ANAEROBIC DISSIMILATION OF GLUCOSE BY *ERWINIA AMYLOVORA*[1]

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Received for publication March 10, 1959

Erwinia amylovora has been described as fermenting glucose and other sugars with the formation of acid but not gas. The nature of the acids and other products of fermentation has not been reported previously in this species; the fermentation pattern exhibited by the quite different soft-rot phytopathogen *Erwinia carotovora* has received some attention (Kraght and Starr, 1952).

Fermentations of glucose by the enteric bacteria and other microorganisms have sometimes been studied by analysis of the products formed by anaerobically growing cultures, as in the classical studies of Scheffer (1928). This approach to a study of fermentation by *E. amylovora* was precluded by the complexity of the medium necessary for the growth of this organism in the absence of air. Accordingly, an alternative approach—fermentation of glucose by suspensions of nongrowing cells—was used. This report will set forth the products formed by cells of *E. amylovora* during anaerobic dissimilation of glucose and the effects of some environmental factors on the course of the fermentation. A brief account of this work has been given previously (Sutton and Starr, 1958).

MATERIALS AND METHODS

Cultivation. Cultures of *E. amylovora* were taken from the lyophilized collection of phytopathogenic bacteria maintained in this department. Because of the instability of slime production in this species (Ark, 1937), these cultures produced polysaccharide slime in varying amounts, the cultures ranging from smooth to mucoid. When cells from a single colony of either type are plated, some of the daughter colonies are smooth, some mucoid. Since mucoid cultures were difficult to suspend, the culture EA137S used in most of this study was maintained in a predominantly smooth state by periodic selection of smooth colonies.

This strain was taken from a parent culture EA137, originally isolated from fire blight of apple by Dr. P. A. Ark. Both strains EA137 and EA137S were still pathogenic when tested by Dr. Ark during the present study; they were typical of *E. amylovora* in their biochemical reactions.

E. amylovora strain EA137S was grown in 9-L carboys containing 7 L of medium. The medium used in most experiments contained per L: NH_4Cl, 1 g; KH_2PO_4, 4 g; K_2HPO_4, 4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; a mixture of trace inorganic salts (Starr, 1946); glucose (autoclaved separately), 4 g; nicotinic acid, 2 mg; sodium malate, 4 g. This chemically defined medium was sometimes supplemented with casamino acids (Difco), 2 g per L, and with the amino acids plus yeast extract, 1 g per L. Aerobic growth was obtained by sparging with air; semianaerobic growth was obtained in static carboys. Aerobic cultures were harvested by Sharples centrifugation after 9 to 12 hr growth at 28 C, static cultures after 36 hr growth. The cells were washed twice by centrifugation and vigorously aerated in buffer for 2 hr to reduce endogenous metabolism.

Analysis. The fermentation pattern of *E. amylovora* was studied using cell suspensions in 125-ml Warburg flasks. Carbon dioxide production was followed manometrically, correction being made for gas retention by the buffer. In bicarbonate buffers, the carbon dioxide derived from glucose was determined after making a correction for gas released from bicarbonate by acid formation (Umbreit *et al.*, 1949). The reaction was stopped by tipping in excess sulfuric acid. The flasks were then chilled to minimize loss of volatile products; the cells were

[1] This investigation was supported in part by U. S. Public Health Service Research grant E-1067 from the National Institutes of Health, and by a grant-in-aid from the Monsanto Chemical Company.

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removed by centrifugation in the cold. In the large-scale experiments, the supernatant solutions were fractionated by the procedures outlined by Neish (1952). Ethanol was removed as the neutral distillate and determined by microdiffusion into dichromate. Some acetoin appeared in the distillate; the remainder was removed by continuous extraction with ether. The acetoin was determined in both fractions by a quantitative Voges-Proskauer reaction. 2,3-Butanediol appeared in the neutral extract, the quantities formed being near the limit of estimation by the method used (Neish, 1952). The residue was acidified and the organic acids were removed by continuous extraction with ether for 16 hr; nearly all the acids were extracted in the first 8 hr. The acids were then placed on a column of silicic acid prepared according to Bulen *et al.* (1952) and were eluted with the solvents-sequence of Neish (1952). The eluted fractions separated well and their positions corresponded to the predetermined positions of a known mixture of organic acids. The amount of acid in each fraction was measured by titration. The positions of the lactate and succinate fractions were confirmed by paper chromatography of the ammonium salts (Isherwood and Hanes, 1953). Residual glucose was estimated colorimetrically after reaction with dinitrosalicylic acid (Sumner and Somers, 1949).

