METABOLISM OF α - METHYL - D - MANNOSIDE AND TREHALOSE BY BACILLUS POPILLIAE

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ABSTRACT

METABOLISM OF α-METHYL-D-MANNOSIDE
AND TREHALOSE BY *BACILLUS POPILLIAE*

By

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Previous data have shown that oligosporogenous strains of *Bacillus popilliae* would sporulate in the presence of high levels of α-methyl-D-mannoside (α-MM) while much lower levels of glucose or trehalose strongly repressed sporulation. The objectives of the present study were to characterize the initial steps in the metabolism of α-MM and of trehalose by this organism. α-MM was used by all acetate-oxidizing strains of *Bacillus popilliae* tested at a growth limiting rate after a prolonged induction period. In contrast, trehalose utilization was constitutive in this organism, and the rates of growth and oxidation of trehalose were higher than with glucose as substrate. The utilization of α-MM was very dependent on concentration; i.e., the length of the induction period was decreased and the rate of growth increased as the α-MM concentration increased. Studies with resting cells and cell extracts demonstrated that an α-glucosidase was induced in *B. popilliae* by either α-MM or α-methyl-D-glucoside (α-MG), and that the latter compound was the more efficient inducer. The data indicate

that both α-MM and α-MG may be hydrolyzed by the same enzyme, but that the affinity of the enzyme for α-MG is much higher than for α-MM. The slow growth response to the mannoside is apparently due to the slow rate of hydrolysis.

Studies of trehalose metabolism indicated that this disaccharide is transported into *B. popilliae* cells via a phosphoenolpyruvate:sugar phosphotransferase system (PTS). The evidences for this are as follows. (a) Both the oxidation and uptake of trehalose were completely inhibited by sodium fluoride. In contrast, glucose oxidation was only inhibited by about 50 percent. (b) No enzyme which would cleave trehalose *per se* was detectable in cell extracts, but a phosphotrehalase was present which hydrolyzed T6P into equal moles of glucose and glucose-6-phosphate. This enzyme was partially purified and characterized. (c) A mutant culture lacking phosphotrehalase accumulated T6P. (d) Sugar phosphate formation from trehalose by cell extracts was dependent on phosphoenolpyruvate. No other potential phosphoryl donor tested had any significant effect. Therefore it appears that *B. popilliae* cells form T6P during transport by the PTS system, and the T6P is then hydrolyzed by a phosphotrehalase.

Trehalose was also found to repress the synthesis of β-glucosidase, an inducible enzyme in *B. popilliae*. Glucose had little or no effect on this induction. Repression of β-glucosidase by trehalose could not be overcome by adding exogenous cyclic adenosine-3',5'-monophosphate (cAMP).

It appears that *B. popilliae* has evolved a unique system for metabolism of trehalose which is the primary source of carbon and energy for the growth of this pathogen in its natural host, the larvae of the Japanese beetle (*Popilliae japonica*).

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INTRODUCTION

Bacillus popilliae is a causative agent of milky disease of Japanese beetle larvae (*Popilliae japonica*). The use of this organism in biological control has not been possible because of the failure to obtain high populations of spores *in vitro*. The types and quantities of carbohydrates present in the media have been found to affect the ability of many bacilli to sporulate (3,79). In the hemolymph of the Japanese beetle, *B. popilliae* has been found to sporulate in the presence of large amounts of trehalose (69). However, trehalose at levels above 0.1% inhibits *in vitro* sporulation of an oligosporogenous strain (81). Some oligosporogenous strains of *B. popilliae* were recently found to sporulate in the presence of high levels of α-methyl-D-mannoside (α-MM) (16). These findings suggested that there must be major differences in the utilization of trehalose and α-MM by *B. popilliae*, and instigated the present investigation. Studies of the metabolism of these two compounds constitute the major parts of this thesis.

It has been previously proposed by Schaeffer *et al.* (79) that certain essential spore specific enzymes may be regulated by a mechanism similar to the regulation of β-galactosidase synthesis by catabolite repression in *Escherichia coli*. Cyclic adenosine-3',5'-monophosphate (cAMP) has been shown to relieve catabolite repression of the lactose enzymes in *E. coli* (67), and to be of great importance in the

differentiation of *Dictyostelium* (72). Therefore, a few experiments were conducted to determine if it was possible to demonstrate a regulatory role of cAMP in the sporulation or in the repression of an inducible enzyme in *B. popilliae*.

This thesis is organized into four sections. The first is a literature review including discussions of the biology of *Bacillus popilliae*, known pathways of trehalose metabolism, phosphoenolpyruvate: sugar phosphotransferase systems involved in the active transport of lactose in *Staphylococcus aureus*, and the control of sporulation by catabolite repression. The second section consists of a published manuscript on the utilization of α-methyl-D-mannoside by *B. popilliae*, and the third section is comprised of a manuscript prepared for publication on the metabolism of trehalose by *B. popilliae*. The last part of the thesis is an appendix detailing the studies of the effects of cAMP.

LITERATURE REVIEW

Biology of *Bacillus popilliae*

Bacillus popilliae was first reported by Dutky (18) in 1940 as a causative agent for milky disease of the Japanese beetle (*Popilliae japonica*). Reviews of the biology of this disease and of the potential of using *B. popilliae* for biological control have been published (19,89). Large scale production of spores of *B. popilliae* is the key to extensive use of this organism in biological control. However, attempts to obtain large spore yields of the organism *in vitro* have been unsuccessful. Some specific strains sporulate at low frequency under specific conditions (48,71,81). Also, growth in a liquid medium containing activated carbon results in limited formation of spores (28). Furthermore, in one medium (15), as much as 50% to 100% of the population formed refractile bodies which appeared to be abortive spores (52).

Growth requirements of *B. popilliae*. Various media and conditions to grow *B. popilliae* have been established (15,80,88). Only limited growth results in synthetic media (91). Complex nitrogen sources are required for optimal growth, and there is but little growth in a carbohydrate free medium (4,15,19,70). The addition of glucose, fructose, or trehalose to a complex medium supports good growth (15,19,70). α-Methyl-D-glucoside, α-methyl-D-mannoside, and salicin have also been found to support growth of *B. popilliae*

(4; and Costilow, unpublished results). Pyruvate may be utilized to some extent, since addition of this compound induces growth equal to about half that obtained with an equivalent amount of glucose. Arabinose, ribose, xylose, rhamnose, galactose, lactose, sucrose, cellobiose, melibiose, raffinose, melezitose, starch, inulin, and glycerol are not utilized or attacked. The very limited growth in a trypticase, yeast extract medium in the absence of a fermentable carbohydrate indicates that peptones do not serve as a satisfactory source of energy or as a carbon source for cell proliferation even when present at high concentrations (19).

Glucose catabolism in *B. popilliae*. *B. popilliae* catabolizes glucose to produce lactic acid, acetic acid, and CO_2 as major products; and a small but measurable amount of ethanol, glycerol, and traces of acetoin, and acetaldehyde (65). The ratio of acetic acid and lactic acid can be varied over a wide range by changing the oxygen level in the atmosphere. As the percentage of oxygen increases, acetic acid production increases and lactic acid production decreases. Pepper and Costilow (65) demonstrated that the organism catabolizes glucose through Embden-Meyerhof-Parnas and hexosemonophosphate pathways. There was no evidence of Entner-Doudoroff or phosphoketolase pathways. No glucose oxidase or glucose dehydrogenase activities were detected in the cell extracts. More recently, Bulla *et al.* (5) investigated the catabolism of glucose by proliferating vegetative cells of *B. popilliae* and obtained similar results.

The presence of tricaboxylic acid (TCA) cycle in *B. popilliae* was examined by McKay *et al.* (48). They postulate that *B. popilliae* has a complete TCA cycle but the activity of the cycle is strongly repressed in wild type strains under the usual conditions used for *in vitro* cultivation. Somewhat different conclusions on the presence of a complete TCA cycle were reported by Bulla *et al.* (6). The different strains of *B. popilliae* used and the differences in methods of cultivation of the organisms may account for the conflicting results. However, *B. popilliae* cells harvested from larval hemolymph were found to oxidize acetate (personal communication, NRRL., Peoria, Illinois). A functional glyoxylate cycle was not found in *B. popilliae* (6,65).

B. popilliae was originally reported to be a facultative anaerobe and to grow best in highly poised reducing media (18). However, Pepper and Costilow (65) failed to obtain significant growth in the complete absence of oxygen, and found that aeration of broth cultures was very stimulatory to growth (15). Glucose was not dissimilated under anaerobic conditions, although pyruvate was dissimilated anaerobically to approximately equimolar amounts of acetic and lactic acid and CO_2. No hydrogen was evolved (65).

There is a cytochrome dependent electron transport in *B. popilliae* utilizing cytochrome b but not cytochrome c (66). The cytochrome dependent system is particulate, whereas the independent electron transport system is soluble and is stimulated by flavin nucleotides. There is a soluble NADH oxidase which produces H_2O_2;

and the soluble system is relatively more active than the particulate oxidase in older cells (66). *B. popilliae* does not have catalase or peroxidase (66), however, it does contain high levels of superoxide dismutase (14,95).

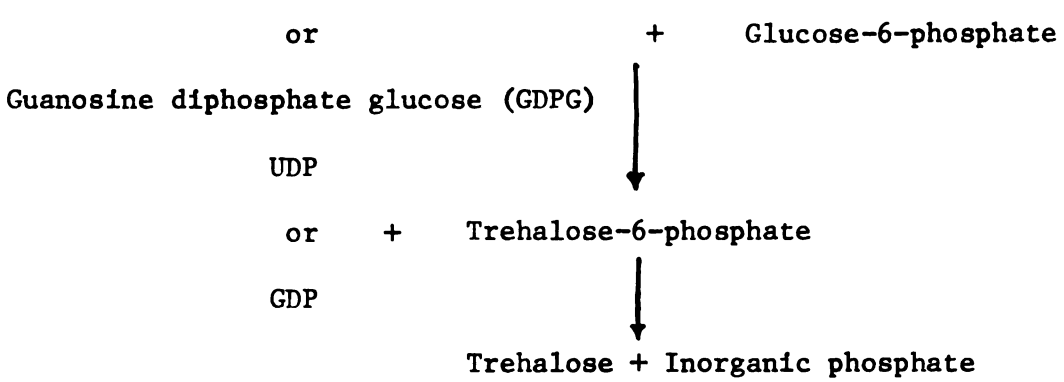
Physiological changes in hemolymph of Japanese beetle larvae during infection. A relatively small amount of work has been done on comparing the physiological changes in infected and non-infected hemolymph of Japanese beetles. The pH of the larval hemolymph was unchanged during the course of the disease (87). The dissolved oxygen content of larval hemolymph containing proliferating *B. popilliae* was one-third that of normal larvae (93). Organic acids (90), and amino acids (82) in healthy and diseased larvae have been examined. The amount of lactate and acetate remained constant throughout the infectious period, and that of pyruvate doubled (90).

Trehalose metabolism in *B. popilliae*. Trehalose is the predominant carbohydrate present in the hemolymph of many insects (94). The hemolymph of third instar Japanese beetle larvae does not contain a significant amount of glucose but does contain a relatively large amount of trehalose (69). The amount of trehalose in the hemolymph of diseased larvae was approximately two-thirds the amount in healthy larvae, and may indicate the use of trehalose for proliferation of the pathogen in the host (69). Growth of *B. popilliae* in liquid medium indicates that trehalose is readily utilized, and the products of its fermentation appear to be similar to those from glucose (70). However,

a small amount of glucose in an acetate agar medium severely restricted spore formation, while trehalose was found to be less inhibitory to sporulation of *B. popilliae* than glucose (71). Costilow and Coulter (16), and Rhodes (69) have suggested that most of trehalose in the hemolymph may not be available to the cells.

Trehalose

Synthesis of trehalose. Trehalose is a disaccharide (1-α-D-glucopyranosyl-α-D-glucopyranoside) which is widely distributed in insects (94), fungi (34), and in bacteria (26). Mostly, trehalose exists in these organisms as a storage compound. Synthesis of trehalose has been worked out in locusts (9), *Dictyostelium discoideum* (38,76), and in *Mycobacterium smegmatis* (41). The steps in trehalose synthesis involve trehalose-6-monophosphate as an intermediate. It proceeds as follows:



A number of enzymes are known which synthesize trehalose-phosphate but most of these have not been examined in detail. Those reported to utilize UDPG as the glucosyl donor have been isolated in yeast (8,62),

silkmoth (55), *Dictyostelium discoides* (76), locusts (9), and *Mycobacterium tuberculosis* (26). The synthesis of trehalose in *Streptomyces hygroscopicus* was found to utilize GDPG as the glucosyl donor (23).

Friedman (25) has isolated trehalose-6-phosphate phosphatase from *Phormia regina*. The enzyme catalyzes the hydrolysis of trehalose-6-phosphate into free trehalose and inorganic phosphate. The enzyme is very specific. It does not hydrolyze any other phosphoryl containing compound except that it attacks glucose-6-phosphate at the rate of about 8% that of its normal substrate (25).

Pathways in trehalose metabolism. Metabolism of trehalose, as of present, has been found to proceed in two separate ways. The first and probably the most common route is the hydrolysis of trehalose into two glucose molecules by the enzyme trehalase. Friedman (24) found that the trehalase of *Phormia regina* is specific for trehalose with no activity toward the following substrates: maltose, sucrose, melibiose, lactose, raffinose, and turanose. Saito (77), and Carnie and Proteous (10) have isolated trehalase from pupae of the silkworm, *Bombyx mori*, and rabbit small intestine respectively. Furthermore, trehalase has been examined in several insect species (94), Neurospora (34), and *Dictyostelium discoideus* (11). Generally, trehalase is found to be quite specific for the α,α linkage of trehalose and not to be a general α-glucosidase (24,47). Clements *et al.* (13) examined trehalase in fleshfly *Sarcophage barbata*, and reported the enzyme to have an optimal pH of 5.3, and a K_m of 1.4×10^{-3} M. These were the same for both a

soluble and a particulate enzyme. Hey and Elbein (33) studied the trehalase from *Streptomyces hygroscopicus*. This enzyme had a K_m of 1.8×10^{-2} M and an optimal pH of 6.5. Tris (hydroxymethyl) aminomethane buffer was found to inhibit many trehalases (24,33,47). The trehalase from *Streptomyces hygroscopicus* was purified 80 fold and was found to be very specific for trehalose, with only a slight activity in trehalosamine. The enzyme is present in all of the species of *Streptomyces*. The trehalase from *Drosophila melanogaster* is constitutive (47).

Recently an alternative pathway for metabolism of trehalose has been described in *Euglena gracilis* (1). The enzyme trehalose phosphorylase catalyzes the following reaction:

Trehalose + Inorganic phosphate $\rightleftharpoons$ β-Glucose-1-phosphate + Glucose

This enzyme has been partially purified. The reaction is reversible and either 6-deoxy-glucose or xylose can be substituted for glucose in the reverse reactions forming 6-deoxy-α-D-glucopyranosyl-α-D-glucopyranoside and α-D-xylopyranosyl-α-D-glucopyranoside respectively (2).

Trehalose is absorbed and respired by *Myrothecium verocaris*. It has been proposed that the enzyme trehalase of *M. verocaris* may be involved in trehalose transport (45). Further studies with the same organism indicate that trehalose can also be absorbed by another transport system which can be induced by exposure to turanose or nigerose (45). This induction needs oxygen and trehalose has no activity as an inducer. Turanose induces the system specific for α-linked glucosyl-glucose or glucosyl-fructose. Trehalose is absorbed against a

concentration gradient by the constitutive as well as by induced system. The non-induced system is associated with trehalase, and the rate of absorption of trehalose by the induced system is affected by trehalose concentration.

Phosphoenolpyruvate:sugar phosphotransferase systems

Only two different pathways for the metabolism of trehalose, involving either hydrolysis or phospholysis have been described. However, two other possible pathways for metabolism of disaccharides have been reported. Both involve phosphorylation of the disaccharide prior to cleavage. The main differences in the two pathways are with respect to the phosphoryl donor, and the nature of the transported disaccharide molecule. Cellobiose and gentibiose have been found to be phosphorylated with adenosine-5'-triphosphate (ATP) by a soluble, inducible β-glucoside kinase after they enter the cell. The disaccharide monophosphates are then subsequently cleaved by a phospho-β-glucosidase (58,59,60,61). Both enzymes from *Aerobacter aerogenes* were partially purified and characterized by Palmer and Anderson (59,60). In the other pathway in which a disaccharide is phosphorylated, the phosphorylation occurs concomitantly with the transport of the molecule inside the cell via the phosphoenolpyruvate (PEP) dependent phosphotransferase (PTS) of Kundig and Roseman (39). The PTS system is widely distributed in bacteria (73), and the extensive work on this system has been reviewed (27,74,75). This portion of the review will include only the PTS systems which involve phosphorylation of lactose in *Staphylococcus aureus* and *Streptococcus lactis*.

PTS system in *Staphylococcus auerus*. Using a lactose negative strain of *S. aureus*, Hengstenberg *et al.* (30) showed that this strain of *S. aureus* accumulates lactose phosphate (a phosphoryl group is on the "6" position of the galactose moiety) when lactose is used as a substrate. They also demonstrated that *S. aureus* has an inducible enzyme which hydrolyses lactose phosphate into glucose and galactose-6-phosphate. Phosphoenolpyruvate stimulates the hydrolysis of o-nitro-phenyl-β-D-galactoside (ONPG) (31), and 5.0×10^{-2} M sodium fluoride inhibits ONPG hydrolysis by intact cells of *S. aureus* (36). The data indicate that a continuing supply of PEP formed via the enolase reaction is needed for the hydrolytic process (36). Galactose-6-phosphate is the best inducer and isopropyl-β-D-thiogalactoside (IPTG) at 1×10^{-3} M inhibits induction (54). Using specific mutants of PTS systems, Hengstenbert *et al.* (32) partially purified the four components of the PTS system; Enzyme I, HPr, Factor III, and Enzyme II. Laue and MacDonald (42) found that the product of thiomethyl-β-galactopyranoside (TMG) accumulated in *S. aureus* is TMG-6-phosphate. The accumulation of TMG as TMG-6-phosphate in whole cells and the phosphorylation of TMG by cell extract were compared in terms of the effect of temperature, inhibitors, and genetic change (43). Egan and Morse (20,21,22) isolated and characterized a car^- mutant of *Staphylococcus aureus* which cannot utilize lactose, maltose, sucrose, galactose, fructose, mannitol, ribose, or trehalose. The ability to ferment glucose was retained but the rate of fermentation was slower in car^- mutant. The car^- mutant does not accumulate any sugar derivatives

(30) and was suggested to be an Enzyme I mutant from a mixing experiment with an Enzyme I mutant of *E. coli* (31).

