Production of a Sporulation Pigment by 
*Streptomyces venezuelae*

H. E. SCRIBNER III,¹ TERRY TANG, AND S. G. BRADLEY

Department of Microbiology, Virginia Commonwealth University, Richmond, Virginia 23298

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*Streptomyces venezuelae* S13 produced a pH-indicating sporulation pigment on a glucose-salts-agar medium consisting of glucose, KNO₃, MgSO₄, and Na₂HPO₄, pH 7. Pigmentation on this medium appeared to be closely associated with sporulation, which normally required 5 to 7 days at 30°C. The pigment was soluble in water as well as in a number of organic solvents. Butanol-extracted pigment exhibited absorption maxima at 430 and 520 nm at pH 3 and 12, respectively. Although many salts of organic acids and amino acids could replace glucose as the sole carbon source in basal salts-agar medium for growth and pigmentation, most sugars that were tested supported good growth but negligible pigmentation. Among the nitrogenous substances tested, KNO₃ was most desirable for pigmentation. The organism did not exhibit any specific requirements for divalent cations with respect to growth and pigmentation. In the absence of MgSO₄, however, glucose-salts-agar prepared by autoclaving all components together failed to support growth. The production of the sporulation pigment on glucose-salts-agar was comparable to that obtained on tomato paste-oatmeal-agar medium. Incorporation of partially purified pigment material into broth medium that did not normally support sporulation induced sporulation, and amino acid-salts-agar medium could induce vegetative mycelia to pigment when transferred from medium that did not support either pigmentation or sporulation.

Tewfik and Bradley (15) observed that deoxyribonucleic acid (DNA) from spores of *Streptomyces venezuelae* S13 possessed unique properties that were not exhibited by mycelial DNA. DNA from *S. venezuelae* S13, as compared with mycelial DNA, had an increased thermal denaturation temperature, a decreased buoyant density in cesium chloride, and a distinctive absorption spectrum in the visible light region. Subsequently, Enquist and Bradley (3) extended these observations by showing that the gradual change in these properties of DNA correlated with the chronological age of the spores. In addition to the increase in melting temperature and decrease in buoyant density, these investigators also reported that the ability of denatured spore DNA to anneal with denatured mycelial DNA decreased from 100 to 30% as the spores aged. The spore DNA also contained 15 to 20% protein, based upon the Folin phenol assay. Considering all of the evidence, we concluded that *S. venezuelae* S13 spore DNA is complexed with a sporulation pigment material that may play a significant role in controlling the overall cellular regulatory mechanisms.

Essentially all studies using *S. venezuelae* S13 have employed complex media for spore and pigment production. Because this organism grows and pigments well on complex media, such media are good sources of large quantities of spores and pigment. However, the individual components in the complex medium cannot be accurately determined; thus it is not possible to control the composition of the medium effectively. Because pigment production by *S. venezuelae* S13 is associated with sporulation, which in turn involves changes in metabolic patterns, it is desirable to study the nutritional requirements for growth and pigment production by this organism in a defined medium. Defined media have been developed that are as effective as the complex medium in supporting both growth and pigmentation of *S. venezuelae* S13.

**MATERIALS AND METHODS**

Stock cultures of *S. venezuelae* S13 were maintained on glucose-salts-agar containing 20 g of glucose, 2 g of each of KNO₃, MgSO₄·7H₂O, and Na₂HPO₄, 1 liter of distilled water, and 15 g of agar

¹Present address: Department of Biology, Washington University, St. Louis, Missouri 63130.
(Difco Laboratories, Detroit, Mich.), with pH of 6.8 to 7. Because the method of medium preparation affected both growth and pigment production, three methods were used in this study to prepare the glucose-salts-agar. Method A: glucose and agar were sterilized as one solution. Each of the remaining medium components was made up as concentrated individual solutions and sterilized separately. The final medium was obtained by mixing appropriate amounts of each concentrated solution. The pH of the medium was adjusted to 6.8 to 7 immediately prior to dispensing the molten medium into petri dishes. Method B: two stock solutions were made and sterilized separately. Solution I contained glucose, MgSO₄·7H₂O, and agar. Solution II contained the remaining medium components. The final medium was obtained by combining the two solutions. Method C: all medium components were made in a single solution and sterilized. Unless specifically indicated, agar medium used in the present study was prepared by method A.

