Synthesis of Lipoic Acid by *Streptococcus faecalis* 10C1 and End-products Produced Anaerobically from Low Concentrations of Glucose

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SUMMARY

Streptococcus faecalis 10C1 and 6783 grew anaerobically on glucose in media lacking lipoic acid and acetate, and assays with *Streptococcus cremoris* KH indicated that extracts of *S. faecalis* 10C1 contained lipoic acid activity equivalent to 200 ng of α -D,L-lipoic acid/mg protein. The Y_{glucose} value for a continuous culture of strain 10C1 grown anaerobically in lipoic acid-deficient medium was 37.2, about the same as that for 10C1 in lipoic acid-sufficient medium (35.5). Strains 10C1 and 6783 metabolized low concentrations of glucose (1 to 8 $\mu\text{mol/ml}$) non-homolactically and produced per mol of glucose about 1.0 mol lactate, 1.2 mol acetate, and 0.4 mol ethanol. Appreciable amounts of pyruvate, formate, glycerol, acetoin, diacetyl, and 2,3-butylene glycol were not detected. The Y_{glucose} values for strain 10C1 were lower (25, 17 and 21, respectively) if 10^{-4} M-arsenite or 0.5 % acetate was added or the pH value in the fermentor was decreased to 5.3. With acetate added or the pH value reduced, the glucose was metabolized mostly to lactate. Results indicate that *S. faecalis* 10C1 used the pyruvate-dehydrogenase-enzyme complex rather than the phosphoroclastic mechanism in metabolizing anaerobically to acetate approximately half of the pyruvate produced from low concentrations of glucose.

INTRODUCTION

The roles of acetate and lipoic acid (Reed, DeBusk, Gunsalus & Hornberger, 1951*a*; Reed, DeBusk, Johnson & Gelzendaner, 1951*b*) in the nutrition of *Streptococcus faecalis* are not completely understood (Deibel, 1964; Kamihara, 1969). Strain 10C1 and certain other strains of this species can grow on glucose in partially defined media containing acid-hydrolysed casein without lipoic acid, acetate, or lipid substances (O'Kane & Gunsalus, 1948; Deibel, 1964; Kamihara, 1969). Growing bacteria, resting suspensions, or extracts of this organism, however, require lipoic acid for pyruvate oxidation (O'Kane & Gunsalus, 1948; Gunsalus, Dolin & Struglia, 1952*a*; Reed, Leach & Koike, 1958; Deibel, 1964).

Propionate inhibited growth of *Streptococcus faecalis* 10C1 on 1 % (w/v) glucose in a casein-hydrolysate medium lacking lipoic acid, and this inhibition was reversed by the addition of lipoic acid or acetate (Stokstad, Seaman, Davis & Hutner, 1956; Kamihara, 1969). Acetate can relieve growth inhibition by propionate because this organism possesses the acetate kinase-phosphotransacetylase enzyme system (Rose, Grunberg-Manago, Korey & Ochoa, 1954), via which it can activate free acetate first to acetyl-phosphate and then to acetyl-coenzyme A (acetyl-CoA) and thereby obtain the C_2 units needed for synthesis. In manometric studies with suspensions of bacteria with lipoic acid present, the inclusion of pro-

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pionate in amounts equimolar to pyruvate reduced oxidation of extracellular pyruvate by 50 % (Kamihara, 1969). Kamihara (1969) postulated that propionate was activated to propionyl-CoA, which interfered with oxidation of pyruvate to acetyl-CoA, and concluded that pyruvate oxidation is necessary for growth of *S. faecalis* 10C1 and is important in lipid synthesis.

Collectively, the above reports raise the question of how *Streptococcus faecalis* is able to obtain the C₂ units needed for synthesis when grown in media lacking lipoic acid, acetate, or other lipid substances. The data reported in this paper indicate that *S. faecalis* 10C1 growing in lipoic acid-deficient media under anaerobic conditions synthesizes lipoic acid, which permits it to metabolize intracellular pyruvate derived from glucose to acetate. Exogenous acetate in large concentrations (0.5 %) or a low pH value (5.3) prevents the anaerobic metabolism of intracellular pyruvate to acetate and hence the formation of energy from pyruvate.

