ACTION OF METAL CHELATES ON GROWTH INITIATION OF *BACILLUS SUBTILIS*

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ABSTRACT

MAYER, GERALD D. (University of Southwestern Louisiana, Lafayette) AND R. W. TRAXLER. Action of metal chelates on growth initiation of *Bacillus subtilis*. J. Bacteriol. **83**:1281–1286. 1962.—Certain compounds which have a potential as metal chelates are stimulatory for *Bacillus subtilis* at low concentrations. If the concentration of these compounds is increased beyond the optimum for prompt growth initiation, they become inhibitory. It is demonstrated that this organism requires a critical concentration of manganese for growth initiation. If the manganese concentration is increased there is a corresponding increase in the lag time and, therefore, in the growth time of the culture. At a manganese concentration of 10 μg per ml growth is completely inhibited. The manganese inhibition can be reversed by the addition of a chelate.

Dialysis of cells with chelate presumably removes a metal ion(s) essential for the initiation of growth. Supplementing kojic acid-dialyzed cells with additional manganese reverses this inhibitory effect to some extent, indicating that the removal of manganese by the chelate dialysis is partially responsible for the increased time for growth initiation.

Our knowledge of the physical factors which influence growth initiation has been fairly complete for many years. However, the effect of the chemical environment on cells in the lag phase still is in need of extensive investigation. One particular area for investigation is the role of metal ions in growth initiation. Mineral metabolism is difficult to study since the metal requirements are so minute, and if increased often lead to an inhibition of growth. Chelating agents permit a more definitive study of the metal ions and their relation to growth initiation. Substances produced by organisms are capable of acting as chelating agents (Weinberg, 1954, 1957) and will suppress the toxicity of copper and zinc for *Bacillus subtilis*. Lankford, Kustoff, and Sergeant (1957) demonstrated that certain compounds with a potential chelating activity are stimulatory in low concentration but rapidly become inhibitory as the concentration is increased beyond the optimum. Further, it was demonstrated that chelate inhibition was reversed by the incorporation into the medium of additional critical metal ions. This study outlines the preliminary work with certain chelating compounds and metal ions and their influence on growth initiation of *B. subtilis* var. *niger*.

MATERIALS AND METHODS

B. subtilis var. *niger* was grown in the medium described by Sergeant, Lankford, and Traxler (1957). The chelating supplements were adjusted to pH 7.0, sterilized by filtration through an HA Millipore filter, and added aseptically to the culture medium. Stock cultures were grown and maintained on Trypticase soy agar and transferred at 12-hr intervals for the inoculum to minimize the number of spores present. A thrice-washed basal medium suspension of agar-grown cells was used as the inoculum for all experiments. Growth was followed by turbidity measurements with the Klett-Summerson photoelectric colorimeter, equipped with the 400 to 465 mμ (blue) filter. In some experiments, turbidity measurements were correlated with plate counts in Trypticase soy agar. Inoculum size was determined for all experiments by dilution plate counts in the same medium. In some of the experiments, the degree of growth stimulation is expressed as the reduction of "growth time," which is defined as the time in hours required for a culture to attain an arbitrary degree of turbidity.

For dialysis, 30 ml of the cell suspension were

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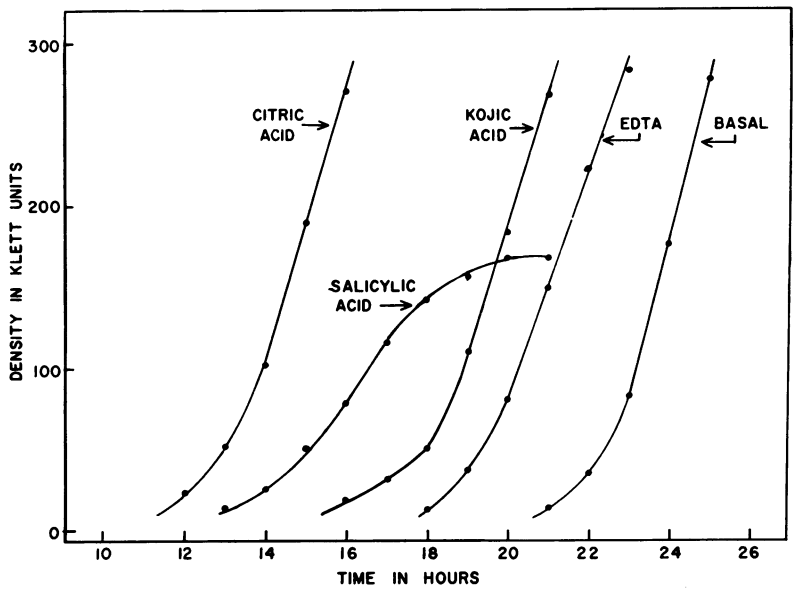

