JOURNAL OF BACTERIOLOGY, Jan., 1967, p. 286-294
Vol. 93, No. 1

Iron-chelating Hydroxamic Acid (Schizokinen) Active in Initiation of Cell Division in *Bacillus megaterium*

B. R. BYERS, M. V. POWELL,[1] AND C. E. LANKFORD
Department of Microbiology, The University of Texas, Austin, Texas

Received for publication 3 June 1966

ABSTRACT

Bacillus megaterium ATCC 19213 secretes a cell division-initiating "schizokinen" (SK) which accumulates during its culture cycle to a concentration inversely proportional to the iron added to a sucrose-mineral salts medium. Secreted SK was purified from culture filtrates as a red Fe (III) chelate, and a fraction with similar biological properties was obtained from whole cells. Infrared spectra of SK, and analyses of unhydrolyzed and acid-hydrolyzed preparations indicated it to be a secondary hydroxamate; visible absorption maxima of the ferric complex showed *p*H dependency typical of ferric monohydroxamates. Schizokinen preparations from cultures grown at "normal" and at low Fe concentrations were similar biologically and in certain of their chemical properties, but their R_F values and infrared spectra suggested nonidentity. Significant lag reduction of *B. megaterium* was effected by 0.2 mμg of SK per ml; the Fe (III)-SK chelate and "iron-free" SK were equally effective. A 50-mμg amount produced half-maximal growth response of the siderochrome auxotroph, *Arthrobacter* JG-9. Schizokinen also overcame ferrimycin A inhibition of three *Bacillus* species. These properties relate the *B. megaterium* schizokinen to the trihydroxamate siderochromes, although SK appears to be a monohydroxamate.

An inverse relation between duration of cell division lag and initial cell populations of microbial cultures has been attributed to a requirement for a critical concentration of diffusible endogenous factors (*see* 18). An inoculum-dependent and an inoculum-independent component of division lag of *Bacillus megaterium* in a chemically defined medium recently were distinguished (22). Exponential division was initiated in such cultures coincidentally with accumulation of a factor in culture fluid to a concentration which, if added to a fresh culture, eliminated its inoculum-dependent lag. Evidence was adduced for a requirement of a population-dependent critical concentration of the factor both for initiation and for maintenance of exponential division (22; Walker and Lankford, Bacteriol. Proc., p. 43, 1963; Arceneaux and Lankford, Texas Rept. Biol. Med. 23:630, 1965). The term schizokinen was suggested for endogenous factors of bacteria so involved in cell growth and division. The isolation and general chemical characteristics of a substance having the biological properties of the *B. megaterium* schizokinen are described here.

[1] Present address: Department of Biology, Portland State College, Portland, Ore.

MATERIALS AND METHODS

Abbreviations. The following abbreviations will be used: SK for schizokinen, specifically the *B. megaterium* schizokinen; Fe-SK for the Fe(III)-SK chelate; desferri SK for "iron-free" SK; CFU for colony-forming units; a "unit of SK activity," previously defined (22) as that amount of culture filtrate producing a growth response of *B. megaterium* equivalent to 25 μg of meconic acid, is redefined here as equivalent to 0.2 mμg of purified SK.

Cultures. B. megaterium Texas (ATCC 19213), *B. subtilis* var. *niger*, and *B. subtilis* were described previously (22). The siderochrome-requiring organism, *Arthrobacter* JG-9, was obtained from H. A. Barker of the University of California, Berkeley, and *Staphylococcus aureus* FDA 209P, from the departmental culture collection.

Culture media and procedures. The *Bacillus* species were cultivated in the media and by the methods described previously (22). The trace metal content of the basal medium designed for *B. megaterium* was reduced for some experiments by a modification of the method of Donald et al. (9). Sucrose (10 g), $(NH_4)_2HPO_4$ (1.0 g), K_2HPO_4 (2.5 g), KH_2PO_4 (2.5 g), and $MgSO_4 \cdot 7H_2O$ (0.2 g) were dissolved in 1 liter of demineralized water; the *p*H was adjusted to 7.3 to 7.4 with 1 N KOH, and 10 g of chromatographic-grade alumina (Alcoa Chemicals Division, Pittsburgh, Pa.) was added. The suspension was autoclaved for 15

min at 121 C, swirled while hot, and allowed to stand for 12 hr. The alumina was removed by filtration through a sterile membrane filter (0.45 μ; Millipore Filter Corp., Bedford, Mass.) assembly previously rinsed with 6 N HCl and sufficient demineralized water to remove the acid. The medium was adjusted to *p*H 7.0 with filter-sterilized HCl, prepared by isothermic distillation, and supplemented with filter-sterilized solutions of Specpure (Johnson, Matthey, & Co., Inc., New York, N.Y.), $MnSO_4 \cdot H_2O$ (2 μg of Mn per ml), $FeSO_4 \cdot 7H_2O$ (0.02, 0.2, or 2.0 μg of Fe per ml), and $ZnSO_4 \cdot 7H_2O$ (0.02 μg of Zn per ml). The purified basal medium was dispensed aseptically to Pyrex culture vessels which had been rinsed in 6 N HCl, and then with sufficient water to remove the acid.

Culture filtrates containing SK were obtained by culturing *B. megaterium* to maximal stationary phase in the regular basal medium (2.0 μg of Fe per ml), or in alumina-treated basal medium at a lower Fe concentration, in Erlenmeyer flasks at a ratio of medium to flask volume of 1:6. These cultures were inoculated with 10^4 to 10^5 CFU per ml of *B. megaterium* which had been harvested from the second of two successive 12-hr Brain Heart Infusion Agar (Difco) slant cultures and washed three times by centrifugation from the growth medium. The cultures were agitated on a reciprocating shaker at 37 C. Cells were removed at maximal growth by centrifugation at 3 C in a Sorvall RC2 centrifuge, washed twice in 0.05 M phosphate buffer (*p*H 7), and stored at −20 C. The culture supernatant fluid was filtered and refrigerated until purification was begun.

