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FUMARATE REDUCTION AND ITS ROLE IN THE DIVERSION OF GLUCOSE FERMENTATION BY *STREPTOCOCCUS FAECALIS*[1]

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ABSTRACT

DEIBEL, R. H. (American Meat Institute Foundation, Chicago, Ill.), AND M. J. KVETKAS. Fumarate reduction and its role in the diversion of glucose fermentation by *Streptococcus faecalis*. J. Bacteriol. 88:858-864. 1964.—Fumarate diverts the normal fermentation of glucose by *Streptococcus faecalis* FB82, as shown by the production of increased amounts of CO_2, formate, acetate, and acetoin, and decreased formation of lactate and ethanol. Experiments with D-glucose-*1*-C^{14}, in which low levels of labeled CO_2 were recovered, indicated that C-1 cleavage of the glucose molecule was not involved. The presence of fumarate afforded consistently larger cell crops in growth studies with glucose and other energy sources. On a molar growth-yield basis, anaerobically grown, glucose-fumarate cultures were equivalent to aerobically grown, glucose cultures. The reduction of fumarate by cell suspensions indicated that glucose, gluconate, and, to a lesser extent, glycerol and mannitol could serve as hydrogen donors. Several common metabolic inhibitors had no effect upon the fumarate reductase system in cell suspensions, although some sensitivity to acidic pH was noted. Significant levels of succinate oxidation activity were not detected. Fumarate reductase activity was demonstrated in all five *S. faecalis* strains tested. Distribution of this ability in *S. faecium* strains was variable, ranging from activity comparable with that of *S. faecalis* to total inactivity. The observations support the conclusion that fumarate functions as an alternate hydrogen acceptor, thus allowing pyruvate to participate in the energy-yielding phosphoroclastic and dismutation pathways.

In a previous communication (Deibel, 1964*b*), data were presented which suggested the ability of fumarate to divert the normal homofermentative dissimilation of glucose by *Streptococcus faecalis*. In the presence of fumarate, the fermentation of glucose yielded significantly increased quantities of CO_2, formate, acetate, and acetoin. To account for this radical alteration of end products, it was assumed that fumarate substituted for pyruvate in the regeneration of oxidized nicotinamide adenine dinucleotide, and thus averted the reduction of pyruvate to lactate. The subsequent catabolism of pyruvate via the dismutation and phosphoroclastic pathways would then account for the observed alteration of end products.

It is the purpose of this communication to substantiate and to extend the above hypothesis and observations, as well as to integrate these findings with those of previous investigators.

MATERIALS AND METHODS

Strains. One strain of *S. faecalis* (FB82) was used extensively in this study. It possessed the majority of physiological characteristics associated with this species (Deibel, Lake, and Niven, 1963). The source and characteristics of this and other enterococcal strains used were described previously (Deibel et al., 1963).

Media. The strains were maintained by daily transfer in APT medium (Difco). Most of the experiments were performed with a complex medium of the following composition: Tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled water, 1 liter; pH 7.2 to 7.4. The energy source was variable, but unless otherwise indicated it was added at the 1.0% level.

In some experiments a semisynthetic, casein-hydrolysate medium was employed (Deibel and Niven, 1964). Methionine was added to the medium, as Brodovsky, Utley, and Pearson (1958) showed that commercial preparations of

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casein hydrolysates may be deficient in this amino acid.

Chemicals. D-Glucose-*1*-C^{14}, sodium fumarate, and sodium pyruvate were obtained from Calbiochem.

Analytical methods. In fermentation-balance and in radioisotopic studies, the clarification procedure described by Neish (1952) was used. Volatile neutral products were determined by distilling a 50-ml sample of the clarified broth (pH 7.6 to 7.8). Approximately 200 ml of distillate were collected over a period of 2.5 to 3.5 hr. The reaction flask was then acidified, and the distillation was continued for the volatile acids. This sequence was employed to prevent some acetoin from distilling with the volatile-acid fraction and interfering in the subsequent determination of formate. Formate was estimated by the macrogravimetric procedure (Anonymous, 1940). Acetate was calculated by difference from the total volatile acids. Previously, it was observed that formate and acetate were the only volatile acids produced in the fermentation of pyruvate by *S. faecalis* (Deibel and Niven, 1964).