Roseman and co-workers (29,57,83,84,85,86) have characterized the PTS system in *Staphylococcus aureus* in much detail. The system catalyzes the transfer of a phosphoryl group from PEP to β-galactosides to yield corresponding galactoside-6-phosphate esters. This transfer requires the participation of four protein components, Enzyme I, two phospho-carrier proteins (HPr and Factor III^{lac}) and Enzyme II^{lac}. These components were separated from one another and subjected to purification procedures (29,86). The four protein components of the PTS system are required for the PEP dependent phosphorylation of lactose and its analogs. The overall reaction proceeds by an ordered sequence of phosphoryl transfer steps, each of which is studied in detail by Simoni *et al.* (83). The initial phosphoryl transfer from PEP to the phospho-carrier protein HPr is catalyzed by Enzyme I; phospho-Enzyme I appears to be an intermediate in this reaction. Only one phosphoryl group is transferred to each HPr molecule. The subsequent reversible phosphoryl transfer from phospho-HPr to the sugar specific phospho-carrier protein Factor III^{lac} is entirely self-catalyzed. The phosphoryl groups in both phospho-carrier proteins are linked to the imidazole moieties of histidine residues; to N-1 in HPr and N-3 in Factor III. The final step, formation of galactoside-6-phosphate, is catalyzed by the membrane bound Enzyme II. Kinetic studies indicate that the reaction does not proceed by a 'ping-pong' mechanism, but that a tenary

complex is formed by the two substrates, phospho-Factor III, and lactose, and Enzyme II^{lac}. No evidence for a phospho-Enzyme II intermediate is observed.

Simoni and Roseman (85) presented evidence for the physiological role of the PEP:lactose phosphotransferase system in the utilization of lactose by *Staphylococcus aureus*. This organism takes up lactose by group translocation, i.e., the sugar is phosphorylated while it passes through the cell membrane, and that the phosphotransferase system is responsible for this process. Studies of mutants defective in proteins of the PTS systems provide the evidence for this conclusion. Mutants lacking Enzyme I are unable to ferment a variety of sugars, and cannot take up methyl-α-glucopyranoside or thiomethyl-β-galactopyranoside. Mutants defective in Enzyme II or Factor III of the PTS system are also incapable of transporting thiomethyl-β-galactopyranoside, but transport methyl-α-glucopyranoside at normal rates. The mutants do not catalyze a facilitated diffusion process, therefore indicating that the phosphotransferase system is responsible for translocation *per se*, and that it does not act as a "sugar trap" (85).

There are some speculations (75) that all carbohydrates are transported and phosphorylated by PTS system in *S. aureus*. However, Button *et al.* (7) have shown that maltose is metabolized principally via diffusion and hydrolysis of the non-phosphorylated molecule.

PTS system in *Streptococcus lactis*. McKay *et al.* (50) demonstrated that various strains of *Streptococcus lactis* have an

enzyme, β-D-phosphogalactoside galactohydrolase which hydrolyses lactose-phosphate. The latter compound is formed by the PTS system. Lactose utilization by intact cells of *S. lactis* C_2F is inhibited by 3×10^{-2} M NaF; and phosphoenolpyruvate but no other phosphoryl donors stimulate the hydrolysis of ONPG by extracts of cells of this organism. Also, toluene treated cells need PEP to hydrolyse ONPG. The uptake of $[^{14}C]$TMG was studied, and the accumulated product shown to be TMG-phosphate. Complementation studies between cell extracts of *S. lactis* and of various PTS mutants of *Staphylococcus aureus* showed that the components in *S. lactis* are essentially interchangeable with that of *S. aureus* (49). Molskness *et al.* (53) studied β-D-phosphogalactoside galactohydrolase from *S. lactis*; and Premi *et al.* (68) partially purified β-D-phosphogalactoside galactohydrolase from *Lactobacillus casei*. Using ONPG-6-phosphate as substrate the enzyme was found to have a K_m of 1.59×10^{-3} M, a pH optimum of 5.0, a temperature optimum of 37 C, and a molecular weight of 1.3×10^{-5}.

Catabolite Repression and Sporulation

Controls in sporulation. The differentiation of vegetative cells to endospores requires changes in morphological events which undoubtedly arise from well controlled changes in biochemical events. The biochemical events in vegetative cells and sporulating cells are believed to be quite different both in the catabolic and biosynthetic pathways. These changes result in the production of enzymes, substances, and structures new or different from vegetative materials (3). The

types of controls involved have to account for the ability of an organism to repress the activities of spore specific enzymes during vegetative growth, to shut off certain vegetative enzyme activities and turn on the spore specific enzymes during sporogenesis (46). It has been previously proposed by Schaeffer *et al.* (79) that certain essential spore specific enzymes may be regulated by a mechanism similar to the regulation of β-galactosidase synthesis in *Escherichia coli*. The spore specific enzymes may be repressed by a catabolite(s) during vegetative growth. When the amount of the catabolite(s) in the cell decreases, the repression of a key sporulation specific enzyme(s) would be released. The synthesis of a spore specific enzyme may then lead to sequential induction of several other spore specific enzyme(s) needed for the formation of the various spore components (56).

Cyclic adenosine-3',5'-monophosphate (cAMP) and catabolite repression. Makman and Sutherland (44) demonstrated the effect of glucose on the level of cAMP in *E. coli* in 1965. Since then, cAMP and its roles in the control of enzyme synthesis have been demonstrated in several other genera of bacteria (37,64,72). The finding that cAMP relieved catabolite repression and transient repression of the synthesis of β-galactosidase in *E. coli* (67) led to the discovery of a positive control on the transcription of the lactose operon. Several other enzyme systems beside β-galactosidase have also been found to be affected by cAMP (17) either at the transcriptional level (51,92), translational level (40,63) or at the level of enzyme activity (78). Furthermore, cAMP has been shown to be associated with control of differentiation in *Dictyostelium* (72).

Cyclic adenosine-3',5'-monophosphate and controls in sporulation. The ability of cAMP to relieve catabolite repression and transient repression, and its effect on certain types of differentiation has led to the speculations that cAMP may be involved in the control of sporulation. So far, there have been very few reports on cAMP in *Bacillus* species. Ide (35) looked for adenyl cyclase and phosphodiesterase in four *Bacillus* species and was unable to detect any activities of either enzyme. However, Clark and Bernlohr (12), working with *B. licheniformis* found that the level of cAMP in the cell increased at the time of sporulation. Yet, they found no significant difference in the activities of adenyl cyclase or phosphodiesterase during sporulation.

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ARTICLE 1

UTILIZATION OF α-METHYL-D-MANNOSIDE BY *BACILLUS POPILLIAE*

By

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Utilization of α-methyl-D-mannoside by *Bacillus popilliae*[1, 2]

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α-Methyl-D-mannoside (α-MM) was used by all acetate-oxidizing strains of *B. popilliae* tested at a growth limiting rate after a prolonged induction period. Those strains which did not oxidize acetate could not use α-MM for growth unless it was present at a high concentration. The period of induction was decreased, the rate of growth increased, and the extent of oxidation decreased as the α-MM concentration was increased. Theoretical levels of O_2 uptake and CO_2 evolution for the complete oxidation of the mannose moiety of α-MM were observed when the concentration of α-MM was low. Studies with resting cells and cell extracts demonstrated that an α-glucosidase was induced in *B. popilliae* by either α-MM or α-methyl-D-glucoside (α-MG). The enzyme was induced more rapidly by α-MG than by α-MM. Similarly, the ability of cells to oxidize α-MM was induced more efficiently by α-MG than by the mannoside. The data indicate that the hydrolysis of both α-MG and α-MM may be catalyzed by the same enzyme, but that the affinity for α-MG is much higher than that for the mannoside. The slow hydrolysis of α-MM by this enzyme is believed to account for the type of growth response observed.

BHUMIRATANA, A., et R. N. COSTILOW. 1973. Utilization of α-methyl-D-mannoside by *Bacillus popilliae*. Can. J. Microbiol. **19**: 169–176.

L'*alpha*-méthyl-D-mannoside (*alpha*-MM) est utilisé par toutes les lignées de *Bacillus popilliae* qui oxident l'acétate et testées à un taux limitant à la croissance après une période d'induction prolongée. Les lignées qui n'oxident pas l'acétate ne peuvent pas utiliser l'*alpha*-MM pour la croissance à moins qu'il soit présent à une forte concentration. Lorsque la concentration de l'*alpha*-MM est augmentée, la période d'induction décroît, le taux de croissance augmente et l'intensité de l'oxidation diminue. Les valeurs théoriques de la consommation d'O_2 et de l'évolution du CO_2 pour l'oxidation complète de la partie mannose de l'*alpha*-MM ont été observées lorsque la concentration de l'*alpha*-MM est diminuée. Des études avec des cellules au repos et des extraits cellulaires démontrent qu'une *alpha*-glucosidase est induite chez *B. popilliae* par l'*alpha*-MM ou l'*alpha*-méthyl-D-glucoside (*alpha*-MG). L'enzyme est induit plus rapidement par l'*alpha*-MG que par l'*alpha*-MM. Semblablement, l'habilité des cellules à oxider l'*alpha*-MM est induite plus efficacement par l'*alpha*-MG que par le mannoside. Les données indiquent que l'hydrolyse de l'*alpha*-MG et de l'*alpha*-MM peut être catalysée par le même enzyme, mais que l'affinité pour l'*alpha*-MG est beaucoup plus élevée que pour le mannoside. Nous croyons que l'hydrolyse lente de l'*alpha*-MM par cet enzyme explique le type de croissance observé.

[Traduit par le journal]

Introduction

An oligosporogenous strain of *Bacillus popilliae* (NRRL B-2309M) forms spores during colonial development on agar plates of a special medium devised by Sharpe *et al.* (12). Spore formation occurs in colonies during a prolonged period of a decelerating growth rate (1). The glucose level in the medium is very low during this time and the slow diffusion of the remaining sugar to the cells probably results in the prolonged period between exponential and maximum growth. When α-methyl-D-mannoside (α-MM) was substituted for the 0.05% glucose in this medium, it was found that a much higher level (0.5%) of the mannoside was required to attain a sporulation frequency equivalent to that in glucose medium. It is known that sporulation of *Clostridium thermosaccharolyticum* occurs in media with carbon sources which greatly limit growth or when glucose is added to the medium at a growth limiting rate (4, 5). It appeared possible that α-MM could be functioning in a similar manner during sporulation of the oligosporogenous strain of *B. popilliae*. Thus, we decided to study the use of this sugar in some detail.

The data herein demonstrate that the use of α-MM is inducible in *B. popilliae* and that the sugar is used at a growth limiting rate. This probably explains the ability of an oligosporogenous strain to sporulate in the presence of a high level of the mannoside. It is believed that the reason for the slow rate of α-MM utilization by this organism is that the hydrolytic enzyme induced is actually an α-glucosidase with a very low affinity for the mannoside.

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[2]Journal Article No. 5659, Michigan Agricultural Experiment Station.

Materials and Methods

Cultures and Media

The sources and characteristics of all the strains of *B. popilliae* used in this study have been described elsewhere (9). Unless otherwise specified, all experiments were conducted with *B. popilliae* NRRL B-2309MC. This is an asporogenic strain derived from the oligosporogenous strain NRRL B-2309M described by Sharpe *et al.* (12). This strain was selected for study since the type of growth response to α-MM was similar to that with 2309M but the cell yields were higher with 2309MC. The basal medium used routinely contained 1.5% trypticase, 0.5% yeast extract, 0.6% K_2HPO_4 (TY medium). The phosphate was left out of the medium in some experiments so that pH changes could be clearly observed. Unless otherwise indicated, carbon sources were added at a level of 0.2%. Cells used in these experiments were grown in TY medium containing 0.2% α-MM (TYM) unless otherwise stated. Cultures were incubated at 30°C on a rotary shaker.

Growth Studies

Growth was followed by measurements of the optical density (O.D.) at 620 nm using a Gilford model 2000 spectrophotometer. The pH was measured with a Beckman, model G, pH meter.

Oxidation Experiments

The oxidation of various carbon sources by resting cells was assayed manometrically. The direct procedure (14) was used for determining total CO_2 production. The $^{14}CO_2$ produced from [1-^{14}C] acetate was determined by the procedure of McKay *et al.* (9).

The level of α-MM was estimated by measuring the concentration of reducing sugar before and after hydrolysis. Hydrolysis was accomplished by adding 0.4 ml of concentrated HCl to a 4-ml sample and incubating 1 h at 100°C. The sample was neutralized and made to a standard volume before assaying. Reducing sugar was determined by the method of Neish (10). The amount of free reducing sugar released from α-MM during the oxidation experiments was estimated by determining the amount present initially and at the end of the reaction period.

Enzyme Assays

Crude extracts were prepared from cells harvested from cultures at the time of maximum growth response to the carbon source added. Cells were disrupted by ultrasonic oscillation for four 15-s intervals with an MSE-100 probe (Measuring and Scientific Equipment) at 220 V and a peak to peak amplitude of ~ 7 micrometers (μm). Large particles were removed by centrifugation at 3000 × *g* for 15 min, and the supernatant was dialyzed overnight against 500 volumes of distilled water. Protein was estimated by the method of Lowry *et al.* (8).

The hydrolysis of α-MM by the extracts was followed by measuring the amount of reducing sugar released (see above for assay). Reaction mixtures of 1.5 ml total volume contained cell extract, 0.05 *M* potassium phosphate (pH 7.2), and various levels of α-MM. Substrate was added to start the reaction at 30°C. At various intervals, 0.25-ml aliquots were removed, diluted to 2 ml, and boiled at 100°C for 10 min. The precipitate was removed by centrifugation and the supernatant solution assayed.

Total α-glucosidase was assayed by following the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucoside (PNPG). The increase in O.D. at 420 nm was continuously measured using a Gilford model 2000 recording spectrophotometer. The assay system was similar to that

TABLE 1

Utilization of α-methyl-D-mannoside (α-MM) by various strains of *B. popilliae*

Strain	Optical density, 620 nm		pH	
	No sugar	+ α-MM	No sugar	+ α-MM
Experiment 1[a]				
Uninoculated	—	—	7.3	7.3
2309	0.216	0.207	7.4	7.3
2309PA	0.241	0.235	7.3	6.8
2309S	0.325	0.357	6.8	6.9
2309MC	0.372	1.462	7.5	8.6
2309M[b]	0.424	0.667	7.2	8.2
Experiment 2[c]				
2309M[b]	0.074	0.627	7.1	8.25
MS-9[b]	0.124	0.697	7.4	8.5
MS-54[b]	0.252	0.605	8.1	8.5
MS-69[b]	0.453	0.937	8.5	8.4
MS-85[b]	0.459	0.629	8.5	8.7
Experiment 3[d]				
2309S	0.173	1.439	7.45	6.6

[a]The basal medium in Experiment 1 contained 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, and 0.01% $MnSO_4$. Where indicated, 0.2% α-MM was added. Readings were taken after 13 days incubation.
[b]Oligosporogenous strains.
[c]A 1.5% trypticase, 0.5% yeast extract broth was the basal medium in Experiment 2, and 0.2% α-MM was added where indicated. Readings were taken after 7 days incubation.
[d]Same as c except that 1% α-MM was added.

of Halvorson and Ellias (3). However, no glutathione or $MgCl_2$ was added since these reagents failed to stimulate the hydrolysis. Hydrolysis of α-methyl-D-glucoside (α-MG) was followed by coupling the reaction with hexokinase and glucose-6-phosphate dehydrogenase and measuring the rate of reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$). Reaction mixtures of 0.5 ml in 5 m*M* phosphate buffer (pH 7.2) contained 2 m*M* $MgCl_2$, 2 m*M* adenosine triphosphate (ATP), 0.4 m*M* $NADP^+$, excess hexokinase, excess glucose-6-phosphate dehydrogenase, cell extract, and variable α-MG concentrations. The cell extracts contained some ATPase activity and low levels of an NADPH oxidase but the reactions were linear for over 1 min.

Chemicals

α-Methyl-D-mannoside was purchased from General Biochemicals, α-MG was from Pfanstiehl Chemical Co., and PNPG and *p*-nitrophenyl-α-D-mannoside were from Mann Research Laboratories.

Results

Growth Response

A number of asporogenous and oligosporogenous cultures of *B. popilliae* were tested for their ability to use α-MM for growth. All of the latter cultures and one asporogenic strain (2309MC) derived from 2309M showed a growth response to this sugar (Table 1). Also, the final pH of the medium was higher than that of the control (no added sugar) medium. In contrast, the other three asporogenic strains tested failed to respond to the presence of 0.2% α-MM. When 1% α-MM was added, the optical density of the culture of strain 2309S did increase greatly, but the final pH of the medium was less than that of the control. The ability to use α-MM at a low concentration is correlated with the ability to oxidize acetate. McKay *et al.* (9) found that all of the oligosporogenous strains and strain 2309MC would oxidize acetate and that strains 2309 and 2309S would not. While 2309PA was originally found to oxidize acetate, we found that it would no longer do so.

With some of the cultures tested, there was an extended lag period of growth (up to 4 days) in an α-MM medium after the optical density had attained the level of a control with no added sugar. This lag was eliminated when the cultures were successively transferred in an α-MM

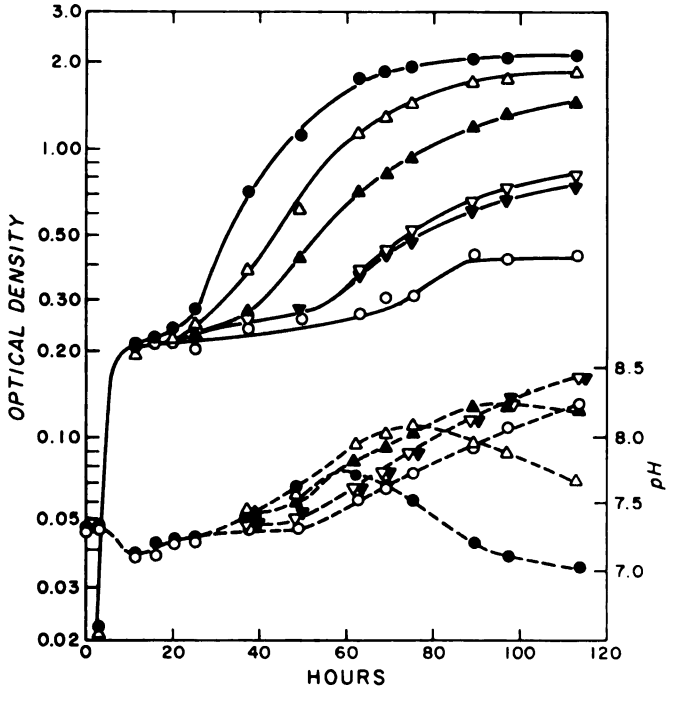

FIG. 1. Effect of various concentrations of α-methyl-D-mannoside (α-MM) on the growth of *B. popilliae* and the pH changes during growth in a trypticase – yeast extract (TY) medium with the phosphate omitted. The solid lines represent growth and the dashed lines pH. The concentrations of α-MM were: (○), zero; (●), 1%; (△), 0.6%; (▲), 0.4%; (▽), 0.2%; (▼), 0.1%.

medium, but was observed again when cells grown in TYM were subcultured in a glucose medium and then subcultured again in TYM. Thus, it did not appear that the prolonged lag was due to the selection of mutants.

