GENETIC STUDIES ON THE REGULATION OF AMYLASE PRODUCTION IN BACILLUS SUBTILIS

YOSHINAMI UEDA AND SHOHACHIRO YUKI

Department of Biology, Faculty of Science and Department of Biology, College of General Education, Kobe University, Kobe 657

Received May 4, 1971

A large amount of amylase, an extracellular enzyme of Bacillus subtilis, is produced at the stationary phase of bacterial growth. It has been reported that B. subtilis produces no substantial amounts of amylase during the exponential phase of growth (Nomura et al. 1956), but produces the enzyme after the bacterial growth has entered the stationary phase, as a result of de novo synthesis (Coleman 1967). This phenomenon seems to involve a problem of regulation of gene expression at the different phase of growth cycle, but it is not known how such a unique pattern of development of amylase activity during bacterial growth is controlled.

This pattern of amylase production resembles the pattern of production of the other extracellular enzymes of B. subtilis, such as protease and ribonuclease (Schaeffer 1969), and the pattern of synthesis of several intracellular enzymes such as arginase, malic dehydrogenase, ornithine transcarbamylase and purine nucleoside phosphorylase, which are absent or present at very low levels in vegetative cells and start to increase during sporulation process of B. subtilis (Deutscher et al. 1968). The nature of the mechanism controlling synthesis of such enzymes is also still obscure.

Assuming that some controlling mechanisms may be involved in specifying the unique pattern of development of amylase activity, we have started experiments to inquire the mechanism, using a transformable B. subtilis 168 to analyse the problem genetically. In this paper, we will report the finding that, when B. subtilis 168 was grown at 52°C, amylase production did not take place after the bacterial growth had ended. This may imply that the mechanism controlling the pattern of amylase production is affected at 52°C and amylase production comes to develop at the same manner as the pattern of synthesis of the cellular proteins essential for the cell growth. We will also describe a type of mutants in which sporulation as well as the mechanism controlling the pattern of development of amylase activity seems to be affected by a single mutation.

MATERIALS AND METHODS

Bacteria. B. subtilis 1-168 (trp2) was used throughout this investigation. Derivatives of 1-168 strain, 1-18 (his2) and 1-114 (his2 aro114) were used as recipients in the transformation experiments. Strain 1088 was used as a donor. 1-85 (trp2 amy12) was an
amylase negative derivative of 1-168. These strains have been described (Yuki 1967; Yuki et al. 1968). HL-3, a mutant of 1-114, produced amylase at a higher rate than 1-168.

**Media and culture conditions.** Nutrient broth (NB) contained 10 g of meat extract, 10 g of polypeptone and 2 g of NaCl per liter. NBS medium was NB containing 2% starch. Nutrient sporulation medium (NSMP) contained 5 g of meat extract, 5 g of polypeptone, 1 g of NaCl, 5 ml of metal mixture containing CaCl$_2$ (0.14 M), MnCl$_2$ (0.01 M) and MgCl$_2$ (0.2 M), 0.5 ml of FeCl$_3$ (2×10^{-3}M in 0.01 N HCl) and 50 ml of potassium phosphate buffer (2 M, pH 6.5) per liter. These media were adjusted to pH 7.0 by 10 N NaOH before autoclaving. A solid medium was prepared by adding 15 g of agar per liter of liquid medium. Nutrient sporulation agar medium contained NSMP without phosphate buffer, plus 2% agar.

Bacteria were grown overnight at 37°C in NB medium and inoculated into NB or NSMP medium. Since cells of 1-168 lost rapidly viability when their growth temperature was suddenly shifted to 52°C from 37°C, we used the following procedure to culture this strain at 52°C. Bacteria were grown overnight at 37°C in NB and diluted in the same medium prewarmed at 45°C. The culture was then shifted to 52°C gradually, taking 45 minutes.

To determine the number of spores, the culture was heated for 15 minutes at 75°C, diluted and plated on NB agar.

**Transformation.** Method of extraction of transforming DNA and procedure of transformation were described previously (Yuki 1967).

**Assay of amylase activity.** Assay method of amylase activity was described previously (Yuki 1967). In some cases, the enzyme assay was carried out at 65°C, instead of 40°C, to obtain higher activity. At 65°C, amylase of 1-168 showed about a 2-fold higher activity than at 40°C.