FIG. 1. *Stimulatory effects of chelates on growth in basal medium. The concentration of chelate is 100 μg per ml for citric acid, salicylic acid, and kojic acid, but 1.0 /μ per ml for EDTA. Inoculum size is 1,000 organisms per ml and incubation at 37 C with shaking.*

placed in a cellophane dialysis tube, sealed at both ends to prevent leakage, and the tube was suspended in a 250-ml flask containing the chelate for dialysis. The dialyzing solution was removed each hour and replaced with a fresh solution. Viable cell counts were run on the cell suspension after dialysis; dilutions were prepared to yield the desired inoculum size; and the cells were then inoculated into fresh media for growth-response studies. Viable cell counts were made from the final flasks at zero time, in Trypticase soy agar, to obtain an accurate inoculum size. Dialysis was carried out at room temperature (24 to 25 C) on a New Brunswick controlled environment incubator-shaker model G26 at 130 rev/min.

RESULTS

The stimulatory effects of the compounds used in this study are shown in Fig. 1 and 2. Results with citric acid, kojic acid, and ethylenediaminetetraacetate (EDTA) were typical of the results obtained with most of the chelating compounds studied. The stimulatory effect was on the initiation of growth rather than on the rate of growth once initiated or on the total cell crop. This was confirmed with lag-time studies by dilution plate counts in Trypticase soy agar. Salicylic acid,

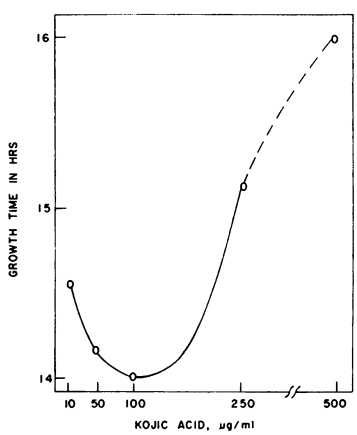

FIG. 2. *Growth time plot of the effect of concentration of kojic acid on Bacillus subtilis. Growth time calculated as the time required to reach 150 Klett units. Inoculum size was 1,000 organisms per ml; incubation at 37 C with shaking.*

however, must act in a different manner since this material exerted an effect not only on growth initiation but also on the rate of growth and total cell crop (Fig. 1). Cells grown with salicylic acid had a slower growth rate in the logarithmic phase than those grown in basal medium, and a much lower total cell crop. With the other agents

the rate of growth, once initiated, was the same as in basal medium, and the total cell crop was essentially the same. It was concluded that in the stimulatory range the effect of citric acid, kojic acid, and EDTA was exerted primarily on growth initiation. As the concentration of kojic acid was increased beyond the optimum (Fig. 2), there was a corresponding increase in growth time which indicated inhibition of growth initiation.

In an earlier paper (Sergeant et al., 1957), the mineral requirements of the organism were shown to be satisfied by manganese, magnesium, and iron. Manganese must be present in the basal medium at a critical concentration (0.01 μg per ml). When manganese was increased beyond this concentration an increase in growth time was noted (Table 1), and at a concentration as high as 10 μg per ml gave complete inhibition of growth. The inhibitory action of manganese was reversed by adding salicylic acid (Table 1). There was an apparent mutual relationship between the manganese and salicylic acid. Inhibitory concentrations of manganese were reversed by adding chelate, and inhibitory concentrations of chelate were reversed by adding additional manganese to the basal medium.

It is apparent from the above data that there is a relationship between the chelating compound and manganese (possibly other metals) which is important to the cell in growth initiation. If one accepts the hypothesis that the chelate acts by sequestering an essential metal ion then it should be possible, by dialysis of cells against the

TABLE 1. *Reduction of manganese toxicity by salicylic acid*

Manganese	Growth time*		
	Salicylic acid (μg/ml)		
	0	500	1,000
μg/ml			
1.0	18.5	14.1	13.1
0.1	18.0	12.0	14.6
0.01	15.7	13.6	14.8
0.001	17.3	14.7	14.9

* Growth time computed in hr required to reach 50 Klett units. Inoculum: 1,000 organisms per ml.