The basal medium devised by Burnham (5) for *Arthrobacter* JG-9 was modified by reducing the trace-metal supplements (to avoid occasional formation of a flocculent precipitate) to final concentrations (grams per liter) of: $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.007; $ZnSO_4 \cdot 7H_2O$, 0.002; $CuSO_4 \cdot 5H_2O$, 0.001. Stock cultures and seed inocula were cultivated on this medium, supplemented with 0.1% dried beef hemoglobin (Difco) and solidified with 1.5% agar.

The dose-response assay for SK (22) which is based on the proportionality of lag reduction of *B. megaterium* to SK concentrations was used without modification. Dose-response assays with *Arthrobacter* JG-9 were prepared by adding to each tube (22 by 175 mm) of unsupplemented basal medium the desired amount of filter-sterilized supplement and the inoculum to a total volume of 5 ml. A 5-day slant culture on hemoglobin medium was harvested and washed three times by centrifugation from the unsupplemented basal medium. Dilutions were made to give a final concentration of about 10^5 CFU per ml in each tube. Incubation for 4 days at 25 C on a reciprocating shaker resulted in growth which was proportional to the concentration of ferrichrome or ferrioxamine B supplement up to 1 mμg/ml of either. The optical density of each culture tube was determined in a Bausch & Lomb Spectronic-20 colorimeter at 600 mμ, and the activity of the test preparation was estimated by comparison with the standard (ferrioxamine B).

Chemical assays. For determination of its Fe content, SK was ashed for 12 hr at 600 C in Vycor crucibles. The ash was dissolved in 2 N HCl, the solution transferred to 5- or 10-ml volumetric flasks, and its volume was made up with demineralized water. The Fe assay method of Smith et al. (29) was employed with bathophenanthroline (G. F. Smith, Co.) as a chromogenic reagent. The absorbancy at 533 mμ of the colored complex was determined in a Beckman DU spectrophotometer, with a standard of ferrous ammonium sulfate (hexahydrate).

Bound hydroxylamine was determined according to the method of Csáky (8) with unhydrolyzed and acid-hydrolyzed (12 hr in 6 N HCl at 106 C) samples. Hydroxylamine hydrochloride served as the standard. Absorbancy of the mixtures was read in a Beckman DU spectrophotometer at 520 mμ. The presence of NH_2OH in hydrolysates was confirmed by ascending paper chromatography in four solvents recommended by Bremner (4) and Bickel et al. (3). Hydroxylamine was located on the dried papers with sprays of diacetyl monoxime Ni salt, picryl chloride, the Csáky reagents (4), and tetrazolium (30).

Sources of siderochromes and hydroxamic acids. Acethydroxamic acid was prepared by a combination of the methods of Fink and Fink (13) and Wise and Brandt (32); aspartyl hydroxamate was provided by Joanne Ravel, The University of Texas, and hadacidin (19), by R. F. Pittillo, Southern Research Institute, Birmingham. The siderochromes and their sources were as follows: coprogen (17), ferrichrysin (20), and ferrioxamine B (1) from I. H. Zähner, Eidg. Technischen Hochschule, Zurich, Switzerland; ferrirubin and ferricrocin (20) from H. Bickel, Ciba Research Laboratory, Basel, Switzerland; ferrimycin A (2) and Desferal (methane sulfonate of iron-free ferrioxamine B) from S. S. Barkulis, Ciba Pharmaceutical Co., Summit, N.J.; ferrichrome (24) from J. B. Neilands, The University of California, Berkeley; and mycobactin (14) from N. E. Morrison, Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory, Baltimore, Md.

RESULTS

Preparation of excreted SK. Concentration and purification of SK secreted by *B. megaterium* into its culture fluid was achieved initially from filtrates of cultures in the regular basal medium. The low yields of SK from relatively large culture volumes precluded detailed investigations of the partially purified preparation, although clues were obtained as to its general chemical nature. An observation that synthesis and excretion of the *B. megaterium* SK is enhanced by growth in alumina-treated basal medium at reduced Fe concentrations provided means for obtaining sufficient active material for the development of better purification methods and for definitive chemical tests. Reduction of added Fe from 2.0 to 0.2 μg/ml did not diminish the final cell crop, but it increased the final SK accumulation about 100-fold, as determined by assay with *B. megaterium* (Table 1). The addition of Zn (0.02 μg/ml) doubled this yield. The medium found to give

TABLE 1. *Secretion of Bacillus megaterium schizokinen in relation to the concentration of metals added to alumina-treated medium*

Metal supplement (μg/ml)				Maximal optical density at 600 mμ[a]	SK (units/ml of filtrate)
Fe	Mn	Zn	Mg		
2.0[b]	2.0[b]	—	20.0[b]	1.1	2,900[b]
0.2	2.0	—	20.0	1.0	21,000
0.02	2.0	—	20.0	1.1	210,000
0.02	2.0	0.2	20.0	1.1	460,000
0.02	2.0	0.02	20.0	1.1	480,000
0.02	2.0	0.02	—[c]	1.1	470,000

[a] Inoculum = 10^4 to 10^5 CFU/ml.

[b] Regular basal medium, not alumina-treated; usual range of activity, 2,000 to 3,000 units per ml.

[c] Prior to treatment with alumina, 20.0 μg of Mg per ml was added.

best SK production included supplements of 0.02 μg of Fe per ml, 0.02 μg of Zn per ml, and 2.0 μg of Mn per ml; 20 μg of Mg per ml was added prior to alumina treatment.

