CHARACTERIZATION OF NEW VIRIDOMYCINS AND REQUIREMENTS FOR PRODUCTION IN CULTURES OF *STREPTOMYCES GRISEUS*

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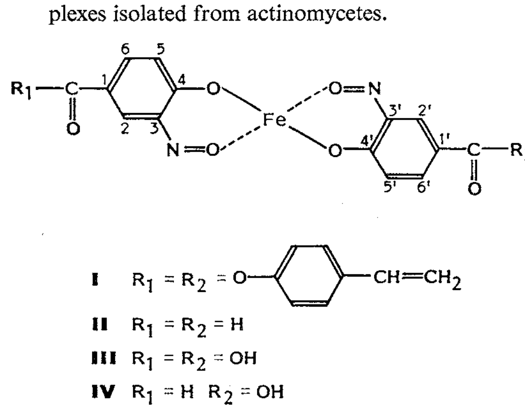
(Received for publication November 1, 1986)

Cultures of *Streptomyces griseus* grown under phosphate-limiting conditions produced a complex of green products. Three of these were separated from the mixture and characterized. One was identified as viridomycin A, the ferrous chelate of 4-hydroxy-3-nitrosobenzaldehyde; the second (actinoviridin A) was the corresponding carboxylic acid chelate and the third (viridomycin E) was a hybrid chelate containing both the aldehyde and acid ligands. Only two out of nine strains of *S. griseus* examined produced viridomycins and the ligands were biosynthesized only in media from which phosphate had been exhausted. Optimization of the production medium showed that fructose and alanine were the most favorable carbon and nitrogen sources and that relatively high concentrations of ferrous ions were necessary. The results suggest that viridomycins are not produced by *S. griseus* as iron scavengers in response to iron deficiency but as secondary metabolites that are stabilized adventitiously in the broth by metal ion chelation.

During studies on nutritional control of streptomycin production by *Streptomyces griseus*, we observed that cultures grown in certain low-phosphate nitrogen-sufficient media became bright green during the late growth phase. Of the green pigments of microorganisms, several from actinomycetes are ferrous chelates of *p*-substituted *o*-nitrosophenols. Ferroverdin (I, Fig. 1) was isolated by CHAIN and co-workers from the mycelium of an undesignated *Streptomyces* species[1,2] while viridomycin A (II, Fig. 1), the main component in a group of green antibiotic pigments excreted by *Actinomyces* (*Streptomyces*) *viridaris*[3] has been characterized by KHOKHLOV and BLINOVA[4]. The structures of viridomycins A_1, A_2, B, C and D which were extracted from the culture broth along with viridomycin A and separated from it by countercurrent distribution[3] do not appear to have been determined. However, BLINOV[5,6] have reported that actinoviridin A has the structure III. Similar compounds have been implicated as the agents responsible for green pigmentation in cultures of *Actinomyces roseoviridis*, *Actinomyces atroolivaceus*, *Actinomyces olivoviridis*, *Actinomyces viridans*, *Actinomyces viridobrunneus*, *Actinomyces intermedius*, *Actinomyces streptomycini* and *Actinomyces herbaricolor*[3,7].

Fig. 1. Structures of ferrous *o*-nitrosophenol complexes isolated from actinomycetes.



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Investigation of the *S. griseus* cultures has shown that the green color is caused by a mixture of compounds, two of which have been identified as **II** and **III**. Among the remaining components was a hybrid ferrous chelate (**IV**) of the ligands in **II** and **III**. We have examined nine strains designated as *S. griseus* for the formation of these metabolites and determined the physiological requirements for their optimum production.

Materials and Methods

Chemicals

4-Hydroxy-3-nitrobenzaldehyde and 4-hydroxy-3-nitrobenzoic acid were purchased from Aldrich Chemical Company Inc., Milwaukee, Wisconsin.