METHODS

Organisms. *Streptococcus faecalis*, strains NCTC 6783 and 10C1 (ATCC 11700; Gunsalus & Razzel, 1957), were obtained from Dr S. R. Elsdén, University of Sheffield, and Dr I. C. Gunsalus, University of Illinois, U.S.A., respectively. Since the pyruvate-dehydrogenase-enzyme complex of *S. faecalis* is inducible (Deibel & Niven, 1964), stock cultures were kept as agar stabs in the complex medium of Deibel & Niven (1964) containing 1 % (w/v) sodium pyruvate as an energy source. Ability to use pyruvate as an energy source separates these two strains from *S. faecium* (Deibel, 1964; Kandler, Schleifer & Dandl, 1968). *Streptococcus cremoris*, strain KH, was maintained in litmus milk and transferred monthly. The incubation temperature was 37 °C for *S. faecalis* and 30 °C for *S. cremoris*.

Inocula (1 to 2 %, v/v) for experiments to determine molar growth yields were prepared by growing each test organism on the homologous energy source in the medium to be used in determining growth yields. Bacteria were collected on a 0.45 µm membrane filter (Sartorius Division, Brinkman Instruments, Westbury, New York, U.S.A.), washed with one volume of 0.01 M-phosphate buffer (pH 7.0), and resuspended in one volume of buffer.

Media. For testing the ability of *Streptococcus faecalis* or *S. cremoris* to grow on glucose in the absence of lipoic acid and acetate and for assaying samples for lipoic acid, a modification of the partially defined HCM medium of Moustafa & Collins (1968) was used. This completely defined medium, designated HCM-AA, was prepared by omitting sodium acetate and replacing casein hydrolysate with the following 21 amino acids used by Niven (1944): the DL forms of alanine, aspartic acid, glutamine, isoleucine, leucine, lysine, methionine, norleucine, phenylalanine, serine, threonine and valine (0.1 g of each/l), the L-isomers of arginine hydrochloride, cystine, glutamic acid, histidine, hydroxyproline, proline, tryptophan and tyrosine (0.05 g of each/l), and L-asparagine (0.1 g/l). D,L-Glutamine and L-asparagine were sterilized by passage through microporous porcelain filters (no. VFA-54-03, Sela Flotronics, Spring House, Pennsylvania, U.S.A.) and added aseptically to autoclaved and cooled media. These two amino acids, required by most strains of *S. cremoris*, *S. diacetylactis* and *S. lactis* (Niven, 1944; Collins, Nelson & Parmelee, 1950; Collins & Bruhn, 1970), are heat labile and must be filter sterilized to support optimal growth of organisms that require them.

Molar growth yields were determined with the partially defined medium of Bauchop & Elsdén (1960), designated here and by Moustafa & Collins (1968) as BEO medium, and with the partially defined, lipoic acid-deficient medium of Gunsalus *et al.* (1952*a*), modified by omission of sodium acetate and addition of 1 % (w/v) vitamin-free acid-hydrolysed casein

(Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and the 21 amino acids listed above. The BEO medium contained Oxoid acid-hydrolysed casein (Consolidated Laboratories, Chicago Heights, Illinois, U.S.A.). In one experiment, 10 % (w/v) of vitamin-free acid-hydrolysed casein replaced the Oxoid casein hydrolysate (BEV medium).

Media in 5 or 10 ml and 300 ml quantities were autoclaved at 121 °C for 11 and 15 min, respectively. Larger volumes were autoclaved for 30 min. A stock solution of glucose was autoclaved separately and appropriate amounts were added aseptically to the media.

Extraction of lipoic acid from casein hydrolysate. A solution of Oxoid casein hydrolysate sufficient for 1 l of BEO medium was prepared according to the method of Bauchop & Elsdon (1960). This solution and some NBC casein hydrolysate were separately adjusted to pH 3.0 with hydrochloric acid, 25 ml samples were steam-distilled by the method of Neish (1950) to remove acetate, and the residues were extracted with 12 ml quantities of chloroform (Stokstad *et al.* 1956). The chloroform extracts were evaporated just to dryness in a hot-water bath, dissolved in 10 ml of 95 % ethanol, and sterilized by filtration through microporous porcelain filters. Samples were assayed for lipoic acid.

