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Pyruvate Production and Excretion by the Luminous Marine Bacteria

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During aerobic growth on glucose, several species of luminous marine bacteria exhibited an incomplete oxidative catabolism of substrate. Pyruvate, one of the products of glucose metabolism, was excreted into the medium during exponential growth and accounted for up to 50% of the substrate carbon metabolized. When glucose was depleted from the medium, the excreted pyruvate was promptly utilized, demonstrating that the cells are capable of pyruvate catabolism. Pyruvate excretion is not a general phenomenon of carbohydrate metabolism since it does not occur during the utilization of glycerol or maltose. When cells pregrown on glycerol were exposed to glucose, they began to excrete pyruvate, even if protein synthesis was blocked with chloramphenicol. Glucose thus appears to have an effect on the activity of preexisting catabolic enzymes.

Light emission by the luminous bacteria results from the activity of bacterial luciferase. This enzyme is present in the cells throughout the growth cycle but often undergoes a reduced in vivo activity during the later phases of growth. This phenomenon is readily seen with colonies of luminous bacteria appearing on petri plates. Within a day or two of plating, the cells reach a maximum intensity of light production; however, after several days, the colonies become dim and eventually dark, although the cells themselves remain viable for many weeks.

Bright luminescence is often restored in old cultures in the region surrounding a chance fungal contaminant that arises on the plate (9). This interaction was described by Hill and his associates (14), who found that during growth, the luminous bacteria gradually lower the pH of the medium to a point at which the luciferase can no longer function. The utilization of these excreted acids by the fungus returns the medium to neutrality and allows the resumption of light emission.

Several workers have demonstrated acid excretion as a result of anaerobic fermentation by the luminous bacteria (7, 27). However, observations of significant acid formation by young petri plate cultures suggest that acid excretion may also accompany aerobic growth. Accordingly, we examined the luminous bacteria with regard to acid production during aerobic metabolism. One of the major products of aerobic metabolism was pyruvate, a rarely encountered excretion product whose kinetics of appearance was investigated.

MATERIALS AND METHODS

Bacterial strains. Strains of the genus *Beneckea* used in this study were identified by Baumann et al. (2) and Reichelt and Baumann (27) as *B. harveyi* (RB-130, RB-376, RB-392), *B. splendida* (RB-378), *B. parahaemolytica* (RB-113), and *B. natriegens* (RB-107). Strains of the genus *Photobacterium* were characterized by Reichelt and Baumann (27) as *P. fischeri* (RB-61), *P. leiognathi* (RB-480, RB-225), and *P. phosphoreum* (RB-404). Other strains used in this study were identified by the method of Reichelt and Baumann (27). Four were isolated as symbionts from the light organs of luminous fishes: MJ-1 and CG-1 (*P. fischeri*) (31), PL-721 (*P. leiognathi*) (29), and NZ-1 (*P. phosphoreum*). One luminous strain of *P. phosphoreum* (MB7-38), and two unidentified nonluminous, gram-negative rods (NL-1 and NL-2) were isolated from seawater. The nonluminous marine strains *Vibrio marinus* (ATCC-15381), *V. costicolus* (NCMB-701), and *V. anguillarum* (strain 629) as well as the aerobe *Alcaligenes pacificus* (strain 62) were also supplied by Baumann and Reichelt. The strain of *Serratia marinorubra* was obtained from A. Carlucci.

Media. The seawater media used in this study were prepared with artificial seawater consisting of 0.4 M NaCl, 0.1 M $MgSO_4 \cdot 7H_2O$, 0.02 M KCl, and 0.02 M $CaCl_2 \cdot 2H_2O$ (18). The basal medium broth (HM) contained 50 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5), 19 mM NH_4Cl, 0.33 mM $K_2PO_4 \cdot 3H_2O$, 0.01 mM $FeSO_4$, and half-strength artificial seawater. For a complex medium (HC), 5 g of peptone (Difco) and 3 g of yeast extract (Difco) were added to 1 liter of HM. Carbohydrate carbon sources were added as indicated.