The cell-free supernatant fluid of maximal stationary cultures of *B. megaterium* grown in medium containing 0.02 μg of Fe per ml turned red upon addition of dilute ferric chloride, but this color change was not obtained with filtrates of cultures grown in regular basal medium (2.0 μg of Fe per ml). Batches of 6 to 10 liters of pooled filtrates of low-Fe cultures were treated with 0.3% (w/v) Norit A charcoal, cleaned before use by successive washes with ethyl alcohol, acetone, ethyl alcohol-acetone-7% ammonia (50:25:10), and water. Adsorbed SK was recovered by elution with ethyl alcohol-acetone-7% ammonia (50:25:10). These charcoal eluates contained 50 to 70% of the SK activity, in addition to most of the ferric chloride-reactive material.

Charcoal eluates were saturated with Fe at *p*H 4 by addition of ferric chloride (5 μg/ml) until there was no further increase in optical density at 440 mμ. The preparation th n was extracted three times with 0.5 volume of 85% phenol, the only effective solvent found, and the phenol layer was washed twice with 1 volume of 1 N HCl; 0.2 volume of water and 5 volumes of petroleum ether-ether-acetone (50:25:10) were added to the phenol, and the mixture was shaken for 5 min. The resulting organic layer was washed four times with 0.2 volume of water, and the aqueous phases were pooled and washed twice with 1 volume of ether. Dissolved ether was evaporated from the red solution, which contained 80 to 90% of the activity present in the charcoal eluate.

The red material and the SK activity (at *p*H 3) passed immediately through Dowex 1 × 8 (200- to 400-mesh, chloride form), which had been washed successively with ethyl alcohol, acetone, 1 N HCl, and sufficient water to remove the organic solvents and the acid. Elevation of the *p*H above neutrality (which changed the color from red to yellow) allowed retention of both color and activity by the resin. Phenol extracts obtained from charcoal eluates of low-Fe culture filtrates were adjusted to *p*H 11 with 1 N KOH, and amounts representing about 3 liters of filtrate (700 million units) were exchanged onto a column (5.5 by 9 cm) of Dowex 1 (Cl). The column was washed with 500 ml of demineralized water and 100 ml of 0.01 M NaCl, and elution was begun with 0.1 M NaCl. The elution pattern of the Fe-binding material was determined from the optical density at 400 mμ of 5-ml fractions collected with a Research Specialties model 1205 fraction collector at a flow rate of 20 ml per hr. Activity assays of these fractions with *B. megaterium* revealed a corresponding major peak after the passage of about 500 ml of 0.1 M NaCl. Several other minor peaks of corresponding optical density and activity (containing a total of 15 to 20% of the activity applied to the column) also were found. SK in the major peak was recovered in 2 to 3 ml of water by phenol extraction and evaporation in a vacuum at 42 C, with an average recovery of 70% of the activity applied to the column. Addition of 5 to 10 volumes of acetone to this concentrate resulted in the formation of a brick-red precipitate after 6 hr at 5 C. The precipitate was collected by centrifugation at 15,000 × *g* and dissolved in a minimum of demineralized water. After a second precipitation from acetone-water, the red precipitate was washed three times with acetone and dried over P_2O_5. An average of 20 to 25 mg, with a specific activity of 5,000 units per μg, was obtained from 1 liter of filtrate from low-Fe medium. This represented a recovery of about 30% of the original filtrate activity.

The purification method utilized for SK present in low-Fe cultures was applied to filtrates of cultures grown in untreated basal medium at a higher Fe concentration (2 μg of Fe per ml). Chromatography of a phenol extract from 10 liters of filtrate on Dowex 1 (Cl) separated 70% of the activity into a single fraction in the 0.1 M NaCl effluent. Precipitation of the Fe complex from acetone-water yielded about 150 μg per liter of filtrate, with a specific activity equivalent to SK synthesized in the low-Fe medium (5,000 units per μg).

Extensive efforts to crystallize the Fe-SK complex from a variety of solvents and solvent mix-

tures were unsuccessful, a difficulty which has been encountered with certain other biological hydroxamates (for example, 11). Precipitation of the Fe-SK complexes from acetone-water up to four times did not increase their specific activity. Their purity also was indicated by paper chromatographic and paper electrophoretic analyses and by the constancy of the infrared spectra of different preparations of the same type (to be described below).

Preparation of desferri SK. Desferri SK was prepared either by alkaline precipitation of the Fe (24), or by purification of the desferri SK without prior saturation with Fe. In the latter method, 1 N KOH was added to a final concentration of 0.1 N to a charcoal eluate from 3 liters of low-Fe culture filtrate, and after 30 min in ice the solution was centrifuged to collect precipitated Fe. The *p*H was adjusted to 4 with 1 N HCl, and after phenol extraction the desferri SK was chromatographed on Dowex 1 (Cl) by the method used for Fe-SK complexes. Progress in elution was followed by determining the reactivity of each fraction with ferric chloride. About 70% of the desferri SK eluted in a single peak with 0.1 M NaCl, although a smaller faster-moving peak was found. The major peak was desalted by phenol extraction, and lyophilization yielded about 65 mg of a slightly orange material with a specific activity equal to that of the Fe complex. This desferri SK was extremely hygroscopic and relatively insoluble in a variety of organic solvents except methanol. Attempts to crystallize it were unsuccessful.

Preparation of SK from cells of B. megaterium. Previous experiments (22) demonstrated the presence of substances with biological properties similar to excreted SK in extracts of cells disrupted by sonic oscillation. The stimulatory activity of sonic extracts of cells grown in low-Fe medium (9,000 units per mg, dry weight) was about five times that found in cells grown in regular basal medium (2,000 units per mg).