Later experiments on the effects of certain variables were made in 20-ml Warburg flasks. Since three products—lactate, ethanol, and carbon dioxide—accounted for nearly 90 per cent of the glucose utilized, these products were measured without prior fractionation. Carbon dioxide, ethanol, and glucose were determined as previously stated; lactate was estimated colorimetrically (Neish, 1952).

EXPERIMENTAL RESULTS

Preliminary manometric studies showed that cells of *E. amylovora* evolved gas during fermentation of glucose in bicarbonate, as was shown also by Katznelson (1955). These results do not, however, show whether the gas was carbon dioxide released from the buffer by acid production, or whether the gas was a product of metabolism of the substrate. Production of gas from glucose itself was shown in phosphate buffers at pH 6.0 and 6.8, where fermentation of 5 μmoles glucose yielded up to 7.5 μmoles of gas. Since flasks in which carbon dioxide was trapped by 10 per cent KOH showed no net pressure increase, the gaseous product is considered to be carbon dioxide alone.

Fermentation of higher levels of glucose was considered necessary to obtain sufficient amounts of the products for fractionation and analysis. In the first attempts to do this, however, the rate of fermentation declined before the substrate was consumed. Calcium carbonate was added to the buffer system to achieve complete fermentation of 100 μmoles glucose. The products recovered after such an experiment are listed in table 1. The amount of carbon dioxide shown there was corrected for release of gas from the buffer by the acids formed. These products accounted for 94 per cent of the glucose utilized; the ratio of oxidized to reduced products was 1.07.

The rates of formation of the three major products were ascertained by stopping the fermentation in replicate flasks at various times. Values (table 2) show that carbon dioxide, ethanol, and lactate are formed concurrently during the disappearance of glucose. There is some indication, however, that some carbon dioxide and ethanol were formed at the expense of lactate after the substrate was exhausted.

The fermentation pattern of *E. amylovora* was influenced, though not markedly, by the hydrogen ion concentration. Data in table 3 indicate generally increased production of carbon dioxide

TABLE 1

*Products of fermentation of glucose by resting cells of Erwinia amylovora**

Products	Micromoles	Microgram-atoms Carbon
Ethanol	155	310
Carbon dioxide	161	161
Lactic acid	18	54
Acetic acid	7	14
Formic acid	6	6
Succinic acid	2	8
Acetoin	1	4
2,3-Butanediol	1	4

* Cells were grown for 12 hr at 28 C in defined medium sparged with air. The reaction vessel contained 3 g wet cells (0.38 g dry weight); bicarbonate, 200 μmoles; $CaCO_3$, 200 μmoles; glucose, 100 μmoles; fluid volume, 25 ml; atmosphere, 50 per cent CO_2 in nitrogen; temperature, 30 C.

TABLE 2

*Time course of fermentation of glucose by Erwinia amylovora**

Time	Glucose Utilized	Metabolic CO_2	Lactic Acid	Ethanol	Recovery†
min	*μmoles*	*μmoles*	*μmoles*	*μmoles*	%
22	4.4	4.6	2.5	5.6	88
40	8.0	9.4	4.5	8.9	85
80	10.0	14.5	5.7	10.9	89
150	10.0	14.3	5.8	11.8	92
200	10.0	14.9	5.3	12.4	93
200	Endogenous	1.5	0.2	0.6	−5

* Cells were grown statically 36 hr at 28 C in defined medium. Each Warburg flask contained 0.75 g wet cells in bicarbonate buffer, 0.007 M final concentration; 10 μmoles glucose; fluid volume, 3.0 ml; atmosphere, 5 per cent CO_2 in nitrogen; pH, 7.0; temperature, 30 C. The reaction was stopped with 600 μmoles H_2SO_4.

† Carbon.