Extraction of lipoic acid from Streptococcus faecalis. *Streptococcus faecalis* 10C1 was grown anaerobically on glucose in continuous culture in the partially defined, lipoic acid-deficient medium of Gunsalus *et al.* (1952*a*), modified as described above. The bacteria were washed twice with distilled water and disrupted by shaking with 0.2 mm glass beads in a Mickle tissue disintegrator for 1 h at 2 °C. The extract was clarified by centrifuging for 30 min at 15000 g and dialysed against 0.05 M-phosphate buffer (pH 6.7) at 2 °C for 24 h (four 1-l volumes, changed every 6 h). The dialysed extract was sterilized by passage through a Sartorius membrane filter (pore size, 0.22 µm), and 1 ml amounts were acid hydrolysed by adding 2 ml of 9 N-H₂SO₄ to each and autoclaving them for 2 h at 121 °C (Gunsalus, Struglia & O'Kane, 1952*b*). The hydrolysates were extracted with chloroform; the chloroform fractions were separated, evaporated to dryness in a warm water bath, and dissolved in ethanol.

Assays for lipoic acid. The modified HCM-AA medium containing 0.5 % (w/v) glucose was used in assaying samples for lipoic acid. The test organism, *Streptococcus cremoris* KH, proved unable to grow in the basal medium unless lipoic acid or acetate was added – a result that substantiated previous reports (Collins *et al.* 1950; Lytle & O'Kane, 1951). Inocula were prepared by growing *S. cremoris* as 5 ml cultures in screw-cap test tubes (13 × 100 mm) at 30 °C for 24 h in the HCM-AA medium supplemented with 1 ng/ml of α-D,L-lipoic acid. The bacteria were washed once with 0.01 M-phosphate buffer and resuspended in buffer before 0.05 ml amounts were transferred to 5 ml quantities of the basal medium containing the material to be tested for lipoic acid. Growth (extinction at 600 nm) in the medium (70 h at 30 °C) was compared to that in tubes of the basal medium containing 0.0 to 10 ng α-D,L-lipoic acid/ml. The stock solution of lipoic acid from which the standards were made was prepared by the method of Gunsalus & Razzel (1957).

Cultivation methods. The continuous culture device (chemostat type; working volume, 300 ml) and the gassing train were modifications of those employed by Moustafa & Collins (1968). The flow rate of medium to the growth vessel, controlled by a Buchler Polystaltic Pump (model 2-6100, Buchler Instruments, Fort Lee, New Jersey, U.S.A.), was monitored by measuring the flow of effluent from the growth vessel over timed intervals and found to vary no more than ± 2 %. Alkaline pyrogallol, prepared just before use by combining 25 ml 40 % (w/v) pyrogalllic acid and 25 ml 20 % (w/v) Na₂CO₃ for each litre of medium and headspace, was used to remove any traces of oxygen from the nitrogen gas (99.99 % purity). In some experiments the nitrogen was passed over heated copper subsequent to passing it

through the alkaline pyrogallol. Lower bacterial yields did not result, indicating that fresh alkaline pyrogallol alone adequately removed any traces of oxygen.

Effluent samples, conveyed via latex rubber tubing to stoppered flasks in a mixture of ice and water, were prepared for chemical analysis by centrifuging them to remove bacteria, adding sufficient 10 N-H₂SO₄ to reduce the pH value to 2, and storing the samples at 4 °C until tested.

Batch cultures were grown in 150 ml of medium in 160 ml prescription bottles partially immersed in a constant-temperature water bath. For anaerobiosis the bottles were fitted with rubber stoppers and 3 mm glass tubing and attached via a manifold to nitrogen passed through a gassing train of alkaline pyrogallol.

Growth and yield measurements. Growth was followed by measuring extinction (*E*) at 600 nm with a Beckman spectrophotometer, model DB, with 1 cm cuvettes. Bacterial mass was determined gravimetrically for each concentration of energy source using four to eight 30-ml samples. Bacteria were pelleted by centrifugation at 8000 *g* for 30 min at 2 to 4 °C, washed once with one volume of cold distilled water, resuspended in one volume of water, and dried in aluminium weighing dishes to constant weight at 105 °C.

Standard curves relating *E* to dry wt were prepared by the method of Moustafa & Collins (1968). *Streptococcus faecalis* was harvested from continuous cultures growing anaerobically on glucose (1 to 8 µmol/ml) at a dilution rate of 0.25 h⁻¹. Regression analyses were used to determine the lines of best fit. With the partially defined media, *E* units corresponding to 1 mg dry wt *S. faecalis* strains 6783 and 10C1/ml were 2.91 and 2.64 respectively.