### RESULTS

**The pattern of development of amylase activity when 1-168 was grown at 37°C and 52°C**

As can be seen in Fig. 1 and Fig. 2, when 1-168 was grown in NB medium at 37°C, amylase production began after the exponential cell growth was over, and continued thereafter for more than 6 hours. No substantial production of amylase was observed during the exponential phase of growth. This shows that 1-168 produces amylase at the same pattern as that so far reported with different strains of *B. subtilis* (Normura et al. 1956; Coleman 1967).

Meanwhile, when grown at 52°C, amylase activity in the culture medium was found to increase during the exponential phase but, after the cessation of growth, no increase but slight decrease of amylase activity was observed (Fig. 2). The growth rate at 52°C was slightly lower and growth stopped at the lower level of cell density. The decrease of viable cell counts observed during the stationary phase may be attributable to the lysis of cells, since the decrease in turbidity paralleled. Since amylase produced during the exponential phase was found to be stable at 52°C, we may conclude that 1-168 can
Fig. 1. Production of amylase by 1-168 into the culture medium. 1-168 was cultivated with shaking in NB at 37°C and amylase activity in the culture medium was assayed at intervals. -O---O-, optical density; -O-----O-, amylase activity.

Fig. 2. Production of amylase by 1-168 into the culture medium at 37°C and 52°C. 1-168 was cultivated with shaking in NB at 37°C and 52°C, and amylase activity in the culture medium was assayed at intervals. -O---O-, Cells/ml at 37°C; -•----•-, cells/ml at 52°C; -O-----O-, amylase activity at 37°C; -•-----•-, amylase activity at 52°C.
not produce amylase at the stationary phase at 52°C. In addition, it was found that 1-168 could not form spores on nutrient sporulation agar at 52°C. Production of protease was detected at 52°C on NB agar containing 2% casein, but we have not yet examined the pattern of development of this enzyme activity.

The effect of temperature shift from 52°C to 37°C on the amylase production is shown in Fig. 3. When the temperature was shifted down from 52°C to 37°C at the exponential phase, bacteria began to grow faster than at 52°C, and amylase production stopped immediately (Fig. 3a). But, after the cell growth was over, amylase production was observed to be restored. On the other hand, when the temperature shift was performed after the cell growth had ended, the cell growth started again after about 40 minutes lag, and continued for about 80 minutes. Amylase production was restored during this period and continued thereafter (Fig. 3b).

Anamylase negative mutant did not produce amylase at both temperatures. In addition, heat stabilities and electrophoretic mobilities of amylase from 1-168 cultured at 37°C and 52°C did not show any difference. This indicates that amylase produced at the exponential phase at 52°C is controlled by the same gene as amylase produced at the stationary phase at 37°C. This may be also supported by the observation that, HL-3, a mutant which produced amylase at a higher rate (3 to 4-fold) than 1-168 during the stationary phase at 37°C, and whose mutation site was closely linked to amy locus, also produced the enzyme at about 2-fold higher rate than 1-168 during the exponential
We may conclude that the pattern of development of amylase activity of *B. subtilis* 1-168, during growth in a complex medium at 37°C, is the same as those of other strains reported so far and it can be altered at 52°C, where 1-168 produces amylase during the exponential phase, but not at the stationary phase. From these results, it may be assumed that there is working a temperature sensitive regulatory machinery for amylase production of 1-168, and amylase production seems to have a close correlation with cell growth. If this assumption were the case, it might be possible to obtain a mutant with alteration in pattern of development of amylase activity at 37°C.

Isolation of mutants with alterations in pattern of development of amylase activity when cultured at 37°C

The isolation of mutants with alterations in pattern of development of amylase activity when cultured at 37°C, was facilitated by the observation of some morphological change of colony grown at 52°C, which seemed to have connection with the ability to produce amylase during cell growth. When 1-168 was grown at 52°C on NB agar containing 2% starch (NBS agar), it formed a white colony which was distinguished from a translucent colony on NB agar containing no starch at the same temperature. On the contrary, at 37°C, the colonies of 1-168 on the above two media did not show any marked difference. It seems that the morphological difference of colonies of 1-168 on NBS and NB agar has connection with the ability to produce amylase, since an amylase negative mutant of 1-168 was found to form translucent colony on the above two agar plates at 52°C. We utilized these observations in the attempt to isolate mutants whose pattern of amylase production was altered. Thus, we isolated mutants whose colonies on NBS agar differed from their colonies on NB agar. N-methyl-N'-nitro-N-nitrosoguanidine (200 μg/ml) was used as a mutagen. Among the mutants, we found a group of mutants whose colonies on NBS agar were white during initial 12 hour incubation at 37°C, but then became reddish-colored, and we examined them further, since they formed translucent colonies on NB agar.