For purification of cellular SK, thawed cell suspensions (20 mg per ml, dry weight) were diluted with 95% ethyl alcohol to a final concentration of 50%. The *p*H was raised to 11 with ammonium hydroxide, and the mixtures were boiled twice and allowed to stand for 3 hr with frequent stirring. Debris was removed by centrifugation in stainless-steel vessels. The supernatant fluid was adjusted to *p*H 3 with 2 N HCl, and a heavy precipitate was centrifuged and discarded. After removing the ethyl alcohol in a vacuum at 42 C, the aqueous residue was filter-sterilized for dose-response assays, which indicated a recovery of only about 20% of the activity found in sonic extracts of cells. This low recovery of cellular SK may be more apparent than real if other cell materials in the sonic extracts contribute additively to stimulation. However, the extractions of cells with alkaline ethyl alcohol may have destroyed a portion of the cellular SK or extraction may have been incomplete. SK present in ethyl alcoholic cell extracts was processed by the method given previously for purification of filtrate SK, and the same elution pattern found with filtrate SK was obtained from Dowex 1 columns.

Infrared spectra of SK. The infrared spectra of the SK preparations, incorporated in KBr wafers at a concentration of 0.5%, were determined by the infrared laboratory on a Baird model 4-55 spectrophotometer, and were interpreted by P. D. Gardner of the Department of Chemistry. The spectra of six different preparations of SK obtained from low-Fe culture filtrates were essentially identical and showed strong absorption peaks at 6.1 and 6.35 μ, in the position for amidic or substituted amidic linkages (Fig. 1). The infrared tracing of three different preparations of SK from regular basal medium were identical, but they were sufficiently different from those of "low-Fe SK" to suggest nonidentical, but related, molecules (Fig. 1). Preparations obtained by removal of Fe from SK by alkaline precipitation, or by purification of SK without Fe saturation yielded an absorption peak at 5.8 μ (Fig. 1). Thus, in the absence of the metal the carbonyl frequency became normal. The appearance of this peak suggests formation of a free carboxylic acid function upon removal of iron.

Visible and ultraviolet spectra of SK. The visible

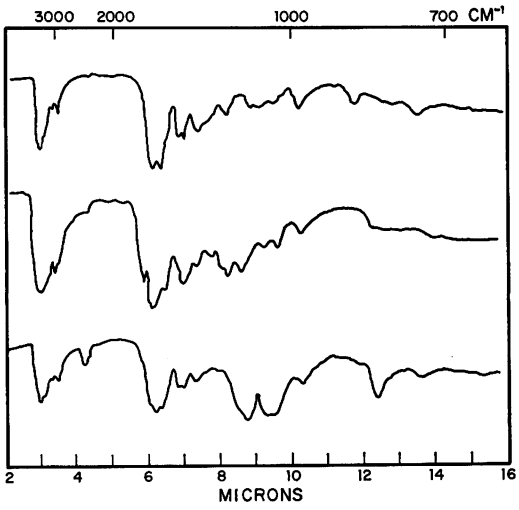

FIG. 1. *Infrared spectra of SK from culture filtrate. Top: Fe-SK from low-iron medium. Middle: Desferri SK from low-iron medium. Bottom: Fe-SK from regular medium.*

absorption maximum of Fe-SK, determined with aqueous solutions in the Cary model 14 spectrophotometer, was markedly *p*H-dependent. Fe-SK complexes from both regular and low-Fe medium, and SK purified from cells in low-Fe medium, displayed identical visible spectra, which are illustrated by the example in Fig. 2. The Fe-SK complex behaved much like Fe(III) complexes of monohydroxamic acids, which undergo a change from yellow, to red, to violet, as the *p*H is adjusted downward from 7, corresponding to a shift from 3:1 to 1:1 ligand-metal complexes (10). The siderochromes, which are ferric trihydroxamates, have a broad visible maximum from 420 to 440 mμ, and do not exhibit the marked *p*H-dependent spectral shift of ferric monohydroxamates. Purified desferri SK, examined at concentrations up to 140 μg/ml and at *p*H values from 2.5 to 7.0, absorbed in the ultraviolet range only below 240 mμ. The Fe-SK complex, however, absorbed rather strongly in the ultraviolet, with a peak appearing at 256 mμ above *p*H 5.1, and increasing to maximal absorbance at about *p*H 6.0 ($E^{1\%}_{1cm}$ = 184 at *p*H 7.0). This absorption peak is believed to be consequential to the predominant Fe-chelate structure in this *p*H range.

Metal chelation. The major Fe-SK fraction obtained by Dowex 1 chromatography of SK from low-Fe medium contained about 10% Fe after precipitation from acetone-water. The visible spectral data indicate that the Fe content of SK may be *p*H-dependent, possibly owing to formation of Fe-SK chelates greater than 1:1 at elevated *p*H. To determine the maximal Fe-binding capacity of SK, 20 mg of SK containing 10.1% Fe were dissolved in water and 10 mg of Fe was added as ferric chloride. The SK was recovered by phenol extraction and the complex was reprecipitated twice from acetone. No change was observed in the infrared spectrum of this "saturated" SK, but its Fe content was 14.1%. The maximal Fe-binding capacity of SK also was examined by adding Fe^{3+} in small increments to a 100-μg sample of desferri SK at *p*H 3. No further increase in optical density at 440 mμ occurred after the addition of 16 μg of Fe, indicating that the Fe saturation level of SK at low *p*H is about 14%.

A solution containing 1 mg of Fe-SK per ml was reduced instantly to a colorless state by sodium hydrosulfite, and shaking in air restored the color. Addition of bathophenanthroline to reduced SK produced the pink color of the ferrous-bathophenanthroline chelate, with nearly quantitative transfer of the Fe to bathophenanthroline. Less than 0.01% of the Fe in SK was bound by bathophenanthroline in unreduced solutions. These observations support the conclusion that SK, like the siderochromes, binds Fe^{3+}, and that Fe^{2+} is only weakly bound.

