Nonutilization of Beta-Glucosides for Growth by Fluorescent Pseudomonads

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ABSTRACT

Although *Pseudomonas syringae* hydrolyzed large amounts of glucosides such as arbutin and salicin, the hydrolysis products were not utilized for growth. The maximum amount of growth in media containing 0.01, 0.04, and 0.1% glucose was correlated with the glucose present. The addition of 1-2% of a glucoside had no influence on growth, although nearly complete hydrolysis of the glucoside occurred. Growth was supported by the hydrolysis products of glucosides hydrolyzed by almond β-glucosidase. Oxygen uptake was very slight with arbutin as a substrate ($<$ 3.0 μliter O_2/μmole substrate per hr maximum compared with $>$ 60 μliter/hr with glucose). Only trace amounts of glucose were formed during hydrolysis, and inorganic phosphate was not required. Also, cell-free extracts hydrolyzed nonphosphorylated glucosides. These data indicate that the pseudomonad β-glucosidase system is different from that occurring in the Enterobacteriaceae. Phytopathology 60:502-505.

Many of the phytopathogenic fluorescent pseudomonads hydrolyze naturally occurring β-glucosides such as arbutin or salicin (4). The sequence of this hydrolysis has not been described, but it appears to be different from the reported system (2, 7) of *Escherichia coli*. One indication of this is that acids are formed when *Erwinia* sp. (organisms closely related to *E. coli*) are grown in the presence of glucosides, but not when pseudomonads are grown (4). Also, sonicated cell preparations of pseudomonads hydrolyze β-glucosides, although similarly treated cells of *Erwinia* do not. Consequently, an investigation was initiated to investigate some of the properties of this pseudomonad system.

METHODS.—*Glucoside utilization studies.*—Cells of *Pseudomonas syringae* were grown in a mineral medium consisting of KH_2PO_4, 1.5 g; Na_2HPO_4, 3.0 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; and H_2O, 1,000 ml; and containing various amounts of glucose (0.1, 0.4, or 1.0 g/liter). In addition to the glucose, 0.2 or 2.0 g/liter of arbutin, 1.0 g/liter of salicin, or 2.0 g/liter of cellobiose was added. All carbohydrates were sterilized separately. Fifty ml of the medium were dispensed into 250-ml flasks having a sidearm for insertion into a Klett-Summerson colorimeter. Incubation was at 28 C on a rotary shaker. Growth was measured using the Klett colorimeter (green filter) or an electronic particle counter (Coulter Counter Model B).

Chromatographic techniques.—Chromatograms were run in *n*-propanol: ethyl acetate: H_2O (7:1:2) or *n*-butanol: acetic acid: H_2O: ethanol (61:10:26:3) solvent systems. Arbutin and hydroquinone were detected using a diazotized sulfanilic acid reagent. Other compounds such as glucose were detected using the nonspecific acetone-$AgNO_3$ reagent (9).

Respirometry.—Oxygen uptake was measured in a Gilson differential respirometer according to the methods described by Umbreit et al. (10). The water bath temperature was 28 C. Each vessel contained 1.7 ml of bacterial suspension in the main compartment, 0.2 ml of a freshly prepared solution containing 10 μmole of glucoside substrates or 5 μmole of glucose in the sidearm, and 0.1 ml of a 20%-KOH solution with a strip of filter paper in the central well. The gas phase in the manometers was air. The substrate was added after a preincubation period of 15-20 min at 28 C. All figures were corrected for endogenous respiration, and the results expressed as μliter O_2/μmole substrate.

The cells used were grown either in the medium described above (4 g/liter glucose) or on the surface of King B medium slanted in 500-ml medicine flasks. To induce β-glucosidase synthesis, 5 g/liter salicin was added to the medium. The cells were collected after incubation for 28 hr at 28 C and washed twice with 0.005 M phosphate buffer pH 6.8. They then were suspended in 0.02 M phosphate buffer pH 6.8 to give a final concentration of 50 mg cells/1.7 ml (wet w/v).