The reddish colony forming mutants did not require any nutrient other than tryptophan for the growth. In every case tested, reddish colony formers showed altered pattern of development of amylase activity. The time course of growth and amylase production of one of them named J-0 is shown in Fig. 4. As can be seen in the figure, J-0 produced amylase during the exponential phase, and when the cell culture entered the stationary phase, amylase production ceased. As shown in Fig. 4, J-0 could grow to only lower level of cell density, and the lysis of cells was found to take place at the stationary phase. It was observed that J-0 grew to much higher level of cell density, when grown in NBS medium, comparing to that attained when grown in NB medium. Higher level of cell density was also attained at the stationary phase when 1-168 was grown in NBS medium at 52°C than when grown in NB medium at 52°C. It was shown that, J-0, when an amylase negative mutation was introduced to it by transformation, formed translucent colonies on NBS agar, indicating that the morphological difference of colonies of J-0 on NBS and NB agar had connection with amylase production. Thus, from all these observations, it may be said that the properties of J-0 investigated at 37°C seem to mimic those observed with 1-168 at 52°C,
Fig. 4. Production of amylase by J-0 into the culture medium. J-0 was cultivated with shaking in NB at 37°C and amylase activity in the culture medium was assayed at intervals. Amylase activity was assayed at 65°C. -○---○-, Optical density; -●---●-, cells/ml; -○----○-, amylase activity.

Fig. 5. Production of amylase by J-27, J-11 and J-51 mutants into the culture medium. J-27, J-11 and J-51 were cultivated with shaking in NB at 37°C and amylase activity was assayed at intervals. Amylase activity was assayed at 65°C. (a) J-27. (b) J-11. (c) J-51. -○---○-, Optical density; -○----○-, amylase activity.
except that colonies of J-0 color reddish on NBS agar. However, it was observed that, when J-0 was grown at 52°C on NBS agar, colonies did not color reddish and remained white. Similarly, when J-0 was grown at 20°C, colonies remained uncolored on NBS agar. Another property of J-0 to be noted is that the cells in the reddish colony on NBS agar lost completely the colony forming ability, when kept at the room temperature for a long time, even though the reddish colony did not assume any lytic appearance.

Through above investigation on J-0, it may be assumed that J-0 might be a sporulation mutant. This was confirmed by the following experiments. When J-4 and J-8, which showed almost the same properties as J-0, were cultured for 24 hours in NSMP, the frequencies of heat-resistant spores per colony forming cells of J-4 and J-8 were $4.7 \times 10^{-5}$ and $0.9 \times 10^{-4}$, respectively. The time course of formation of heat-resistant spores of J-8 showed that the significant increase of the spore frequency (to $1.8 \times 10^{-4}$), by a factor of more than 100, took place during 2 hours after the end of exponential growth, and the decrease occurred thereafter. Since it was observed that J-0 could not utilize glutamate as a carbon source, but could utilize lactate, J-0 might be characterized as a type of sporulation mutant which had a defect in the citric acid cycle (Freese et al. 1967; Fortnagel et al. 1968).

Among other reddish-colony forming mutants, which produced amylase at the exponential phase, in J-27, amylase production continued for 2 hours after the cessation of growth, and then completely stopped (Fig. 5a), and in J-11, amylase production at a significantly lower rate was observed during the stationary phase (Fig. 5b). J-51 produced amylase during the stationary phase at a considerably high rate, though it was lower than that of amylase production at the exponential phase (Fig. 5c). On these mutants, further analyses have not been carried out.