Cultures

Streptomyces griseus strains were obtained from the following sources: MA8 and MA45 from Merck, Sharp and Dohme, Rahway, New Jersey; 11798 and 7-455.F3 from Pfizer International, Inc., Groton, Connecticut; IMRU 3570 from the Waksman Institute of Microbiology, Rutgers University, New Brunswick, New Jersey; NRRL 3851 from the Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois; ATCC 27001 and ATCC 13733 (subspecies *spiralis*) from the American Type Culture Collection, Rockville Maryland; S104 from L.A. JONES, Wayne State University, Detroit, Michigan.

Cultures were maintained on V-8 agar (V-8 vegetable juice 20%, $CaCO_3$ 0.3%, agar 2%). A vegetative inoculum was grown in glucose - Soytone - corn steep - yeast extract medium[8] at 26°C on a rotary shaker (220 rpm, 3.8 cm eccentricity) for 3 days. Production cultures comparing the effects of medium composition received 4% of washed vegetative inoculum and were incubated in 250-ml Erlenmeyer flasks containing 50 ml of medium under the conditions used to grow the inoculum. The composition of the basal medium was (per liter): K_2HPO_4 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $FeSO_4 \cdot 7H_2O$ 0.25 g, $CuSO_4 \cdot 5H_2O$ 1.6 mg, $MnSO_4 \cdot 4H_2O$ 1.2 mg, $ZnSO_4 \cdot 7H_2O$ 3.0 mg and $CaCl_2$ 5.0 mg. Carbon and nitrogen sources were added as described in the text and tables.

Production and Isolation of Viridomycins

For routine production of viridomycins, FA medium in which the basal medium was supplemented with fructose (20 g/liter) and alanine (4.2 g/liter) received 2 ml of unwashed vegetative inoculum and were incubated for 9 days as described above. Cultures were harvested by filtration. The filtrate, adjusted where necessary to a pH value above 8.0 with $NaHCO_3$, was extracted with BuOH to give a non-acidic fraction. Acidic components were recovered from the aq phase by acidifying to pH 2 with HCl and extracting with BuOH. Evaporation of the acidic and non-acidic extracts yielded dark green residues that, by reversed phase TLC (Whatman silica $KC_{18}F$; EtOAc - MeOH - H_2O, 35 : 30 : 35), each gave multiple green zones. The non-acidic product showed a dominant component (compound **X**) at Rf 0.68. The main component of the acidic product was at Rf 0.98 (compound **Y**) with another substantial component at Rf 0.94 (compound **Z**).

The non-acidic product was fractionated by partition chromatography on a 2.5×95 cm column of dextran gel (Sephadex LH-20 fine beads, Pharmacia Limited, Uppsala) with a solvent containing benzene - EtOAc - MeOH - H_2O (12 : 16 : 4 : 1). Fractions (10 ml) were collected and examined by TLC. Compound **X** was eluted as a dark green zone in fractions 55~78. It was recovered as an amorphous green solid by fractional precipitation from MeOH - EtOAc with ether.

The acidic product was also fractionated on a column (2.5×95 cm) of Sephadex LH-20 but with EtOAc - MeOH - H_2O (20 : 10 : 1) as the developing solvent. Examination by TLC of the 10-ml fractions collected showed compound **Y** as the sole component in fractions 130~200. Compound **Z** was the principal component in fractions 58~72. The latter fractions were pooled and rechromatographed by the same procedure to give a homogeneous product. Compounds **Y** and **Z** were recovered as amorphous green solids by fractional precipitation from MeOH - EtOAc with ether. The properties of compounds **X**, **Y** and **Z** are listed in Table 1.

Analyses

The concentration of viridomycins was estimated from the absorbance of solutions at 690 nm; it was calculated as viridomycin A, based on a specific extinction ($E^{1\%}_{1cm}$) of 168 at this wavelength. Yields of viridomycin in cultures were measured in clarified broth supplemented to 2 mM with $FeSO_4$ before filtration. To measure the amount of viridomycins in the mycelium, the filtration residue was extracted with methanol at ambient temperature and the absorbance at 690 nm was determined.

Growth was determined as the dried weight of mycelium recovered by filtration. Orthophosphate was measured by the procedure of CHEN *et al.*[9]. Sugars were determined colorimetrically after reaction with phenol in H_2SO_4[10] using appropriate standards. Alanine concentrations were estimated with alanine dehydrogenase[11]. Protein was measured by the procedure of LOWRY *et al.*[12].