Chemical methods. Glucose was determined by the glucostat method (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) as modified by Chaykin (1966). Pyruvate was determined by the method of Friedmann & Haugen (1943) and by the pyruvate stat-pack method (Calbiochem, Los Angeles, California, U.S.A.). Formate was determined by the method of Wood & Gest (1957); protein by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Chaykin (1966); acetoin and diacetyl by the method of Westerfeld (1945); ethanol by the alcohol stat-pack method (Calbiochem); total lactate by the method of Barker & Summerson (1941) as modified by Barker (1957); and glycerol and 2,3-butylene glycol were determined by the method of Neish (1950).

Acetate was determined by gas-liquid chromatography with an Aerograph Hy-Fi, model 600 D, with stainless steel column (Varian Aerograph, Walnut Creek, California, U.S.A.). The column was 152.4 × 0.32 cm (5 ft × $\frac{1}{8}$ in.), packed with 60- to 80-mesh Chromosorb W (DMGS treatment) containing 20 % neopentylglycol succinate and 2 % H₃PO₄ as the liquid phase. The injection and column temperatures were 220 and 155 °C, respectively. The carrier gas, 100 % nitrogen, and the hydrogen flame were supplied at gas flow rates of 26 and 20 ml/min, respectively. Standards were run in triplicate, and 0.8 µl amounts of unknown samples were injected in duplicate or triplicate. When sodium acetate was added to growth media, net acetate production was not determined.

RESULTS

Synthesis of lipoic acid by Streptococcus faecalis. Neither the modified completely defined HCM-AA medium nor the lipoic acid-deficient medium of Gunsalus *et al.* (1952*a*), modified by omission of acetate and addition of 21 amino acids and NBC casein hydrolysate, supported growth of *Streptococcus cremoris* KH unless either lipoic acid or acetate was added. Nevertheless, each of the media supported growth of *S. faecalis*, and each of the strains (10C1 and 6783) was transferred serially at 24 h intervals in the media in the absence of

added lipoic acid or acetate. These results suggested that the strains of *S. faecalis* were able to synthesize lipoic acid and to use it in forming the C₂ units from pyruvate required for synthesis. This possibility was tested by attempting to extract lipoic acid from *S. faecalis* 10C1 and to use the extracted lipoic acid in place of commercially obtained lipoic acid for growing *S. cremoris* KH.

Prior to testing extracts of *Streptococcus faecalis* for lipoic acid, the growth response of *S. cremoris* KH to increasing known concentrations of lipoic acid was determined. The lowest concentration of α -D,L-lipoic acid to which this strain of *S. cremoris* responded was 0.1 ng/ml medium, and the growth response (increase in *E* at 600 nm) to concentrations of 1.0 to 10.0 ng (the highest concentration tested) was linear and similar to that reported for *S. cremoris* HI-2 by Lytle & O'Kane (1951) and *S. faecalis* 8034 by Stokstad *et al.* (1956).

Chloroform extracts of Oxoid and NBC casein hydrolysates that had been steam distilled at pH 3.0 by the method of Neish (1950) to remove any acetate were tested for lipoic acid. The extract of Oxoid casein hydrolysate promoted growth of *Streptococcus cremoris* equivalent to 500 to 1000 ng α -D,L-lipoic acid/g dry wt. However, the extract prepared from NBC casein hydrolysate did not promote growth of *S. cremoris*. The latter result substantiated the finding of Collins & Bruhn (1970) that NBC casein hydrolysate does not contain lipoic acid.

Streptococcus faecalis 10C1 was grown on glucose anaerobically as a continuous culture in the medium of Gunsalus *et al.* (1952a) modified as indicated above, and chloroform extracts were prepared (see Methods). The extracts served in place of lipoic acid or acetate for growing *S. cremoris* KH in the modified HCM-AA medium. Calculations showed that the extracts had growth promotional activity equal to 200 ng α -D,L-lipoic acid/mg protein. Gas chromatographic analyses of the extracts did not reveal acetate or other short chain fatty acids, and it was concluded that the extracts contained some form of lipoic acid.

End-products and molar growth yields. Y_{glucose} values and end-products produced anaerobically from glucose by continuous cultures of *Streptococcus faecalis* 10C1 in lipoic acid-deficient and lipoic acid-sufficient media were determined (Table 1). In our modification of the medium of Gunsalus *et al.* (1952a), which we had found devoid of lipoic acid, the end-products produced were the same and molar growth yields were not greatly different from those produced in BEO medium, which according to our results contains lipoic acid. In each of these cases the fermentation was non-homolactic, and approximately half the glucose was converted to acetate. Some ethanol was produced, pyruvate did not accumulate, and only the following trace amounts of other compounds were found (mol/mol glucose): formate (0.05 to 0.10), acetoin plus diacetyl (0.008), glycerol (0.00 to 0.05), and 2,3-butanediol (0.00 to 0.17).