Fumarate was measured spectrophotometrically at a wavelength of 240 mμ in appropriately diluted samples of the clarified fermentation broth (Racker, 1950). Succinate was estimated manometrically after continuous ether extraction of the sample for 48 hr (Umbreit, Burris, and Stauffer, 1957). Ethanol (volatile neutral distillate) was determined enzymatically by the procedure of Bonnicksen and Lundgren (1957). The enzyme preparation used in this method was obtained from Sigma Chemical Co., St. Louis, Mo. Lactate was also estimated enzymatically, by the method of Scholz et al. (1959). The lactic dehydrogenase preparation was purchased from Calbiochem. The excellent agreement between this method and the chemical method of Barker and Summerson (1941) prompted continued use of the less laborious enzymatic procedure. Acetoin was estimated by the procedure described by Neish (1952).

Carbon dioxide was precipitated in a series of 0.1 N barium hydroxide traps after acidification of the culture medium and flushing with helium. The precipitate was collected by centrifugation, washed with cold distilled CO_2-free water, and dried to constant weight at 110 C.

Radioisotopic procedures. The $C^{14}O_2$ produced from the fermentation of D-glucose-*1*-C^{14} was trapped in a series of tubes containing 0.1 N sodium hydroxide; 1-ml portions of these samples and of the fermentation media, suitably diluted in 0.1 N sodium hydroxide, were dispensed on stainless-steel planchets. Radioactivity was measured in a gas-flow counter.

Preparation of cell suspension. Cultures were grown in the complex medium, supplemented with 1.0% sodium fumarate and 1.0% glycerol (or other energy source). The cells were harvested by centrifugation, resuspended in phosphate buffer (pH 7.0), and rapidly frozen in an ethylene glycol bath at −20 C. Prior to use, the frozen cells were rapidly thawed and resuspended in appropriate buffer. The cell concentration was adjusted by dilution and comparison to a standard curve relating optical density to dry weight of the cells.

Miscellaneous methods. Growth was estimated by optical-density determinations in a Bausch & Lomb Spectronic-20 colorimeter at a wavelength of 600 mμ. Methods of incubating under aerobic and anaerobic conditions were described previously (Deibel, 1964*a*). All cultures were incubated at 37 C.

RESULTS

Fate of C_1 in glucose-1-C^{14}. This experiment was performed to ascertain the possible diversion of the glucose fermentation by fumarate by a pathway other than the hexose diphosphate scheme. The fate of the various carbon atoms in alternate pathways of glucose metabolism has been reviewed (Gunsalus, Horecker, and Wood, 1955). Characteristically, the C_1 atom of glucose is found in the methyl group of lactate (and presumably pyruvate) in the hexose diphosphate scheme, and in the CO_2 if metabolism occurs via the hexose monophosphate scheme. Even if the fermentation is diverted by fumarate as anticipated, the decarboxylation of pyruvate would not be expected to yield labeled CO_2 from glucose-*1*-C^{14}.

S. faecalis FB82 was grown in 50 ml of the complex medium containing 1.0% glucose and 2.0 mc of glucose-*1*-C^{14} (specific activity = 7.4 mc/mmole). A duplicate culture was prepared and supplemented with 1.0% sodium fumarate. Another set of cultures was prepared in an identical manner but with the omission of the radioactive glucose. These cultures served as controls for subsequent analyses to determine the extent of diversion of the fermentation effected by

TABLE 1. *Glucose fermentation balances with Streptococcus faecalis FB82, illustrating the effect of fumarate*

Substrate	Product	Amt*
		mmoles
Glucose†		100
	Lactate	177.9
	Acetoin	0.7
	Ethyl alcohol	5.8
	Acetate	1.3
	Formate	2.9
	CO_2	6.8
Glucose‡		100
Fumarate		93.8
	Succinate	98.7
	Lactate	126.8
	Acetoin	9.2
	Ethyl alcohol	0.09
	Acetate	45.9
	Formate	7.3
	CO_2	52.7

* All values corrected for endogenous metabolism.