The concentration of α-MM in the medium had a pronounced effect on the length of the lag period in the growth curve, the rate and extent of growth during the use of α-MM, and on the pH changes observed in the medium (Fig. 1). With 1% α-MM present the lag period was less than 24 h, the generation time during the initial response was from 8 to 10 h, and the final optical density was about 2.0. Corresponding values in the presence of 0.1 and 0.2% α-MM were about 52 h, 24 h, and 0.8 optical density, respectively. It is also evident from Fig. 1 that acid accumulated in cultures when α-MM was present in great excess, but there was no accumulation evident when the sugar was added at low levels (0.1 and 0.2%). When 0.2% α-MG was used as the carbon source, there was only a 4- to 5-h lag in the growth response, and the generation time during exponential growth was the same as in a glucose medium (3 to 4 h). Also, the pH of the medium decreased rapidly during the period of rapid growth.

Oxidation of α-MM

Cells harvested from TYM oxidized α-MM with a respiratory quotient (RQ) of 1, and, when the sugar was present at low levels, apparently oxidized the mannose moiety to completion (Table 2). However, as the concentration of α-MM was increased, the amounts of O_2 consumed and CO_2 produced per μmole of α-MM used were progressively lowered. At the highest α-MM level tested, only 2 μmoles of each gas was consumed or produced per μmole of sugar oxidized. However, there was a significant amount of free reducing sugar released into the medium.

The rates of O_2 uptake by resting cells were also dependent on the α-MM concentration. The Q_{O_2} of one lot of cells from TYM medium was ~ 19 μliters O_2 per hour per milligram (dry weight) when measured in the presence of 13 m*M* α-MM while with either 44 or 130 m*M* α-MM the Q_{O_2} was ~ 38. However, with the highest substrate level the respiration was linear for only ~ 1 h while it was linear for over 2 h with the two lower levels tested.

Since the foregoing data indicated that the mannose moiety of α-MM was oxidized completely if the α-MM was at a reasonably low level and that the pH of the growth medium increased throughout the incubation period, it appeared possible that the presence of α-MM might stimulate acetate oxidation. This proved to be true (Table 3). Cells from the stationary phase of a culture in TYM oxidized about 4 times more [1-^{14}C] acetate in the presence of α-MM than in the absence of the sugar. Also, the presence of acetate stimulated the degradation of α-MM by about 4 times. Although the O_2 uptake data were corrected for endogenous levels, the levels of O_2 uptake observed in the presence of either [1-^{14}C] acetate or α-MM were higher than theoretical. This may have been due to the enhancement of endogenous activity by the presence of added substrate.

Respiration of *B. popilliae* was completely inhibited by iodoacetate and arsenite (Table 4). Since only a small amount of reducing sugar was detectable in the presence of iodoacetate, it is probable that the hydrolytic enzyme was strongly inhibited. α-Glucosidase from yeast is partially inhibited by iodoacetate (3). Arsenite did not inhibit the degradation of the mannoside, but sharply reduced the amounts of O_2 uptake and CO_2 evolved per equivalent of sugar used. This inhibitor completely inhibits acetate oxidation by this organism (11).

Induction of α-MM Oxidation

Cells were harvested at various periods during the growth of *B. popilliae* in TYM with 0.01% glucose added and tested for their ability to oxidize α-MM and acetate. The glucose was added to the growth medium to obtain higher cell yields during early stages of growth. The shape of the growth curve was not changed by the addition of this level of glucose. Cells from very young cultures (10 h) failed to show any activity on either substrate (Fig. 2). The maximum Q_{O_2} on acetate was observed with cells harvested at the first stationary phase of the diauxie growth curve and decreased thereafter. Some oxidation of the α-MM was observed initially at the same point as acetate, but the Q_{O_2} for the sugar continued to increase throughout the incubation period. The increase in the Q_{O_2} did not vary significantly from a linear function, but there was some evidence of an

increase in the rate corresponding to the time of the secondary growth response in the diauxie growth curve.

Cells produced in media containing glucose or trehalose were unable to oxidize either α-MM or α-MG (Table 5). However, α-MG-grown cells oxidized α-MM more rapidly than α-MM-grown cells, and cells grown on α-MM oxidized the glucoside more rapidly than the mannoside. Thus, it appeared that α-MG was the preferred inducer and substrate for the enzyme(s) involved. The oxidation of maltose was constitutive and cells grown with this sugar did not oxidize α-MG except after a prolonged lag.

TABLE 2

Effect of concentration on the extent of oxidation of α-methyl-D-mannoside (α-MM) by *B. popilliae* 2309MC[a]

μmoles α-MM		O_2 uptake, μmoles	CO_2 produced, μmoles	μmoles O_2/CO_2 per μmole α-MM utilized	Reducing sugar released, μmoles
Added	Degraded				
Experiment 1					
10	2.1	12.5	12.1	6.0/5.8	—[b]
50	10.7	65.6	65.0	6.1/6.1	—[b]
Experiment 2					
30	9.8	51.4	51.1	5.4/5.3[c]	0.1
100	22.2	76.2	81.5	3.5/3.8[c]	0.5
300	24.0	33.0	33.6	2.0/2.0[c]	7.2

[a]Reaction mixtures in Warburg vessels contained 7 to 8 mg (dry weight) of cells, and the indicated level of α-MM in 0.05 *M* potassium phosphate buffer (pH 7.2). Those in Experiment 1 were incubated for 6 h and those in Experiment 2 were incubated for 5 h.
[b]Not determined.
[c]The amount of α-MM degraded was corrected for the amount of free reducing sugar released during the experiment (column 2 minus column 6).

TABLE 3

Effect of added acetate on α-methyl-D-mannoside (α-MM) oxidation and of added α-MM on acetate oxidation by *B. popilliae*[a]

Substrate(s)	O_2 uptake, μmoles[b]	α-MM degraded, μmoles	$^{14}CO_2$ from [1-^{14}C] acetate, cpm	Acetate used, μmoles[c]
[1-^{14}C] Acetate (9 μmoles)	3.8	—	9.11×10^4	1.42
α-MM (30 μmoles)	9.6	1.5	—	—
Both of the above	26.0	6.1	3.46×10^5	5.39

[a]The reaction mixtures were as described in Table 2. Reaction mixtures were incubated for 2.5 h.
[b]Values reported were corrected for endogenous O_2 uptake.
[c]Calculated from the amount of $^{14}CO_2$ using the specific activity of the [1-^{14}C] acetate added (6.43×10^4 cpm/μmole).

TABLE 4

Effect of arsenite and iodoacetate on the oxidation of α-methyl-D-mannoside (α-MM) by *B. popilliae*[a]

Additive	α-MM degraded, μmoles	Reducing sugar produced, μmoles	O_2 uptake, μmoles	CO_2 produced, μmoles
None	10.25	0.03	50.3	49.7
Arsenite (10 m*M*)	12.51	0	20.1	10.6
Iodoacetate (10 m*M*)	0	0.55	0	0

[a]Reaction mixtures in Warburg vessels contained 8.09 mg (dry weight) of cells grown in TYM, 50 m*M* potassium phosphate, pH 7.2; inhibitors where indicated; and 30 μmoles of α-MM. They were incubated for 5 h. All values are corrected for endogenous controls.

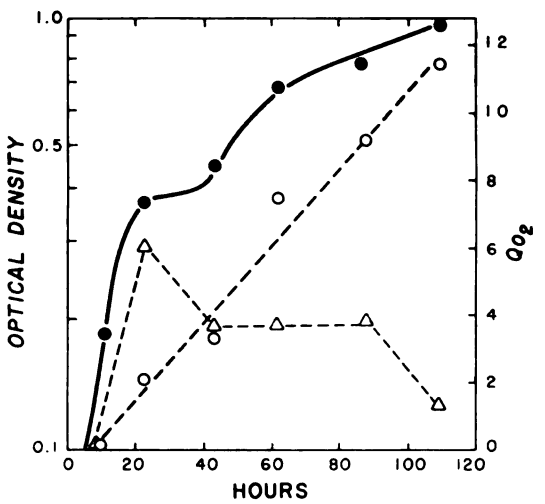

FIG. 2. Respiration rates of *B. popilliae* cells on α-methyl-D-mannoside (α-MM) and acetate at various stages of cultural development. Cells were harvested from TYM + 0.01% glucose medium at the points indicated on the optical density curve (●), and the rates of oxidation of the two substrates determined manometrically using the same types of reaction mixtures described in Table 2. (○), α-MM (30 μmoles); (△), acetate (9 μmoles).

In a separate experiment, we observed that glucose-grown cells oxidized mannose as rapidly as glucose.

Hydrolysis of α-MM and α-MG

Crude extracts of *B. popilliae* cells failed to hydrolyze *p*-nitrophenyl-α-D-mannoside (PNPM) to a significant extent under the same conditions used for α-D-glucosidase assays. There was no increase in the O.D. at 420 nm with 1×10^{-3} *M* PNPM and only a very slight increase with 1.4×10^{-2} *M*. Addition of Zn^{2+} to the reaction mixture failed to enhance the activity. Similarly, there was no detectable hydrolysis of PNPM when the assay was conducted by the procedure used for mammalian α-mannosidase (7). The rate of release of reducing sugar from α-MM by crude (nondialyzed) extracts of *B. popilliae* cells was very low except at high substrate levels (Fig. 3). Obviously, the affinity of the enzyme for α-MM was quite low. A Lineweaver–Burk plot of the data for the four highest substrate levels tested indicated a K_m of $\sim 5 \times 10^{-1}$ *M*. The reason for the 5- to 7-min lag observed in all reactions is obscure. The addition of adenosine triphosphate or of phosphoenolpyruvate to reaction mixtures similar to those in Fig. 3 had no effect on the lag or the rates of hydrolysis.

Lineweaver–Burk plots for the hydrolysis of PNPG by extracts of cells grown in either α-MM or α-MG were essentially identical, and the hydrolysis in both instances was inhibited by α-MG in a competitive manner. The apparent K_m for PNPG with both extracts was $\sim 1 \times 10^{-3}$ *M*, and the average apparent K_I for α-MG was $\sim 2 \times 10^{-1}$ *M*. The apparent K_m for α-MG as determined with the coupled dehydrogenase assay was $\sim 1.9 \times 10^{-2}$ *M*. Thus, the apparent affinity of the hydrolytic enzyme for α-MG is $\sim 25 \times$ higher than that for α-MM. There was no significant inhibition of PNPG hydrolysis by α-MM in concentrations up to 0.4 *M*. However, this could be due to the great difference in the affinities for the two substrates.

TABLE 5

Effect of carbon and energy source in growth medium on the rates of oxygen uptake by *B. popilliae* cells using various sugars[a]

Growth substrates	Q_{O_2}[b] with:				
	glucose	trehalose	α-MG	α-MM	maltose
Glucose	17.1	31.4	1.0	0.0	37.0
Trehalose	16.0	33.0	0.0	0.0	—[d]
α-MG[c]	34.8	—[d]	33.5	10.8	18.0
α-MM[c]	22.4	—[d]	23.2	8.8	—[d]
Maltose	44.5	—[d]	0.0[e]	0.0	28.3

[a]Reaction mixtures in Warburg vessels contained cells (7 to 10 mg dry wt.) and the indicated sugars (30 μmoles/vessel) in 50 m*M* potassium phosphate buffer (pH 7.2).
[b]Q_{O_2}, μliters oxygen uptake/h per milligram dry weight of cells.
[c]α-MG = α-methyl-D-glucoside; α-MM = α-methyl-D-mannoside.
[d]Not determined.
[e]There was some oxidation after a 30-min lag but this may have been due to induction.

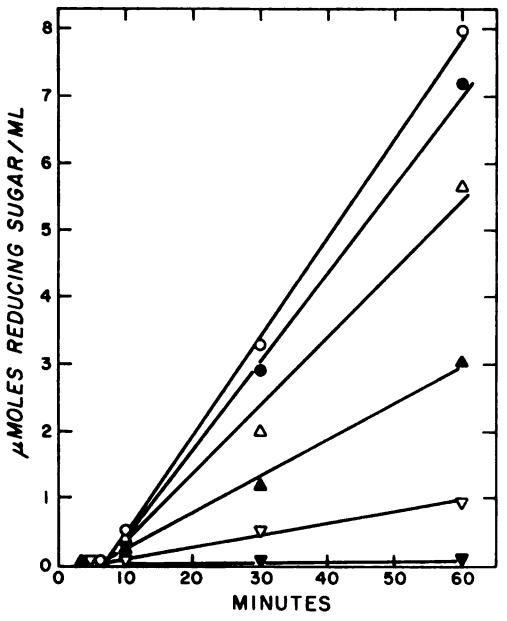

FIG. 3. Hydrolysis of α-methyl-D-mannoside by a crude nondialyzed extract of *B. popilliae* cells. Reaction mixtures of 1.5 ml total volume contained 1 ml cell extract (26.7 mg protein); 33 m*M* potassium phosphate, pH 7.2; and the following m*M* concentrations of α-MM: (○), 102; (●), 76.2; (△), 50.8; (▲), 25.4; (▽), 5.1; (▼), none added.

Extracts of cells produced in a glucose medium hydrolyzed PNPG at a slow rate but there was no detectable hydrolysis of α-MG or α-MM. Therefore, the PNPG hydrolysis in extracts of these cells was probably catalyzed by a constitutive maltase enzyme. Both maltase and α-methyl glucosidase from yeast hydrolyze PNPG (6).

Discussion

It is apparent that α-MM is used by *B. popilliae* at growth limiting rates, and the rate is dependent on the concentration of the mannoside. However, the minimum generation time achieved with 1% α-MM (~ 8–10 h) was quite slow compared to the time in a glucose medium (3–4 h). This provides a reasonable explanation for the observations that an oligosporogenous strain of this organism sporulates in the presence of 0.5% α-MM (1) while sporulation is strongly inhibited by readily utilizable carbon sources at concentrations over 0.1% (12). While there was a prolonged period of a decelerating growth rate in the α-MM broth cultures similar to that observed in colonies on agar plates (1), there were no detectable spores produced in the broth. Thus, there are undoubtedly factors other than the limitation of growth rates by the availability of the carbon source which are important to the sporulation of *B. popilliae*.

The data all indicate that *B. popilliae* has an inducible α-glucosidase which can be induced at low efficiencies by α-MM. It appears likely that this enzyme will hydrolyze α-MM but has a low affinity for it. These observations are adequate to explain the type of growth response to α-MM observed. Thus, the prolonged lag in growth is correlated with the slow induction of the glucosidase, and the growth rate is limited by the rate of hydrolysis of the mannoside. When α-MG is used in the growth medium the lag is greatly reduced and the rate of exponential growth is essentially equivalent to that in a glucose medium. Mannose is used as readily as glucose.

It is apparent that α-MM at low concentrations does not inhibit the oxidation of acetate by certain strains of *B. popilliae*. Theoretical levels of O_2 uptake and CO_2 evolution for the complete oxidation of the mannose moiety of α-MM were observed with 10 m*M* α-MM. However, with 100 m*M* α-MM, only sufficient gas exchange was noted to account for the oxidation of a hexose to the level of acetate. The pH changes observed in an α-MM medium were correlated with these observations. Thus, at low substrate levels, the pH continued to increase throughout the growth period, while, at high α-MM levels, the pH of the medium decreased. *B. popilliae* oxidizes glucose during growth in the presence of unlimited oxygen to acetate and CO_2 (11), but with some strains the cells start oxidizing acetate at relatively low rates as they approach the stationary phase of growth (9). Apparently only those strains which can oxidize acetate obtain sufficient energy to support growth from the oxidation of α-MM when it is present at a low level (0.2%). Those strains which cannot oxidize acetate only grow when a high level of α-MM is present in the medium.

The role of α-MM in the stimulation of acetate oxidation by *B. popilliae* may be in supplying four carbon intermediates of the tricarboxylic acid cycle. Previous data indicate that this organism lacks a glyoxalate cycle, and produces a considerable amount of glutamate

from acetate (9). The mechanism for the stimulation of α-MM utilization by the presence of acetate is not apparent. Inhibition of acetate oxidation by arsenite had no effect on the extent of α-MM hydrolysis by intact cells. Thus, it is not likely that the stimulation is due to the availability of additional energy.

It appears probable that both α-MM and α-MG are hydrolyzed by the same enzyme in *B. popilliae*. If there was a specific α-methyl mannosidase present, one would expect it to hydrolyze *p*-nitrophenyl-α-D-mannoside, but this was not observed. Also, one would expect that a specific mannosidase would be induced more efficiently by α-MM than by α-MG; but the opposite effect was observed. It would be unique for a single enzyme to be induced which hydrolyzes both α-MG and α-MM. Spiegelman *et al.* (13) reported the induction by maltose of two enzymes in yeast. One is specific for maltose and the other for α-MG, but both enzymes hydrolyze PNPG (6). There are also two α-glucosidases induced by either maltose or α-MG in *Streptococcus pyogenes* (2). However, the maltase from this organism does not hydrolyze PNPG while the other enzyme hydrolyzes both PNPG and α-MG. The induction of an α-glucosidase by α-MM and the hydrolysis of α-MM by this enzyme would be unique. Studies of α-glucosidases indicate that their specificity is controlled by the entire molecule but there is less tolerance for variation in the sugar moiety than in the aglycone (3). We have found no reports of an α-glucosidase which can hydrolyze α-MM or which is induced by a mannoside.