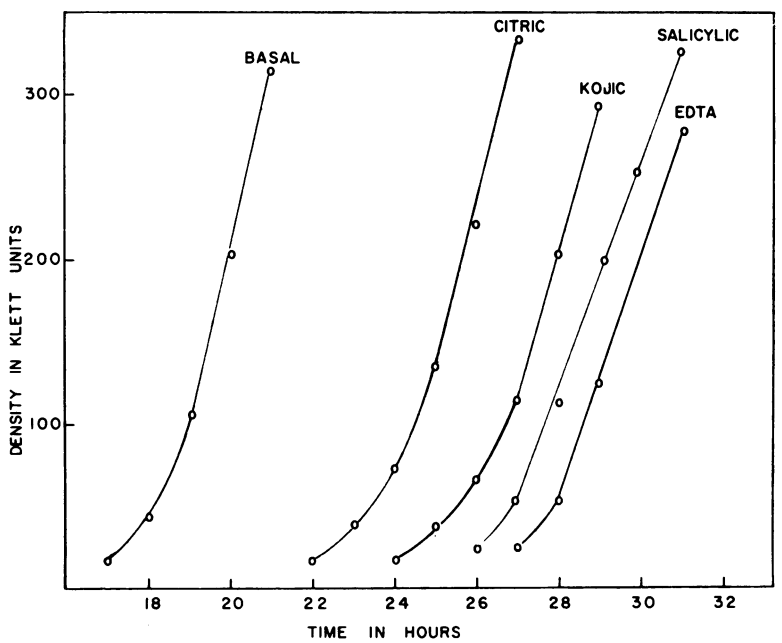

FIG. 3. *Growth response of cells after dialysis against chelate compounds. The cells were dialyzed at pH 6.0 against basal medium as a control, 100 μg per ml of citric acid, kojic acid, salicylic acid, and 1 μg per ml of EDTA for 6 hr, washed in phosphate buffer (pH 7.0), and then inoculated into fresh basal medium for growth studies. Inoculum size, 1,000 organisms per ml, and incubation at 37 C with shaking.*

chelate, to remove this required metal and prevent or at least prolong growth initiation. Cells grown in basal medium, dialyzed against chelating agents, and inoculated into fresh basal medium showed an increase in time for growth initiation (Fig. 3). Dialysis of cells against basal medium served as a control and gave identical growth initiation as nondialyzed cells. In all cases, the concentration of the chelate used (100 μg per ml) has been demonstrated to be not only nontoxic but actually stimulatory for the test organism. EDTA is active at a much lower concentration than the other chelates; therefore, 1 μg per ml was used for dialysis. We concluded that a metal essential for growth initiation was being removed from the cells or drastically diminished by the dialysis. The pH at which the cells were dialyzed was important in demonstrating the effect. The results in Table 2 show the effect of pH during dialysis on growth initiation. The optimal pH varied with the chelating agent. The best results, for example, were obtained with citric acid at pH 4, kojic acid at pH 6, EDTA at pH 4, and salicylic acid at either pH 4 or 6. It is interesting to note that the agents were inactive or showed little activity at pH 7.0 and gave es-

TABLE 2. *Effect of pH on dialysis with chelates*

Dialyzate (100 μg/ml)	Growth time (hr)*				
	pH				
	4	6	7	8	9
Basal	19.5	19.5	19.5	19.5	19.5
Salicylic acid	29.2	29.2	19.8	28.7	29.2
Citric acid	27.5	25.2	—	25.5	23.0
Kojic acid	26.7	27.2	20.8	26.5	27.4
EDTA†	31.0	29.4	20.7	29.8	29.5

* Growth time computed as time for the culture to reach 150 Klett units.

† Concentration = 1 μg per ml.