Spore inocula were prepared by harvesting the aerial growth from glucose-salts-agar cultures. The mycelia were suspended in sterile, distilled water and homogenized. One loopful of this suspension was used to streak a plate. In general, crowded but isolated colonies developed after 30 to 48 h of incubation at 30 C. The culture eventually became densely pigmented after 7 to 10 days at 30 C. The amount of growth was scored on an arbitrary scale from 0 (no growth) to 4+ (maximal growth).

Cultures on agar medium with equivalent growth were used for pigment extraction. The agar medium was suspended in an equal volume of n-butanol for 24 h in the dark at room temperature (24 C). The solution was filtered by gravity through Whatman no. 2 filter paper. The amount of pigment was measured as the absorbance at 430 nm (Spectronic 20).

Tomato paste-oatmeal-agar was prepared as described by Gottlieb (5). Salts of metallic cation solutions incorporated into the tomato paste-oatmeal-agar were sterilized by membrane filtration. Portions of the concentrated stock solution were added to the medium to obtain a final concentration of 0.1% (wt/vol).

**RESULTS**

Tomato paste-oatmeal-agar has been used previously as the standard medium for spore production by *S. venezuelae* S13. In order to gain insight into the nutritional requirements for growth and pigmentation of the organism, a basal salts medium containing glucose and KNO₃ as the sole carbon and nitrogen sources was developed. This medium was used to assess the growth-supporting ability of each component. Various carbon sources were evaluated by replacing glucose with a number of amino acids, sugars, and carboxylic acids. In addition, each carbon source was tested at three concentrations (wt/vol), i.e., 0.1, 0.5, and 2.0% (Table 1). On glucose-salts-agar, pigment production was best at the highest concentration of glucose tested, but growth was approximately equal at all three concentrations. Generally, the amino acids tested were similar in ability to support growth at the three concentrations examined. On the other hand, higher concentrations of the amino acids favored pigment production. This phenomenon was especially prominent in asparagine-salts and proline-salts-agar media. The ability of phenylalanine or succinate as the carbon source to support growth in the basal salts-agar medium, however, was inversely related to the concentration supplied; consequently, the organism grew better at the lower

| Carbon source | 0.1% concn | 0.5% concn | 2% concn |
|---------------|------------|------------|----------|
| Growth* | Pigment* | Growth | Pigment | Growth |
| 5 days | 7 days | 5 days | 7 days | 5 days | 7 days |
| Glucose | 3+ | 0.10 | 0.10 | 4+ | 0.58 | 0.84 | 4+ | 0.64 | 1.53 |
| Asparagine | 3+ | 0.13 | 0.15 | 3+ | 0.76 | 0.78 | 3+ | 0.54 | 0.51 |
| Aspartate | 3+ | 0.34 | 0.45 | 3+ | 0.74 | 1.17 | 1+ | 0.08 | 0.12 |
| Succinate | 4+ | 0.49 | 0.66 | 3+ | 0.74 | 0.96 | 4+ | 0.78 | 0.81 |
| Glutamate | 3+ | 0.19 | 0.21 | 4+ | 0.86 | 0.96 | 4+ | 1.08 | 1.56 |
| Proline | 4+ | 0.18 | 0.21 | 4+ | 0.43 | 0.69 | 4+ | 0.03 | 0.03 |
| Phenylalanine | 4+ | 0.27 | 0.25 | 4+ | 0.19 | 0.45 | 1+ | 0.15 | 0.15 |
| Tyrosine | 4+ | 0.13 | 0.21 | 4+ | 0.15 | 0.29 | 4+ | 0.19 | 0.33 |
| Arabinose | 3+ | 0.14 | 0.16 | 3+ | None | 0.15 | 4+ | 0.10 | 0.15 |
| Mannitol | 4+ | 0.15 | 0.14 | 4+ | None | 0.09 | 4+ | 0.15 | 0.10 |

*Growth after incubation for 5 and 7 days was scored as 0 (no growth) to 4+ (maximal growth).