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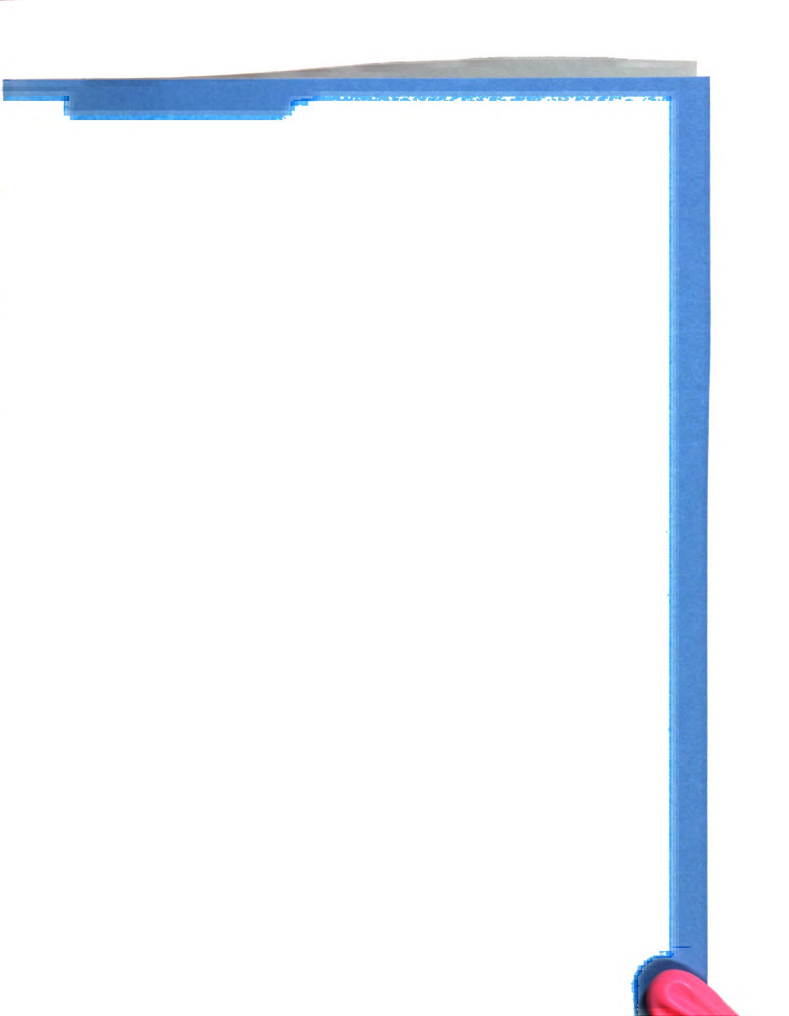

ARTICLE 2

TREHALOSE METABOLISM BY *BACILLUS POPILLIAE*

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Journal of Bacteriology

ABSTRACT

TREHALOSE METABOLISM
BY *BACILLUS POPILLIAE*

By

Amaret Bhumiratana and Ralph N. Costilow

Results from growth and oxidation studies indicated that trehalose was a better carbon source for *Bacillus popilliae* than glucose. Synthesis of β-glucosidase in *B. popilliae* was also found to be strongly repressed by trehalose, whereas glucose only exerted a slight effect. Cyclic adenosine 3',5'-monophosphate did not overcome the trehalose repression. The kinetics of [^{14}C]trehalose uptake and its inhibition by sodium fluoride suggested that trehalose was transported by an energy dependent system. The crude cell extract of *B. popilliae* had no trehalase or trehalose phosphorylase activities. However, cell extracts contained a phosphotrehalase which cleaved trehalose 6-phosphate (T6P) into equal moles of glucose and glucose 6-phosphate (G6P). The enzyme was purified 34 fold, and found to have an apparent Km of 1.8×10^{-3} M for T6P, and a pH optimum between 6.5 and 7.0. A *B. popilliae* mutant lacking phosphotrehalase was isolated, and found to accumulate T6P when trehalose was used as a substrate. Cell extracts of this mutant also produced T6P when it was incubated with trehalose and phosphoenolpyruvate (PEP). PEP was

found to be the only phosphoryl donor which significantly stimulated the production of [^{14}C] sugar phosphate from [^{14}C] trehalose by an extract of wild type cells. The data demonstrate that *B. popilliae* metabolizes trehalose by first phosphorylating it to form T6P, and subsequently hydrolyzing the T6P into glucose and G6P. Although not completely established, it appears likely that trehalose is transported into the cells by a PEP:sugar phosphotransferase system. Cell extracts of various strains of *B. popilliae* were found to produce [^{14}C] sugar phosphate from [^{14}C] trehalose and to have phosphotrehalase activities.

INTRODUCTION

Bacillus popilliae is a pathogen of Japanese beetle (*Popilliae japonica*) larvae and was first described by Dutkey (6). It grows readily in the hemolymph of the larvae and high populations of endospores are accumulated. Numerous attempts to mass produce spores in the laboratory have failed. Studies of hemolymph composition revealed that there is not a significant amount of glucose present but that trehalose is present in excess (26,28). The trehalose level decreased by only about one-third during the growth of *B. popilliae* in the larvae (28). Many studies have shown that *B. popilliae* utilized trehalose readily (2,26,28), and it appears likely that this is the primary source of carbon and energy for growth and sporulation in the natural host.

Glucose metabolism by *B. popilliae* has received considerable attention (3,25) and there is no reason to believe that there should be any difference in trehalose dissimilation after the degradation into two molecules of hexose. Rhodees *et al.* (27) reported that the products formed during growth of the organism in a trehalose containing medium were similar to those from glucose. However, we observed that the rate of respiration of *B. popilliae* with trehalose was about twice that with glucose as substrate (2). This observation prompted us to further studies of the utilization of trehalose by this organism.

To our knowledge, only two types of cleavage reactions have been described for trehalose. A number of organisms have been found to have a trehalase, which hydrolyzes the disaccharide into two molecules of glucose (10,12). A recent report (1) described the phosphorolytic cleavage of trehalose into glucose 1-phosphate and glucose by a phosphorylase from *Euglena gracilis*. Preliminary data indicated that trehalose was not readily hydrolyzed by cell extracts of *B. popilliae*.

Data in this report indicate that trehalose is utilized more readily as a source of energy by *B. popilliae* than is glucose. Trehalose is transported into the cell by a system forming trehalose 6-phosphate (6-O-phosphoryl-α-D-glucopyranosyl-α-D-glucopyranoside) which is then cleaved by a phosphotrehalase with the formation of glucose and glucose 6-phosphate.

MATERIALS AND METHODS

Organisms and culturing procedures. *Bacillus popilliae* NRRL 2309MC was used in most of the studies. All of the cultures used, including their original source and methods for maintenance, have been described elsewhere (17). The basal media (TY) used throughout the studies contained 1.5% trypticase, 0.5% yeast extract, and 0.6% K_2HPO_4 (2). The carbohydrates were filter sterilized separately and added to the autoclaved basal medium. In all cases, 50 ml, 100 ml or 200 ml of media were dispensed in 125 ml, 250 ml or 500 ml Erlenmeyer flasks respectively. The cultures were routinely aerated by shaking on a rotary shaker, and the temperature was maintained at 30 C.

Growth and oxygen uptake experiments. Each culture contained 50 ml of medium in 125 ml flasks. Samples of 2.0 ml were taken at various intervals and growth was monitored by measurements of the optical density at 620 nm. One optical density unit equaled approximately 1.6 mg dry weight of cells per ml.

For oxygen uptake studies, *B. popilliae* 2309MC was grown to mid-exponential phase in TY + 0.2% trehalose. The cells were harvested by centrifugation at 10,000 x g and washed twice using 5×10^{-2} M potassium phosphate buffer, pH 7.2. The pellet was suspended in the same buffer. Oxygen uptake was measured in a Warburg apparatus by standard manometric techniques (35).

Enzyme assays. β-Glucosidase activity was assayed according to the method of Duerksen and Halvorson (5) with slight modifications. Each reaction mixture contained 0.8 ml of cell suspension (125 to 250 µg protein per ml) in 5 x 10^{-2} M potassium phosphate buffer, pH 7.2, and 0.2 ml of 5 x 10^{-2} M p-nitrophenyl-β-D-glucopyranoside (PNPβG). The reaction was incubated at 30 C for 20 min and stopped by adding 0.5 ml of cold 1.0 M Na_2CO_2. The cells were centrifuged and the absorbancy was measured at 400 nm. The increase in absorbancy at 400 nm was linear with time for over 60 min. One unit of β-glucosidase was defined as the amount of enzyme which produced 1 µmole of p-nitrophenol per min. The molar absorbance of p-nitrophenol under these conditions is 1.8 x 10^4 M^{-1} cm^{-1}.

Phosphotrehalase was assayed spectrophotometrically by coupling the reaction to glucose 6-phosphate dehydrogenase. Each reaction contained 10 µmoles of glycylglycine buffer, pH 7.5; 1 µmole $MgCl_2$; 0.1 µmole nicotinamide adenine dinucleotide phosphate ($NADP^+$); 0.5 µmole T6P; a non-rate limiting amount of glucose 6-phosphate dehydrogenase; and a rate limiting amount of the protein to be assayed. The final volume was 0.16 ml. The rate of reaction was determined by monitoring the increase in absorbancy at 340 nm. The rate of increase in absorbancy was constant with time and proportional to the enzyme concentration. Unless indicated otherwise, all assays were run at 30 C. One unit of the enzyme was defined as the amount reducing 1 µmole $NADP^+$ per min. The molar absorbance for reduced $NADP^+$ (NADPH) is 6.2 x 10^3 M^{-1} cm^{-1}.

Partial purification of phosphotrehalase. All enzyme preparations were in standard 0.1 M tris(hydroxymethyl)aminomethane (tris)-hydrochloride buffer, pH 7.2, containing 1 mM dithiothreitol (DTT). The temperature was maintained at 0-4 C during purification.

Step. 1. Preparation of crude extract. *B. popilliae* was grown to late exponential phase in TY + 0.2% trehalose medium. Five liters of cells were harvested by centrifugation at 10,000 x g for 15 min, and washed twice with 5×10^{-2} M potassium phosphate buffer pH 7.2. The pellet was suspended in 10 ml of standard buffer. The cells were disrupted by ultrasonic oscillation as described previously (2). The cell debris was removed by centrifugation at 10,000 x g for 10 min. The supernatant fraction was referred to as the crude extract. Where indicated, the crude extract was dialyzed against standard buffer at 4 C for 8 h.

Step 2. Streptomycin sulfate precipitation. To 40 ml of crude extract, an equal volume of 5.0% streptomycin sulfate in standard buffer was added slowly with stirring, and the mixture stirred for an additional 60 min. The precipitate was removed by centrifuging at 40,000 x g for 30 min and discarded. The supernatant solution was made up to 100 ml with standard buffer.

Step 3. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant solution from step 2 to obtain 40% saturation. The precipitate was removed by centrifuging at

40,000 x g for 30 min and discarded. Additional ammonium sulfate was added to obtain 60% saturation, and the suspension centrifuged as above. The precipitate was dissolved in 14.5 ml of standard buffer and dialyzed overnight against 1 liter of standard buffer with two changes.

Step 4. DEAE-cellulose chromatography. A DEAE-cellulose column (2 x 27 cm) was equilibrated with standard buffer to the same pH and the same conductivity as the starting buffer. The dialyzed sample was then loaded on the column, and the column was washed with 160 ml of standard buffer. The protein was eluted with 300 ml of a continuous gradient from 0 to 0.2 M KCl in standard buffer. The flow rate of the column was 50 ml per h, and 4.0 ml was collected per fraction. Phosphotrehalase was eluted from the column at approximately 0.14 M KCl. Fractions containing phosphotrehalase activities were pooled and concentrated to 7.5 ml by ultrafiltration through a UM 10 Diaflo membrane (Amicon Corporation, Cambridge, Massachusetts). The concentrated preparation was dialyzed against 500 ml of standard buffer for 15 h with three changes.

Step 5. Sephadex G-200 chromatography. The sample obtained from step 4 was divided into two equal portions and each chromatographed on Sephadex G-200. A G-200 Sephadex column (2.3 x 37 cm) was equilibrated with standard buffer. The sample was applied to the column and eluted with standard buffer. The flow rate of the

column was 18 ml per h and 3 ml fractions were collected. The fractions containing phosphotrehalase activities were pooled and concentrated by ultrafiltration as described above. This solution was divided into 1.0 ml portions, sealed in ampules, and stored in liquid N_2 until used.

Isolation of phosphotrehalase negative mutants. *B. popilliae* strain 2309MC was grown to mid-exponential phase in TY + 0.2% trehalose medium and the cells harvested. The cells in 5×10^{-2} M potassium phosphate buffer pH 7.2 were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at a final concentration of 200 μg/ml for 90 min (approximately 2% survival). The suspension was centrifuged to remove NTG, and the pellet was resuspended in potassium phosphate buffer. Samples of the suspension were plated on TY + 0.5% trehalose (TYT) and TYG agar plates. The colonies which grew on TYG but not on TYT were picked and purified by restreaking on TYG. Growth of the potential mutants was tested in liquid media containing glucose and trehalose. The trehalose negative mutants were screened for ability to take up $[^{14}C]$trehalose. The mutants which did not grow on trehalose but did take up $[^{14}C]$trehalose were tentatively identified as phosphotrehalase negative mutants. The crude extracts of these mutants were then assayed for the presence of phosphotrehalase activity.

Paper chromatography. Sugars and sugar phosphates were separated by paper chromatography. Samples were spotted on either

Whatman No. 1 or 3 MM paper, and descending chromatograms were developed for 12 to 15 h. The two solvent systems used were: system A, butanol-pyridine-water (9:5:4); system B, ethyl acetate-water-glacial acetic acid-formic acid (18:4:3:1). Solvent system A was used to separate glucose (Rf 0.33) from trehalose (Rf 0.22) and sugar phosphates (Rf 0). System B separated trehalose phosphate (Rf 0.36) from glucose 6-phosphate and trehalose (Rf 0.45) and from glucose (Rf 0.54).

Uptake of $[^{14}C]$trehalose. Uptake as used in this paper refers to the amount of radioactivity accumulated in cells; and thus, would include both the sugar and metabolic products from it. Cells grown in TY + 0.2% trehalose medium to mid-exponential phase. The cells were harvested, washed twice with 5×10^{-2} M potassium phosphate buffer, pH 7.2, 110 µg/ml dry weight of cells, and 1×10^{-5} M $[^{14}C]$trehalose (2.6×10^{6} cpm/µmole). Where indicated, 1×10^{-2} M NaF was added. The reactions were run at 30 C. Samples of 1.0 ml each were taken at intervals, filtered through millipore filters (0.45 µm pore size), and washed with 5.0 ml of cold potassium phosphate buffer. The filter was dried at 60 C for 15 min, and placed in 5.0 ml of toluene based scintillation liquid to determine the radioactivity.

Miscellaneous. Protein was routinely determined by the method of Lowry *et al.* (16) using bovine serum albumin, fraction V, as standard. For determination of protein of the whole cells, the

cells were first extracted with hot (90 C) 5% trichloroacetic acid (TCA). The precipitate from the hot TCA extraction was then treated with 1 N sodium hydroxide and held at 40 C for 2 h. The preparation was then neutralized with 2 N hydrochloric acid, and protein was determined using the method of Lowry *et al.* (16).

All optical absorbancy measurements were made with a Gilford, model 2000 spectrophotometer. Radioactivity was determined with a Packard Tri-Carb, model 3320, using a toluene based scintillation fluid (0.01 g 1,4-tris- 2-(5-phenyloxazolyl) -benzene and 6 g 2,5-diphenyloxazole in 1 liter of toluene).

RESULTS

Repression of β-glucosidase synthesis by trehalose. β-Glucosidase in *Bacillus popilliae* 2309MC was found to be inducible by salicin. Activity of the enzyme in the induced culture was ten fold higher than that of the non-induced. The plot of the β-glucosidase activity as a function of growth remained linear after the culture was fully induced.

Trehalose, but not glucose, was found to strongly repress the synthesis of β-glucosidase (Fig. 1). In the presence of glucose and salicin, β-glucosidase synthesis was delayed but the differential rate of synthesis only decreased slightly as compared to the culture without glucose added. However, when trehalose was added together with salicin, the differential rate of β-glucosidase synthesis decreased by 88% as compared to the culture with salicin alone. In other experiments, it was noted that trehalose also repressed β-glucosidase when trehalose was added after the culture was fully induced. This repression was not affected by the addition of 5×10^{-3} M cyclic adenosine 3',5'-monophosphate to the medium. The results of these experiments and those of previous studies (2) indicated that trehalose was more readily utilized by *B. popilliae* than was glucose.

Growth and oxidation of various concentrations of glucose trehalose by *B. popilliae* 2309MC. The rate and extent of growth of *B. popilliae* 2309MC varied greatly when the concentration of

glucose added to the medium was varied from 2.8×10^{-3} M to 2.8×10^{-2} M. The maximal rate of growth was obtained with glucose at a concentration of 2.8×10^{-2} M with a growth rate of 0.25 generation h^{-1}. However, a growth rate of 0.4 generation h^{-1} was obtained when only 1.3×10^{-3} M trehalose was used as the carbon source.

It has been previously reported that *B. popilliae* 2309MC grown with either glucose or trehalose as the carbon source oxidized trehalose about twice as fast as glucose when the two sugars were present at the same concentration (0.01 M) (2). When we varied the concentration of the two sugars, it was observed that the rate of O_2 uptake by resting cells with glucose as substrate increased as the glucose concentration increased up to a maximum uptake rate at 5×10^{-2} M glucose (Table 1). In contrast, the rate of uptake was maximal with only $5 \times 10^{-2}4$ M trehalose. These data suggest that the difference in growth response to the two sugars might be due to differences in their manner of uptake by the cells.

Sodium fluoride is known to inhibit the enzyme enolase in the Embden-Meyerhof-Parnas pathway (13), and previous data (25) showed that it inhibited the rate of glucose oxidation by *B. popilliae* by 50%. When this inhibitor was tested on trehalose oxidation by resting cells of *B. popilliae* grown in trehalose, the oxidation was completely inhibited (Fig. 2). Glucose oxidation was only inhibited by 50%, which is the same level of inhibition

reported previously with a lower concentration of glucose (25). Concentrations of trehalose and glucose were adjusted in this experiment so that maximal rates of O_2 uptake was obtained in the controls. The results suggested that the uptake of trehalose was energy dependent and dependent on phosphoenolpyruvate (PEP).

Inhibition of $[^{14}C]$trehalose uptake by NaF. In order to clarify the complete inhibition of trehalose oxidation by NaF, uptake experiments using $[^{14}C]$trehalose with and without NaF added were studied. As illustrated in Fig. 3, the uptake of $[^{14}C]$trehalose by resting cells of *B. popilliae* 2309MC was almost completely inhibited by 1×10^{-2} M NaF. In the absence of the inhibitor, the uptake of $[^{14}C]$trehalose was found to have saturation kinetics (Fig. 4).