NMR spectra were obtained with Varian model CFT 80 and Bruker model MSL 300 Fourier transform spectrometers. Measurements (δ) are reported in ppm downfield from tetramethylsilane as the internal standard.

Synthesis of Viridomycin A and Actinoviridin A

The cupric complexes of 4-hydroxy-3-nitrosobenzaldehyde and methyl 4-hydroxy-3-nitrosobenzoate were prepared as described by CRONHEIM[13]. Copper was replaced with iron by stirring the products in excess aq $FeSO_4$. The dark green suspensions were extracted with BuOH and the evaporated extracts were chromatographed on columns of silicic acid (BioSil A, Biorad, California) using a gradient of $(CH_3)_2CO$ in EtOAc as eluant. The ferrous chelate of 4-hydroxy-3-nitrosobenzaldehyde (viridomycin A) was recovered from fractions eluted with 10% $(CH_3)_2CO$ and purified by partition chromatography as described for the isolation of viridomycin A from culture broths. The ferrous chelate of methyl 4-hydroxy-3-nitrosobenzoate (ν^{KBr}_{max} 1675 cm^{-1}), also eluted from the silicic acid column with 10% $(CH_3)_2CO$, was dissolved (200 mg/20 ml) in MeOH and hydrolyzed with 5 M NaOH (5 ml) at room temp for 24 hours. Acidification and extraction with EtOAc gave actinoviridin A (120 mg) which was purified as a dark green amorphous solid by fractional precipitation from MeOH with EtOAc.

Reductive Acetylation

To a viridomycin sample (5~50 mg) in MeOH (5 ml) was added acetic anhydride (0.5 ml) and zinc dust (0.1 mg). The mixture was agitated until the green color had disappeared, and after a further 5 minutes was filtered, diluted with water (10 ml) and extracted with EtOAc. For viridomycin A or actinoviridin A samples, the extract was concentrated and applied directly to a preparative (1 mm) layer of silica gel (Sil 60 with fluorescent indicator, E. Merck, Darmstadt). The chromatogram was developed with EtOAc or $CHCl_3$ - MeOH (9 : 1). For viridomycin E, neutral and acidic products in the EtOAc extract were separated by back extraction with 1% aq $NaHCO_3$. Each fraction was then subjected to preparative layer chromatography (PLC) as above. Fluorescence-quenching zones were scraped from the plate and the product was eluted with $(CH_3)_2CO$. For qualitative TLC analysis, resolution of the reductive acetylation products was improved by impregnating 0.25-mm silica gel layers with citric acid.

Results

Characterization of Metabolites

The *S. griseus* metabolites exhibited absorption maxima near 690 nm which is comparable to the values reported for ferroverdin (670 nm) and for the viridomycins (680 nm[3] and near 700 nm[7]). In addition, their absorption spectra were similar over the entire wavelength range to the published spectrum of viridomycin A[7]. The IR absorption spectrum of compound X showed a peak at 1675 cm^{-1} which can be attributed to an aromatic aldehyde group. In the ^1H NMR spectrum, a singlet at δ 9.82 is assigned to the aldehyde hydrogen atom. Signals consisting of a doublet of doublets at δ 7.98 and doublets at δ 7.60 and 7.16 can be ascribed to an aromatic ABM system and assigned to H-6 (H-6′), H-2 (H-2′) and H-5 (H-5′), respectively. These spectral data are similar to those reported for viridо-

Table 1. Properties of viridomycin components from *Streptomyces griseus* MA45.