*Pigmentation is expressed as the absorbancy at 430 nm of the n-butanol layer after extraction of the culture and medium culture with an equal volume of solvent.
concentrations tested. Mannitol-salts- and arabino-salts-agar media supported good growth, but the organism did not produce much pigment. Results similar to those with mannitol-salts and arabino-salts-agar media were also obtained with galactose-salts- and rhamnose-salts-agar media. Moreover, when citrate, leucine, lysine, and glycerol were employed as the sole carbon source, the organism grew relatively well but did not produce pigment. Acetate was unable to support growth when used at a concentration of 2% in the basal salts-agar medium.

By using glucose-salts-agar as the standard medium, the ability of a number of nitrogenous substances to support growth and pigmentation by *S. venezuelae* S13 was studied. The organism grew well on all nitrogen sources tested (KNO₃, NH₄Cl, NH₄NO₃, urea, or asparagine at 2 g per liter, or 1 g each of KNO₃ and NH₄Cl per liter), but KNO₃ supported pigment production better than ammonium salts, asparagine, or urea. *S. venezuelae* S13 exhibited different colonial morphologies on glucose-salts media containing different nitrogen sources. For example, on medium containing NH₄Cl, the organism grew less than it did on standard glucose-salts-agar with KNO₃ as the nitrogen source, and the average size of a colony was approximately 1 mm in diameter on NH₄Cl-medium, compared with 3 to 4 mm on KNO₃-medium. Furthermore, there was no visible aerial growth on NH₄Cl medium, and the culture was not pigmented, although there were a few colonies that were distinctively light yellow. Interestingly enough, on glucose-salts-agar medium containing both KNO₃ and NH₄Cl as the nitrogen source, the colonial characteristics were similar to those where NH₄Cl alone served as the nitrogen source.

The requirement for an inorganic salt for growth and pigmentation of *S. venezuelae* S13 was assessed on glucose-salts medium that contained no added MgSO₄. On this medium, the organism grew and pigmented essentially to the same extent as on the standard glucose-salts-agar medium (Table 2). However, the ability of glucose-salts-agar medium containing no MgSO₄ to sustain growth and pigmentation depended on the method of medium preparation. Media prepared by methods A and B supported growth and pigmentation to the same extent. Medium lacking MgSO₄ and prepared by method C, however, was not satisfactory for growth. Further experiments indicated that the organism grew and pigmented on medium lacking MgSO₄ if the glucose was sterilized separately from other components (method C). Presumably, autoclaving medium components together may lead to inactivation of biological activity of some component or to the production of growth inhibitory substances (Table 2).

Although *S. venezuelae* S13 grew and pigmented well on glucose-salts medium containing no MgSO₄, the question arises whether different inorganic salts may enhance growth

**Table 2. Growth and pigmentation of *S. venezuelae* S13 with different inorganic salts in glucose-salts medium**

| Salts (2 g/liter) | Method of medium prep | Growth | Pigment |
|-------------------|-----------------------|--------|---------|
|                   |                       | 5 days | 7 days  | 5 days | 7 days  |
| MgSO₄·7H₂O (control) | A                     | 4+     | 4+     | 0.25   | 0.68   |
|                   | B                     | 3+     | 3+     | 0.33   | 0.53   |
|                   | C                     | 4+     | 4+     | 0.28   | 0.58   |
| None              | A                     | 4+     | 4+     | 0.26   | 0.54   |
|                   | B                     | 4+     | 4+     | 0.33   | 0.58   |
|                   | C                     | 4+     | 4+     | 0.58   | 0.70   |
|                   | C*                    | 0      | 0      | None   | None   |
| CaCl₂             | A                     | 4+     | 4+     | 0.42   | 0.64   |
| MgCl₂             | A                     | 4+     | 4+     | 0.47   | 0.70   |
| KCl               | A                     | 3+     | 4+     | 0.11   | 0.24   |
| NaCl              | A                     | 3+     | 4+     | 0.11   | 0.32   |
| NH₄Cl             | A                     | 1+     | 3+     | None   | None   |
|                   | B                     | 4+     | 4+     | 0.41   |        |
| MnCl₂*            | A                     | 0      | 0      | None   |        |

