Studies on the Formation of Bacitracin by *Bacillus licheniformis*: Role of Catabolite Repression and Organic Acids

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SUMMARY

Bacitracin production by *Bacillus licheniformis* ATCC10716 is markedly inhibited by glucose. This inhibitory effect is due to the lowered pH and undissociated organic acids produced by the metabolism of glucose. The possibility that catabolite repression is an incidental result of low pH and the production of organic acids from glucose during growth is discussed.

INTRODUCTION

Since the initial observation by Epps & Gale (1942), the inhibitory effect of glucose and related compounds upon a variety of microbial enzymes has been reported by numerous investigators. An excellent review is given by Paigen & Williams (1970). This glucose effect was termed catabolite repression by Magasanik (1961) who postulated that the phenomenon was a regulatory mechanism, a view which has found general acceptance (Mandelstam, 1962; Moses & Prevost, 1966; Demain, 1968; Schaeffer, 1969; Gallo & Katz, 1972; Meers, 1972).

The mechanism of catabolite repression is not completely understood. The phenomenon involves interference with the induction of certain enzymes by the catabolites of a rapidly used carbon source. The catabolites which are supposed to interfere are termed effectors, although the effector has not been positively identified in any system (Paigen & Williams, 1970).

Bacitracin production by *Bacillus licheniformis* is reported to be controlled by catabolite repression due to glucose (Laishley & Bernlohr, 1968). The inhibitory effect of glucose upon bacitracin formation by *B. licheniformis* has been examined and the results are discussed in this paper.

METHODS

Organism. The bacitracin-producing strain of *B. licheniformis* ATCC10716 was kept as a spore suspension at 4 °C throughout the investigation.

Media and growth conditions. The chemically defined media, M2 and M20, used for growth and bacitracin production are described by Haavik (1974a). The inoculum consisted of spores.

Microbial assay of bacitracin. Bacitracin was determined by an agar diffusion method according to Haavik & Thomassen (1973).

Growth. Bacterial growth was measured as extinction at 650 nm using a Spectronic 20 spectrophotometer. Before E_{650} was measured, the culture fluid was appropriately diluted with distilled water. Growth was then expressed as $E_{650} \times$ dilution factor. Growth measured

Table 1. *Effect of glucose upon bacitracin production by Bacillus licheniformis in M20 medium*

Time of incubation (h)	Glucose absent			Glucose (1.0 %)		
	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)
10	0.32	7.58	0.2	0.30	6.90	n.d.
11	0.62	7.75	0.4	0.68	6.50	n.d.
12	1.10	7.93	0.5	1.20	5.84	n.d.
13	1.70	7.98	1.1	2.00	5.50	n.d.
14	2.35	8.06	2.4	2.60	5.46	n.d.
16	3.90	8.26	4.2	4.43	5.52	0.3
18	5.90	8.35	8.0	6.00	6.30	1.8
20	7.40	8.44	10.1	7.20	7.00	3.8
22	8.64	8.60	10.8	8.45	7.28	6.0
24	9.55	8.82	11.4	9.90	7.50	10.1
26	9.60	8.90	12.0	10.00	7.85	10.0
28	8.50	9.20	8.3	9.80	8.10	8.0

n.d., Not detected.

in this manner showed an excellent correlation with bacterial viability (Haavik, 1974*b*). Bacterial viability was determined by spreading a suitable dilution of the culture upon nutrient agar (Difco) plates and counting the colonies formed after 24 h of incubation at 37 °C.

Determination of organic acids in the fermentate. The bacteria were removed by sterile filtration (Millipore type HA, 0.45 μ m). The filtrate was acidified with 1 M-HCl to pH 2.0, and the organic acids extracted with ether. The ether fraction was evaporated to dryness, and the residue was redissolved in chloroform. Samples from this solution were injected into the gas chromatograph (Varian Gas Chromatograph 1400). The column was a 1.8 m coiled-steel column (3.2 mm inside diam) filled with Porapack Q (80 to 100 mesh, Waters Ass. Inc., Massachusetts, U.S.A.). The column temperature was 200 °C. The compounds were detected by a flame ionization detector. The peaks were identified by running authentic compounds both separately and together with the sample. Acetic acid was the only acid detected by this method. Pyruvate was determined enzymically in the filtrate with a Biochemica Test Combination (Boehringer, Mannheim, Germany).