The metal-binding properties of desferri SK were investigated by alkaline titration (in 0.1 M KCl), using a Metrohm model E 300 *p*H meter. Aqueous solutions of desferri SK (1 mg/ml) were about *p*H 4; titration with freshly prepared KOH, standardized with potassium acid phthalate, demonstrated two buffer regions, the first having a pK_a of about 4.2 and the second having a pK_a of about 9.5 (Fig. 3). The release of protons associated with chelation was measured by titration

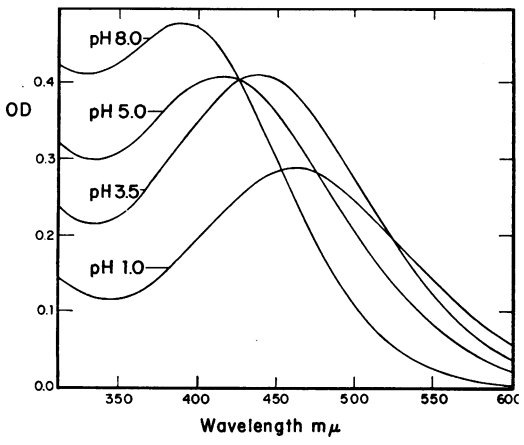

FIG. 2. *Visible spectra of an aqueous solution of Fe-SK (100 μg per ml, 10% Fe) as a function of pH.*

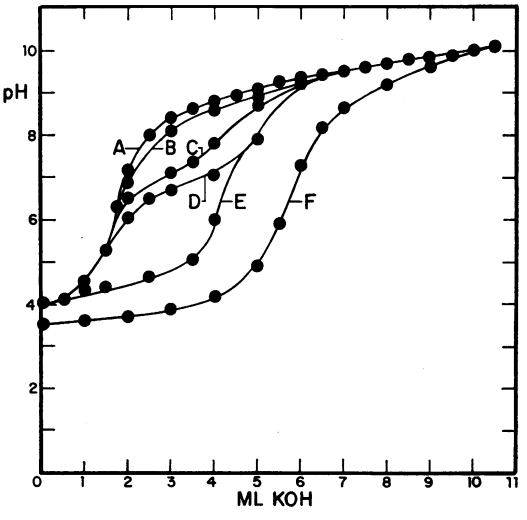

FIG. 3. *Titration of SK after addition of metal salts. A 10-mg amount of desferri SK was titrated with 0.008 M KOH in 0.1 M KCl, with 11.2 μmoles of each metal added. A, no metal; B, Mn^{2+}, Mg^{2+}; C, Co^{2+}; D, Zn^{2+}; E, Cu^{2+}; F, Fe^{3+}.*

with KOH after the addition to desferri SK of Specpure metal salts to a concentration of metal equivalent to about 42% of the maximal Fe content. The relative affinity of SK for the metals was deduced from the position and shape of the titration curves. Fe^{3+} and Cu^{2+} were strongly bound over the entire *p*H range; Co^{2+} and Zn^{2+} were bound above *p*H 6; Mn^{2+} and Mg^{2+} were not appreciably complexed. Addition of Cu^{2+} to desferri SK produced a green solution, but an insoluble complex, typical of unsubstituted monohydroxamates (11), was not formed.

Hydroxylamine content of SK. The hydroxylamine content of samples of SK was compared with that of aspartyl and acethydroxamic acids, ferrioxamine B, and iron-free ferrioxamine B (Table 2). Theoretical yields of NH_2OH were obtained from unhydrolyzed aspartyl and acethydroxamic acids. Unhydrolyzed desferri SK gave low values, but 2.3 to 2.9% NH_2OH was obtained from both hydrolyzed desferri SK and Fe-SK. Since recovery of hydroxylamine from hydrolysates of secondary hydroxamates may be low (Table 2), these data are considered to have qualitative significance only.

The nonreactivity of unhydrolyzed SK suggested that its hydroxamate group(s) may be of secondary configuration. Confirmation of this assumption was obtained by application of the test for secondary hydroxamates developed by Emery and Neilands (12). Treatment of a solution of desferri SK with periodic acid generated an ultraviolet peak at 267, typical of *N*-alkylhydroxylamino compounds (12). Studies are now underway to identify the acyl and amino components, or the amino acid components, or both, of SK.

Paper chromatographic examinations. Several purified SK samples were examined by ascending paper chromatography with isopropanol-water (4:1) and *n*-butanol-acetic acid-water (60:15:25) as solvent mixtures. The spots, which were ninhydrin-negative, were located by their intrinsic color or by spraying with 1% ferric chloride. Desferri SK purified from low-Fe cultures gave a single spot of R_F 0.67 in the isopropanol solvent and 0.70 in the butanol-acetic acid solvent, whereas the R_F values of the corresponding Fe-SK chelate (10 or 14% Fe) were 0.30 and 0.35, respectively, in the two solvents. Acethydroxamic acid also moved farther in both solvents than its ferric complex. The R_F values of samples of Fe-SK (13% Fe) isolated from cultures at 2 μg of Fe per ml were 0.08 in the isopropanol solvent and 0.38 in the butanol-acetic acid solvent, reinforcing data obtained from infrared analyses, which indicated that the chemical nature of the predominant SK species in the culture filtrate may be dependent upon the Fe concentration in the medium.

Paper chromatograms of up to 20 μg of the several SK samples used in the preceding studies, prepared with the above solvents and two others (methanol-6 N HCl, 7:3, and *t*-butyl alcohol-methyl ethyl ketone - water - diethylamine, 10:10:5:1), showed only a single iron-binding component (SK); there was no evidence of other acidic or tetrazolium-reactive components or of ninhydrin-positive compounds. Their purities also were indicated by the appearance of a single band moving toward the anode when up to 250 μg was subjected to paper electrophoresis at *p*H 7.0 in 0.1 M acetate buffer.