* See Materials and Methods.
* Growth after incubation for 5 or 7 days was scored as 0 (no growth) to 4+ (maximal growth).
* Pigmentation is expressed as the absorbancy at 430 nm of the n-butanol layer after extraction of the culture medium with an equal volume of solvent.
* All medium components were autoclaved together except for glucose, which was autoclaved separately.
* CoCl₂ and CuCl₂ showed no growth.
and pigmentation of the organism. To test this possibility, MgSO₄ was either replaced by a test salt (Table 2) or was used in combination with a test salt (Table 3) in the standard glucose-salts-agar medium. Generally, chlorides of divalent metallic ion, such as CaCl₂ and MgCl₂, potentiated pigmentation, whereas chlorides of monovalent cations, such as KCl and NaCl, supported relatively good growth but less culture pigmentation (Table 2). When NH₄Cl replaced MgSO₄ in the standard glucose-salts-agar medium, the response of the organism was again affected by the method of medium preparation. Medium containing NH₄Cl and lacking MgSO₄ prepared by method A supported growth, but pigmentation was nil (Table 2). Furthermore, a series of glucose-salts-agar media with NH₄Cl replacing MgSO₄ was prepared by method A. These media contained varying concentrations of NH₄Cl, ranging from 0.2 to 2.0 g/liter, and the initial medium pH was adjusted to pH 5, 6, and 7. In this case, equally good growth was obtained with all NH₄Cl media having an initial pH of 6 and 7, but considerably less growth occurred at pH 5. On the other hand, pigment was produced in the presence of 2.0 g of NH₄Cl/liter with initial medium pH adjusted to neutrality, but no pigment was produced at pH 5 or 6. Glucose-salts-agar medium lacking MgSO₄ and prepared by method B containing either 0.2 or 2.0 g of NH₄Cl/liter supported growth and pigmentation at pH 7 (Table 2). Salts of cobalt, manganese, and copper were either inhibitory or toxic when 2.0 g/liter was used in the glucose-salts medium. The inhibitory or toxic effects of Co, Mn, and Cu salts could be overcome by reducing their concentration and adding MgSO₄ to the medium (Table 3).

Glucose-salts-agar and tomato paste-oatmeal-agar media were comparable in ability to support pigmentation of S. venezuelae S13 (Fig. 1). Pigmentation steadily increased from 6 to 10 days until the maximum amount of pigment was produced. Inorganic salts added to tomato paste-oatmeal-agar did not have an observable beneficial effect on either growth or pigmentation.

A number of amino acids were capable of inducing pigment formation in growing vegetative hyphae of S. venezuelae S13. A vegetative culture was obtained by inoculating spores into peptone-yeast extract-broth and incubating them at 30 C for 18 to 24 h with agitation. A 0.2-ml portion of the vegetative growth was inoculated onto Whatman no. 2 filter paper placed on top of a peptone-yeast extract-agar plate. After 24 h of incubation at 30 C, the filter paper was transferred to a basal salts-agar medium containing an amino acid as the sole energy source. The filter paper was used to facilitate shifting of mycelia from the rich medium to the defined medium. Growth and pigmentation of the culture were evaluated after 3 days of incubation at 30 C. On control medium, i.e., glucose-salts-agar, an appreciable amount of pigment was formed (Table 4). Cultures on asparagine-salts- and proline-salts-agar pigmented extensively. Good pigmentation was also obtained on aspartate-salts- and glutamate-salts-agar media. In general, the ability of the amino acids to induce the formation of sporulation pigment in vegetative cells paralleled their ability to support growth and pig-