RESULTS

Effect of glucose and CaCO₃

Bacitracin production closely paralleled growth in the medium without glucose, but was markedly retarded when glucose (1 %) was added (Table 1). pH decreased during the first period of growth when glucose was a constituent of the medium.

Analysis of the culture filtrate revealed that the pH drop was due to the presence of acetic and pyruvic acids. This is in agreement with Speck & Freese (1973) who examined acid production from glucose by *Bacillus subtilis*.

When the culture fluid was neutralized with CaCO₃, both the drop in pH and the inhibition of bacitracin production by glucose were prevented (Haavik, 1974*a*).

Effect of organic acids and low pH

When the pH of the culture fluid was lowered to about 5.5 with sterile hydrochloric acid (1 M) 12 h after the initiation of growth in the M20 medium without glucose, bacitracin production was not inhibited (Table 2). However, when the pH was lowered to about 5.5 with a mixture of acetic and pyruvic acids (about 0.3 ml of a 1:1 by volume mixture of concentrated acids was added to 60 ml culture fluid), bacitracin production was markedly inhibited (Table 2).

When acetate and pyruvate (5.0 g of each/l) were added to the M20 medium at neutral conditions a few hours after the initiation of growth, bacitracin production was not inhibited (Table 2).

Epps & Gale (1942) examined the effect of low pH upon several enzyme activities by using a phthalate buffer medium of pH 5.0. Therefore 0.5 % (w/v) phthalic acid was introduced into the M20 medium and the pH was then lowered with either hydrochloric acid or a mixture of acetic and pyruvic acids. Bacitracin production was only inhibited when the pH was lowered with acetic and pyruvic acids (Table 3). The inclusion of phthalic acid had no effect.

When the pH was lowered to 5.5 with acetic acid alone (0.3 ml/60 ml culture fluid), both bacitracin production and growth were severely inhibited. When the pH was lowered to 5.5 with pyruvic acid alone (about 0.3 ml/60 ml culture fluid) only bacitracin production was inhibited (Table 4).

DISCUSSION

Bacitracin production was inhibited when glucose was added to the medium, an effect also shown by Laishley & Bernlohr (1968) who suggested that bacitracin production is controlled by catabolite repression by glucose. The inhibitory effect of glucose, however, can be prevented by neutralizing the culture fluid with CaCO_3 (Haavik, 1974*a*), which indicates that the effect of glucose upon bacitracin production might simply be an effect of the low pH produced by glucose metabolism.

It is generally believed that the glucose effect or catabolite repression is not an incidental result of acid production, although a drop in pH is frequently observed as the glucose is metabolized (Paigen & Williams, 1970). This belief is mainly based on the work of Epps & Gale (1942) who examined the effect of low pH and glucose upon several enzyme activities of *Escherichia coli*. When glucose was added to their medium, the pH dropped to about 5.0 during its consumption. However, a comparison of organisms grown in a medium maintained at pH 5.0 with those grown in a glucose medium revealed a marked inhibition of several enzyme activities in the glucose medium as compared with the low pH medium, and they concluded that glucose has an inhibitory effect on the enzyme activities far greater than any effect that can be ascribed to pH.

Epps & Gale (1942) did not determine which acids from glucose metabolism caused the drop in pH, and they made a low pH medium by the addition of phthalic acid buffer. Consequently they compared the effect of some unknown organic acids from glucose metabolism with the effect of phthalic acid upon the enzyme activities at pH 5.0. We have examined the effect of low pH upon bacitracin production by lowering the pH with the organic acids actually formed as the glucose is metabolized by the bacteria. The low pH resulting from the addition of acetic acid and pyruvic acid produced a marked inhibition of bacitracin production, whereas a low pH produced by an inorganic acid (HCl) and phthalic

Table 2. *Effect of low pH and organic acids on growth and bacitracin production in M20 medium*

Time of incubation (h)	Control culture			pH lowered with HCl			pH lowered with acetic and pyruvic acid			Acetate and pyruvate added at neutral conditions		
	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)
11	0.32	7.10	0.1	0.32	7.12	0.1	0.30	7.10	0.1	0.33	7.10	0.1
12	0.64	7.30	0.3	0.60	5.51	0.3	0.62	5.55	0.3	0.66	7.34	0.3
13	1.20	7.43	0.8	1.30	5.64	0.9	1.04	5.65	0.4	1.36	7.39	0.7
14	1.90	7.58	1.6	1.64	5.88	2.0	1.20	5.80	0.5	1.80	7.53	1.7
15	2.96	7.80	3.2	2.50	6.20	3.8	1.84	6.10	0.7	2.60	7.68	3.3
16	3.44	8.12	4.3	3.60	6.38	4.6	2.80	6.40	1.2	3.04	7.92	4.9
17	4.10	8.25	8.2	3.96	6.80	7.7	3.24	6.92	1.7	3.65	8.27	8.5
18	5.10	8.30	12.7	5.25	7.05	13.4	5.00	7.04	2.1	5.10	8.33	12.0
20	6.20	8.52	14.5	6.30	7.19	15.0	6.10	7.30	5.4	6.10	8.50	13.7