Phosphotrehalase in *B. popilliae* 2309MC. A number of unsuccessful attempts were made to detect activity of an enzyme(s) in the crude extract of *B. popilliae* 2309MC which would cleave trehalose. Various methods were used to detect trehalase (7,9) and trehalose phosphorylase (1), but no cleavage of trehalose was detected ($<$0.0005 μmoles glucose formed per min per mg protein). Therefore, it appeared likely that this organism did not contain an enzyme that attacked trehalose *per se*. However, these extracts did contain an enzyme which cleaved T6P into equimolar amounts of glucose and glucose 6-phosphate (G6P). This enzyme, phosphotrehalase,

was partially purified (34 fold) using the purification steps outlined in Materials and Methods and summarized in Table 2. The kinetic studies of the partially purified phosphotrehalase indicated that the enzyme had an apparent K_m for T6P of 1.8×10^{-3} M. The pH optimum of the enzyme was found to be between 6.5 and 7.0. The buffer used in the assay system affected the activity of the enzyme; the activities in tris-HCl and in potassium phosphate buffers were only about 1% and 60% respectively of the activity in glycylglycine buffer at the same pH. As shown in Table 3, the partially purified preparation produced equimolar amounts of glucose and G6P from T6P.

Characterization of phosphotrehalase negative mutant of *B. popilliae* 2309MC. The above data implied that T6P was probably an intermediate in the metabolism of trehalose by *B. popilliae*. To confirm that T6P was a true intermediate, and that phosphotrehalase was an essential enzyme in trehalose metabolism, a *B. popilliae* mutant lacking the phosphotrehalase was isolated. The isolation procedure is described in the Materials and Methods section.

The mutant, 2309MC100, did not grow with trehalose as the carbon source but it did take up [^{14}C]trehalose. Also, the crude extract of 2309MC100 did not contain detectable phosphotrehalase activity ($\ll$0.0002 µmole NADPH formed per min per mg protein by the assay procedure). The mutant behaved similarly to the wild type with respect to carbohydrates other than trehalose (Table 4).

In addition, it was similar to the wild type culture in that it was catalase negative, a unique characteristic of this *Bacillus*.

Not only did the mutant fail to grow in TY medium + 0.2% trehalose, but also it did not grow in this medium containing both 0.2% trehalose and 0.5% glucose. To show the inhibition of trehalose on growth of the mutant, a culture of 2309MC100 was allowed to grow in TY medium + 0.5% glucose to mid-exponential phase, and 0.2% trehalose was then added to the culture. As shown in Fig. 5, the growth of the mutant stopped shortly after the addition of trehalose. Addition of trehalose to the wild type culture growing in glucose did not affect growth of the organism. Trehalose was not found to interfere with uptake of $[^{14}C]$glucose in wild type cells, even when the concentration of unlabelled trehalose was four times greater than that of the $[^{14}C]$glucose. Perhaps, the T6P which was found to accumulate in this mutant was inhibitory. Previous studies have demonstrated the inhibition of microbial growth by the accumulation of sugar phosphates from L-arabinose (9), L-rhamnose (7,8), and D-galactose (20).

Identification of T6P accumulation by the phosphotrehalose negative mutant of *B. popilliae*. Since trehalose was found to be metabolized readily in wild type cells, it appeared likely that the T6P was hydrolyzed as rapidly as it was formed. Therefore, we looked for the accumulation of T6P in the phosphotrehalase negative mutant. A resting cell suspension of the mutant 2309MC100 was incubated with

[^{14}C]trehalose and the cells extracted with 5% cold TCA. The TCA extract was subjected to chromatography with two different solvent systems, and the radioactive spots in both solvent systems were found to migrate to the same location (same Rf's) as authentic T6P. After treating the TCA extract with alkaline phosphatase, the radioactivity appeared at the location corresponding to free trehalose. Furthermore, when it was treated with partially purified phosphotrehalase, the radioactivity appeared equally in both the glucose and G6P spots. These data are summarized in Table 5.

The same types of assays indicated that a crude cell extract of mutant 2309MC100 also accumulated [^{14}C]T6P in reaction mixtures containing [^{14}C]trehalose, $MgCl_2$, and PEP. Furthermore, the production of T6P from trehalose was indicated by a coupled enzymatic assay (Table 6). In the complete assay mixture with the phosphotrehalase, hexokinase, ATP, G6P dehydrogenase, and $NADP^+$, over 27 nmoles of NADPH were formed, while only 5.5 nmoles and 17.8 nmoles were formed on omission of phosphotrehalase and the hexokinase system respectively. If one corrects for the amount of reduction in the absence of phosphotrehalase, which may have been due to some side reaction(s) in the crude extract, the corrected amount of $NADP^+$ reduced in the complete assay system (Rx. 1, 22 nmoles) was approximately double that reduced in the absence of added hexokinase and ATP (Rx. 3, 12.3 nmoles). This would be expected since the phosphotrehalase cleaves T6P to glucose and G6P.

Effect of PEP and other phosphorylated compounds on the accumulation of sugar phosphates from trehalose. Since all the previous data indicated that the phosphorylation of trehalose might well occur during transport, we examined the effect of a number of possible phosphoryl donors on the formation of sugar phosphate from [^{14}C]trehalose by a crude cell extract (Table 7). Of the compounds tested, only PEP was found to have a significant effect, although there appeared to be a slight stimulation by ATP and guanosine 5'-triphosphate.

Although we have not studied this system in detail, preliminary experiments have demonstrated that the enzyme(s) responsible for the phosphorylation of trehalose is associated primarily with the particulate fraction of cell extracts of *B. popilliae*. Washed cell particles prepared as described by McKay *et al.* (18) and the soluble fraction of the extract were assayed by the procedure outlined in Table 7. The amount of sugar phosphate accumulated from [^{14}C]trehalose by the washed particles was about 60% of the amount produced with the combined particulate and soluble fractions, while the activity of the soluble fraction was only 10 to 20% that of the particles alone.

Assays of various strains of *B. popilliae* for the production of sugar phosphate from trehalose and for phosphotrehalase. A number of strains of *B. popilliae* in our collection including a culture recently initiated from spores harvested from larval hemolymph were

tested for the unique activities observed with 2309MC. All of these cultures were catalase negative, which is characteristic of this *Bacillus*. Cell extracts of all strains were shown to produce sugar phosphate from [^{14}C]trehalose and to have phosphotrehalase. The phosphotrehalase activities were about the same in all the extracts, but the amount of sugar phosphate accumulated varied considerably.

DISCUSSION

B. popilliae is well adapted to the utilization of trehalose which is the principal carbon source available in its natural host, Japanese beetle larvae. The utilization of this disaccharide is constitutive in this organism (2). The comparative rates of growth were about 2x higher in the presence of trehalose than with glucose even when glucose was present in a much higher concentration than the disaccharide. The respiration rate of *B. popilliae* with 5×10^{-4} M trehalose as substrate was as high as that attained with much higher levels of glucose. Also, strong repression of the synthesis of an inducible enzyme was evident when trehalose was present along with the inducer while glucose had only a slight effect, if any. Thus, it appears likely that trehalose is the preferred energy source for this organism.

The hydrolysis of trehalose by *B. popilliae* is particularly interesting. No trehalase (10,12) or trehalose phosphorylase activity (1) such as reported in other organisms were detected. Rather, all of the data indicate that trehalose is probably taken up by the cell via a PEP:sugar phosphotransferase system forming T6P; and the T6P is hydrolyzed by a phosphotrehalase. The evidence for a PEP:sugar phosphotransferase system is as follows: (a) PEP-dependent formation of T6P by crude cell extracts and by the particulate fraction of cell extracts, (b) accumulation of T6P in a phosphotrehalase negative mutant, (c) complete inhibition by

1×10^{-2} M NaF of [^{14}C]trehalose uptake by cells, and of respiration with trehalose as substrate, and (d) the failure of ATP to significantly stimulate formation of sugar phosphates from trehalose by cell extracts. Glucose did not appear to be transported by the same system, since there was only a 50% inhibition of respiration by NaF with glucose as substrate. Neither was there any inhibition of [^{14}C]trehalase uptake by high concentration of glucose.

The PEP:glucose phosphotransferase system is widely distributed in bacteria including members of the genus *Bacillus* (29). The involvement of a similar system in the transport of lactose is also well established in *Staphylococcus aureus* (11,14,15,30,31,32) and in *Streptococcus lactis* (18,19). Therefore, it would not be unexpected to find such a transport mechanism for other disaccharides. Although phosphorylated intermediates are involved in the metabolism of the disaccharides cellobiose (21,22,23) and gentibiose (24) in *Aerobacter aerogenes*, the phosphorylation is catalyzed by a soluble ATP-dependent kinase. No significant kinase activity was observed in these studies. Most of the trehalose phosphorylating activity was associated with the particulate fraction of the extracts of cells of *B. popilliae*.

The cleavage enzyme for which we propose the trivial name phosphotrehalase is a new enzyme which has not been observed previously. The failure of a mutant deficient in this enzyme to metabolize trehalose; the presence of the enzyme in all strains of

B. popilliae studied; and the absence of a trehalase or trehalose phosphorylase certainly indicates that the metabolism of trehalose by *B. popilliae* involves an initial phosphorylation before cleavage.

The significance of these findings with respect to the ability of *B. popilliae* to sporulate in the hemolymph of Japanese beetle larvae is not clear. High concentrations (0.2% of either glucose or trehalose inhibit sporulation of oligosporogenous strains of this organism *in vitro* (4,27), although trehalose is reported to be less inhibitory than glucose (27). The concentration of trehalose in the hemolymph of the larvae is higher than 0.2% throughout sporulation. It has been proposed (4,26) that the trehalose may be mostly in an unavailable form in the larvae. This should be investigated more thoroughly.

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TABLE 1. *Rate of oxygen uptake by B. popilliae with various concentrations of glucose and trehalose as substrates*[a]

Substrate concentration (M)		µl O_2 per min per mg dry wt.
Glucose:	2.0×10^{-3}	0.201
	5.0×10^{-3}	0.406
	1.0×10^{-2}	0.602
	2.0×10^{-2}	0.738
	5.0×10^{-2}	0.820
	1.0×10^{-1}	0.840
	2.0×10^{-1}	0.840
Trehalose:	5.0×10^{-4}	0.850
	1.0×10^{-3}	0.850
	5.0×10^{-3}	0.850

[a]Oxygen uptake was measured in a Warburg apparatus equilibrated at 30 C. The uptake rate at each substrate concentration was calculated from linear plots of oxygen uptake vs. time. All the values were corrected for the endogenous oxygen uptake which was less than 0.05 µl O_2 per min per mg dry wt. The cells used were grown in the TY basal medium plus 0.2% trehalose to exponential phase, harvested by centrifugation, and washed twice with 5×10^{-2} M potassium buffer, pH 7.2. Each Warburg flask contained 8.49 mg (dry weight) of cells.

TABLE 2. *Partial purification of phosphotrehalase*[a]

Step	Preparation	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
1.	Crude extract	40	1260	100	0.080
2.	Streptomycin sulfate	100	1300	101	0.075
3.	Ammonium sulfate (40% to 60%)	14.5	378	156	0.412
4.	DEAE-cellulose	8.0	136	94	0.688
5.	Sephadex G-200	7.5	30	85	2.83

[a]See Materials and Methods section for detailed procedures.

TABLE 3. *Stoichiometry for cleavage of trehalose-6-phosphate phosphotrehalase*[a]

Trehalose-6-phosphate (nmoles)[b]	Products (nmoles)	
	Glucose	Glucose-6-phosphate
3.2	3.2	3.4
6.4	7.1	7.0
12.8	14.1	15.4
19.2	22.9	20.1

[a]Each reaction mixture (0.25 ml total volume) contained 17 μmoles glycylglycine buffer (pH 7.5), 1.7 μmoles $MgCl_2$, 0.85 umole adenosine-5'-triphosphate (ATP) 0.17 μmole $NADP^+$, the indicated amount of trehalose-6-phosphate, partially purified phosphotrehalase (40 μg protein), and excesses of hexokinase and glucose-6-phosphate dehydrogenase. Reactions were incubated at 30 C. The amount of glucose and glucose-6-phosphate produced were determined by calculating the amount of NADPH formed. The reactions were first incubated without hexokinase and ATP, and the maximal absorbancy at 340 nm was determined. This value represented the amount of glucose-6-phosphate formed. Then, hexokinase and ATP were added and maximal absorbancy at 340 nm was again measured. The latter measurement represented the total amount of glucose and

glucose-6-phosphate formed from trehalose-6-phosphate. The changes in volume upon addition of hexokinase and ATP were corrected for. Control reactions without added phosphotrehalase, or without trehalose-6-phosphate showed no NADPH formation.

[b]Calculated on the basis of 91% purity of the trehalose-6-phosphate as determined by the anthrone reaction (34) for total carbohydrate using trehalose as the standard.

TABLE 4. *Growth response of B. popilliae 2309MC and the phosphotrehalase negative mutant (2309MC100) to various carbon sources*[a]

Carbohydrates (0.5%)	2309MC (Generation/hour)	2309MC100 (Generation/hour)
None	No growth	No growth
Trehalose	0.400	No growth
D-Glucose	0.308	0.250
Maltose	0.133	0.129
Salicin	0.133	0.167
Methyl-α-D-glucoside	0.100	0.154
D-Fructose	0.333	0.200
Sucrose	No growth	No growth
Cellobiose	No growth	No growth
D-Galactose	No growth	No growth
D-Ribose	No growth	No growth
D-Xylose	No growth	No growth
L-Arabinose	No growth	No growth

[a]All the carbohydrates were filter sterilized before addition to the basal TY medium. The inoculum was grown in a glucose medium overnight, centrifuged, and the cells resuspended in basal medium before inoculation. Growth rates were calculated from the exponential phase.

TABLE 5. *Identification of trehalose-6-phosphate as an accumulated product in mutant 2309MC100*[a]

Treatments	Glucose (cpm)	Trehalose (cpm)	Glucose -6- phosphate (cpm)	Trehalose -6- phosphate (cpm)
No treatment	0	0	116	4492
Boiled phosphotrehalase	0	0	78	4571
Alkaline phosphatase	208	4476	0	8
Phosphotrehalase	1914	0	1586	31

[a] *B. popilliae* mutant 2309MC100 was grown to mid-exponential phase in TY medium plus 0.5% glucose. The cells were harvested and washed twice with 5×10^{-2} M potassium phosphate buffer, pH 7.2. The reaction mixtures of 0.4 ml contained 20 µmole potassium phosphate buffer, pH 7.2, 2.38 mg (dry weight) of cells, and 0.2 µmole [^{14}C]trehalose with a specific activity of 2.01×10^{6} cpm/µmole. The reaction mixtures were incubated at 30 C for one hour. The cells were centrifuged, and the pellet extracted twice with 2.0 ml of 5% trichloroacetic acid (TCA) at 0 C for 30 min. The TCA was removed from the combined supernatant solutions by extracting three times with an equal volume of ether. The resulting solution was evaporated under reduced pressure at 40 C to a small volume. Sugar phosphates were separated from any free sugar by preparative paper chromatography using solvent system A. After elution from the paper with water, the

accumulated sugar phosphate was chromatographed in two different solvent systems (A and B) along with the products from alkaline phosphatase treated sub-samples. Glucose, glucose-6-phosphate, trehalose, and trehalose-6-phosphate were co-chromatographed as standards. The chromatograms were dried and radioactive spots located using a Packard model 7201 radiochromatogram scanner. The standard sugars and sugar phosphates were made visible by alkaline silver nitrate method (35). The areas corresponding to the peak activities were compared to the standards and cut out to quantitate the amount of radioactivity in toluene based scintillation liquid. The data were corrected for background values.

TABLE 6. *Production of trehalose-6-phosphate by a crude extract of cells of the mutant 2309MC100*[a]

Omissions from assay reaction mixtures	μmole NADPH produced
None	27.5
Phosphotrehalase	5.5
Hexokinase and ATP	17.8
Phosphotrehalase, hexokinase, ATP, and G6P dehydrogenase	0.9

[a]For the production of T6P, reaction mixtures contained 0.5 μmole $MgCl_2$, 1.0 μmole PEP, 1.0 μmole trehalose, and dialyzed crude extract of cells (1.8 mg protein) in a total volume of 0.1 ml. After 30 min at 30 C, reactions were stopped by boiling for 5 min, and the precipitate centrifuged down. A control reaction with boiled enzyme (crude extract) was run concurrently. The supernatant solutions were assayed for T6P as follows. The complete reaction mixture contained 0.03 ml of the test solution, 17 μmoles glycylglycine buffer, pH 7.5, 1.7 μmole $MgCl_2$, 0.85 μmole ATP, 0.17 μmole $NADP^+$, and excesses of G6P dehydrogenase, hexokinase, and phosphotrehalase. The final volume was 0.25 ml. Omissions were as indicated in the table. The μmoles NADPH formed were calculated from the change in the absorbancy at 340 nm. The values were all

corrected for the amount of NADPH formed under the same conditions by supernatant solutions of the control reaction mixture referred to above.

TABLE 7. *Effect of potential phosphoryl donors on the production of sugar phosphates from trehalose by cell extracts of B. popilliae 2309MC*[a]

Phosphoryl donors (1×10^{-2} M)	pmoles/mg protein/min.[b]
No phosphoryl donor	40
PEP	470
ATP	57
Guanosine 5'-triphosphate	69
Glucose 6-phosphate	28
Acetyl phosphate	30
Carbamyl phosphate	27

[a]The crude cell extract was dialyzed for 8 hours at 4 C in 0.1 M Tris, 1 mM DTT buffer, pH 7.2. Each reaction with a total volume of 0.1 ml contained 6 µmoles of tris-HCl buffer pH 7.2, 0.5 µmole $MgCl_2$, 1 µmole of the tested compounds, 2.2 mg cell extract protein, and 1 µmole $[^{14}C]$trehalose with a specific activity of 2.2×10^5 cpm/µmole. The reactions were started by adding the substrate and incubated at 30 C for 30 min. They were stopped by boiling for 5 min. The precipitate was spun down and 10 µliters of the supernatant solution was spotted on Whatman No. 1 filter paper and chromatographed with solvent system A. The spots corresponding to

sugar phosphate were cut out and the radioactivity determined in toluene based scintillation fluid. All values were corrected for background.

[b] Calculated equivalents of [^{14}C]trehalose.