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**Table 3. Growth and pigmentation of S. venezuelae S13 with MgSO₄ used in combination with a test salt**

| Salt          | Growth | Pigment |
|---------------|--------|---------|
|               | 5 days | 7 days  | 5 days | 7 days |
| MgSO₄·7H₂O    | 4+     | 4+     | 0.25   | 0.68   |
| (control)     |        |        |        |        |
| FeCl₂        | 4+     | 4+     | 0.50   | 1.00   |
| MgCl₂        | 4+     | 4+     | 0.50   | 1.32   |
| ZnCl₂        | 2+     | 3+     | 0.24   | 0.29   |
| MnCl₂        | 2+     | 3+     | 0.18   | 0.25   |
| CaCl₂        | 2+     | 3+     | 0.03   | 0.19   |
| CoCl₂        | 2+     | 2+     | None   | None   |

*One gram of MgSO₄·7H₂O plus 1 g of test salt per liter of medium.

* Growth after incubation at 30 C for 5 or 7 days was scored as 0 (no growth) to 4+ (maximal growth).

* Pigmentation is expressed as the absorbancy at 430 nm of the n-butanol layer after extraction of the culture and culture medium with an equal volume of solvent.

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**Fig. 1. Pigment production by S. venezuelae S13 on glucose-salts-agar (Δ) and tomato paste-oatmeal-agar (▲).**
mentation with a spore inoculum (Table 1).

The indicator pigment produced by S. venezuelae S13 was extracted from glucose-salts-agar cultures with n-butanol and was partially purified. The n-butanol extract was first concentrated by flash evaporation. Upon adding five volumes of acetone to the concentrated pigment preparation, a dark-purple precipitate formed. The precipitate was collected, air-dried, and weighed. A weighed amount of the precipitate was dissolved in a defined, small volume of sterile, distilled water. Portions of the aqueous pigment solution were added to peptone-yeast extract-broth to give a range of pigment concentrations (Table 5), and 24-h-old vegetative inoculum was used to inoculate peptone-yeast extract-broth containing the pigment. The results indicated that the partially purified pigment material was able to initiate sporulation in peptone-yeast extract-broth, which had not been observed to support sporulation. Apparently, both the amount of the pigment and the time of incubation affected the extent of sporulation (Table 5). Spores produced in response to the partially purified pigment in the broth medium were morphologically similar to those produced on agar medium as judged by phase-contrast microscopy examination (Fig. 2).

**DISCUSSION**

Proteins and amino acids are preferred to carbohydrates for growth by a large number of actinomycetes. However, most actinomycetes are also able to use organic acids, sugars, starches, and cellulose (2, 16). The results from this study indicate that S. venezuelae S13 has the ability to utilize a number of organic compounds for growth. Moreover, each compound may possess a unique optimal concentration to support the best growth of the organism.

Mineral nutrition in streptomycetes has been studied primarily in connection with antibiotic production. Spicher (11) reported that soil extracts promoted growth of a number of Streptomyces, apparently because of the trace elements in the extract. By using a chemically defined medium, Heim and Lechevalier (8) studied the effect of salts of iron, zinc, manganese, and calcium on eight strains of Streptomyces. Zinc and iron were beneficial for all eight strains, whereas manganese was beneficial only for S. coelicolor. Gallichio and Gottlieb (4) reported that elimination of zinc and iron from a glycerol-lactate medium suppressed the production of chloramphenicol by S. venezuelae R-1. In our studies, FeCl₃ also promoted pigmentation of S. venezuelae S13 (Table 3). Two alternatives may account for this pigmentation in the absence of added metallic salts: (i) either the organism does not require a specific metallic cation for growth and pigmentation, (ii) the minute amounts required are already present in the growth medium. It should be noted that no attempt was made to free the agar and glassware of trace salts.