The pH was lowered after 12 h incubation by addition of 1 M-HCl or a mixture of equal parts by volume of concentrated acetic and pyruvic acids (see text).

Table 3. *Effect of low pH and organic acids on growth and bacitracin production when phthalic acid (0.5 %) is a constituent of the medium*

Time of incubation (h)	pH lowered with HCl			pH lowered with acetic and pyruvic acids		
	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)
12	0.86	5.62	0.3	0.84	5.68	0.2
13	1.14	5.68	0.7	1.04	5.72	0.2
14	1.70	5.75	1.3	1.50	5.80	0.3
15	2.30	5.95	2.0	2.00	5.90	0.6
16	3.16	6.12	4.5	2.86	6.00	1.0
17	3.84	6.52	7.2	3.66	6.25	1.3
20	6.10	6.90	12.5	6.04	6.37	3.2
24	8.20	7.20	14.0	8.15	6.78	4.5

The pH was lowered after 12 h incubation by addition of 1 M-HCl or a mixture of equal parts by volume of concentrated acetic and pyruvic acids (see text).

Table 4. *Effect of acetic acid or pyruvic acid and low pH on growth and bacitracin production*

Time of incubation (h)	pH lowered with acetic acid			pH lowered with pyruvic acid		
	Growth (E_{650})	pH	Bacitracin (i.u./ml)	Growth (E_{650})	pH	Bacitracin (i.u./ml)
12	0.80	5.60	0.2	0.89	5.55	0.3
13	0.80	5.58	0.3	1.24	5.83	0.3
14	0.84	5.62	0.3	2.14	5.95	0.6
15	0.88	5.60	0.3	2.84	6.20	0.9
16	0.79	5.63	0.3	3.59	6.34	1.3
17	0.98	5.66	0.4	4.40	6.42	2.5

The pH was lowered to about 5.5 with concentrated acetic acid or concentrated pyruvic acid 12 h after the initiation of growth.

acid had no inhibitory effect. This indicates that the inhibitory effect of glucose upon bacitracin production was due to the organic acids produced from glucose metabolism.

Acetate and pyruvate have no inhibitory effect upon bacitracin production when added at neutral pH. Consequently the organic acids inhibit only in an acidic environment. Dagley, Dawes & Foster (1953) showed that the toxicity of organic acids is pH-dependent. They observed that the total growth was dependent on the initial pH of the medium: lower initial pH resulted in low total growth.

Our data indicate that the effect of low pH is dependent on the acid used to lower the pH. Total growth was not influenced by lowering the pH to 5.5 except when acetic acid was added, causing severely inhibited growth.

Organic acids exist in their undissociated state at low pH values; such undissociated small molecules penetrate the bacterial membrane relatively easily and hence create a low internal pH upon dissociation. This lower internal pH is probably strongly inhibitory to bacitracin production, in agreement with Ishihara, Sasaki & Shimura (1968) who found an optimum pH of 7.4 for the incorporation of radioactive histidine into bacitracin by a cell-free preparation. At lower pH, markedly less histidine was incorporated. This may explain why both organic acids and low pH are required to inhibit bacitracin formation.

Since acetic acid has a higher dissociation constant than pyruvic acid, more undissociated

molecules of acetic acid than of pyruvic acid are present in the medium at pH 5.5. Acetic acid is therefore able to create a lower internal pH than pyruvic acid. Bacitracin production is more sensitive to low pH than is growth (Haavik, 1974a). This may explain why acetic acid added to pH 5.5 inhibits both growth and bacitracin production, whereas pyruvic acid only inhibits bacitracin production.

Since the breakdown of glucose or other rapidly metabolized carbohydrates is often associated with a drop in pH due to the formation of organic acids, in some cases the glucose effect or catabolite repression may simply be an incidental inhibitory effect of low pH and undissociated organic acids.

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