Bacterial β-glucosidase preparation.—Cells were grown in the mineral-glucose medium (4 g/liter glucose) with or without added salicin (5 g/liter) for 24 hr. After centrifugation, they were suspended in distilled water (1 g wet-wt cells/10 ml H_2O), centrifuged, and resuspended 3 times in the same water. The final cell-free supernatant was used as the bacterial β-glucosidase preparation. The activity of this preparation depended upon whether the cells had been induced for the enzyme or not.

RESULTS.—*Nonutilization of β-glucosides for growth.*—The nonacid formation upon hydrolysis of glucosides by pseudomonads suggested that pseudomonads might not have the ability to utilize hydrolysis products for growth. This was investigated by measuring the amount of growth of pseudomonads on several glucosides in the presence of small, limiting quantities of glucose. Growth was measured for 4 days, and the maximum turbidity and cell numbers were recorded. Two pseudomonads were tested, a β-glucosidase synthesizing organism (*P. syringae* S-3) and a β-glucosidase negative organism (*P. putida* A3.12).

Neither of the two pseudomonads tested utilized the glucosides for growth. The maximum growth obtained

TABLE 1. Growth of *Pseudomonas syringae* and *P. putida* on limiting amounts of glucose in the presence of glucosides

Glucoside	Glucose 0.01%	Glucose 0.04%	Glucose 0.1%
Pseudomonas syringae (S-3)			
Glucose	1.1×10^8 [a]	3.2×10^8	5.6×10^8
Glucose + 0.02% Arbutin	1.5	3.7	7.0
Glucose + 0.2% Arbutin	1.1	3.1	6.8
Glucose + 0.1% Salicin	1.3	3.4	7.0
Glucose + 0.2% Cellobiose	1.2	3.1	6.9
Pseudomonas putida (A3.12)			
Glucose	1.3	4.4	7.7
Glucose + 0.02% Arbutin	1.1	3.5	9.2
Glucose + 0.2% Arbutin	1.2	4.4	9.2
Glucose + 0.1% Salicin	1.0	4.5	9.9
Glucose + 0.2% Cellobiose	1.0	4.0	9.1

[a] Maximum number of cells/ml during a 72-hr period.

always corresponded to the amount of glucose present, regardless of the presence or absence and quantity of a glucoside (Table 1). Chromatographic analysis of the arbutin growth mixtures after 4 days indicated that large amounts of the glucoside had been hydrolyzed.

The aglycons of many glucosides are toxic to microorganisms. To determine whether the nongrowth might be due to the toxicity of the aglycon, an experiment was conducted in which salicin hydrolyzed by either the bacterial enzyme or by commercial β-glucosidase (emulsin, obtained from almond) was added to the glucose-limiting medium. Hydrolysis was achieved by adding 1 ml of an emulsin solution (containing 5 mg emulsin) or bacterial β-glucosidase solution (which hydrolyzes approximately 40 μmole *p*-nitrophenol-β-glucoside/min) to 50 ml of the glucose medium, and incubating the mixture at 28 C for 3 hr before seeding the flasks with the bacterium (*P. syringae* S-9).

The results obtained with this experiment were similar to the previous results, in that the salicin hydrolyzed by the bacterial enzyme did not support additional growth beyond the limiting glucose (Table 2). The emulsin-hydrolyzed salicin, however, supported considerable additional growth, indicating that aglycon toxicity was not the cause of nongrowth.

Oxygen uptake with glucosides as substrates.—The preceding experiments indicated that hydrolysis products of β-glucosides did not support growth of the bacteria. Consequently, it was of interest to determine if O_2 uptake occurred during hydrolysis of glucosides. *P. syringae* S-9 was used for this investigation. Substrates included α- and β-methyl glucosides, the disaccharides, cellobiose and trehalose, and the naturally occurring β-glucosides, arbutin, amygdalin, indican (indoxyl-β-D-glucoside), phoridzin, and salicin.