TABLE 3

*Effects of buffer and pH on glucose fermentation by Erwinia amylovora**

Buffer	pH	Glucose Utilized	Metabolic CO_2	Lactic Acid	Ethanol	Recovery†
		μmoles	*μmoles*	*μmoles*	*μmoles*	%
Tris	8.8	10	17.5	8.0	8.8	99
Tris	8.0	10	17.2	10.9	7.0	106
Tris	7.2	10	12.9	12.0	4.4	96
Tris	7.2	Endogenous	6.9	1.0	1.2	−20
Phosphate	7.8	10	16.2	12.8	8.0	118
Phosphate	7.0	10	15.4	10.9	5.4	98
Phosphate	6.2	10	11.7	11.5	4.2	91
Phosphate	6.2	Endogenous	7.0	0.8	1.8	−21

* Cells were grown 11 hr at 28 C in the casamino medium sparged with air. Each Warburg flask contained 0.75 g wet cells and 10 μmoles glucose in 3.0 ml 0.01 M buffer; atmosphere, nitrogen; temperature, 30 C.

† Subtraction of endogenous metabolism would reduce the reported recoveries of carbon by approximately 20 per cent.

and ethanol with increasing pH values, whereas lactic acid production decreased at pH 8.0 and higher. The kind of buffer used also influenced the fermentation, as more ethanol was formed in phosphate buffers at pH 7.0 and 7.8 than in Tris (tris(hydroxymethyl)aminomethane) buffers at pH 7.2 and 8.0, respectively. In flasks containing 5 per cent carbon dioxide over concentrations of bicarbonate calculated to form buffers at pH 7.8 and 8.5, the major products appeared in ratios similar to that found in Tris buffer at pH 8.8.

In Tris, phosphate, or bicarbonate buffers the fermentation, as measured by carbon dioxide evolution, was essentially complete in the first hour. Phthalate was also used as a buffer, but seemed to be inhibitory. In 0.01 M phthalate buffer at pH 6.3, only half the substrate was fermented in 2 hr; the ratio of the products was similar to that observed in phosphate buffer at pH 6.2. Only one fourth of the substrate was fermented in 2 hr in phthalate buffer at pH 5.4; practically none at pH 4.5.

Comparison of the ratio of products formed by five different isolates of *E. amylovora* showed that the cultural history of the cells had as great an influence on the course of the fermentation as any other variable tested. The amounts of slime material associated with the cells were inversely related to the rates of fermentation. Variability in slime formation has already been mentioned; of the five isolates studied comparatively, strain EA123 was the least mucoid, EA107 the most mucoid. Cells of strain EA123 fermented 10 μmoles glucose in less than 2 hr, a rate comparable to that of the selected strain EA137S. The more mucoid isolates did not completely dissimilate the substrate in 2 hr. The variability in the relative yields of the products is seen in table 4. The least mucoid isolate EA123 formed lactate and ethanol in a ratio of 1:1; the most mucoid isolate EA107 yielded a ratio of 4:1. These data could be interpreted to imply that the proportionate yield of ethanol is a function of the over-all rate of fermentation.

Other experiments were designed to show the effects of the age of the culture and the medium used for growth. Although the results were not striking quantitatively, they did show a general trend consistent with the above interpretation.

Aerobic cultures were harvested after approximately 9 hr and 12 hr growth. Comparison of the cultures by phase contrast microscopy showed senescent changes in the older culture; many distorted cells and some protoplast-like spheres were seen. Cells of the younger culture were more active in fermentation than cells of the older culture. The faster fermentation of the younger

TABLE 4

*Fermentation patterns of different isolates of Erwinia amylovora**

Isolate	Glucose Utilized	Metabolic CO_2	Lactic Acid	Ethanol	Recovery†
	μmoles	*μmoles*	*μmoles*	*μmoles*	%
EA123	10.0	10.1	10.2	9.9	101
EA145	8.9	5.9	12.0	6.0	101
EA137	8.2	3.8	12.2	4.1	99
EA146	8.0	4.2	12.2	4.5	104
EA107	8.0	4.5	10.9	2.5	88

* Cells were grown on agar plates of the casamino medium. Each Warburg flask contained 0.75 g wet cells and 10 μmoles glucose in 3.0 ml 0.011 M bicarbonate buffer; calculated pH, 7.2; atmosphere, 5 per cent CO_2 in nitrogen; temperature, 30 C. The yield of products from endogenous metabolism was about one fifth that of exogenous metabolism.