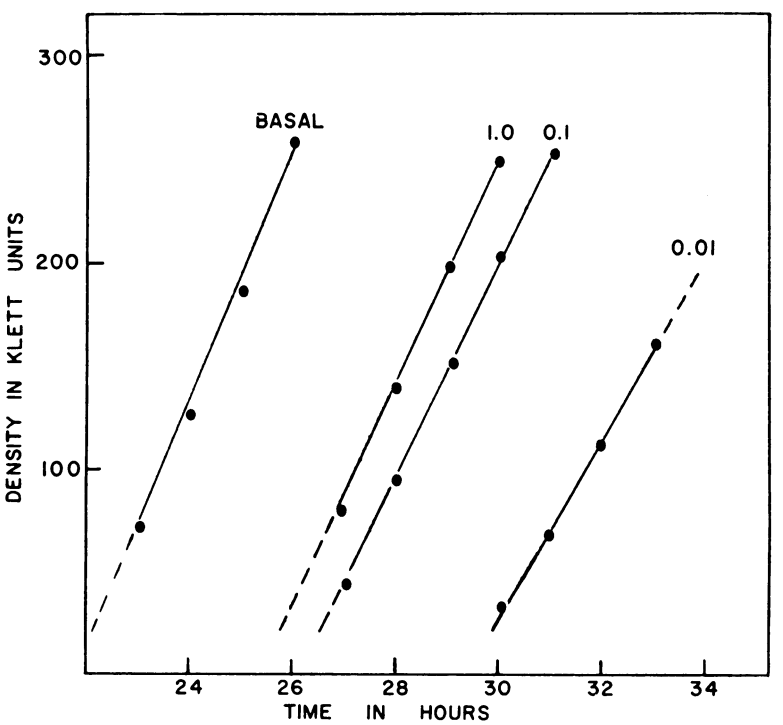

FIG. 4. *Growth response of dialyzed cells supplemented with additional manganese. Cells dialyzed against kojic acid and inoculated into basal medium with an increased concentration of manganese. The numbers above the line refer to the manganese concentration in μg per ml in the basal medium. The basal response is for cells dialyzed against basal medium, and 0.01 μg per ml of manganese represents the usual concentration of manganese in basic medium. Inoculum size, 1,000 organisms per ml, and incubation at 37 C with shaking.*

sentially the same response as the basal medium. To determine if manganese removal could account for the increased time for growth initiation, cells were dialyzed against kojic acid and then inoculated into basal medium supplemented with additional manganese (Fig. 4). This treatment did partially reverse the inhibition of growth initiation. If manganese was increased to 10 μg per ml, the time for growth initiation was increased to a considerable degree, but growth did occur; and in nondialyzed cells 10 μg per ml of manganese was inhibitory (Table 1). This failure to completely reverse the dialysis-imposed inhibition with manganese indicated that possibly other metal ions besides manganese were removed by the dialysis. Work is still in progress to determine if other metals will completely reverse this effect of dialysis.

DISCUSSION

The stimulatory action of chelating compounds on the growth initiation of *Bacillus* has previously been described (Lankford et al., 1957), as has the action of chelating compounds and metal ions on other microbial systems (Weinberg, 1954, 1957). Van Eys and Pearson (1954) showed that *Streptococcus faecalis* requires both manganese and citrate for maximal growth in manganese-deficient medium. The reversal of the inhibitory action of natural materials which function as chelating compounds by metal ions has been demonstrated (Newton, 1953; Weinberg, 1954; Saz and Slie, 1954). There is little doubt that chelation has an important role in the physiology of growth initiation. The actual mechanisms and functions of the chelate are still unknown.

With the potential chelating agents studied, all except salicylic acid exert their stimulatory action on growth initiation and not on the rate of growth or on the total cell crop (Fig. 1). The one exception is the action of salicylic acid, which apparently affects all three stages of growth. This can most likely be linked with the metal ion(s) associated with the various chelates. With this same culture system, it has been demonstrated (Traxler, 1958) that manganese and iron exert their primary effect on the initiation of growth of *Bacillus*. Magnesium, which is also required for growth, has not only an effect on growth initiation but also on the rate of growth and total cell crop. It would appear, therefore, that salicylic acid in this system has an effect on magnesium metabolism and also possibly on manganese and iron. This assumption would then indicate the involvement of kojic acid, citric acid, and EDTA primarily with manganese, and possibly iron metabolism. Dialysis experiments further substantiate the relation between kojic acid, citric acid, EDTA, and manganese. Dialysis removes some metal ion(s) essential to growth initiation from the cell environment. The inhibition imposed by this treatment is partially reversed by addition of manganese to the growth medium. The data on pH of dialysis indicate that the pH at which dialysis is carried out will influence the results, since chelate stability constants vary widely for different metal ions, as well as at different pH values.

The failure of manganese supplements to completely reverse the inhibition imposed by dialysis with kojic acid indicates that manganese is not the only metal ion removed by chelation. As pointed out earlier (Martell and Calvin, 1952; Lankford et al., 1957), the stability constants of EDTA and kojic acid with manganese and magnesium are higher than those of salicylic acid with these same metal ions.

Several theories have been postulated for the action of the chelate stimulation (Lankford et al., 1957). The data at present are still not definite enough to decide which of these effects is operative, but it is believed that these data do support to some extent the theory that the chelating compounds function to provide the cell with essential metal ions in a nontoxic form.

Further studies on this problem should provide evidence for the role of these chelates in cell division and growth initiation.

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