These respirometric studies further indicated that glucosides were not utilized for growth by fluorescent pseudomonads. With arbutin as a substrate, some uptake of O_2 appeared to occur with β-glucosidase-induced cells (Fig. 1). The amount was very small ($<$ 3 μliter O_2/mole substrate per hr). This was approximately two times that occurring with noninduced cells. Also, little O_2 uptake occurred with the other glucoside substrates. The amount of O_2 uptake in 150 min using noninduced cells was 4.6 μliter/μmole substrate for amygdalin, 0.9 for indican, 1.2 for phloridzin, 0.6 for salicin, 0.1 for trehalose, and 0.8 for cellobiose. The amount in 105 min was 1.9 for β-methyl glucoside and 1.2 for α-methyl glucoside. Over 60 μliter O_2/μmole substrate were taken up with glucose in 60 min.

Glucose as a hydrolysis product.—Glucose is one of the reaction products formed upon hydrolysis of glucosides by β-glucosidases such as emulsin. The lack of growth of pseudomonads on glucosides hydrolyzed by their β-glucosidase, however, suggested that glucose was not a hydrolysis product. A chromatographic examination was made, therefore, of the reaction products of the hydrolysis of *p*-nitrophenol-β-glucoside.

The reaction mixture consisted of 1 ml enzyme preparation, 1 ml 0.02 M phosphate buffer pH 6.8, 1 ml *p*-nitrophenol-β-glucoside solution (5 mg/ml), and 1 ml H_2O. Controls included reaction mixtures containing

TABLE 2. Maximum growth of *Pseudomonas syringae* (S-9) on salicin hydrolyzed by almond and *Pseudomonas* β-glucosidases

Treatment	Carbon sources: 0.2% Salicin	0.2% Salicin + 0.01% glucose	0.2% Salicin + 0.04% glucose	0.01% Glucose	0.04% Glucose
None	0[a]	20	58	19	58
Bacterial enzyme[b]	15	21	64		
Bacterial enzyme, boiled immediately	15	27	81		
Emulsin[c]	103	104	129		
Emulsin, boiled immediately	27	41	59		

[a] Maximum OD obtained during a 72-hr period as measured with a Klett-Summerson colorimeter with a green filter.
[b] One ml of β-glucosidase solution (which hydrolyzes approximately 20 μmole *p*-nitrophenol β-glucoside per min) added 3 hr prior to seeding the flasks.
[c] Five mg added in 1 ml H_2O 3 hr prior to seeding the flasks.

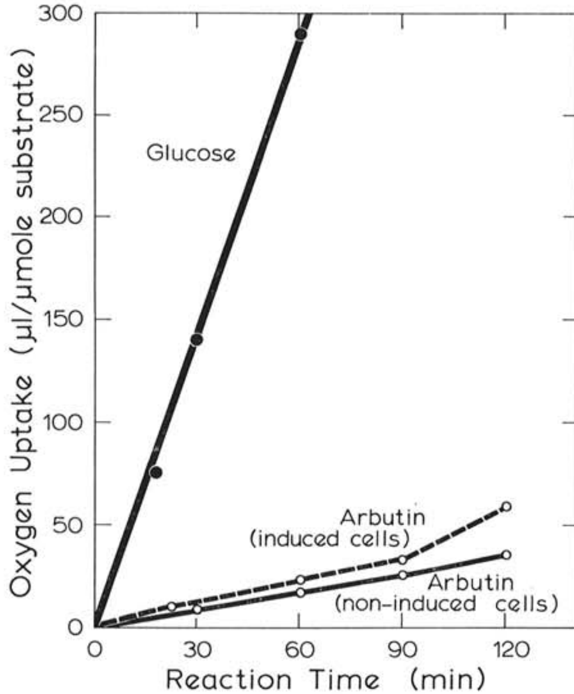

Fig. 1. Oxygen uptake by *Pseudomonas syringae* cells with arbutin as a substrate. Bacterial cells induced and non-induced for β-glucosidase were used.

no *p*-nitrophenol-β-glucoside, one with no β-glucosidase, and one with emulsin (0.5 mg/ml) in place of the bacterial enzyme. The mixtures were incubated for 5 hr at 30 C before being spotted on the chromatography paper.