† Carbon.

cells yielded a greater proportion of ethanol than did the senescent cells. The difference was small, though, in comparison to the differences shown in table 4.

Static cultures grew slowly and yielded small populations but the cells were more active, on a wet weight basis, than cells grown aerobically. Cells grown in the minimal medium were smaller but more active in fermentation than cells grown in the supplemented medium. Again, there was some correlation between the rates of fermentation and the relative yields of ethanol.

DISCUSSION

Three products—lactate, ethanol, and carbon dioxide—accounted for most of the glucose dissimilation by *E. amylovora* under a variety of experimental conditions. This fermentation pattern can be compared with the patterns of other bacteria that form these products.

The same three products are formed in the heterolactic fermentation of glucose by *Leuconostoc mesenteroides*. The latter pattern is uniquely constant, the three products being formed in an equimolar ratio. Current knowledge of the heterolactic pathway, reviewed by Gunsalus *et al.* (1955) reveals the basis for this constancy. Since the three products arise from precursors that are not interconvertible, the relative yields cannot deviate from an equimolar ratio. In contrast, the ratios of lactate to ethanol (and CO_2) in the fermentation pattern of *E. amylovora* varied from 1:8 to 4:1. In view of this and other significant differences between the two organisms, the similarity in products is considered only coincidental.

For various reasons *E. amylovora* is placed in the family Enterobacteriaceae. Significant comparisons can be made with the fermentation patterns of the well-known coliforms and related enterics. In studies of these organisms two important features stand out. One is the great variety of products that may be formed during fermentation of glucose by these organisms. The products include four organic acids, three alcohols, carbon dioxide, and hydrogen. Scheffer (1928) found that most species of enterics could form significant amounts (more than 0.1 mole per mole glucose) of at least five of these nine products. The other key feature is the ability to form large amounts of formic acid—as much as 1.8 moles per mole of glucose (Tikka, 1935).

Neither of these general features, however, is apparent in the fermentation pattern of *E. amylovora*. Only small amounts of formic acid were formed. On the other hand, ethanol and lactate were produced in greater yield than has been reported from studies of other enteric bacteria. In the experiments reported here the yield of lactate varied from 0.2 to 1.2 moles per mole glucose fermented. Other enteric bacteria form usually less than 1 mole lactate per mole glucose; the highest yield, 1.4 mole, was observed in fermentation by *Salmonella typhi* (calculated from Scheffer, 1928). Formation of ethanol by *E. amylovora* was more striking; yields varying from 0.4 to 1.5 moles per mole glucose were found. The maximal yields of ethanol reported for other enteric bacteria were 0.84 moles per mole glucose by *Escherichia coli* (Stokes, 1949) and 0.75 moles by *Aerobacter indologenes* (Reynolds and Werkman, 1937).

The questions to be considered are the following: Does the high yield of ethanol by *E. amylovora* represent only an extension of our concept of variation in fermentation patterns among the Enterobacteriaceae? Or does *E. amylovora* possess a unique enzymatic pathway which shunts a large amount of the substrate to formation of ethanol and carbon dioxide? Evidence (Sutton and Starr, 1959, *unpublished data*) will be presented later that *E. amylovora* possesses enzymes for dissimilation of glucose to pyruvate via the Embden-Meyerhof

pathway and that the products of fermentation of variously labeled glucose are consistent with operation of this pathway in cells of *E. amylovora*. If the enzymatic makeup of *E. amylovora* is uniquely different from other enteric bacteria, then the differences must lie in the pathway of reduction of pyruvate.

SUMMARY

Suspensions of nongrowing cells of *Erwinia amylovora* fermented glucose to form the products lactic acid, ethanol, and carbon dioxide. Under various experimental conditions the ratio of lactate to ethanol varied from 1:8 to 4:1, whereas the ratio of ethanol to carbon dioxide was essentially 1:1. The fermentation pattern of *E. amylovora* is compared to the patterns of other Enterobacteriaceae.

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