or bipyrrole (MBC) moieties. However, the condensation step was partially functional since simultaneous addition of both moieties resulted in pigment synthesis equivalent to 63% of the

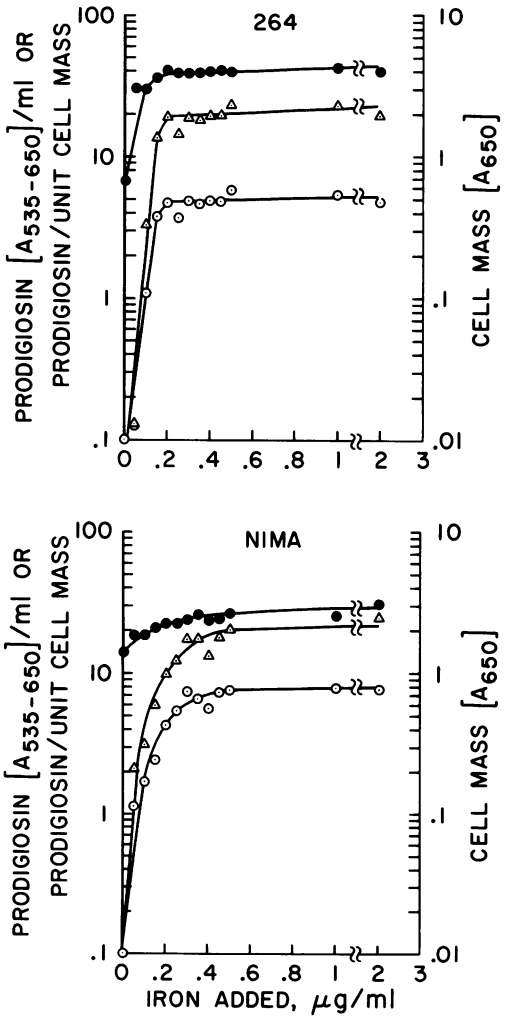

FIG. 2. *Effect of iron added to medium GA on growth and prodigiosin synthesis by S. marcescens wild-types ATCC 264 and Nima. Symbols: ●, cell mass; Δ, prodigiosin per milliliter; ○, prodigiosin per unit of cell mass.*

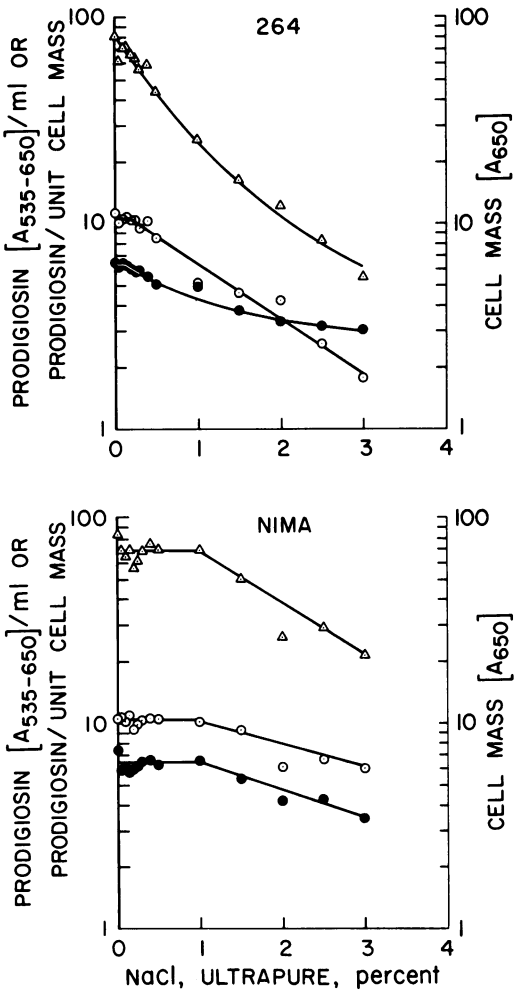

FIG. 3. *Effect of NaCl added to medium GAI on growth and prodigiosin synthesis by S. marcescens wild-types ATCC 264 and Nima. Symbols: ●, cell mass; Δ, prodigiosin per milliliter; O, prodigiosin per unit of cell mass.*

pigment in the salt-free control. This suggests that 0.5% NaCl completely blocked synthesis of MBC and MAP (the natural monopyrrole) but only partially blocked the terminal condensation step.

Wild-type Nima was unable to synthesize pigment when given DEP and MBC simultaneously, indicating a complete salt block of the terminal condensation step. Although Nima also failed to synthesize pigment when given DEP or MBC singly, it was not possible to determine whether 2.5% NaCl blocked the monopyrrole or the bipyrrole pathway, or both, because of the complete block in the condensation step.

Mutant WF (which synthesizes MAP but not MBC), grown in 3% NaCl and given only MBC, synthesized pigment equivalent to 54% of the pigment synthesized in the salt-free control. When given both DEP and MBC, salt-grown strain WF produced pigment equivalent to 61% of that synthesized by the salt-free control. These data indicate a partial salt block in the terminal condensation step. However, it was impossible to determine whether MAP synthesis was unaffected or was partially blocked because of the partial block in the condensation step.

