A SCHIZOKINEN (SIDEROCHROME) AUXOTROPH OF BACILLUS MEGATERIUM INDUCED WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE*

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When grown in a sucrose-salts basal medium, Bacillus megaterium Texas (ATCC 19213) secretes a compound, schizokinen (SK), which is required at a population-dependent critical concentration for initiation and maintenance of exponential cell division (Walker and Lankford, 1963; Arceneaux and Lankford, 1965; Lankford et al., 1966). SK has been isolated and identified as a secondary, monohydroxamic acid which binds ferric iron (Byers et al., 1966) and, hence, is related in certain respects to the complex trihydroxamate siderochromes isolated from fungi and actinomycetes (Emery and Neilands, 1959; Zähner et al., 1962). Mutants of B. megaterium Texas which do not secrete detectable SK have been isolated after treatment with N-methyl-N'-nitro-N-nitrosoguanidine. These mutants require SK or a suitable substituent for growth in basal medium. Although a few wild-type siderochrome-auxotrophic fungi and bacteria have been isolated (cf., Zähner et al., 1962), this is believed to be the first reported isolation of an induced siderochrome-auxotrophic mutant.

Isolation of SK^- mutants. Isolation of SK-auxotrophic mutants presented difficulties in terms of enrichment and selection in

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fluid media, since SK secreted to critical concentration by the parent presumably would cause the mutants to initiate growth and division at the same time as the parent. Isolation of SK^- mutants was achieved in the following manner. Log phase cells from basal medium (Lankford et al., 1966) were washed with the growth medium in which the phosphate buffer was replaced with Tris-maleic acid buffer (both M/20) adjusted to pH 6.5 (Adelberg et al., 1965) and were exposed to N-methyl-N'-nitro-N-nitroso-guanidine[1] (100 µg/ml) for 15 minutes at 37 C. The cells were washed free of mutagen and plated on basal agar plates prepared with washed agar. When small colonies appeared on the plates of the non-treated controls, all colonies on the plates of the treated organisms were marked. After further incubation, the appearance of new colonies was noted. In some cases, plates with only a few colonies were incubated for two days and then sprayed with a sterile solution of SK; new colonies which appeared after this treatment were examined. Of 594 such colonies picked and tested individually for growth in basal medium and in medium supplemented with SK, about 2% grew only in the latter from inocula less than 10^6. Characteristics of one mutant, SK^-_{300}, are described here.

Effect of SK on B. megaterium SK^-_{300}. For all experiments with SK^- mutants, inoculum cells were obtained from the log phase in basal medium supplemented with 10 mµg SK/ml and were washed 3 times with basal medium. Growth responses of B. megaterium SK^-_{300} (Fig. 1) to SK in basal medium indicated that 6 mµg SK/ml is required for a maximal response over a wide range of inoculum size, an effect in contrast to the population-dependent critical

1. Aldridge Chem. Co., Inc., Milwaukee, Wisconsin.

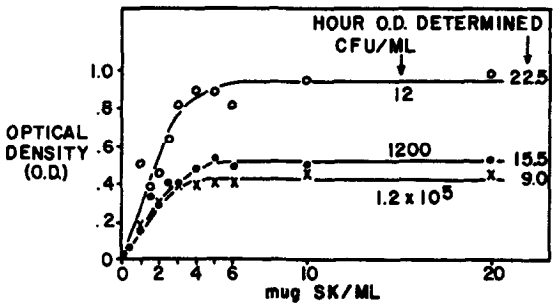

Figure 1. Growth response of B. megaterium SK^-_{300} to SK.

concentration required for growth initiation by the parent culture. Growth response to SK also was determined by viable cell counts (CFU or colony forming units) (Fig. 2). The generation time (84 minutes) of B. megaterium SK^-_{300} was shortest when the medium was supplemented with at least 6 mμg SK/ml. SK became limiting at 1 mμg/ml; the viable count and O.D. did not reach the expected maximum. In unsupplemented medium, only a single division occurred and the cells finally died.

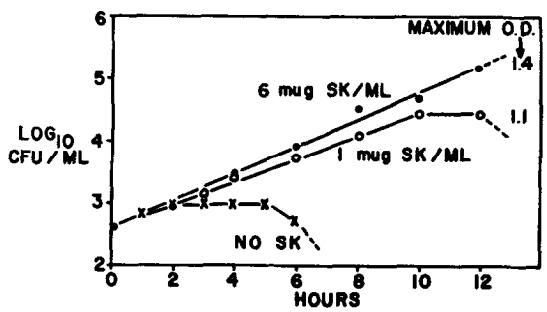

Figure 2. Viable cell counts of B. megaterium SK^-_{300} in relation to concentration of SK. Dilutions made in SK-free basal medium and plated on nutrient agar.