Cell enumeration. Cell material was monitored turbidimetrically with a Coleman Jr. II spectrophotometer at 660 nm. The conversion of optical density

(OD) to total cell protein was 3.0 to 3.5 mg of protein per ml per OD unit for all strains used. Cell number was determined electronically with a ZB-1 model Coulter Counter with a 30-μm aperture. For strain MJ-1, cell number was linear with OD over the range 0.05 to 0.5 OD units, and 0.1 OD unit was equivalent to 2.7×10^8 cells per ml.

Analytical techniques. Cell protein was determined after a 30-min digestion at 100°C in 1 M NaOH by the method of Lowry et al. (17). The concentrations of glucose, pyruvate, and acetate in the spent medium were determined after centrifugation at 8,500 × *g* for 10 min and filtration of the supernatant through 0.45-μm membrane filters (Millipore Corp.). The Glucostat method (Worthington Biochemical Corp.) was used to quantify glucose, pyruvate was measured enzymatically by the method of Lowry and Passonneau (16), and acetate was measured by acetokinase (30).

Conversion of carbon source to pyruvate. A variety of species of luminous and nonluminous marine bacteria were tested for pyruvate excretion. If pyruvate was produced, the percentage of carbon source utilized that was excreted as pyruvate was determined. Cells were grown aerobically to an OD of 0.3 (10^9 cells per ml) in 40 ml of HC with 2 mg of one of several carbohydrates per ml. The cells were harvested by centrifugation at 8,500 × *g* for 10 min. The pellet was washed in sterile seawater and recentrifuged, and the supernatant was removed completely. A 5-ml portion of HM with 3 mg of the carbohydrate (or carbohydrate plus metabolic inhibitor) per ml was added, and the pellet was quickly suspended. Several 1-ml portions were removed to 30-ml-capacity centrifuge tubes and immediately placed in a shaker at 300 rpm, which insured complete aeration of the thick cell suspension. At intervals of 5 to 10 min, a tube was removed and placed in an ice bath to stop cell activity. The tubes were centrifuged, and the supernatant was removed and filtered through 0.45-μm membrane filters (Millipore Corp.). The medium was then assayed for substrate utilized and pyruvate produced during the incubation period. These values were used to calculate the percentage of substrate carbon converted to pyruvate.

RESULTS

When cells of *P. fischeri* (strain MJ-1) were inoculated into a complex liquid medium containing glucose, the pH decreased as a function of aerobic growth (Fig. 1). If the medium was harvested at an OD of 0.3 (10^9 cells per ml), acetate and pyruvate were found as the major acidic excretion products.

To determine if these acids were actually products of glucose catabolism, cells pregrown in an HC with glucose were harvested and suspended in HM containing only glucose. An analysis of the medium during aerobic incubation revealed that 30 to 40% of the glucose-carbon utilized by the cells was excreted as pyruvate (Fig. 2). Acetate accounted for less than 9% of the utilized glucose.

Reports of pyruvate excretion and accumulation in the growth medium are sufficiently unusual to warrant a survey of the luminous bacteria with respect to this property. Table 1 presents the results of such a survey from the four species of luminous bacteria as well as several other species of nonluminous, marine, facultative anaerobes. Pyruvate production occurred to varying degrees in several strains using glucose. Isolates of *B. harveyi*, *B. natriegens*, and *P. fischeri* all excreted into the medium as pyruvate a considerable portion (>10%) of the glucose carbon utilized. A minor percentage (<10%) was present in the medium of strains of *P. phosphoreum*, *V. marinus*, *S. marinorubra*, and several *Beneckea* sp. whereas *P. leiognathi*, *V. anguillarum*, *V. costicolus*, two uniden-

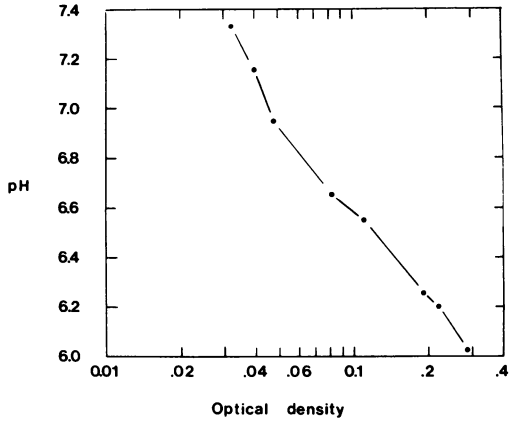

FIG. 1. *Decrease in the pH of complex liquid medium containing aerobically growing cells of strain MJ-1.*