The R_F values in the butanol-acetic acid solvent differed somewhat from those reported earlier

TABLE 2. *Hydroxylamine content of SK*

Sample	Per cent hydroxylamine			Percentage of theoretical recovered after hydrolysis[a]
	No hydrolysis	6 N HCl, 12 hr, 106 C	Theoretical	
SK from low-Fe medium				
Fe-SK	0	2.9	—	—
Desferri SK	0.4-0.6	2.7	—	—
Fe-SK from regular medium	0	2.3	—	—
Controls				
Aspartyl hydroxamic acid	21.5	15.8	22.3	71
Acethydroxamic acid	43.3	33.3	44.4	75
Ferrioxamine B hydrochloride	0	3.7	15.3	24
Desferrioxamine B (Desferal)[b]	0.2	3.5	15.1	23

[a] Recovery of hydroxylamine from acid hydrolysates of hydroxamic acids is variable and often poor; it may be dependent upon the presence of Fe (J. B. Neilands, *personal communication*) and other factors. Bickel et al. (3) recovered about 25% of the hydroxylamine of ferrioxamine B after hydrolysis at 100 C in 1 N HCl for 4 to 5 hr.

[b] Methane sulfonate of iron-free ferrioxamine B.

(0.17 to 0.25) for SK in crude filtrates (22). The amount of Fe bound by the SK in the filtrates may have influenced its R_F, and salts and organic compounds in the preparations, as well as the difficulty in estimating the exact R_F by the bioautographic method, also may have contributed to this difference. Preparations of cellular SK from both high and low-Fe medium contained SK with R_F values similar to the respective filtrate SK. The visible absorption spectrum of Fe-SK from "low-Fe cells" was identical with excreted SK, and both types of cellular SK gave positive tests for secondary hydroxamates. The form of SK found in culture fluid may be identical with that found in cells, but it is possible that some or all cellular SK may be bound to a cell component.

Biological activity of schizokinen for B. megaterium and Arthrobacter JG-9. The dose-response assay used during purification of SK was based upon the degree which it reduced the division lag of *B. megaterium*. One unit of SK activity was defined originally (22) as equivalent to the growth response of *B. megaterium* produced by 25 μg of meconic acid per ml. Equal responses of *B. megaterium* were obtained with Fe-SK and desferri SK, both from low-Fe and regular medium (Fig. 4); 0.2 mμg was found to be equivalent to 1 unit. Maximal stimulation (lag reduction) of an inoculum of 100 to 200 CFU was provoked by a concentration of about 1 mμg/ml, equivalent to 5 units. Walker and Lankford (Bacteriol. Proc., p. 43, 1963) determined by assay of crude culture filtrate that the "critical concentration" for lag termination of an equivalent inoculum was about 5 units, an estimate supported by assays of purified SK.

The lag of *B. megaterium* also was reduced by several siderochromes available for testing (Table 3), but not by mycobactin or by hadacidin (*N*-formyl hydroxyaminoacetic acid); the latter was inhibitory at concentrations above 4 μg/ml. Ferrioxamine B and ferrimycin A were equal to SK in stimulatory units per weight unit, but other siderochromes were less active.

Arthrobacter JG-9 might be described as a siderochrome auxotroph, the growth factor requirement of which is satisfied by certain naturally occurring hydroxamic acids or by heme (6). This organism also was responsive to *B. megaterium* SK, and although SK had only 1% of the activity of ferrioxamine B or ferrichrome (Table 3), its activity was within the range of several other siderochromes (7).

Reversal of ferrimycin A inhibition. The effectiveness of SK as an antagonist of ferrimycin A, a "sideromycin" active against many gram-positive organisms, was tested by the method of Zähner et al. (34). Sterile strips of filter paper soaked in ferrimycin A (160 μg/ml) and Fe-SK (1,000 μg/ml) were crossed on the surface of nutrient agar plates spread with *B. megaterium*, *B. subtilis*, var. *niger*, *B. subtilis*, and *Staphylococcus aureus*. Increased or confluent growth at the juncture of the strips indicated reversal of ferrimycin A inhibition of the *Bacillus* species by SK. Inhibition of *S. aureus* by ferrimycin A was not overcome by this concentration of SK, although ferrioxamine B (160 μg/ml) was effective. This inactivity of SK for *S. aureus* is illustrative of species-specific effects encountered among

TABLE 3. *Stimulatory activities of SK and several siderochromes for Bacillus megaterium and Arthrobacter JG-9*

Sample	Amt equivalent to 1 unit for *B. megaterium*[a]	Amt supporting half-maximal growth of *Arthrobacter* JG-9
	mμg	mμg
B. megaterium schizokinen	0.2	50
Ferrioxamine B	0.2	0.5
Ferrimycin A	0.5	0.5
Ferricrocin	1	
Ferrichrysin	1	
Ferrichrome	2	0.5
Coprogen	10	10
Ferrirubin	190	20

[a] One unit of schizokinen activity, previously defined (22) as an amount inducing a response of *B. megaterium* equivalent to that produced by 25,000 mμg of meconic acid per ml, is redefined here as an amount producing stimulation equivalent to 0.2 mμg of purified SK.