The transformation of the gene controlling the phenotypic traits which J-0 exhibited was exposed to DNA extracted from J-0, and his$_2^+$ transformants were selected on minimal agar. It was found that approximately 3% of the his$_2^+$ transformants formed reddish colonies on NBS agar. The frequency is comparable to the one which is expected when his$_2$ is unlinked to the gene controlling the reddish colony formation. One of the reddish colony forming transformants was examined in regard to its time course of growth and amylase production, and similar results were obtained, in comparison with those of original mutant J-0. From these results, it may be concluded that a single gene might control those phenotypic traits which J-0 exhibited. When 1-114 was exposed to J-0 DNA, no reddish colony former was found among 50 aro$_{116}^+$ transformants. Since amy, the structure gene for amylase, is linked to aro$_{116}$ (Yuki et al. 1968), this result indicates that the gene controlling the reddish colony formation is not linked to the structure gene for amylase. Meanwhile, when J-0 was exposed to DNA of 1088, approximately 5% of trp$_2^+$ transformants formed a normal 1-168 type colony on NBS agar at 37°C. From this result, it may be again concluded that a single gene might control the phenotypic traits of J-0.

Although the exact relation between the properties of J-0 at 37°C and those of 1-168 at 52°C is unknown at present, there seems to be a correlation between the alteration in the pattern of development of amylase activity and the change in the bacterial growth and sporulation. Genetic experiments suggested that all of these
changes of J-0 were caused by a single mutation.

*Amylase production during the sporulation period by 1-168*

Since the correlation between the mechanism controlling the pattern of development of amylase activity and the ability of sporulation was suggested, we interested in the pattern of development of amylase activity during the sporulation period. The time course of growth, amylase production and sporulation of 1-168 in NSMP is shown in Fig. 6. In this experiment, bacteria grew exponentially for about 2 hours and the turbidity was gradually increased during next 2 hours. The cell number did not increase, but decreased during this period after the exponential cell growth. Presumably, this decrease is due to the aggregation of cells. The heat-resistant spores began to appear 6 to 7 hours after the end of exponential cell growth. Amylase production began after the end of exponential cell growth, proceeded during subsequent 4 hours and stopped within next 3 hours, at which time, heat-resistant spores became apparent. Since it has been reported that refractile forespores have been formed in the majority of cell population when heat-resistant spores become apparent (Spudich et al. 1968), it may be said that, once a refractile forespore is formed in a cell, amylase production stops completely.

From these observations, it seems that amylase production of 1-168 may take place in a sporangium, along with the developmental process of spore formation, in a sporulation medium.
DISCUSSION

When 1-168 was grown in a complex medium at 37°C, it produced amylase during the stationary phase, but not at the exponential phase, when the cellular proteins essential for the growth were actively synthesized. We found that, at 52°C, 1-168 produced amylase during the exponential phase, but not at the stationary phase, and could isolate a type of mutants from 1-168 whose pattern of development of amylase activity at 37°C was quite similar to that observed when 1-168 was grown at 52°C. We may say that the expression of amylase gene is governed by a specific regulatory machinery which is temperature sensitive, and the regulatory machinery is modified in the mutants. As the results of the modification, the expression of amylase gene may have come to be regulated at the same manner as the expression of the genes, specifying the cellular proteins essential for the cell growth (Deutscher et al. 1968).

Our mutants exhibited alterations of different phenotypic traits, such as loss of spore forming ability, altered pattern of cell growth and so on, which were also found when 1-168 was grown at 52°C. Genetic studies gave evidences that these phenotypic traits might be controlled by a single gene. One of our mutants, J-0, exhibits many properties which is common with sporulation mutant which has defect in the citric acid cycle (Freese et al. 1967; Fortnagel et al. 1968). Although we do not know which enzyme in the cycle is defective in J-0, it may be said that J-0 has a defect in the citric acid cycle, and consequently that a functional citric acid cycle seems to have an important role on the regulation of expression of amylase gene, especially in specifying the pattern of development of amylase activity. It has been assumed that amylase production is catabolically repressed, but derepressed when either carbon or nitrogen source, or both in the medium, are exhausted, and bacteria stop growing (Schaeffer 1969). Therefore, it seems that amylase production may escape catabolite repression in the exponentially growing cells of J-0, perhaps as the result of having a defect in the citric acid cycle. Presumably, the citric acid cycle seems to be defective in the other reddish colony forming mutants and 1-168 grown at 52°C, and amylase production also seems to escape catabolite repression in their exponentially growing cells.