† Carbon recovery = 93.4%; O/R balance = 1.27; C_1/C_2 = 1.14.

‡ Carbon recovery = 94.9%; O/R balance = 1.02; C_1/C_2 = 0.93.

TABLE 2. *Growth response of Streptococcus faecalis FB82 to increasing levels of glucose in the presence and absence of fumarate*

Glucose in medium	Medium*	
	Basal	Basal + fumarate (1.0%)
%		
0	42†	48
0.1	63	85
0.2	73	100
0.3	85	122
0.4	105	130
0.5	113	140

* The complex medium was employed (see Materials and Methods).

† Optical density × 100.

fumarate. The cultures were incubated anaerobically for 24 hr at 37 C, and the CO_2 produced was trapped and counted.

In the presence of fumarate, only 0.52% of the initial radioactivity was recovered in the CO_2, and 79.9% of the activity remained in the clarified medium. In the absence of fumarate, corresponding values of 0.21% and 77.5% were obtained. Presumably, the remainder of the activity was associated with the cells which were removed in the clarification procedure.

The results indicate that a C_1 cleavage of the glucose molecule is not effected in the presence of fumarate. Thus, the most probable pathway of glucose catabolism in the presence of fumarate is via the hexose diphosphate scheme.

Fermentation balances. Significant increases in the quantities of acetate, formate, acetoin, and CO_2, and decreases in the quantities of lactate and ethanol, were observed in cultures with added fumarate (Table 1). The ratio of glucose utilized to fumarate reduced approached unity, indicating that approximately 50% of the total theoretical C_3-cleavage products of glucose were diverted from the normal pathway which ultimately results in the reduction of pyruvate to lactate. The approximately quantitative recovery of succinate tends to negate any fumarase activity, and the alteration of end products effected by fumarate cannot be associated with this enzyme. *In toto*, the results indicate that fumarate diverts the fermentation by acting as an alternate hydrogen acceptor, thus allowing the subsequent catabolism of pyruvate via the phosphoroclastic and dismutation pathways.

Growth studies. The inclusion of fumarate in the growth medium resulted in small but reproducible increases in the cell crop. A series of increasing glucose concentrations was prepared in the complex medium. A duplicate set was prepared in the same medium supplemented with fumarate. The increased growth response of the fumarate-containing cultures (Table 2) is in keeping with the diversion hypothesis, as additional energy-yielding reactions can be associated with pyruvate catabolism. The studies were extended to include the gluconate, ribose, and pyruvate fermentations, since these compounds also serve as energy sources for *S. faecalis* (Deibel et al., 1963) and involve catabolic pathways which differ from those of glucose. The results obtained with gluconate and ribose paralleled those obtained with glucose, in that the fumarate-containing medium afforded a larger cell crop as compared with the medium without fumarate. The formation of slime, with pyruvate as the energy source, precluded optical density determinations. The production and characterization of this viscous material has been discussed previously (Deibel, 1964*b*).

The increased cell crop effected by culturing the organism with fumarate was measured in terms of molar growth yields. Glucose-containing cultures were grown anaerobically with and without fumarate. In addition, a duplicate set of cultures was incubated aerobically on a reciprocating shaking apparatus. To afford greater stability of the cells in the subsequent repeated washings, hydroxylysine (0.1 mg/ml) was added to the growth medium. Smith et al. (1962) demonstrated superior stability of enterococcal cells when grown in the presence of this amino acid.

Previously, Seeley and VanDemark (1951) demonstrated a 35% increase in the cell crop of aerobically (as compared with anaerobically) grown cultures of *S. faecalis*. Thus, if this increase in cell crop can be equated with an alternative hydrogen acceptor (oxygen), the molar growth yield of aerobically grown, glucose cultures should approximate that of anaerobically grown, glucose-fumarate cultures. The results of the experiment (Table 3) indicate a more efficient anaerobic utilization of glucose when the organism is cultured with fumarate. In addition, the results suggest that Seeley and VanDemark's (1951) observation with aerobic cultures, and the anaerobic diversion effected with fumarate, reflect the same basic mechanism, which differs only in final hydrogen acceptor (oxygen or fumarate). Although the molar growth yield obtained with the anaerobically grown, glucose-containing culture of *S. faecalis* was somewhat higher than that reported by Bauchop and Elsden (1960), the relationships described in this study are not affected.