Property	Compound		
	X (Viridomycin A)	Y (Actinoviridin A)	Z (Viridomycin E)
MP (°C)	>300	>300	>300
UV-visible λ_{max}^{MeOH} nm (ε)	293, 312, 430, 680 (2.14, 1.93, 0.71, 0.60×10^4)	268, 295, 433, 686 (2.61, 2.27, 0.72, 0.67×10^4)	268, 295, 312[a], 434, 685 (2.28, 2.13, 1.60, 0.65, 0.64×10^4)
IR ν_{max} (KBr) cm^{-1}	3050~2850, 1675, 1590	3450~2500, 1690, 1595	3450~2500, 1683, 1595
^1H NMR (MeOH-d_4) δ (J, Hz)	7.16 (d, $J_{5,6}$=9.0), 7.60 (d, $J_{3,5}$=1.9), 7.98 (dd, $J_{3,5}$=1.9, $J_{5,6}$=9.0), 9.82 (s)	7.10 (d, $J_{5,6}$=8.3), 7.69 (d, $J_{3,5}$=1.3), 8.06 (dd, $J_{3,5}$=1.3, $J_{5,6}$=8.3)	7.10 (d, $J_{5,6}$=9.1), 7.16 (d, $J_{5,6}$=8.8), 7.72 (d, $J_{3,5}$=1.3), 7.62 (d, $J_{3,5}$=1.6), 8.06 (dd, $J_{3,5}$=1.3, $J_{5,6}$=9.3), 7.97 (dd, $J_{3,5}$=1.7, $J_{5,6}$=10.6), 9.74 (s)
^{13}C NMR (DMSO-d_6) δ	115.5, 122.4, 123.4, 134.6, 159.1, 181.6, 191.0	112.1, 116.1, 121.3, 136.6, 158.6, 167.0, 180.3	112.3, 114.9, 116.6, 121.4, 122.2, 123.1, 134.2, 136.8, 158.9, 159.1, 167.1, 180.5, 181.3, 190.9

[a] Inflection.

mycin A[3]. In addition, the proton-decoupled ^{13}C NMR spectrum of compound X showed seven resonances (Table 1).

Reductive acetylation of compound X with acetic anhydride and zinc gave a mixture of two components that were separated by PLC with ethyl acetate as the developing solvent. The main product, Rf 0.30, was characterized as 3-diacetylamino-4-hydroxybenzaldehyde from its ^1H NMR resonances in $(CH_3)_2CO$-d_6 at δ 2.14 (s, CH_3), 2.23 (s, CH_3), 9.99 (s, CHO), and ABM system at 7.69 (dd), 7.38 (d) and 8.79 (d) due to three aromatic H's. The second component, Rf 0.36, was similarly characterized from its ^1H NMR signals in $(CH_3)_2CO$-d_6 at δ 2.24 (s, CH_3), 9.58 (s, CHO), and ABM system at δ 7.59 (dd), 7.16 (d) and 8.27 (d) as 3-acetylamino-4-hydroxybenzaldehyde, identical with a specimen prepared by reductive acetylation of 4-hydroxy-3-nitrobenzaldehyde. It was generated from the less stable di-*N*-acetyl derivative during storage in solution or during recovery after PLC. Formation of these products on reductive acetylation of viridomycin A has been described by ROSYNOV *et al.*[14]. The identity of compound X as II was confirmed by synthesis using the Baudisch reaction in a procedure modified from one reported by KHOKHLOV *et al.*[3]. The natural and synthetic products were indistinguishable in their UV, visible and IR absorption spectra as well as their ^1H and ^{13}C NMR spectra.

Compound Y differed from compound X in its acidic character and in showing an IR absorption peak at 1690 cm^{-1} in place of the aldehyde carbonyl absorption at 1675 cm^{-1}. The ^1H NMR spectrum of compound Y lacked the aldehyde proton resonance at δ 9.82 present in compound X. Signals characteristic of an ABM system in the region expected for aromatic ring protons resembled those given by viridomycin A, and the proton-decoupled ^{13}C NMR spectrum contained seven resonances (Table 1). Reductive acetylation of compound Y gave a mixture of two products which were separated by PLC after repeated development in chloroform - MeOH (9:1). The substance at Rf 0.24 (single development) was recognized as 3-diacetylamino-4-hydroxybenzoic acid from its ^1H NMR resonances

in MeOH-d_4 at δ 2.16 (s, CH_3), 2.33 (s, CH_3), and ABM system at 7.24 (d), 7.84 (dd) and 8.47 (d) due to three aromatic H's. The substance at Rf 0.18 was recognized from its ^1H NMR resonances in MeOH-d_4 at δ 2.18 (s, CH_3), and ABM system at δ 6.90 (d), 7.70 (dd) and 8.41 (d) as 3-acetylamino-4-hydroxybenzoic acid, identical with a specimen synthesized by reductive acetylation of 4-hydroxy-3-nitrobenzoic acid. The structure of compound **Y** was established as **III** by synthesis *via* the dimethyl ester as described by BALLIO *et al.*[2].