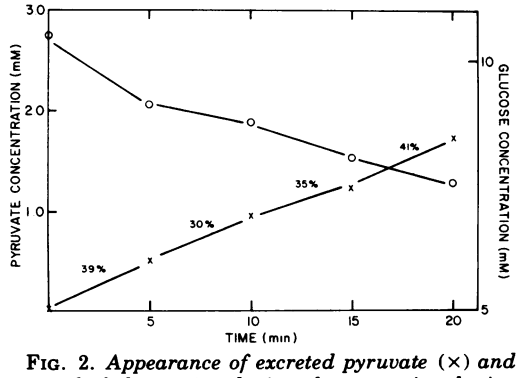

FIG. 2. *Appearance of excreted pyruvate* (×) *and removal of glucose* (○) *during short-term incubation of strain MJ-1 in HM plus glucose (3.5 × 10^9 cells per ml). Numbers indicate the percentage of utilized glucose-carbon that appears in the medium as pyruvate.*

TABLE 1. *Survey of marine heterotrophic bacteria for pyruvate excretion during utilization of glucose as sole carbon source*

Species (strain)		Luminosity[a]	% Glucose-carbon excreted as pyruvate[b]
P. fischeri	RB-61	+	36
P. fischeri	MJ-1	+++	35
P. fischeri	CG-1	++	14
P. leiognathi	PL-721	+++	0
P. leiognathi	RB-480	+	0
P. leiognathi	RB-225	+	0
P. phosphoreum	NZ-1	+++	9
P. phosphoreum	RB-404	+	6
P. phosphoreum	MB7-38	++	3
B. harveyi	RB-376	+	54
B. harveyi	RB-130	−	17
B. harveyi	RB-392	++	14
B. natriegens		−	12
B. splendida		+	3
B. parahaemolytica		−	1
V. marinus		−	2
V. anguillarum		−	0
V. costicolus		−	0
S. marinorubra		−	5
Nonluminous marine isolates	NL-1	−	0
	NL-2	−	0
A. pacificus		−	0

[a] Strains are designated as either moderately (+), brightly (++), or very brightly (+++) luminous; or nonluminous (−).

[b] The percentage of glucose-carbon that is excreted as pyruvic acid is indicated for each strain. No strain was found to excrete pyruvate during glycerol utilization.

tified nonluminous seawater isolates, and the aerobe *A. pacificus* produced no pyruvate. The process of pyruvate production from glucose was thus neither present exclusively in the luminous bacteria nor characteristic of all species of luminous bacteria. However, isolates of the luminous species *P. fischeri* and *B. harveyi* demonstrated an impressive degree of conversion of glucose to pyruvate, suggesting that this process is of some importance for these species. Consequently, a representative strain of *P. fischeri* (MJ-1) was chosen for subsequent study.

In a complex medium containing glucose, pyruvate was produced continuously over several generations of MJ-1 and was associated with balanced cell growth (Fig. 3). Even when a limiting amount of glucose was used as the sole source of carbon and energy, 10 to 20% of the glucose carbon was returned to the medium as pyruvate (Fig. 4). However, when glucose was depleted, the pyruvate was promptly utilized, demonstrating that the cells were capable of pyruvate catabolism (Fig. 4).