The production of the sporulation pigment by S. venezuelae S13 may reflect the response of the organism to both the internal and external cellular environments. In the presence of a sufficiently high level of nutrients, the enzyme

| Carbon source (20 g/liter) | Growth | Pigment |
|---------------------------|--------|---------|
| Glucose (control)         | 4+     | 1.94    |
| Asparagine                | 4+     | 1.44    |
| Proline                   | 4+     | 1.42    |
| Aspartate                 | 4+     | 0.64    |
| Glutamate                 | 4+     | 0.54    |
| Succinate                 | 3+     | 0.34    |
| Tyrosine                  | 2+     | 0.32    |
| Phenylalanine             | 3+     | 0.06    |
| Peptone-yeast extract*    | 4+     | None    |

* A 0.2-ml portion of a 24-h peptone-yeast extract-broth vegetative S. venezuelae S13 culture was inoculated onto a piece of Whatman no. 2 filter paper placed on top of a peptone-yeast extract-agar plate. After 24 h at 30 C, the filter paper was transferred to amino acid-basal salts-agar medium and incubated for 3 days at 30 C.

* Growth after incubation at 30 C for 3 days was scored as 0 (no growth) to 4+ (maximal growth).

* Pigmentation is expressed as the absorbancy at 430 nm of the n-butanol layer after extraction of the medium and filter paper with an equal volume of solvent.

* Peptone-yeast extract-agar: 5 g of peptone and 3 g of yeast extract per liter of medium.

| Table 5. Initiation of sporulation in S. venezuelae S13 by purified pigment in peptone-yeast extract-broth |
|---------------------------------------------------------------|
| **Amount of pigment added (µg/ml)**                           |
| **Extent of sporulation**                                    |
| **(µg/ml)**                                                  |
| **(as absorbancy)**                                         |
| **(at 430 nm)**                                             |
| **Hours at 30 C**                                           |
| 24                                                           |
| 48                                                           |
| 72                                                           |
| 96                                                           |
| 0                                                            |
| 0.75                                                        |
| 2.5                                                         |
| 24.5                                                        |
| 37.5                                                        |
| -                                                            |
| -                                                            |
| ±                                                            |
| ±                                                            |

* Estimated visually under phase contrast microscopy.
system responsible for pigment biosynthesis is probably under catabolite repression. Consequently, substances produced in aged mycelia or metabolites excreted from lysed cells may act as an inducer for the enzyme system involved in pigment biosynthesis. The nature of the inducer is not known; furthermore, such an inducer has not been identified even in a well-studied sporulating organism, i.e., bacillus (1). However, partially purified pigment material added to growing cells in peptone-yeast extract-broth may act as an inducer by binding to a specific site or sites of the vegetative genome, thereby controlling the production of a metabolite that suppresses the enzyme system for pigment biosynthesis. Consequently, the physiology of the cell is shifted from vegetative metabolism to that of sporogenesis. If the metabolic changes occurring during sporulation were generally similar between bacilli and actinomycetes, then the sporulation pigment would be expected to represent one of the first sporulation products in \textit{S. venezuelae} S13, because it has been established in bacilli that antibiotic production occurs at the initial stage of sporulation (6). Sporulation of vegetative cells of \textit{S. venezuelae} S13 transferred from peptone-yeast extract agar to amino acid-salts-agar medium may be analogous to the endotrophic sporulation in bacilli, i.e., sporulation occurring in vegetative cells upon sudden exposure to nutritionally starving conditions (7). Various other substances have been shown to be capable of inducing growing cultures to sporulate; for example, a factor C isolated from \textit{S. griseus} 45 was able to induce spore formation in an asporogenous mutant, \textit{S. griseus} 52-I (14). Sporogen, a low-molecular-weight, ninhydrin-negative sporulation factor, was isolated and purified from \textit{Bacillus cereus} T. The addition of sporogen to \textit{B. cereus} T vegetative cells suspended in a nongrowth supporting medium prevented cell lysis and induced sporulation (12, 13).

The general overall metabolic changes occurring during sporogenesis in \textit{S. venezuelae} S13 are not known. It is probable that the entire process is well-coordinated and sequential. Moreover, other sporulation products may also be produced with the pigment. In this connection, protease represents a good candidate, particularly because it has been shown to be produced by other streptomycetes (9, 10).

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