The ^1H and ^{13}C NMR spectra of compound **Z** (Table 1) contained all of the signals present in the spectra of both compounds **X** and **Y** at intensities indicating that the two ligands were present in equal proportions. The UV, visible and IR absorption spectra were also consistent with this postulate. Reductive acetylation of the product yielded a mixture which, by TLC analysis, contained the diacetyl derivatives of 3-amino-4-hydroxybenzaldehyde and 3-amino-4-hydroxybenzoic acid together with smaller amounts of the monoacetyl derivatives. The acidic and neutral products were separated and the monoacetyl derivatives each purified by PLC to yield compounds identical in melting point and spectral properties with authentic samples. Compound **Z** was therefore concluded to be the hybrid ferrous chelate **IV**.

Production of Viridomycins

The ability of *S. griseus* to produce viridomycins was initially discovered in cultures of strain MA45 growing in the basal medium with glucose (20 g/liter) and glycine (2.6 g/liter) as carbon and nitrogen sources, respectively (GG medium). Of eight other strains of *S. griseus* tested for viridomycin production, only strain ATCC 27001 gave a positive result. A subsequent screening in FA medium which optimized viridomycin production by MA45 showed that the phenotype was restricted to these two strains. The productivity of MA45 and ATCC 27001 in GG medium was similar and they responded similarly when glucose and glycine were replaced with a variety of other carbon and nitrogen sources. Measurement of viridomycins in the cells and filtrate of MA45 cultures at intervals during growth in GG medium showed that less than 5% of the total pigment was associated with the mycelium.

When the initial concentration of glycine in GG medium was varied, viridomycins were produced

Fig. 2. Biomass accumulation, viridomycin production and nutrient utilization in cultures of *Streptomyces griseus* MA45 grown in FA medium.

Cell dry weight (●), viridomycins (○), alanine (▲), phosphate (■). Excess fructose was present throughout the fermentation.

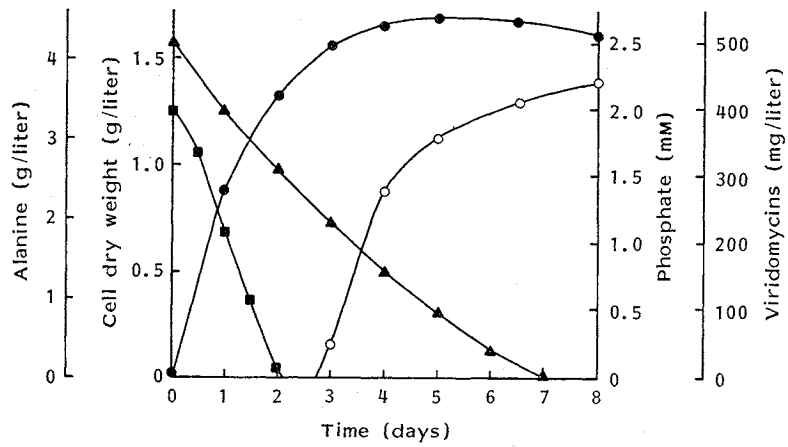
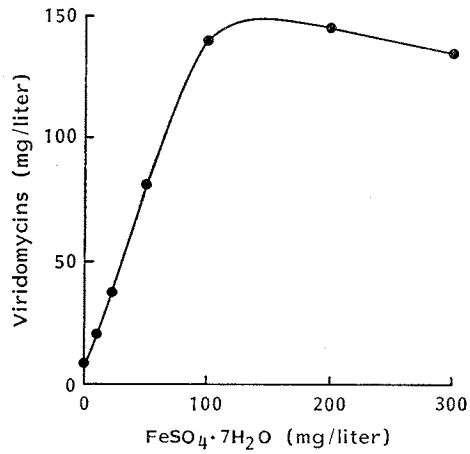


Fig. 3. Effect of iron concentration on the production of viridomycins by *Streptomyces griseus* MA45.