TABLE 8. *Activities of phosphotrehalase and the production of sugar phosphates from trehalose by cell extracts of various strains of B. popilliae*

Strains[a]	Phosphotrehalase[b] (unit/mg protein)	Sugar phosphates[c] (pmole/mg protein/min)
2309MC	.080	470
2309S	.091	930
2309N	.069	490
923B	.051	210
L.S.	.082	117
2309M	.062	283
54	.100	90

[a]Strains 2309MC, 2309S, 2309N, and 923B were maintained as vegetative cells on slants and cultivated in liquid media prior to use. Strain L.S. was obtained from the Northern Utilization Research and Development Division, Peoria, Illinois, as dried spores. Strains 2309M and 54 were maintained as spores on a sporulation medium (17). Strains L.S., 2309M, and 54 were activated according to the method of Splittstoesser and Farkas (33) before cultivating in liquid medium. All strains were grown in TY + 0.2% trehalose to late exponential phase. Crude cell extracts were prepared as indicated in the Material and Methods section.

[b]Phosphotrehalase was assayed as described in the Material and Methods section.

[c]Production of sugar phosphates was determined as described in Table 7. Calculated equivalents of [^{14}C]trehalose.

FIG. 1. *Effect of trehalose and glucose on β-glucosidase synthesis. Three 200 ml cultures in 500 ml Erlenmeyer flasks of B. popilliae were grown on TY medium with the following sugars added:* ● *, 0.2% salicin;* Δ *, 0.2% salicin plus 1.0% glucose;* ■*, 0.2% salicin plus 0.5% trehalose. Ten ml samples were taken from each flask at various intervals, 5.0 ml were used to determine protein, 4.0 ml to measure β-glucosidase activity, and 1.0 ml to follow growth of the culture by measuring the* OD_{620}*. Methods for the determination of protein from whole cells and for the β-glucosidase assay were outlined in the Materials and Methods section.*

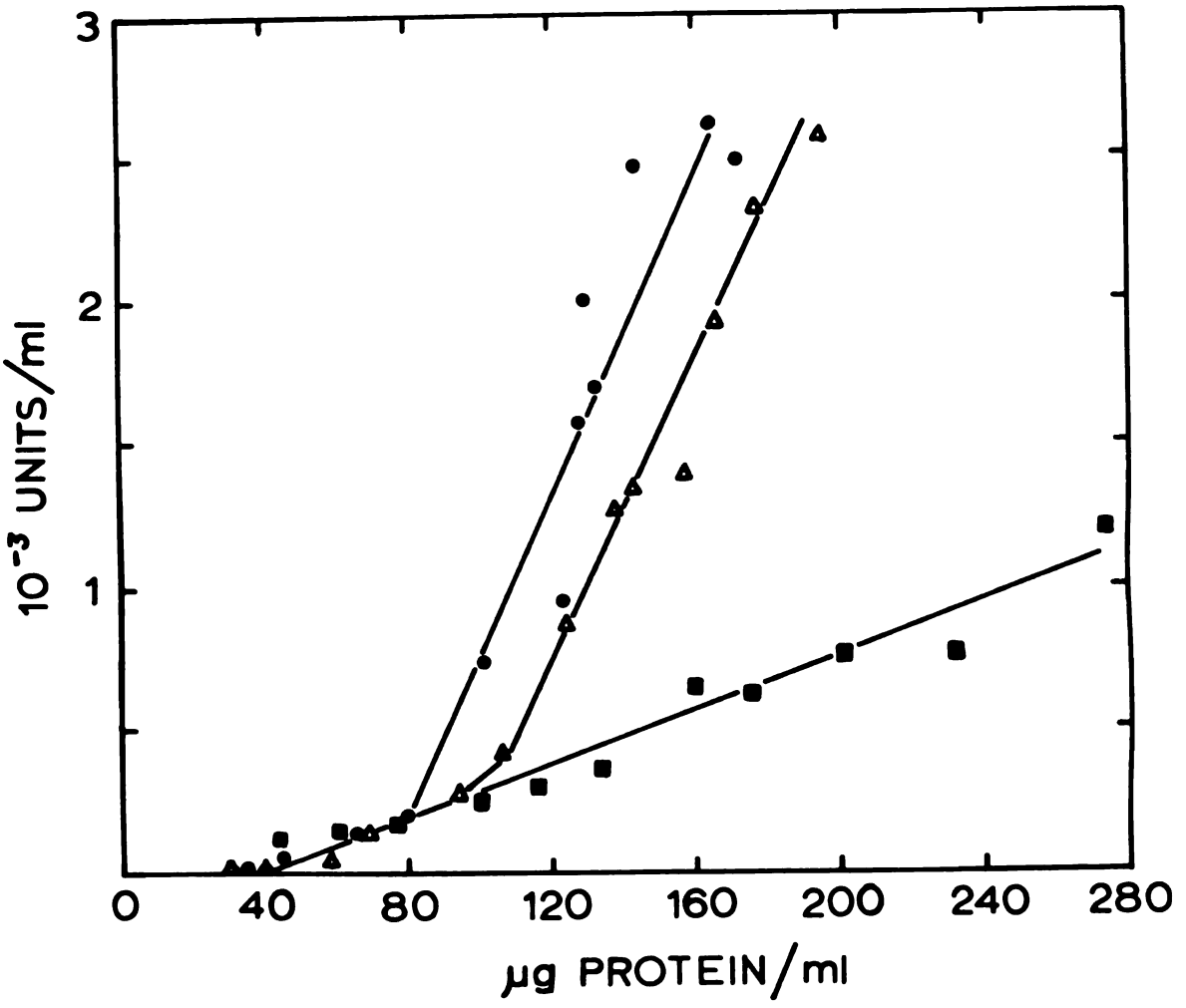

FIG. 1

FIG. 2. *Sodium fluoride inhibition of the oxidation of glucose and trehalose by B. popilliae 2309'1C. The detailed procedures were the same as those outlined in Table 1 except that 1 x 10^{-2} M NaF was added to the indicated Warburg flasks (closed symbols). Final concentrations of glucose (Δ, ▲) and trehalose (○, ●) were 5 x 10^{-2} M and 5 x 10^{-3} M respectively. All the values were corrected for the endogenous oxygen uptake which was less than 0.15 µl O_2 per min. Each Warburg flask contained 5.6 mg dry weight of trehalose grown cells.*

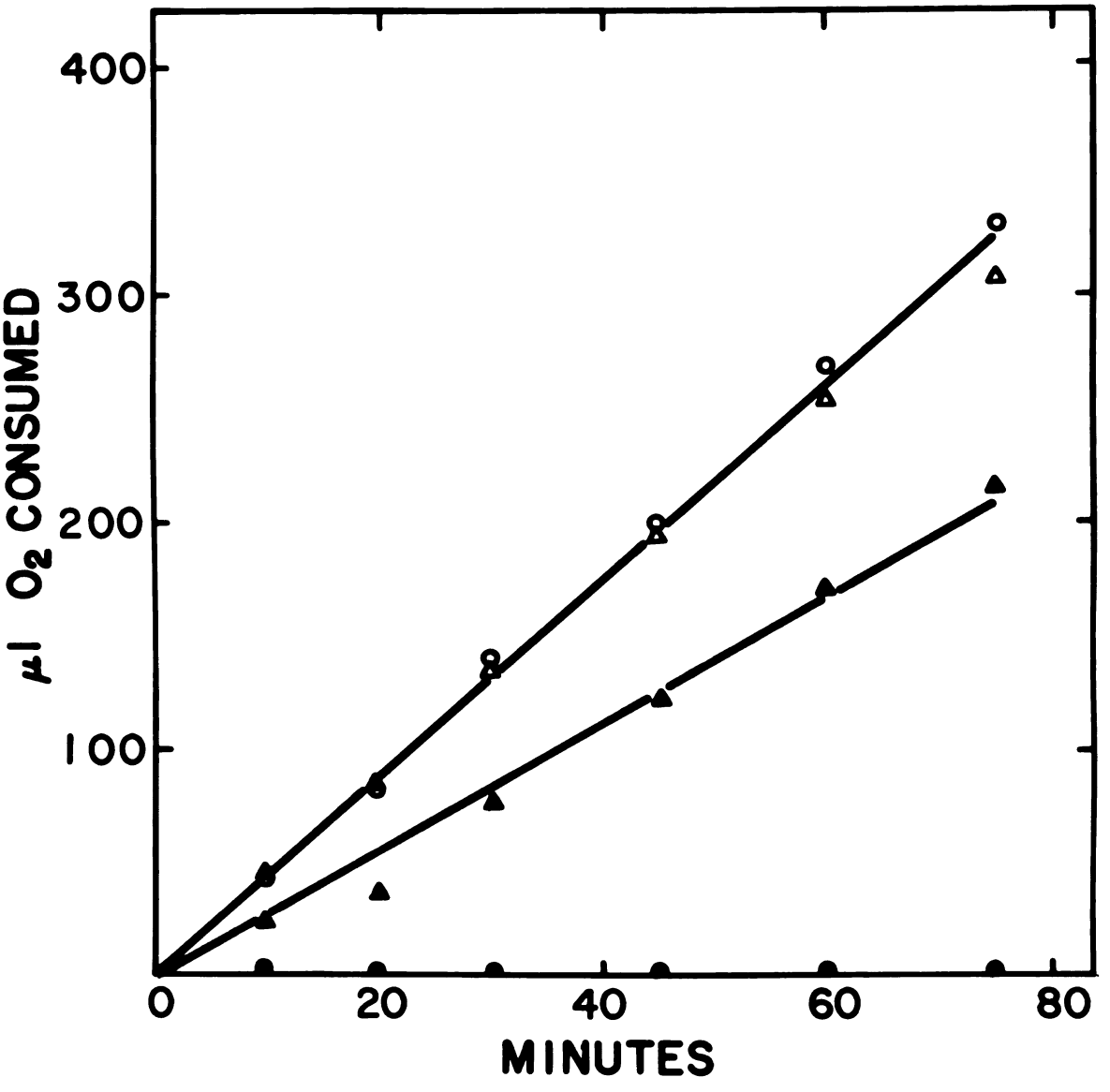

FIG. 2

FIG. 3. *Sodium fluoride inhibition of [^{14}C]trehalose uptake by B. popilliae 2309MC. The detailed procedures were described in the Materials and Methods section. Symbols:* ●, *with NaF;* ○, *without NaF.*

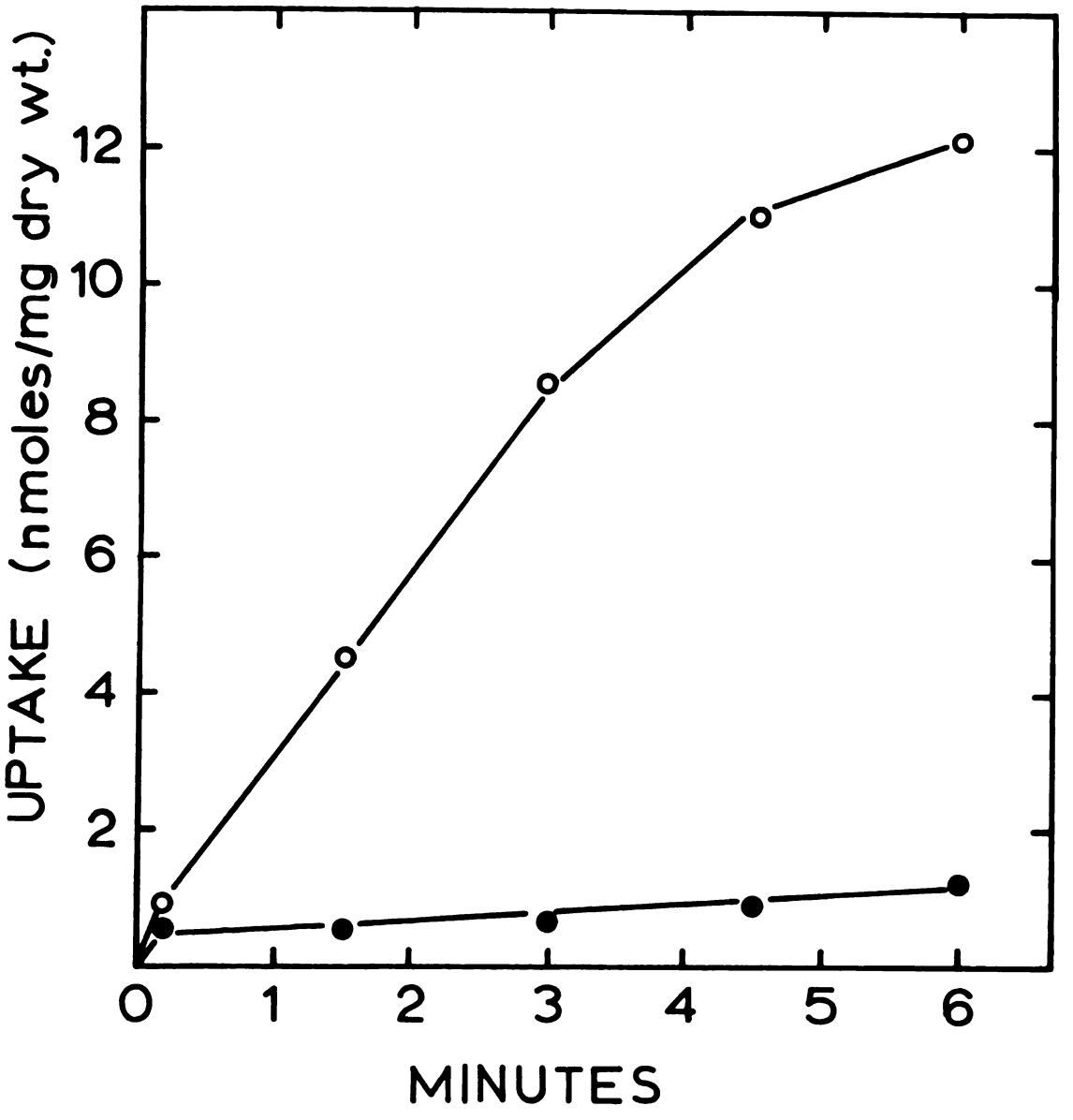

FIG. 3

FIG. 4. *Effect of concentration on* [^{14}C]*trehalose uptake by B. popilliae 2309MC. The general procedure was the same as outlined in Fig. 3 except that no NaF was added, and the trehalose concentration was varied as indicated. Each reaction mixture had a final volume of 2.0 ml and was incubated at 30 C for 1 min.*

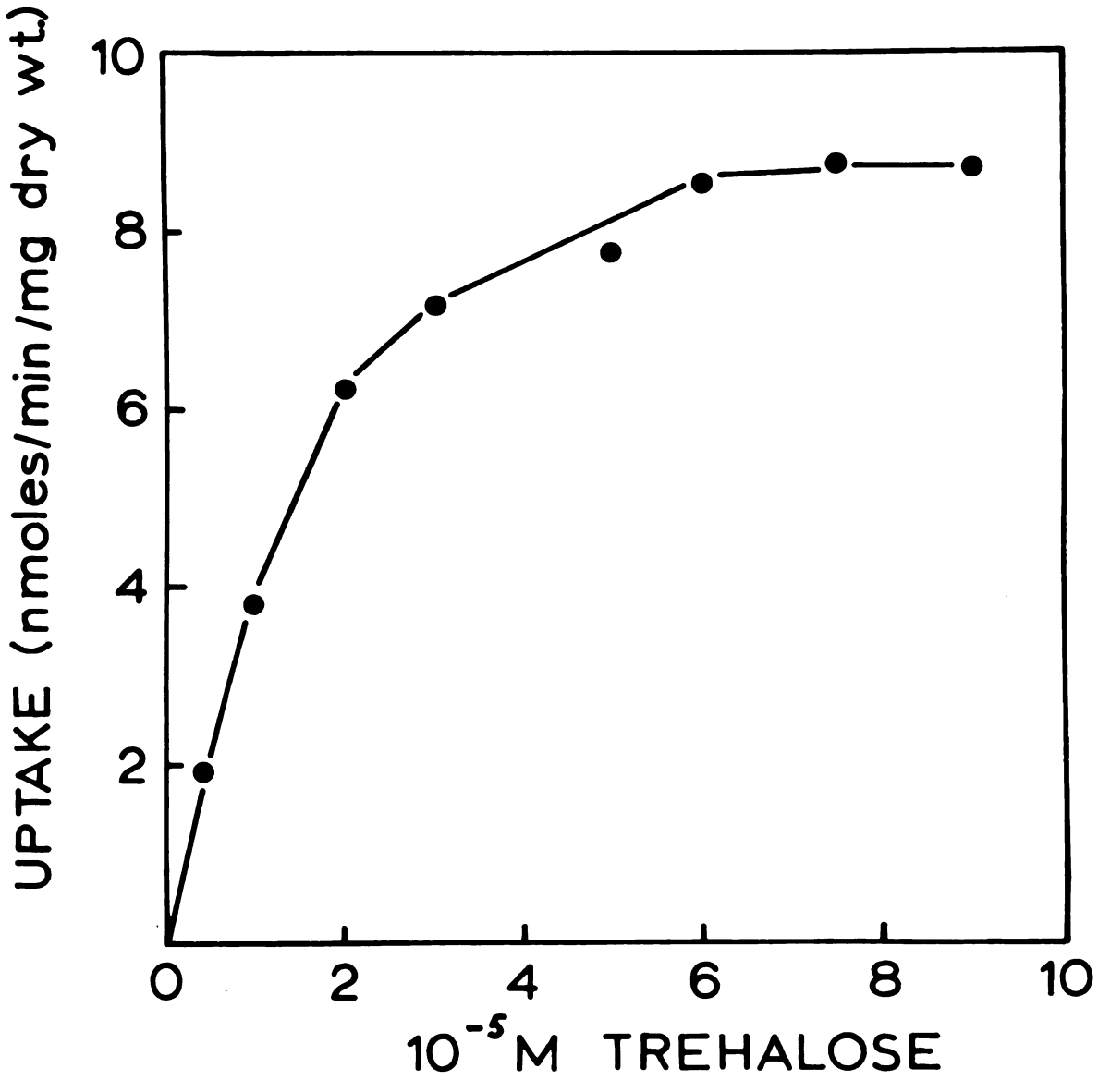

FIG. 4

FIG. 5. *Trehalose inhibition of the growth of mutant 2309MC100. Filter sterilized carbon sources were added to TY medium as follows;* ○, *trehalose (0.2%);* ●, *glucose (0.5%);* ⊖, *trehalose was added at the time indicated (arrow) to the cells growing in the glucose medium.*