Substitution of SK requirement with siderochromes and other chelating agents. Several siderochromes which reduced lag of the parent strain (Byers et al., 1966) were found to satisfy the

SK requirement of B. megaterium SK^-_{300} (Table 1). However, mycobactin (Francis et al., 1953), a complex dihydroxamate (Snow, 1965) produced by Mycobacterium phlei which served as a growth factor for all wild-type siderochrome auxotrophs tested, supported growth of B. megaterium SK^-_{300}, but did not reduce lag of the parent strain (Byers et al., 1966). B. megaterium SK substituted for the siderochrome requirement of Arthrobacter JG-9 (Byers et al., 1966), A. terregens and A. flavescens (Hanks, 1966). Acethydroxamate, reported to be inactive for Arthrobacter JG-9 (Burnham and Neilands, 1961), also satisfied the SK requirement of B. megaterium SK^-_{300}.

Table 1

Test Material	Minimum Growth Time*	Approximate Effective Concentration, µg/ml Optimum	Range
SK	12	0.006->2	0.0004->2
Desferal†	12	0.005->50	0.0005->50
Ferrioxamine B	12	0.008->20	0.0002->20
Ferrimycin A	13	0.006->2	0.0001->20
Ferrichrome	13	<1->20	<0.01->20
Mycobactin	16**	5	
Acethydroxamate	13	75->1250	<19->1250

*Hours for an inoculum of 1-8 x 10^3 to reach O.D. 0.2 at the most effective concentration.
†Iron-free methane sulfonate derivative of ferrioxamine B.
** Corrected for solvent (ethanol) control.

Several chelating agents other than the siderochromes satisfied the SK requirement of B. megaterium SK^-_{300} (Table 2). However, these chelating agents were not as effective as the siderochromes in that higher concentrations were required to substitute for SK and that concentrations above the optimum of several were inhibitory. Salicylaldehyde (1100-0.002 µg/ml), 8-hydroxyquinoline (400-0.01 µg/ml) and acetylacetone (13,000-0.02 µg/ml), which Morrison et al (1965) found to replace the siderochrome requirement of A. terregens and A. flavescens JG-9, did not permit growth of SK^-_{300}. Hemin substitutes for the

siderochrome requirement of A. terregens, A. flavescens JG-9, Microbacterium lacticum 8181, and Pilobolus kleinii (cf., Zähner et al., 1962), but did not substitute for the SK requirement of B. megaterium SK^-_{300} (two-fold increments from 0.004-80 µg/ml). Supplements of peptone, casein hydrolysate or yeast extract permitted growth of B. megaterium SK^-_{300}.

Table 2

Growth Stimulant	Minimum Growth time*	Approximate Effective Concentration, µg/ml Optimum	Range
SK	12	0.006->2	0.0004->2
Disodium EDTA	16	3-10	1-50†
Kojic acid	15	60-300	17-550†
Meconic acid	12	100->800	50->800
Thiomalic acid	11	30-1000	15->3800
Disodium citrate	17.5	900-3500	280->8900
Sodium lactate	10.5	4000-8000	1500->24000
Oxalic acid	12	400-1800	58->1800
Sodium salicylate	18	110-200	40-620†
2,3-dihydroxy-benzoic acid	22	40-65	14-110†

*cf. Table 1.
†Higher concentrations are completely inhibitory.

Since a variety of chelating agents, including those listed in Table 2, was found to reduce the lag of Bacillus spp. (Lankford et al., 1957), it was proposed that the effective compounds substitute for an endogenous factor to supply the cells with metals essential for cell division and growth. The siderochromes are presumed to act as iron transport factors; they also have been assigned a specific cofactor role of iron insertion into protoporphyrin IX (Zähner et al., 1962). However, in view of the variety of chemically-diverse chelating agents which substitute for SK in B. megaterium SK^-_{300}, as well as for its parent, it seems unlikely that SK and siderochromes function as specific cofactors. Since the parent B. megaterium strain is available for direct comparison, SK^- mutants may prove more useful than the wild-type siderochrome auxotrophs for studying the role of

siderochromes and other metal chelating agents in biological systems.

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