Cultures were grown for 10 days in GG medium with different amounts of $FeSO_4 \cdot 7H_2O$.



only when the amount was above 1.86 g/liter. The maximum yield of viridomycins was obtained with glycine concentrations at or above 3.72 g/liter. Production of viridomycins was also sensitive to changes in the initial phosphate content of the medium. In GG medium, the optimum concentration of K_2HPO_4 was 2.0 mM. Cultures receiving this amount depleted the medium of phosphate during the second day of growth with carbon and nitrogen sources remaining in excess (Fig. 2). Viridomycins first appeared in the culture only after phosphate depletion. Measurements of intracellular orthophosphate showed that the concentration declined rapidly from an initial 5 μg/mg protein to 0.8 μg/mg protein after 36 hours and 0.1 μg/mg protein at 2 days when exogenous phosphate had been exhausted. The intracellular concentration recovered to 0.2 μg/mg protein at 3 days and remained constant at this value while viridomycins were being produced.

Table 2. Effect of carbon source on the production of viridomycins by *Streptomyces griseus* MA45*.

Carbon source	Viridomycins (mg/liter)
Fructose	194
Cellobiose	138
Glycerol	91
Mannose	80
Galactose	73
Glucose	29
Maltose	24
Sucrose	2
Arabinose	2

* Cultures were grown in the basal medium supplemented with morpholinopropane sulfonic acid (21 g/liter), asparagine (3.0 g/liter) and various carbon sources at 20 g/liter. The pH of the medium was adjusted to 7.0 with NaOH. The viridomycin concentration in the broth was determined daily for 12 days. Maximum values are given.

Table 3. Effect of nitrogen source on the production of viridomycins by *Streptomyces griseus* MA45*.

Nitrogen source	Viridomycins (mg/liter)
Alanine	106
Glycine	86
Proline	62
Glutamic acid	36
Potassium nitrate	34
Asparagine	27
Histidine	24
Leucine	5
Ammonium sulfate	4
Bacto-peptone	2

* Cultures were grown in the basal medium supplemented with glucose (20 g/liter) and the nitrogen source at 1 g nitrogen equivalent/liter. When the medium contained ammonium sulfate, morpholinopropane sulfonic acid (21 g/liter) was also added and the pH was adjusted to 7.0 with NaOH. Viridomycins were determined as in Table 2.

The yield of viridomycins responded to changes in the initial amount of $FeSO_4$ in GG medium (Fig. 3). With increasing iron concentration, production increased to a maximum at 150 mg/liter $FeSO_4 \cdot 7H_2O$. At low iron concentrations, the assay value for viridomycins increased if cultures were supplemented with $FeSO_4$ before analysis. Comparison of different carbon sources for their ability to support viridomycin production in the basal medium with asparagine supplying nitrogen indicated that fructose was the most effective (Table 2). A similar comparison of nitrogen sources with glucose sup-

plying carbon gave optimum yields with alanine (Table 3). In combination, fructose and alanine gave the highest production (525 mg/liter at 12 days) of any medium tested.

Discussion

Three components in the complex of green products formed by *S. griseus* strain MA45 have been identified as ferrous chelates of 4-hydroxy-3-nitrosobenzaldehyde and 4-hydroxy-3-nitrosobenzoic acid. In two of these compounds, two molecules of the same ligand are associated with a ferrous ion; the third is a hybrid containing one molecule each of the aldehyde and the carboxylic acid ligands. The dialdehyde and diacid complexes have been isolated previously as viridomycin A[3] and actinoviridin A[5,6], respectively. The hybrid may correspond to one of the components designated A_1, A_2, B, C and D in the complex of green products isolated by BLINOVA *et al.*[3] from *S. viridaris*. However, the structures of these components have not been determined and we therefore refer to the hybrid chelate as viridomycin E. Numerous other related compounds are present in the complex produced by *S. griseus* MA45, making purification of individual components a difficult task. Presumably these result from the biosynthesis of additional ligands and their chelation with iron in various combinations.