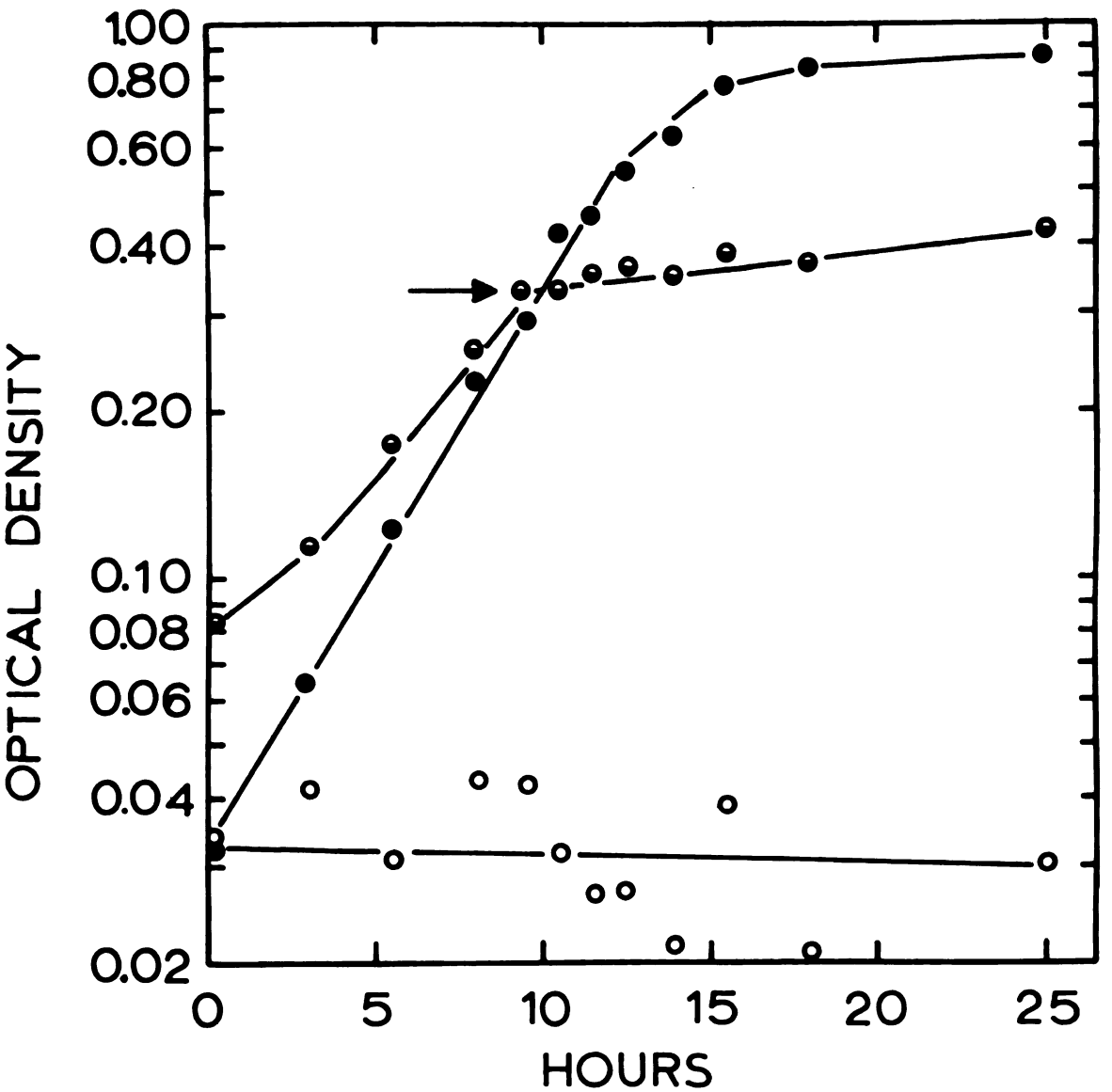
OPTICAL DENSITY
1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
0
5
10
15
20
25
HOURS

FIG. 5

APPENDIX

STUDIES OF THE EFFECT OF CYCLIC ADENOSINE-3',5'-MONOPHOSPHATE ON THE REPRESSION OF β-GLUCOSIDASE IN *BACILLUS POPILLIAE*

By

Amaret Bhumiratana and Ralph N. Costilow

INTRODUCTION

In view of the potential of *Bacillus popilliae* for biological control of the Japanese beetle, many unsuccessful attempts have been made to obtain good spore yields of the organism *in vitro*. As discussed in the Literature Review, catabolite repression may be an important mechanism in controlling sporulation. Therefore, it appeared possible that cyclic adenosine-3',5'-monophosphate (cAMP) might be involved in sporulation.

Attempts to induce sporulation of *B. popilliae* by adding cAMP to the medium were unsuccessful (3). We decided to see if cAMP had any effect on catabolite repression in *B. popilliae*. β-Glucosidase is induced in *B. popilliae* by salicin, saligenin-β-D-glucopyranoside; and the induction is strongly repressed by trehalose. Therefore, this system was utilized for our studies with cAMP.

MATERIALS AND METHODS

Cultures and cultural methods. *Bacillus popilliae* 2309MC was used in all the experiments. The strain used, 2309MC was derived from strain 2309M (7,10). The cultures were maintained on agar slants as outlined by McKay *et al.* (7). The basal medium (TY) used for all of these experiments contained 1.5% trypticase, 0.5% yeast extract, and 0.6% K_2HPO_4. This medium was dispensed in 200 ml volume into 500 ml Erlenmeyer flasks. The carbohydrates were filter sterilized separately and added to the basal medium at the final concentration of 0.5%. All cultures were incubated at 30 C on a rotary shaker.

Growth studies. Growth was measured by determining the optical density (O.D.) at 620 nm with a Gilford spectrophotometer model 2000 using the growth medium clarified by centrifugation as a blank.

β-Glucosidase assay. β-Glucosidase activity was assayed according to the method of Duerkson and Halvorson (4). The standard assay contained 0.8 ml of cell suspension in 5×10^{-2} M phosphate buffer, pH 7.2, and 0.2 ml of 5×10^{-2} M p-nitro-phenyl-β-D-glucopyranoside (PNPβG). The protein content of the cell suspension used ranged from 125 µg/ml to 250 µg/ml. The reaction mixtures were incubated at 30 C for 20 min and stopped by adding 0.5 ml of 1.0 M Na_2CO_3. The reaction mixtures were centrifuged and the optical density was measured at

400 nm using a Gilford spectrophotometer model 2000. One unit of β-glucosidase was defined as the amount of enzyme which produced 1 μmole of p-nitro-phenol per min. The absorbance of p-nitro-phenol under these conditions is 1.8×10^4 M^{-1} cm^{-1}.

Protein of the cell suspension was determined by the method of Lowry *et al.* (6) after prior digestion with 1 N NaOH for 2 h at 40 C (1).

Uptake of $[^3H]$cAMP by *B. popilliae* 2309MC. *B. popilliae* was grown in TY + salicin medium to mid-exponential phase, and the culture used for uptake experiments. Each reaction mixture contained 0.2 ml of $[^3H]$cAMP and 1.8 ml of the culture. The reaction mixtures were incubated at 30 C for 1 min and they were filtered through Millipore filter (0.45 μm pore size), washed with 5.0 ml of cold 5×10^{-2} M phosphate buffer, pH 7.2, and dried at 60 C. The dried Millipore filter was placed in 5.0 ml of toluene based scintillation liquid (1), and the radioactivity determined using a Packard liquid scintillation spectrometer model 3320.

RESULTS

Validity of β-glucosidase assay system. β-Glucosidase was assayed by measuring the ability of the enzyme to cleave p-nitro-phenyl-β-glucopyranoside (PNPβG). The release of the chromogenic group, p-nitro-phenol, from PNPβG was measured spectrometrically at 400 nm. The reaction was found to be linear over 60 min period (Fig. 1). The β-glucosidase activity was directly proportional to the amount of cell protein in the range of 25 to 250 μg/ml (Fig. 2). A Lineweaver-Burk plot (Fig. 3) indicated the apparent K_m of the enzyme was 2.2×10^{-3} M for PNPβG.

Induction of β-glucosidase in *B. popilliae*. The data in Table 1 indicate that β-glucosidase is an inducible enzyme. Very low activity of the enzyme was detected from cells grown in TY + 0.5% glucose medium, whereas high activity was observed from cells grown in TY + 0.5% salicin. When *B. popilliae* was induced for β-glucosidase with 0.5% salicin and the time course of induction was followed, the culture was fully induced after 2-3 h of incubation. The rate of β-glucosidase synthesis became linear after an initial lag period (Fig. 4). The β-glucosidase activity was not detected when salicin was not added.

Repression of β-glucosidase synthesis by trehalose. Data presented earlier in this thesis, demonstrated that the synthesis of β-glucosidase was repressed by trehalose. When trehalose was added to a

culture which had been fully induced to synthesize β-glucosidase, both transient and catabolite repression could be demonstrated (Fig. 5A). However, cAMP added exogenously to the culture at 5×10^{-3} M did not overcome either the transient or catabolite repression (Fig. 5B). Also, 5×10^{-3} M cAMP did not stimulate the rate of β-glucosidase synthesis.

Uptake of $[^3H]$cAMP by *B. popilliae* 2309MC. It was possible that the failure of cAMP to overcome repression of β-glucosidase synthesis by trehalose was due to the inability of cAMP to pass through the cell barrier. Therefore, uptake of $[^3H]$cAMP was studied at various concentrations of $[^3H]$cAMP. The uptake of $[^3H]$cAMP was found to be linear with concentrations of cAMP up to 5×10^{-3} M (Fig. 6).

DISCUSSION

There was no measurable effect of adding cAMP to a growth medium on either sporulation (3) or on the repression of β-glucosidase synthesis in *B. popilliae*. While the cells did take up [^{3}H]cAMP, the data do not rule out the possibility that this compound is involved in the regulation of these processes, since we did not investigate the fate of the cAMP taken up by the cells. It may have been rapidly converted to other forms. There have been few reported studies on the role of cAMP in *Bacillus* species. Ide (5) failed to find either adenyl cyclase or phosphodiesterase in four species of this genus. However, cAMP has been reported to be present in cells of *B. licheniformis*, although the levels reported were very low (2). There are no reports of any physiological effect of this interesting compound in spore-forming bacteria.

While there is no question of the regulatory role of cAMP in a number of systems (8,9), it is increasingly apparent that the observable effects are different in different organisms. Schechter and Krulwich (Bacteriol. Proc., 1972, 176) reported no effect of cAMP on the repression of one inducible enzyme in an *Arthrobacter* species. Even more interesting, they observed that very low levels of this compound repressed the synthesis of another inducible enzyme in the same species. Thus, there is much to be learned about the roles of cAMP in various organisms.

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TABLE 1: Induction of β-glucosidase in *B. popilliae* by salicin.[a]

Sugar in Growth Medium	Specific Activity (Units/mg Protein)
Glucose	.0005
Salicin	.0096

[a]*B. popilliae* was grown in either TY + glucose or TY + salicin medium to mid-exponential phase. The cells were harvested, washed once with 5×10^{-2} M phosphate buffer, pH 7.2, and resuspended in the phosphate buffer. β-Glucosidase was assayed as described in the Materials and Methods section.

FIG. 1. Activity of β-glucosidase versus time. *B. popilliae* was grown in TY + salicin medium to mid-exponential phase, harvested by centrifuging at 10,000 x g for 15 min, and washed twice with 5×10^{-2} M phosphate buffer, pH 7.2. The cells were resuspended in the phosphate buffer. The assay was run as described in Materials and Methods section except that reactions were stopped at various time intervals. Each reaction contained 66 μg protein.

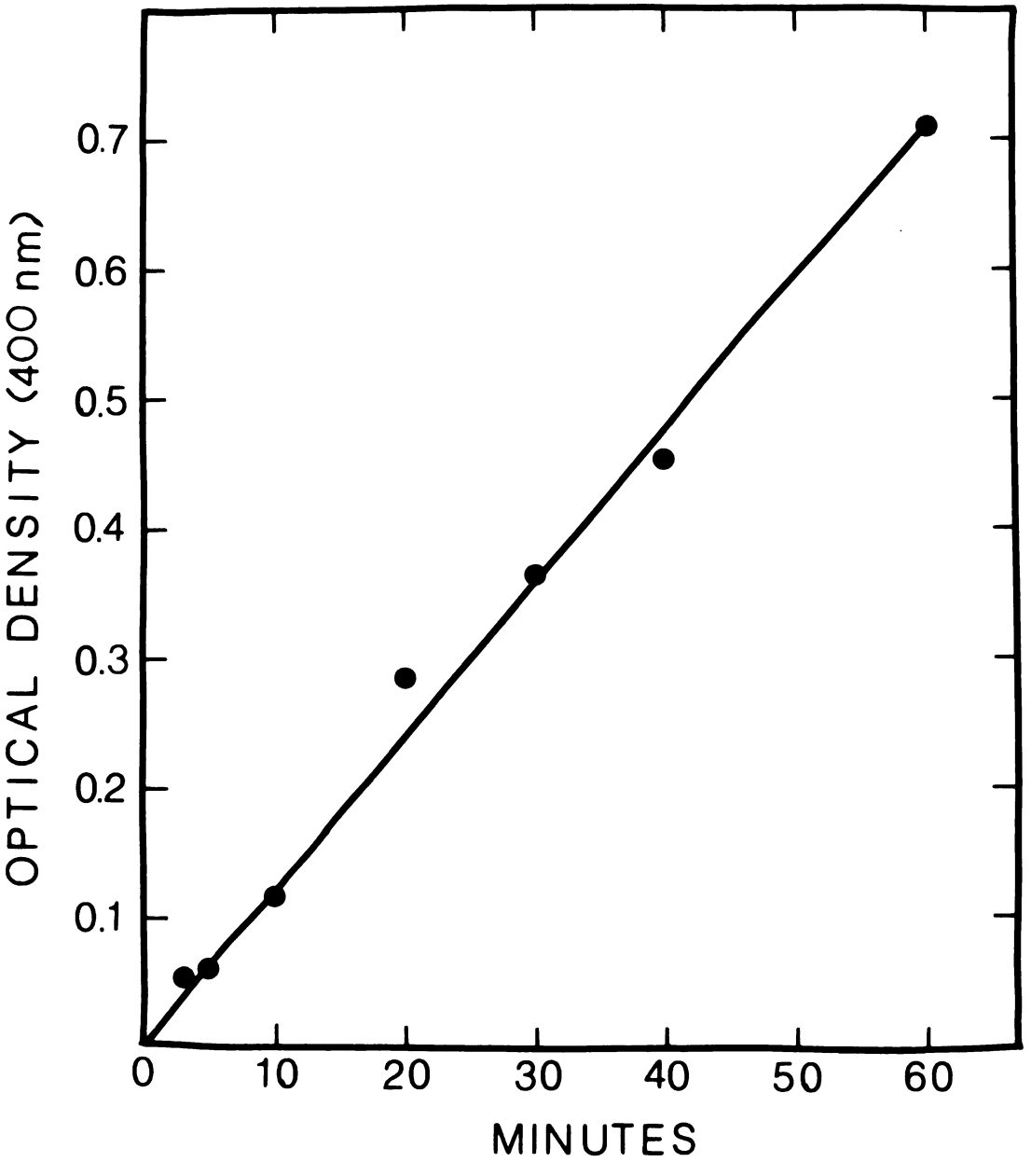
OPTICAL DENSITY (400 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
MINUTES

FIG. 1

FIG. 2. Activity of β-glucosidase versus protein concentration. β-Glucosidase activity was assayed as described in FIG. 1 except that separate reaction mixtures contained variable amounts of protein.

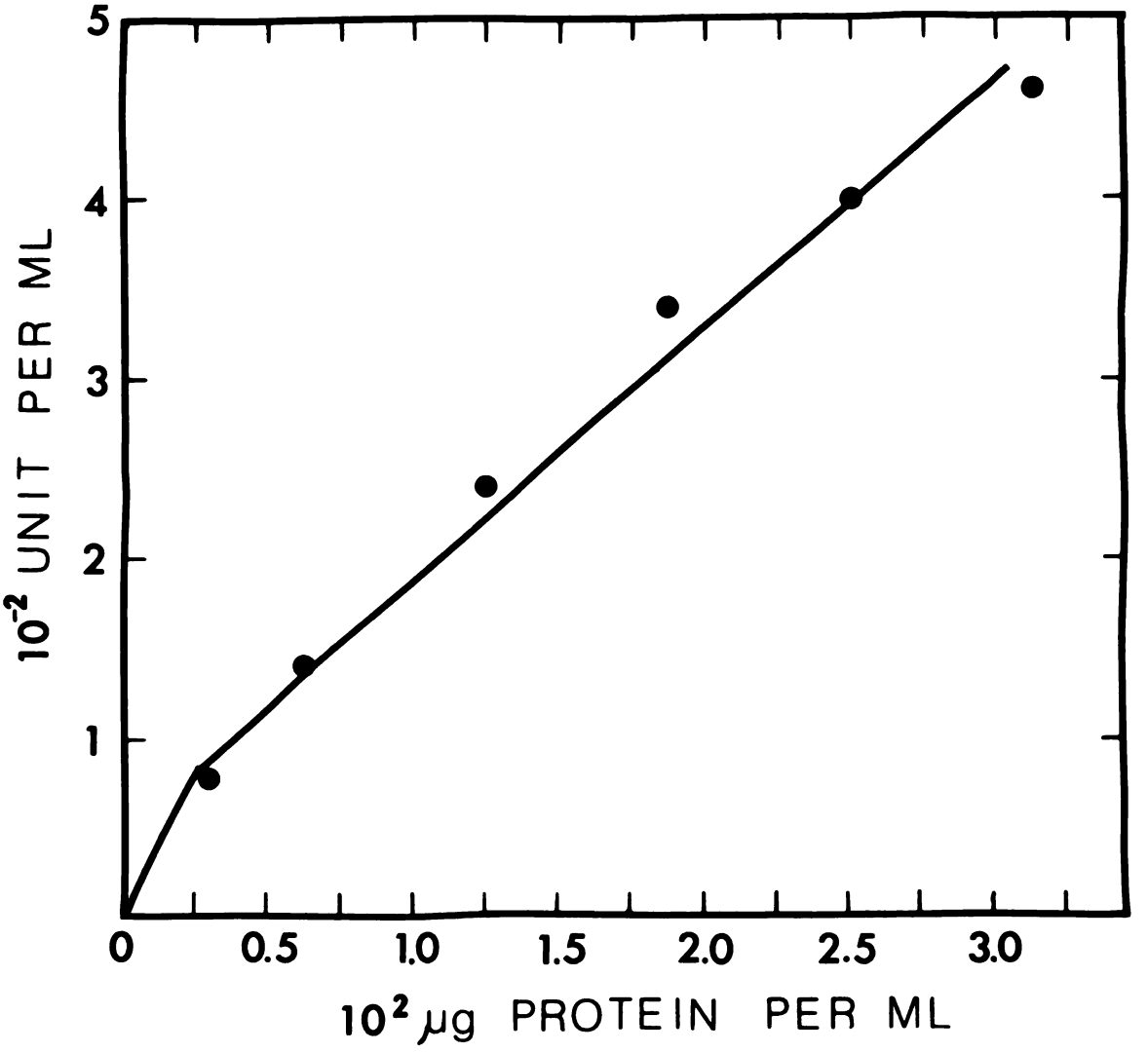

FIG. 2

FIG. 3. Lineweaver-Burk plot of β-glucosidase activity. The assays were conducted as indicated in FIG. 1 except that variable amounts of PNPβG were used in each reaction mixture.