The catabolism of other carbohydrates did not result in pyruvate excretion to the extent seen with glucose (data not shown). None of the strains listed in Table 1 excreted pyruvate during glycerol utilization, suggesting that this process is not a general phenomenon of sugar catabolism. Thus, it was of interest to observe the initiation and kinetics of pyruvate production in MJ-1 cell pregrown in glycerol or glucose when suspended in a glucose medium. Glucose-

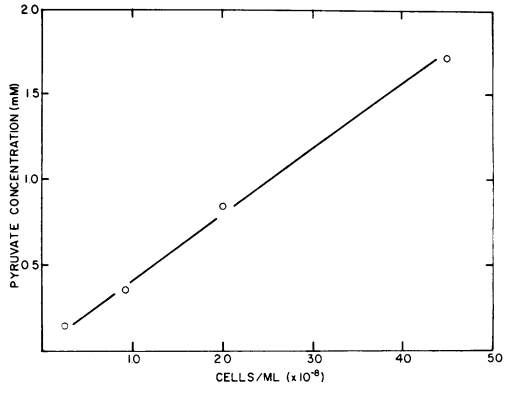

FIG. 3. *Appearance of excreted pyruvate as a function of normal cell growth in HC plus glucose.*

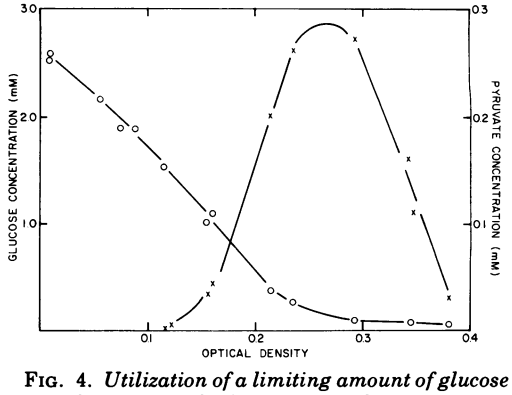

FIG. 4. *Utilization of a limiting amount of glucose in HM during growth of MJ-1* (○) *and appearance in and disappearance from the medium of excreted pyruvate* (×).

grown cells excreted pyruvate immediately upon suspension in a glucose medium (Fig. 5); the same strain however, when pregrown on glycerol and suspended in glucose, began pyruvate production after a lag of 15 to 20 min. This effect was not due to a lag in the initiation of glucose utilization since the cells began to use glucose immediately. Five to ten minutes is the time period typically required for the induction of an enzyme system in this organism (22). However, the lag does not represent a period during which some new enzyme(s) responsible for pyruvate excretion must be synthesized since the onset of excretion is unaffected in cells in which protein synthesis is completely inhibited by chloramphenicol (Fig. 5).

Several other metabolic inhibitors were tested for their effect on pyruvate excretion. Cyanide (10^{-3} M), an inhibitor of oxidative phosphorylation, caused a slight increase in the rate of pyruvate production for glucose-utilizing cells, but had no effect when added to glycerol-metabolizing cells. However, addition of sodium arsenite, a known inhibitor of dehydrogenase complexes such as pyruvate dehydrogenase, produced the pattern seen in Table 2. Incubation of cells with glucose plus arsenite had little effect on the pyruvate excretion rate; however, addition of arsenite to glycerol-utilizing cells initiated a rate of pyruvate excretion comparable to that seen during metabolism of glucose (Table 2).

TABLE 2. *Effect of arsenite on the pyruvate excretion rate of strain MJ-1* [a]

Substrate	Arsenite (mM)	Pyruvate excretion rate (μg/mg of protein/min)
Glycerol	0	0
Glycerol	0.5	95
Glucose	0	120
Glucose	0.5	114

[a] Cells were pregrown in an HC containing either glycerol or glucose and suspended in HM plus the sugar, with or without arsenite.

DISCUSSION

The luminous bacteria have been the subject of several studies into the catabolic processes of microbial intermediary metabolism. Doudoroff (7) demonstrated that two species of the genus *Photobacterium* exhibit a mixed acid fermentation of sugars when grown anaerobically. Baumann et al. (2) demonstrated that isolates of the other genus of luminous bacteria, *Beneckea*, were also facultative anaerobes. When grown aerobically, typical bacterial cytochromes were present, confirming the capacity for oxidative metabolism of substrates described by Johnson (15). This report demonstrates that aerobic acid production occurs in the luminous bacteria and that during oxidative growth on glucose, pyruvate is a major metabolic excretion product in some species.