TABLE 3. *Comparison of growth yields of Streptococcus faecalis FB82 under various conditions of culture*

Conditions of incubation	Additions to basal medium*					
	Glucose			Glucose + fumarate (0.5%)		
	Glucose utilized	Dry wt of cells†	Molar growth yield‡	Glucose utilized	Dry wt of cells†	Molar growth yield‡
	mmoles	*mg*		*mmoles*	*mg*	
Aerobic	1.70	74.4	43.8	1.69	81.6	48.3
Anaerobic	1.75	48.8	27.9	1.75	82.4	47.1

* Complex basal medium. All values corrected for endogenous metabolism. Initial glucose concentration = 1.75 mmoles/150 ml of medium.

† Cell crops were washed five times with distilled water.

‡ Defined as mg (dry weight) of cells per mmole of glucose utilized (Bauchop and Elsden, 1960).

Fumarate reduction with various hydrogen donors. Subsequent experiments were performed to gain additional information on the enterococcal fumarate reductase system. In preliminary studies, cell crops were grown with glycerol as the energy source. Cell suspensions were prepared, and the ability of these preparations to reduce fumarate with glycerol as the hydrogen donor was tested.

The test system consisted of 25 μmoles of sodium fumarate (1 ml), 50 μmoles of glycerol (1 ml), and 1 ml of appropriately diluted cells. (A total volume of 4 ml was attained by addition of buffer.) All substrates and cell suspensions were prepared with phosphate buffer (pH 7.0). Preparations of cells alone, cells plus fumarate, and fumarate alone were included routinely for control purposes. The reaction mixtures were centrifuged (10 to 12,000 × *g*) to remove the cells, and residual fumarate in the supernatant fluid was estimated spectrophotometrically. Generally, 90 to 100% of the fumarate was reduced within 1 hr at pH 7.0 with approximately 10 mg of cells per reaction vessel. In this and subsequent cell-suspension experiments involving fumarate reduction, the reaction vessels were incubated routinely under helium at 37 C.

The activity of other hydrogen donors was compared with that of glycerol. Cell suspensions were prepared from cultures grown with mannitol, glycerol, glucose, or gluconate as the energy source. The concentration of cells employed was adjusted so that less than maximal activity (4.7 mg of cells per reaction vessel) was obtained. All suspensions were adjusted to the same optical density, and the respective substrates (50 μmoles) for each cell suspension were employed. As in previous experiments, a total volume of 4.0 ml was attained by the addition of phosphate buffer (pH 7.0). Glucose and gluconate were found to be the most efficient hydrogen donors, whereas glycerol and mannitol were approximately one-half as active (Table 4).

Initial results with the glucose system indicated complete failure to reduce fumarate. In this experiment, the culture medium contained 1.0% glucose, and the final pH value of the medium dropped to 4.2. As will be amplified below, the

TABLE 4. *Comparison of fumarate reductase activity with various hydrogen donors*[a]

Hydrogen donor[b] (50 μmoles)	Fumarate in cell suspension		Fumarate reduced
	Initial	Residual[c]	
	μmoles	*μmoles*	%
Glycerol	27.5	22.9	16.7
Sodium gluconate	27.5	19.0	31.0
Mannitol	27.5	23.9	13.1
Glucose[d]	27.5	27.5	0
Glucose[e]	26.1	17.6	32.5

[a] All cell suspensions (*Streptococcus faecalis* FB82) were adjusted to give 4.7 mg (dry weight) of cells per reaction vessel. Residual fumarate was determined after 1 hr at 37 C.

[b] The cell crops were grown on the same energy source as the hydrogen donor.