Mutant 9-3-3 (which synthesizes MBC but not MAP), when grown in 2% salt with both DEP and MBC, synthesized pigment equivalent to only 11% of the pigment in the salt-free control. Therefore, there was virtually a complete salt block of the terminal condensation step, which made it impossible to determine whether salt also blocked the bipyrrole synthetic pathway. The conclusions from this series of experiments are that NaCl, at the concentrations tested, partially blocked the terminal condensation step in wild-type 264 and mutant WF and completely or nearly completely blocked it in wild-type Nima and mutant 9-3-3.

Because of the uncertainties with respect to the effect of salt on the monopyrrole and bipyrrole pathways, recourse was had to the syntrophic feeding experiments. These experiments were performed with mutant WF and wild-types 264 and Nima as MAP donors in an attempt to determine whether salt blocked the monopyrrole pathway. It must be emphasized that this technique measures indirectly only the excess MAP produced by the donor strains. The

TABLE 3. *Effect of salt and exogenous pigment precursors on pigment synthesis in S. marcescens wild-types 264 and Nima and in mutants WF and 9-3-3*

Organism	% NaCl	Pigment ($A_{535-650}$) per 10^9 cells[a]			
		None[b]	DEP[b]	MBC[b]	DEP + MBC[b]
264	0	2.0	1.5	3.2	2.9
	0.5	0.1	0.1	0.2	1.8
Nima	0	5.3	7.6	13.9	14.9
	2.5	0.1	0.2	0.6	0.1
WF	0	0.1	0.1	0.9	0.9
	3.0	0.1	0.1	0.5	0.5
9-3-3	0	0.0	7.5	0.0	3.9
	2.0	0.0	0.1	0.0	0.5

[a] Separate 0.2-ml inocula were taken from seed cultures of each strain grown in medium GAI with and without NaCl; other details in Materials and Methods section.

[b] Additions to medium.

data in Table 4 show that strains 264, Nima, and WF all produced greater amounts of excess MAP when grown in the presence of 1.5% or more NaCl than in its absence. This suggests that the bipyrrole pathway and the terminal condensation step were relatively more sensitive to NaCl than the monopyrrole-synthesizing pathway.

Nature of salt inhibition of the condensing enzyme. The data in Table 3 indicate that elevated NaCl concentrations partially or completely blocked the condensing enzyme in the wild types and mutants of *S. marcescens*. High NaCl concentrations might act by (i) partially or completely preventing synthesis of the enzyme or by (ii) permitting synthesis of the enzyme but partially or completely inhibiting its activity. If the second hypothesis were correct, then it might be possible to reverse the inhibition by removing NaCl, thereby permitting pigment synthesis to occur on addition of the monopyrrole and bipyrrole precursors. If the condensing enzyme were absent, as in the first hypothesis, or if the condensing enzyme were present but inhibited irreversibly, then NaCl removal would not result in pigment synthesis on addition of the appropriate precursors.

The results of an experimental test of these hypotheses are given in Table 5. Salt-grown cells of wild types and mutants, washed free of salt and incubated in salt-free buffer, synthesized more pigment from the precursors than salt-grown cells washed in salt and incubated in buffer with 3% NaCl. Therefore, high NaCl concentrations seemed to act by inhibiting the condensing enzyme but not its synthesis during growth in 3% NaCl. The absence of added carbon and nitrogen sources, the presence of inhibiting concentrations of chloramphenicol, and the failure to find an increase in the viable count make it improbable that synthesis of nascent condensing enzyme occurred during the 44-h incubation period.

TABLE 4. *Effect of NaCl on MAP synthesis in S. marcescens wild-types 264 and Nima and in mutant WF*

MAP donor system		Volatile donor MAP captured by 9-3-3[a]
Organism	System	
264	Sterile +0.5% NaCl	0
	Inoculated, no NaCl	0.2
	Inoculated +0.5% NaCl	0.3
264	Sterile +1.5% NaCl	0.1
	Inoculated, no NaCl	0.2
	Inoculated +1.5% NaCl	1.4
Nima	Sterile +2.5% NaCl	0.0
	Inoculated, no NaCl	0.3
	Inoculated +2.5% NaCl	3.7
WF	Sterile +3.0% NaCl	0.2
	Inoculated, no NaCl	4.8
	Inoculated +3.0% NaCl	9.0

[a] Determined as prodigiosin ($A_{535-650}$) per unit of cell mass; other details in Materials and Methods section.

TABLE 5. *Effect of salt removal on pigment synthesis in non-proliferating, salt-grown S. marcescens wild-types 264 and Nima, and mutants WF and 9-3-3*

Organism	Δ Pigment ($A_{535-650}$) per ml[a]	
	3% NaCl	0% NaCl
264	2.2	3.9
Nima	−0.3[b]	4.6
9-3-3	0.6	0.9
WF	3.4	7.3

[a] The data represent the increase in pigment ($A_{535-650}$) per milliliter synthesized from DEP and MBC in 44 h over the pigment ($A_{535-650}$) per milliliter present at zero time. Zero time pigment ($A_{535-650}$) per milliliter for strains 264, Nima, 9-3-3, and WF were 2.0, 9.0, 0, and 0.3, respectively, in 3% NaCl, and 1.8, 8.1, 0, and 0.3, respectively, in 0% NaCl. See Materials and Methods section for complete experimental details.