Production of viridomycins is not a widespread characteristic among *S. griseus* strains and is not correlated with streptomycin production. The conditions for its formation in strain MA45 are defined by a requirement for limiting phosphate concentration. Unlike streptomycin which, in this and other cultures of *S. griseus*, is produced in response to either phosphate or nitrogen deficiency (ref 15 and unpublished results), viridomycin synthesis does not occur in media where nitrogen is exhausted before depletion of phosphate has fostered conditions within the mycelium suitable for secondary metabolism. A variety of carbon and nitrogen sources support production but not equally well. The most favorable for *S. griseus* MA45 was a combination of fructose and alanine but for *S. viridaris* sucrose and nitrate gave the best yields[7]. Other viridomycin producers performed optimally on starch or glycerol as carbon sources[7]. Ferroverdin production by *Streptomyces* strain A-305 was highest in media containing starch, sucrose or lactose and a complex nitrogen source but was also synthesized with nitrate as the source of nitrogen[16]. Such differences suggest that carbon and nitrogen nutrition are not decisive in promoting *o*-nitrosophenol synthesis and that optimum production depends upon subtle physiological characteristics of each producer species. The role of phosphate in controlling the process in strains other than *S. griseus* has not been examined.

In culture media deprived of iron, *S. viridaris* and several other *Streptomyces* species have been reported to produce a yellow proviridomycin that, when isolated and exposed to ferric chloride, gave a substance chromatographically and spectrophotometrically indistinguishable from viridomycin A[7,17]. Unlike the viridomycins, proviridomycin was unstable in solution; consequently, cultures in low-iron media gave low viridomycin titers, even when iron was added at the time of analysis. From the similar behavior of *S. griseus* MA45 in media containing low levels of iron, this species also produces the uncomplexed ligand under such conditions; optimum viridomycin production was obtained only when adequate iron was present during growth of the culture.

Chelating agents are excreted by numerous microorganisms to facilitate uptake of iron[18]. In actinomycetes, ferrioxamines have been shown to fulfil this role[19] and have been isolated from several streptomycetes, including a ferroverdin producer[20]. Where the formation of iron transport substances has been examined, such as with enterochelin[21,22] and pyoverdine[23], the amounts produced are regulated by the iron status of the culture; synthesis and excretion are increased in media depleted of iron salts. This response was not observed for viridomycin synthesis in *S. griseus*, nor in *S. viridaris*[7]. With the ferroverdin producer, the yield increased with increasing iron concentration to a maximum at 8 mg/liter. Beyond this both growth and ferroverdin synthesis were inhibited. In this regard it is of interest that ferroverdin is confined to the mycelium[2] whereas viridomycins accumulate in the culture medium. The absence of ferroverdin excretion and the decreased synthesis of both ferroverdin and viridomycins at low iron levels suggest that these substances are not produced as aids to iron sequestration during iron starvation but as secondary metabolites in response to other cellular or environmental signals. It is possible that the high affinity of *o*-nitrosophenols for iron renders this element inaccessible

to competing organisms. MUSILEK[24] has made the intriguing observation that absence of iron from maintenance media preserves the capacity for streptomycin synthesis in *S. griseus*, suggesting that there may be an interaction between protective agents in this organism.

Acknowledgments

We thank Drs. E. DULANEY of Merck, Sharp and Dohme, J. B. ROUTIEN of Pfizer International Inc., D. P. LABEDA of the Agricultural Research Service, U.S.D.A., L. A. JONES, Wayne State University and R. GORDON, Waksman Institute of Microbiology, Rutgers University for cultures of *Streptomyces griseus*. We are also grateful to Dr. J. A. WALTER, National Research Council of Canada, for NMR spectra and to Mrs. C. L. MOFFORD and Mrs. B. LANGILLE for technical assistance. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada.

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