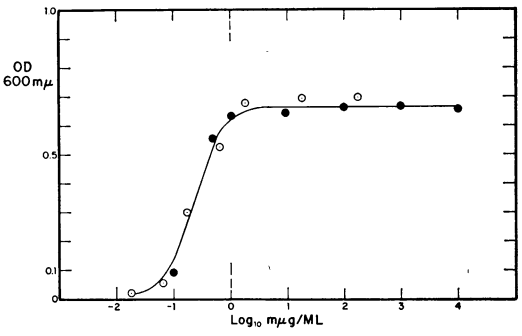

FIG. 4. *Dose response of Bacillus megaterium to Fe-SK (●) and desferri SK (⊙) from low-Fe medium. The inoculum was 100 to 200 CFU per ml with incubation at 37 C for 17 hr. Growth was determined by measuring the optical density at 600 mμ of each culture. Fe-SK from regular medium produced essentially an identical response.*

natural ferric hydroxamates. Within the zone of inhibition of *B. megaterium* around the ferrimycin A paper strip, there were numerous isolated colonies which appeared to be resistant to the antibiotic, since they were not inhibited when similarly retested with ferrimycin A. The prompt stimulation of *B. megaterium* by ferrimycin A in liquid medium (Table 3) seems unlikely to have resulted from selection of resistant mutants in so small an inoculum; possibly spontaneous hydrolysis of ferrimycin A to ferrioxamine B (27) may account for this effect.

DISCUSSION

The evidence presented relates the endogenous lag-reducing factor (SK) present in culture filtrates and in cells of *Bacillus megaterium* to the class of Fe(III)-chelating secondary hydroxamates of microbial origin which have been designated as siderochromes (34). Garibaldi and Neilands (15) judged from characteristics of absorption spectra of culture filtrates that *B. megaterium* excreted a compound similar to ferrichrome. However, the *p*H dependency of the visible absorption maximum of SK suggests a monohydroxamate, rather than a trihydroxamate siderochrome. Two other naturally occurring monohydroxamates, isolated from fungi, are hadacidin (19) and fusarinine (11).

The variation in the quantitative growth response of *Arthrobacter* JG-9 to different siderochromes (7) was confirmed in the present study and extended to include the variable lag-reducing capacity of several siderochromes for *B. megaterium*. In 1957, Lankford et al. (21) suggested that cells depended upon an exogenous or endogenous chelating agent to initiate division. They visualized the role of this stimulant as: (i) solubilization of an essential metal; (ii) transport of the metal across cytoplasmic membranes; (iii) donation of the metal to specific enzymes. These are essentially the roles which have been suggested for the siderochromes (7, 25, 33).

At least one siderochrome (ferrichrome A) has been described as being without biological activity (34). Emery (11) was unable to detect either growth factor activity for *Arthrobacter* JG-9 or inhibition of several bacterial species with fusarinine, whereas hadacidin is inhibitory for many cell types (16, 26). Possibly the best test for physiological activity of a natural hydroxamate could be made with the organism which produces it; tests for lag reduction of small inocula of the source organism by a hydroxamate, especially in low-iron media, are suggested as indices of physiological activity. Reich and Hanks (28) have noted that mycobactin shortened the time before onset of rapid growth of *Mycobacterium phlei*, a mycobactin-producing organism.

Lankford et al. (21) found that a variety of chemicals, all with a known or potential chelating capacity, could substitute for culture filtrates in lag reduction of *Bacillus* spp. It has been reported also (23) that 8-hydroxyquinoline, salicylaldehyde, and acetylacetone substitute for the siderochrome requirement of *Arthrobacter terregens*. Thus, some organisms have a requirement for an endogenous or exogenous chelating agent which can be satisfied by many different compounds, whereas other organisms, such as *Mycobacterium paratuberculosis*, have a more strict preference (31; N. E. Morrison, *personal communication*). Since SK supplies the growth factor requirement of *Arthrobacter* JG-9, it might be speculated that a trihydroxamate configuration is unnecessary, at least with this organism, and that the portion of the molecule other than the iron-binding center determines both the quantitative and qualitative response initiated by a siderochrome.

Zähner et al. (33) predicted that all aerobic microorganisms either produce siderochromes, or are siderochrome-dependent. Lankford et al. (22) postulated that all microbial cells may require an endogenous or exogenous "schizokinen" for initiation and maintenance of cell division. However, identification of the *B. megaterium* schizokinen as a secondary hydroxamate does not presently justify an assumption that all other schizokinens will belong to this category.

ACKNOWLEDGMENTS

This research was supported in part by the University Research Institute and by Public Health Service grant HD 01840 from the National Institute of Child Health and Human Development.

LITERATURE CITED

1. BICKEL, H., R. BOSSHARDT, E. GÄUMANN, P. REUSSER, E. VISCHER, W. VOSER, A. WETTSTEIN, AND H. ZÄHNER. 1960. Stoffwechselprodukte von Actinomyceten, 23 Mitteilung: Uber die Isolierung und Charakterisierung der Ferrioxamine A-F, neuer Wuchstoffe der Sideramin-Gruppe. Helv. Chim. Acta **43**:2118–2128.
2. BICKEL, H., E. GÄUMANN, G. NUSSBERGER, P. REUSSER, E. VISCHER, W. VOSER, A. WETTSTEIN, AND H. ZÄHNER. 1960. Stoffwechselprodukte von Actinomyceten, 25 Mitteilung: Uber die Isolierung und Charakterisierung der Ferrimycine A_1 und A_2, neuer Antibiotika der Sideromycin-Gruppe. Helv. Chim. Acta **43**: 2105–2118.
3. BICKEL, H., G. E. HALL, W. KELLER-SCHIERLEIN, V. PRELOG, E. VISCHER, AND A. WETTSTEIN. 1960. Stoffwechselprodukte von Actinomyceten, 27 Mitteilung: Uber die Konstitution von Ferrioxamine B. Helv. Chim. Acta **43**:2129–2138.
4. BREMNER, J. M. 1954. Identification of hydroxyl-