Little glucose was detected in the reaction mixtures containing the bacterial β-glucosidase, even though large amounts of the glucoside had been hydrolyzed, as indicated by the formation of a yellow color in the reaction mixture (the aglycon *p*-nitrophenol is yellow in solution at pH 6.8) and decrease in intensity of the glucoside spot on the chromatogram. Glucose was not detected unless 500 λ or more were spotted; even then, the spots were faint. None was detected in the controls except for the one containing emulsin where large amounts of glucose occurred. The occurrence of trace amounts of glucose was confirmed in tests of the reaction with the glucostat reagent (Worthington Biochem. Corp.).

Nonrequirement for phosphate.—The β-glucosidase of *E. coli* is a phospho-glucosidase requiring that the glucoside be phosphorylated before it can be hydrolyzed (2, 7). Whether phosphate was required for glucoside hydrolysis by the pseudomonad β-glucosidase was examined. Cells were treated in the same way as with the respirometric studies, except that they were removed from the medium and washed in 0.005 M Tris-maleic acid buffer pH 6.8. After washing, the cells were suspended in Tris-maleic acid buffer 0.02 N with the final concentration of the cell suspension being one-tenth (wet w/v). Only cells induced for β-glucosidase were used. Control cells were prepared with phosphate buffer as in the respirometric studies. The reaction mixtures contained 2 ml of the cell suspension, 2 ml of a salicin solution containing 50 µgm salicin/ml, and 1 ml 0.02 M phosphate buffer pH 6.8 or 1 ml Tris-maleic acid buffer 0.02 M pH 6.8. These mixtures were incubated 5 hr at 28 C.

Phosphate did not appear to be required for glucoside hydrolyses.

The amount of hydrolysis of salicin in both reaction mixtures was approximately the same as indicated by the amount of saligenin formed. This was detected through the use of the 4-aminoantipyrine reagent (3). Chromatographic evidence also indicated that the amount of hydrolysis in both mixtures was approximately equal.

DISCUSSION.—The β-glucosidase system of phytopathogenic fluorescent pseudomonads appears to be different from the *E. coli* system in that apparently the β-glucosidase is not a phospho-β-glucosidase. Decreasing the amount of inorganic phosphate had no effect on hydrolysis, and nonphosphorylated glucosides were hydrolyzed by cell-free extracts. Also, the hydrolysis products of the pseudomonad system can support little or no growth.

The nature of the hydrolysis products other than the aglycon is as yet unknown. β-Glucosidases can have transfer activity. Acceptors in a reaction of this type may be sugars, alcohols (11), or noncarbohydrate (1). The most likely possibility at present is that a transfer reaction is occurring, and that polymerization of the glucose residue is taking place. The trace amount of glucose that we detected in the reaction products probably escaped from the primary reaction. This small amount of glucose may account for the limited O_2 uptake observed.

This pseudomonad β-glucosidase system points out the hazard in relying solely upon nutritional or growth data for taxonomic studies. A number of the phytopathogens possess this system in contrast to the saprophytes (4), yet studies which use growth as an indicator (5, 6, 8) would not detect this difference. Differences in other enzyme systems also may remain undetected because of the emphasis on utilization for growth.

Phytopathogenic pseudomonads generally utilize far fewer compounds for growth than their saprophytic counterparts (5, 6). Perhaps many of these so-called nonutilized substrates are in fact utilized, but utilized for a purpose other than growth. Nongrowth modes of utilization could be major factors in pathogenicity, and thus should be examined in greater detail.

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