On the basis of consideration on the properties of J-0, one explanation for the inability of J-0 to produce amylase after the cessation of growth might be that functional citric acid cycle may be essential for the production of amylase. It has been considered that poor sporulation of citric acid cycle mutants might be owing to a lack of sufficient ATP supply during sporulation (Klofat et al. 1969). Then, poor supply of ATP after the end of growth may be the cause of inability of J-0 to produce amylase after the cessation of growth. This is consistent with the finding that citric acid cycle mutants fail to maintain the capacity of RNA synthesis throughout the developmental period of sporulation (Freese et al. 1967). The alternative explanation is that sporulation process may be essential for the expression of amylase gene; J-0 may fail to produce amylase after the cessation of growth because poor supply of ATP seems to prevent normal development of sporulation process. If this were the case, since J-0 produced amylase during growth, a certain portion of cell population might initiate their developmental process of sporulation (amylase production of 1-168 took place
immediately after the sporulation process had been initiated), but the majority of initiating cells might fail to complete their development because J-0 could not sporulate fully. It was observed that the significant increase of heat-resistant spores, by a factor of more than 100, took place during 2 hours after the end of exponential growth, but the maximum frequency of heat-resistant spores per colony forming cells was $1.8 \times 10^{-4}$. Such an increase at this period was not observed in 1-168. Since sporulation of *B. subtilis* 1-168 has been considered to be under the control of catabolite repression (Schaeffer *et al.* 1965), these considerations for amylase production during growth phase may not be incompatible with the explanation that amylase production seems to escape catabolite repression in an exponentially growing cell. Similarly, we suppose that the stationary phase of the other reddish colony forming mutants and 1-168 grown at 52°C, must be also distinguished from that of 1-168 grown at 37°C by their physiological cell conditions.

Amylase production of 1-168 seems to take place in a sporangium, along with the developmental process of spore formation, in a sporulation medium. Therefore, the fact that our mutants with alterations in the pattern of amylase production fail to sporulate, seems to be reasonable. Although it is not known whether either initiation or developmental process of sporulation, or both, may be essential, we assume that, whenever cells initiate sporulation, amylase can be produced. Thus, we consider that morphological and metabolic changes, which accompany the initiation and developmental process of sporulation, should be taken in consideration in the studies of regulation of amylase production.

Recently, we isolated a sporulation mutant which could produce amylase only during the exponential growth, but at a very low level. In this case, genetic experiments suggested that this alteration in the pattern of amylase production seemed to be controlled by the mutation closely linked to *amy* locus (Yuki 1968), rather than the sporulation mutation (unpublished data). In *Escherichia coli*, recent biochemical studies indicate that catabolite repression-sensitive genes are activated by an activation system, and this activation acts at a promoter site of each genes (Zubay *et al.* 1970). On the other hand, the change in the template specificity of RNA polymerase during sporulation has been suggested (Losik *et al.* 1969; Sonenshein *et al.* 1970; Losick *et al.* 1970). On the basis of our considerations, it seems that either such an activation system or change in RNA polymerase, or both, may be involved in specifying the pattern of development of amylase activity. Experiments are now under way in this direction, using our mutants.

**SUMMARY**

The pattern of development of amylase activity during growth of a transformable *B. subtilis* 168 grown at 52°C was different from that observed at 37°C. Amylase was produced during the stationary phase, but not at the exponential growth phase at 37°C, while, at 52°C, it was produced during the exponential growth phase, but not at the stationary phase. A type of mutants, whose pattern of development of amylase activity
at 37°C was quite similar to that observed when B. subtilis 168 was grown at 52°C, were isolated from B. subtilis 168. They exhibited alterations of different phenotypic traits, such as loss of spore forming ability and altered pattern of cell growth, which were also found when B. subtilis 168 was grown at 52°C. Genetic studies showed that these phenotypic traits might be controlled by a single gene. The fact that one of these mutants was found to have a defect in the citric acid cycle, suggested an important role of functional citric acid cycle on the regulation of amylase production, especially in specifying the pattern of development of amylase activity during growth of B. subtilis 168.

ACKNOWLEDGMENT

The authors wish to express their sincere gratitude to Prof. S. Fujii for his encouragement and criticism.

LITERATURE CITED


Nomura, M., B. Maruo, and S. Akabori, 1956 Studies on amylase formation by Bacillus subtilis. I. Effect of high concentrations of polyethylene glycol on amylase formation by Bacillus subtilis. J. Biochem. 43: 143-152.