amine and hydrazine by paper chromatography. Analyst **79**:198–201.
5. BURNHAM, B. F. 1962. Bacterial iron metabolism: Investigations on the mechanisms of ferrichrome function. Arch. Biochem. Biophys. **97**: 329–335.
6. BURNHAM, B. F. 1963. Investigations on the action of iron-containing growth factors, siderамines; and iron-containing antibiotics, sideromycins. J. Gen. Microbiol. **32**:117–121.
7. BURNHAM, B. F., AND J. B. NEILANDS. 1961. Studies on the metabolic function of the ferrichrome compounds. J. Biol. Chem. **236**:554–559.
8. CSÁKY, T. Z. 1948. On the estimation of bound hydroxylamine in biological material. Acta Chem. Scand. **2**:450–459.
9. DONALD, C., B. I. PASSEY, AND R. J. SWABY. 1952. A comparison of methods for removing trace metals from microbiological media. J. Gen. Microbiol. **7**:211–220.
10. DUTTA, R. L. 1959. Studies on the metal complexes of hydroxamic acids. III. Colored complexes of iron, vanadium and molybdenum with iso-nicotinohydroxamic acid and their analytical uses. J. Ind. Chem. Soc. **36**:285–286.
11. EMERY, T. 1965. Isolation, characterization, and properties of fusarinine, a delta-hydroxamic acid derivative of ornithine. Biochemistry **4**: 1410–1417.
12. EMERY, T., AND J. B. NEILANDS. 1962. Further observations concerning periodic acid oxidation of hydroxylamine derivatives. J. Org. Chem. **27**:1075–1077.
13. FINK, K., AND R. M. FINK. 1949. Application of paper chromatography to quantitative analysis of volatile and non-volatile organic acids. Proc. Soc. Exptl. Biol. Med. **70**:654–661.
14. FRANCIS, J., J. MADINAVEITIA, H. M. MACTUCK, AND G. A. SNOW. 1953. Mycobactin. A growth factor for *Mycobacterium johnei*. Biochem. J. **55**:596–607.
15. GARIBALDI, J. A., AND J. B. NEILANDS. 1956. Formation of iron-binding compounds by microorganisms. Nature **177**:526–527.
16. GITTERMAN, C. O., E. L. DULANEY, E. A. KACZKA, D. HENDLIN, AND H. B. WOODRUFF. 1962. The human tumor-egg system. II. Discovery and properties of a new antitumor agent, hadacidin. Proc. Soc. Exptl. Biol. Med. **109**:852–855.
17. HESSELTINE, C. W., C. PIDACKS, A. R. WHITEHALL, N. BOHONOS, B. L. HUTCHINGS, AND J. H. WILLIAMS. 1952. Coprogen, a new growth factor for coprophilic fungi. J. Am. Chem. Soc. **74**: 1362.
18. HINSHELWOOD, C. N. 1946. The chemical kinetics of the bacterial cell. The Clarendon Press, Oxford, England.
19. KACZKA, E. A., C. O. GITTERMAN, E. L. DULANEY, AND K. FOLKERS. 1962. Hadacidin, a new growth-inhibitory substance in human tumor systems. Biochemistry **1**:340–343.
20. KELLER-SCHIERLEIN, W., AND A. DÉER. 1963. Stoffwechselprodukte von Mikroorganismen, 44 Mitteilung: Konstitution von Ferrichrysin und Ferricrocin. Helv. Chim. Acta **46**:1907–1920.
21. LANKFORD, C. E., T. Y. KUSTOFF, AND T. P. SERGEANT. 1957. Chelating agents in growth initiation of *Bacillus globigii*. J. Bacteriol. **74**: 737–748.
22. LANKFORD, C. E., J. R. WALKER, J. B. REEVES, N. H. NABBUT, B. R. BYERS, AND R. J. JONES. 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s) ("schizokinen"). J. Bacteriol. **91**: 1070–1079.
23. MORRISON, N. E., A. D. ANTOINE, AND E. E. DEWBREY. 1965. Synthetic metal chelators which replace the natural growth-factor requirements of *Arthrobacter terregens*. J. Bacteriol. **89**:1630.
24. NEILANDS, J. B. 1952. A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*). J. Am. Chem. Soc. **74**:4846–4847.
25. NEILANDS, J. B. 1957. Some aspects of microbial iron metabolism. Bacteriol. Rev. **21**:101–111.
26. PITTILLO, R. F., AND F. C. MONCRIEF. 1965. Hadacidin potentiation of lethal action of ionizing irradiation. II. Effect on proliferating gram negative bacteria. Proc. Soc. Exptl. Biol. Med. **118**:525–529.
27. PRELOG, V. 1964. Iron-containing compounds in microorganisms, p. 73–83. *In* F. Gross [ed.], Iron metabolism. Springer-Verlag, Berlin.
28. REICH, C. V., AND J. H. HANKS. 1964. Use of *Arthrobacter terregens* for bioassay of mycobactin. J. Bacteriol. **87**:1317–1320.
29. SMITH, G. F., W. H. MCCURDY, JR., AND H. DIEHL. 1952. The colorimetric determination of iron in raw and treated municipal water supplies by use of 4,7-diphenyl-1,10-phenanthroline. Analyst **77**:418–422.
30. SNOW, G. A. 1954. Mycobactin. A growth factor for *Mycobacterium johnei*. II. Degradation and identification of fragments. J. Chem. Soc., p. 2588–2596.
31. SNOW, G. A. 1965. The structure of mycobactin P, a growth factor for *Mycobacterium johnei*, and the significance of its iron-complex. Biochem. J. **94**:160–165.
32. WISE, U. M., AND W. W. BRANDT. 1955. An investigation of some hydroxamic acids. J. Am. Chem. Soc. **77**:1058–1059.
33. ZÄHNER, H., E. BACHMANN, R. HUTTER, AND J. NÜESCH. 1962. Sideramine, eisenhaltige Wachstumsfaktoren aus Mikroorganismen. Pathol. Microbiol. **25**:708–736.
34. ZÄHNER, H., R. HUTTER, AND E. BACHMANN. 1960. Zur Kenntnis der Sideromycinwirkung. Arch. Mikrobiol. **36**:325–349.