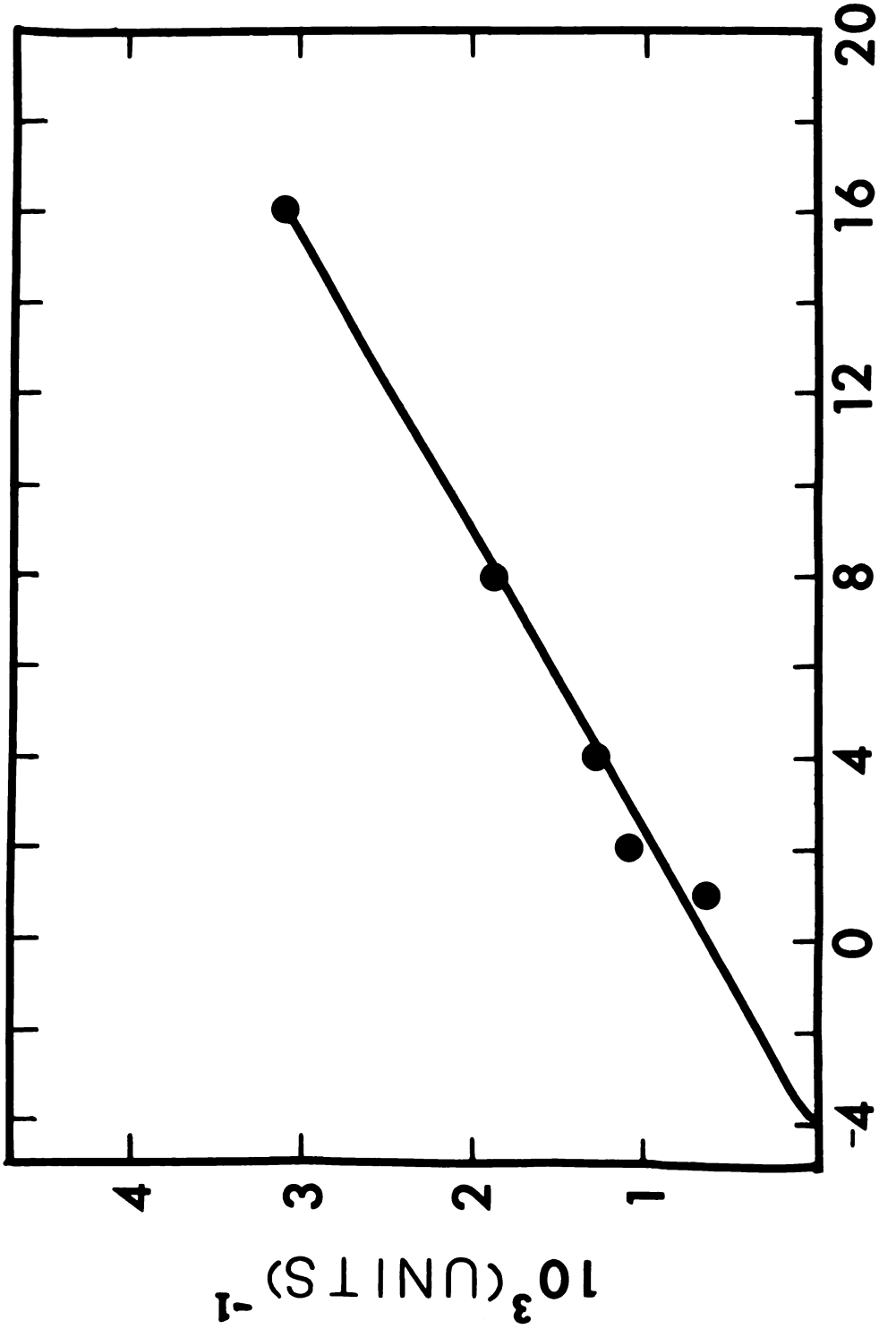

FIG. 3

FIG. 4. Induction of β-glucosidase in *B. popilliae*. *B. popilliae* 2309MC was grown in 50 ml of TY + glucose to late-exponential phase. The cells were harvested by centrifugation at room temperature and resuspended in 8 ml of TY medium. A 1.0 ml inoculum was added to each experimental flask. One experimental flask contained 50 ml of TY with 0.5% salicin (●), and the other with only the basal medium, TY (★). They were aerated by shaking on a rotary shaker at 30 C. Samples were removed at various intervals for measurements of optical density and β-glucosidase activity as described in Materials and Methods.

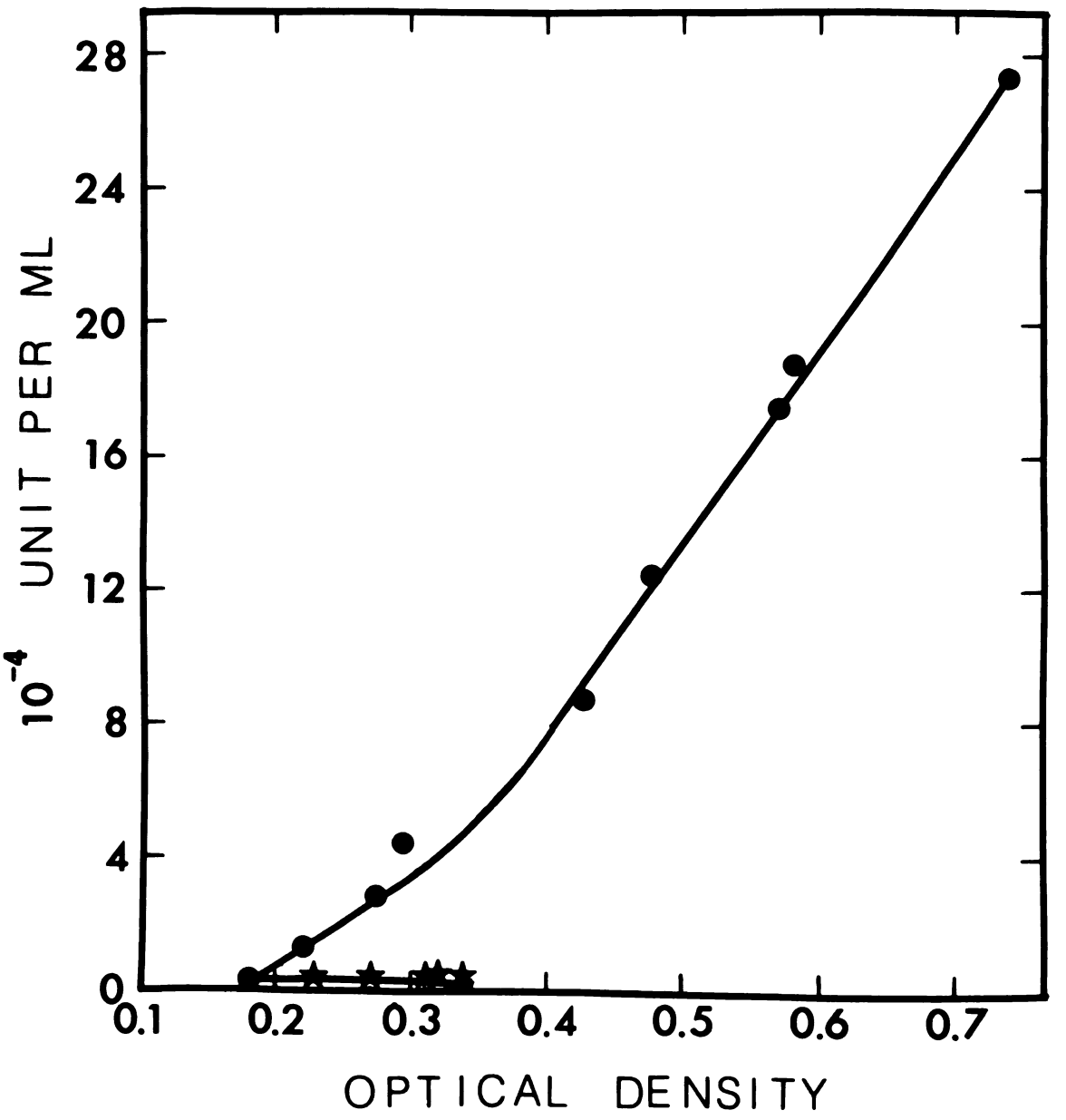
28
24
20
16
12
8
4
0
10^{-4} UNIT PER ML
0.1
0.2
0.3
0.4
0.5
0.6
0.7
OPTICAL DENSITY

FIG. 4

FIG. 5A and 5B. Effect of cAMP on the synthesis of β-glucosidase in *B. popilliae*. Four cultures of *B. popilliae* were grown in TY + 0.5% salicin medium. After the cultures were fully induced to synthesize β-glucosidase, 0.2% trehalose was added to one culture (✿, FIG. 5A), 5×10^{-3} M cAMP was added to the second culture (●, FIG. 5B), and 5×10^{-3} M cAMP and 0.2% trehalose were added to the third culture (★, FIG. 5B). The fourth culture (○, FIG. 5A) served as the control. All additions were made at the time indicated by the arrows, and 1.0 ml of water was added to appropriate flasks to maintain equal volumes. Sampling, β-glucosidase assay, and determination of growth were as described in FIG. 4.

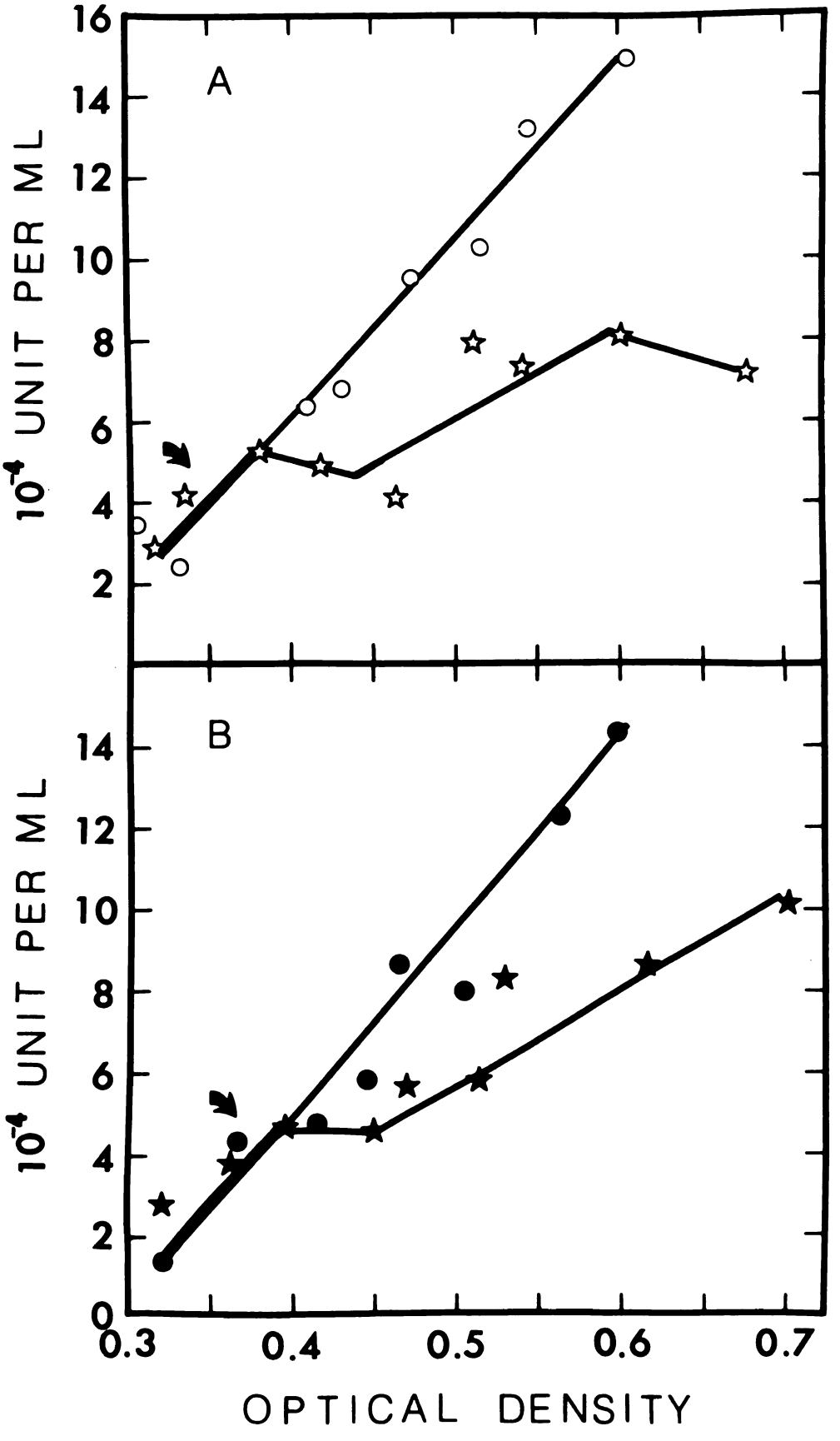
A
B
10^{-4} UNIT PER ML
10^{-4} UNIT PER ML
16
14
12
10
8
6
4
2
0
0.3
0.4
0.5
0.6
0.7
OPTICAL DENSITY

FIG. 5

FIG. 6. Uptake of [^{3}H]cAMP by *B. popilliae* 2309MC. The detailed procedure was outlined in Materials and Methods. The amount of cells in each reaction mixture was 0.89 mg dry weight. The specific activity of the [^{3}H]cAMP was 9.45 x 10^5 cpm/μmole. All counts were corrected for background.

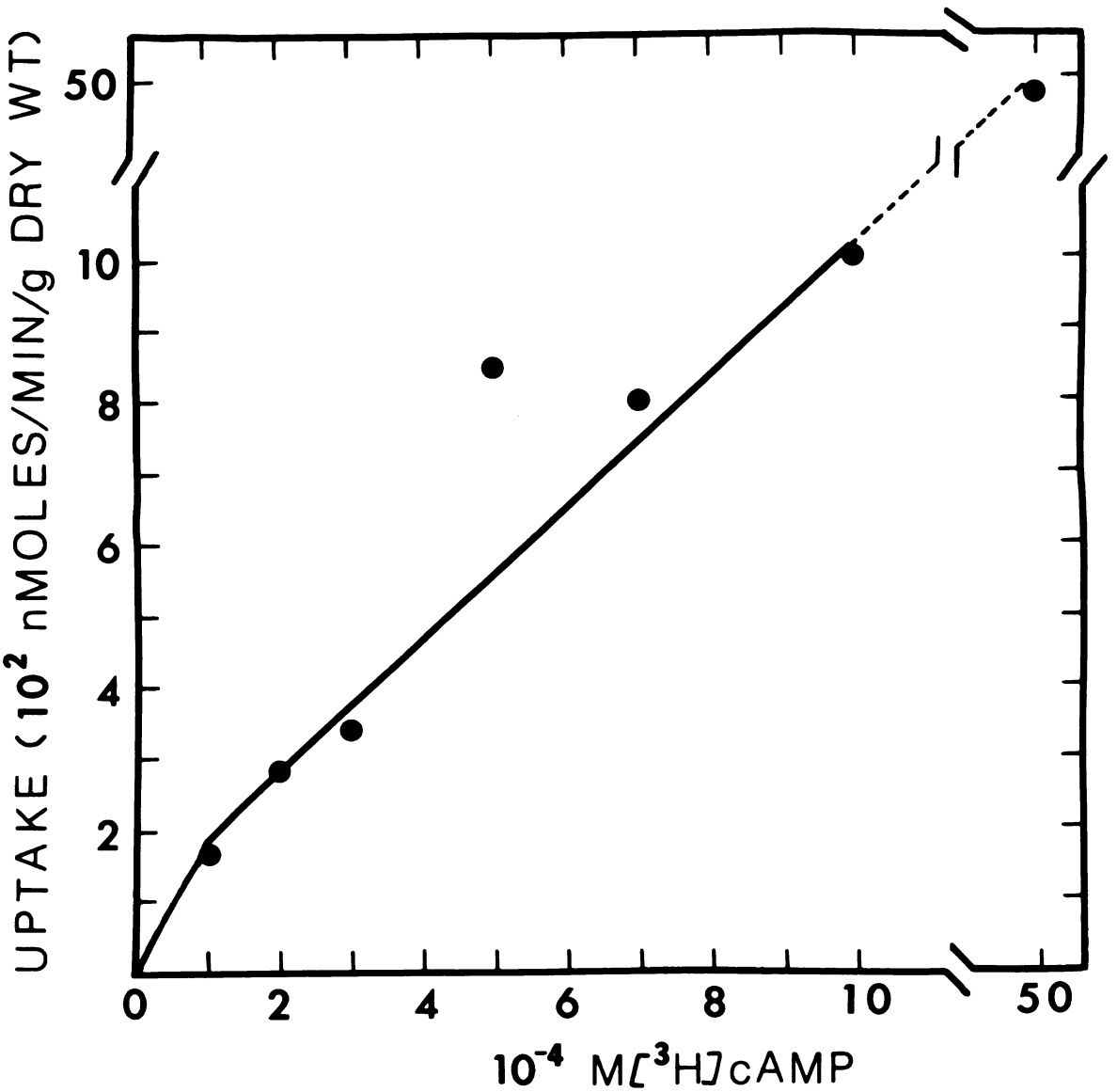
UPTAKE (10^2 nMOLES/MIN/g DRY WT)
50
10
8
6
4
2
0
2
4
6
8
10
50
10^{-4} M[^{3}H]cAMP

FIG. 6