[b] Decrease in pigment per milliliter.

DISCUSSION

Medium GAI supported excellent growth of *S. marcescens* wild-types ATCC 264 and Nima, and mutants WF and 9-3-3, while promoting intense pigmentation in the wild types. However, it may not be suitable for all wild types. Strain HY (obtained from Anne Heuer) did not grow in GAI unless DL-proline or peptone (Difco) were substituted for the L-alanine in GAI, in which case there was excellent growth and pigmentation.

Goldschmitt and Williams (4) reported that thiamine enhanced the production of prodigiosin by wild-type Nima. During the development of medium GAI, we tested the effect of 100 μg of thiamine per ml on prodigiosin production by wild-type 264 grown in medium GA supplemented with 2 μg of Fe per ml, but found no increase in cell mass or the quantity of prodigiosin synthesized per unit cell mass.

Waring and Werkman (13) reported that *S. marcescens* wild-type 2G1, grown in a glucose-$(NH_4)_2SO_4$-mineral salts medium that had been extracted with chloroform-8-hydroxyquinoline to reduce the residual iron content to 0.7 to 3.0 ng per ml, required the addition of 0.02 to 0.03 μg of Fe per ml for maximal growth. Prodigiosin (estimated visually) was produced within the range 0.1 to 2.0 μg of Fe per ml, with no prodigiosin synthesized at iron concentrations

below and above this range. Our results with wild-type 264 (Fig. 2) agree with those of Waring and Werkman (13) only with respect to the quantity of iron required for maximal prodigiosin synthesis. However, wild-type 264 required 10 times more iron for maximal growth than Waring and Werkman's strain. Wild-type Nima required even more iron (0.5 μg per ml) for both maximal growth and prodigiosin synthesis (Fig. 2). Neither strain 264 nor Nima showed loss of pigment synthesis when the iron concentration was raised to as high as 10 μg per ml. Aside from the fact that different strains were used, the discrepancies between our results and those of Waring and Werkman may be explained in part by the different media used. Medium GA with appropriate iron was capable of supporting large populations, whereas the medium used by Waring and Werkman ... "was not entirely adequate for optimal growth" (13). Thus, it would be expected that more iron would be required for an organism growing in a medium that supported a large maximal cell population as opposed to one capable of supporting a smaller cell mass. A specific role for iron in prodigiosin synthesis remains to be elucidated.

The experiments on the location of the salt block (Table 3) indicate that the activity of the terminal condensing enzyme is completely, or nearly completely, blocked in wild-type Nima and mutant 9-3-3 but partially blocked in mutant WF and wild-type 264. These same experiments also suggest that salt completely blocks the synthesis of MBC and MAP in strain 264. The syntrophic feeding experiments (Table 4) suggest that in mutant WF and wild-types 264 and Nima the condensing enzyme and MBC-synthesizing pathway are relatively more sensitive to NaCl than the MAP-synthesizing pathway.

The effect of salt on MAP synthesis in mutant WF is especially noteworthy because almost twice as much MAP was synthesized in the presence of salt than in its absence (Table 4). Since WF is genetically blocked in the synthesis of MBC, we can only surmise that a common precursor must exist for both MAP and MBC, and that salt blocks the abortive MBC pathway at some point beyond the common precursor but before the genetic block, making more common precursor available for MAP synthesis. The existence of such an early step common to both the MAP and MBC pathways was suggested by Santer (Ph.D. thesis, Yale University, New Haven, Conn., 1958). Morrison showed that his class X mutants, which resembled mutants blocked at an early common step, were in reality blocked at two distinct early steps in the MAP and MBC pathways (7).

The conclusions concerning the mechanism of action of salt on the condensing enzyme, i.e., that NaCl reversibly inhibits its activity but not its synthesis, are restricted to that enzyme (Table 5). Salt may affect other steps in the MBC and MAP pathways by the same or other mechanisms. Further clarification must await studies on the effect of salt on cell-free enzyme systems.

S. marcescens wild-type 264 is consistently much more sensitive to elevated NaCl concentrations than wild-type Nima (Fig. 3). One would expect additional *S. marcescens* strains to exist in nature, ranging from those in which pigment synthesis is extremely sensitive to salt to others that display no sensitivity to salt. Some examples of the latter are known. Lewis and Corpe (6) found that two marine isolates grew in sea water media or in media with the chlorides of Na^+, K^+, Mg^{2+}, and Ca^{2+} at the same concentrations found in sea water and produced a pigment apparently identical with prodigiosin. *S. marinorubra*, another marine isolate, synthesized pigment in sea water salt concentrations. These salt-tolerant marine species may have evolved mechanisms to exclude salt from salt-sensitive sites or may have developed pathways resistant to high salt concentrations. Whatever the mechanism may be, our results (Table 5) show that the inhibitory action of salt on at least one enzyme catalyzed step, the condensation reaction, can be reversed by removing the salt.

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