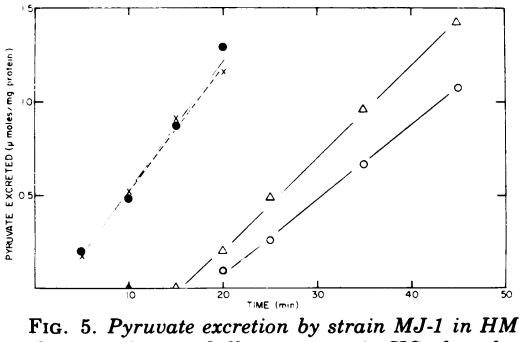

FIG. 5. *Pyruvate excretion by strain MJ-1 in HM with 0.2% glucose. Cells pregrown in HC plus glucose and suspended in HM plus glucose with* (×) *or without* (●) *chloramphenicol (20 mg/ml). Cells pregrown in HC plus glycerol and suspended in HM plus glucose with* (○) *or without* (△) *chloramphenicol.*

The incomplete aerobic catabolism of substrates leading to pyruvate excretion is a rarely reported phenomenon. With perhaps one exception (26), pyruvate has been observed as a major excretory product only in cases of unbalanced growth due to pH stress (22), oxygen limitation (24), or metabolic inhibition (4). The one exception interestingly involves *Pseudomonas natriegens* (8, 26), an estuarine isolate that has subsequently been reclassified as *B. natriegens* and shown to be closely related to *B. harveyi* (28).

Cells often excrete a metabolite if it is produced at a rate greater than it is utilized (23). Thus, the excretion of pyruvate may be a reflection of an imbalance between its glycolytic formation and its subsequent utilization via the tricarboxylic acid cycle (8). For the luminous bacteria, glucose utilization occurs at a moderate rate (0.10 to 0.15 μm/mg of protein per min) for both pyruvate-excreting and nonexcreting species, suggesting that the absolute rate of glucose utilization is not directly related to pyruvate excretion. However, a decrease in the normal activity of the enzymes responsible for the catabolism of pyruvate could also necessitate the buildup and excretion of pyruvate.

The repressive effect of glucose on the synthesis of catabolic enzymes of the tricarboxylic acid cycle is well documented (1, 10, 25). However, the presence of glucose may repress not only the formation but also the activity of postglycolytic enzymes in the luminous bacteria. If

during the exposure to glucose the activity of substrate catabolism is reduced at the level of pyruvate dehydrogenase, pyruvate could accumulate to a point where it must be excreted.

Support for this hypothesis is found in several observations. Cells of MJ-1 excrete pyruvate during the metabolism of glucose (and to a lesser degree glucose-6-phosphate and fructose), but little or no excretion occurs during utilization of glycerol, galactose, mannitol, or maltose. This specificity and the fact that the initiation of pyruvate excretion does not require protein synthesis suggest that glucose is acting on an already established catabolic system and that the effect is a specific rather than a general one. Finally, the addition of arsenite, an inhibitor of catabolic dehydrogenases (6, 11, 32) leads to pyruvate excretion in glycerol-utilizing cells mimicking the effect of glucose addition (Table 2).

Perhaps in those species of bacteria for which pyruvate excretion amounts to only a small percentage of metabolized glucose, it is simply a minor, nonfunctional activity. However, in strains that convert 30 to 50% of their substrate carbon into excreted pyruvate, it is conceivable that the process may have some adaptive significance. Luminous bacteria occur in the light organs of several species of marine fishes (3, 12, 13, 29), these associations being of benefit to both partners. In return for supplying nutrients to the bacterial population, the host fish is provided with a luminescent organ that can be used for one or more of a variety of behavioral purposes (19).

One such association was the source of strain MJ-1 used in this study (31). The excretion of pyruvate by MJ-1 in the light organ may either be a means of minimizing the carbon and energy loss to the host, or function in regulating the physiological state of the symbiosis (30). Both situations are examples of biochemical communication or exchange between partners, a common feature of symbiotic relationships (5, 21, 22). The presence of pyruvate excretion may thus be a specific adaptation by species of luminous bacteria to symbiotic associations.

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