[c] All values corrected for endogenous reduction.

[d] Excessive acid production resulted in loss of reductase activity.

[e] Results obtained with cells grown under pH-controlled conditions. See text.

low pH value probably destroyed the fumarate reductase activity. When the experiment was repeated, with 0.2% glucose in the growth medium, an active system was obtained (Table 4).

An attempt was made to determine the ability of pyruvate to serve as a hydrogen donor for fumarate reduction. However, pyruvate (or perhaps its dimer) was observed to absorb significantly at 240 mμ, and thus further spectrophotometric studies with this substrate were unsuccessful.

The glycerol fumarate reductase system was employed to determine the possible inhibition effected by succinate. Varying the concentration of succinate from 10 to 500 μmoles in the standard system (25 μmoles of fumarate, 50 μmoles of glycerol, and 14.3 mg of cells in a total volume of 4.0 ml; incubation for 1 hr at 37 C) was found to have no effect upon the reduction of fumarate.

Various inhibitors (sodium sulfide, potassium cyanide, sodium arsenite, sodium azide, Atabrine, and malonate) were tested with cells grown in glucose-fumarate and glycerol-fumarate to determine their effect upon fumarate reduction. In the standard system described above, all inhibitors were added at a final concentration of 10^{-3} M, except for malonate (50 μmoles). No significant loss of fumarate reductase activity was demonstrated in either of the two cell suspensions with any of the inhibitors after incubation for 1 hr at 37 C.

To ascertain the adaptive nature of the enterococcal fumarate reductase system, cells were grown in the semisynthetic, casein hydrolysate medium containing 0.15% glucose; their activity was compared with that of cells grown in the same medium supplemented with 1.0% sodium fumarate. The glucose-fumarate-grown cell suspension was diluted to afford equivalent cell density with the glucose-grown cell suspension, and each reaction vessel contained 20 mg (dry weight) of cells. Multiple tubes were prepared in the usual manner and, at periodic intervals, tubes were removed and placed in a boiling-water bath to stop the reaction.

After an initial 5-min lag period, fumarate reduction was essentially linear with both cell suspensions until 25 μmoles of fumarate were reduced. The only significant difference observed was that cells preadapted to fumarate accomplished this reduction in 20 min whereas nonadapted cells required 30 min. It would appear that more conclusive information regarding the adaptive nature of the fumarate reductase must await studies with cell-free extracts.

Effect of pH. In the standard system described above, glycerol-fumarate-grown cells (14.3 mg dry weight per reaction vessel), with glycerol (50 μmoles) as the hydrogen donor, complete reduction of 27.9 μmoles of fumarate was observed at either pH 7 or 8 (phosphate buffer).

Diminished activity was observed at pH 6.0 in phosphate buffer (72.8% of the fumarate was reduced). At pH 5.0 (acetate buffer), almost complete loss of activity was observed (only 3.7% of the fumarate was reduced). These results, in addition to those obtained with a high glucose concentration, indicate that the fumarate reductase system is sensitive to acidic pH. Additional studies with cell-free preparations are indicated.

Succinate oxidation. An experiment was performed to determine the ability of *S. faecalis* FB82 to oxidize succinate. The organism was grown aerobically in the complex medium containing 0.5% glucose supplemented with either 1.0% sodium succinate or 1.0% sodium fumarate. The cell crops were harvested and tested for their ability to oxidize 50 μmoles of succinate under

aerobic conditions in cell suspension. After a 4-hr incubation period, followed by centrifugation to remove the cells, fumarate was estimated spectrophotometrically. Only 2.0 μmoles of fumarate accumulated with the succinate-grown cells, indicating that only 4.0% of the succinate had been oxidized. No activity was observed with the fumarate-grown cells. Consequently, it would appear that the organism has limited ability to oxidize succinate, in contrast with its ability to reduce fumarate.

Distribution of fumarate reductase among enterococci. Ten representative strains of enterococci were cultured in the complex medium containing 0.2% glucose and 0.5% sodium fumarate. Cell suspensions were prepared and tested for fumarate reductase activity, with 50 μmoles of glucose as the hydrogen donor. In this experiment, 20 mg of cells per reaction vessel were used (total volume made to 4.0 ml with phosphate buffer at pH 7.0; incubation for 1 hr at 37 C). *S. faecalis* strains R26, N83, K2A, 26C1, and 10C1 reduced 95 to 100% of the 25 μmoles of fumarate added. The results with the *S. faecium* strains were variable. Strains F24 and K6A were devoid of activity, and strains Igau and R39 reduced 14.1 and 24.1%, respectively, of the added fumarate. Complete reduction of the added fumarate was observed with strain R10.

Further studies with S. faecium (R10). The observation that *S. faecium* R10 possessed the ability to metabolize fumarate prompted a growth experiment identical with that presented in Table 2. However, unlike the *S. faecalis* strains, no increase in the growth response of *S. faecium* R10 was noted when the medium was supplemented with fumarate. To some extent, this result was expected; this strain, like most other *S. faecium* strains, does not possess the ability to utilize pyruvate as an energy source (Deibel and Niven, 1964). Further study with *S. faecium* R10 to determine the fate of fumarate and the end products of the glycerol-fumarate fermentation are warranted.

DISCUSSION

The reduction of fumarate by enterococci was first reported by Gunsalus (1947). He observed that the anaerobic fermentation of glycerol was enhanced significantly by fumarate; the quantitative recovery of succinate supported the conclusion that fumarate served as an alternate hydrogen acceptor. Gunsalus also noted an alteration of end products in the presence of fumarate, with an increased accumulation of oxidized compounds.

Kitahara, Fukui, and Misawa (1960) observed the widespread occurrence of fumarase in various lactobacilli and also detected fumarate disappearance in one of three enterococcus strains. In a previous study (Deibel, 1964*b*), no fumarase activity could be detected when *S. faecalis* FB82 was cultured with labeled fumarate. In addition, the quantitative recovery of succinate tends to reinforce these results. Thus, it is highly unlikely that significant fumarase activity is associated with *S. faecalis* FB82.

The results obtained in this study are in accord with the hypothesis that fumarate diverts the fermentation by acting as an alternate hydrogen acceptor. This affords the further metabolism of the intermediate pyruvate through the phosphoroclastic and dismutation pathways. Therefore, a more efficient utilization of substrate is effected, as demonstrated by an increased cell crop obtained in the presence of fumarate. The ability of *S. faecalis*, but not *S. faecium*, to utilize pyruvate as an energy source is the subject of a previous communication (Deibel and Niven, 1964).

The parallelism between increased cell crop and alternate hydrogen acceptor, as demonstrated in this study and that of Seeley and VanDemark (1951), merits comment. Previously, O'Kane (1950) observed the ability of lipoate (pyruvate oxidation factor) to affect radically the end products of glucose oxidation by cell suspensions of *S. faecalis* 10C1. When lipoate was added, acetate and CO_2 were the chief oxidized products. However, in the absence of lipoate, pyruvate either accumulated or it was further metabolized to form acetoin. It would appear that fumarate as well as oxygen can divert the normal glucose fermentation in such a manner that the subsequent metabolism of the pyruvate intermediate affords a larger cell crop, thus reflecting a more efficient utilization of substrate.

Studies with cell suspensions indicate that the enterococcal fumarate reductase is somewhat sensitive to acid. More definitive studies regarding pH lability and optimum, as well as possible inhibition by various metabolic inhibitors, must await the employment of cell-free preparations. It is interesting that the more reduced substrates

(i.e., glycerol and mannitol) do not effect a more efficient reduction of fumarate than do glucose or gluconate. The data do not offer an explanation for these results.

The enterococcal enzyme catalyzing the reduction of fumarate is somewhat similar to that of *Micrococcus lactilyticus*, as reported by Warringa et al. (1958). Enzyme systems from both species favor the reduction of fumarate and evidence comparatively weak oxidation of succinate. Until cell-free studies are conducted with the enterococcal system, further comparison or contrast with the enzyme obtained from other sources is not possible.

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