Amounts of ATP produced/mol glucose based on end-products were not appreciably different from the amounts calculated by assuming that $Y^{\text{ATP}} = 10.5$ as proposed by Bauchop & Elsdén (1960). Carbon recoveries for the bacteria grown on glucose in the modified medium of Gunsalus *et al.* (1952a) and in BEV medium agreed with those calculated from glucose, but recoveries from continuous cultures of 10C1 and 6783 in BEO medium are about 25 % greater than calculated values. Though the high carbon recoveries suggested that glucose enhanced the production of end-products from some constituent of BEO medium, similar to the requirement of glucose for the metabolism of arginine (Rosenberger & Elsdén, 1960; Moustafa & Collins, 1968), acetate production from the undetermined constituent apparently yielded ATP, and the high carbon recoveries appeared not to invalidate calculating the yield of ATP from the end-products produced. Addition of arsenite, a specific inhibitor of lipoic acid (Losada & Arnon, 1963), or sodium acetate, to decrease the availa-

Table 1. *End-products and molar growth yields produced anaerobically from low concentrations of glucose by Streptococcus faecalis* 10C1 in lipoic acid-deficient and lipoic acid-containing media

Modification of experimental conditions	Medium	End-products/mol glucose			Y_{glucose} (g dry wt/mol)	ATP/mol glucose based on	
		Lactate (mol)	Acetate (mol)	Ethanol (mol)		End-products (mol)	$Y^{\text{ATP}} = 10 \cdot 5$ (mol)
None	BEO	0.90	1.17	0.37	35.5	3.61	3.38
Arsenite (10^{-4} M) added; batch cultures	BEO	Not tested	Not tested	Not tested	25.0	Not determined	2.38
Na acetate (0.5 % w/v) added; batch cultures	BEO	1.87	Not tested	0.13	17.0	2.00	1.62
<i>Streptococcus faecalis</i> 6783	BEO	0.66	1.31	0.47	39.6	3.75	3.77
Batch cultures	BEV	1.30	0.40	0.20	30.6	2.30	2.91
None	MG	1.01	1.07	0.37	37.2	3.52	3.54
pH 5.3	MG	2.05	Not tested	Not tested	21.0	2.05	2.00

Continuous cultures of the organism were grown on glucose (1 to 8 μ mol/ml) in BEO medium, found to contain lipoic acid, and in BEV and modified Gunsalus *et al.* (MG) media, found to not contain lipoic acid, at a dilution rate of 0.22 to 0.26 h^{-1} . End-products produced in the presence of glucose were corrected for the amounts produced in absence of added glucose. In the last experiment the content of K_2HPO_4 in the medium was decreased so that the pH of the continuous culture would be 5.3 instead of 6.3. The formula used for calculating from end-products mol ATP produced/mol glucose was mol lactate + mol ethanol + $2 \times$ mol acetate. For composition of media and details of methods, see text.

bility of CoA by resulting in the formation of acyl esters (Hespell, Joseph & Mortlock, 1969), or operating the chemostat at pH 5.3 rather than pH 6.3, resulted in lower Y_{glucose} values and the production of larger amounts of lactate, indicative of fermentations that were essentially homolactic (Table 1).

DISCUSSION

Some strains of *Streptococcus faecalis* (strain 8034; Stokstad *et al.* 1956) require lipoic acid, as do most strains of *S. cremoris*, *S. lactis* and *S. diacetylactis* (Collins *et al.* 1950; Lytle & O'Kane, 1951; Reed *et al.* 1951*b*; Collins & Bruhn, 1970), and apparently are unable to synthesize this growth factor. Nevertheless, our results, using *S. cremoris* KH as an assay organism, indicate that growth and the production of acetate from low concentrations of glucose by *S. faecalis* 10C1 under strictly anaerobic conditions can be seen as reflecting this organism's ability to synthesize lipoic acid.

Streptococcus faecalis 10C1 ferments glucose by the hexose diphosphate pathway (Sokatch & Gunsalus, 1957). Under strictly anaerobic conditions the organism metabolized to acetate approximately half of the pyruvate produced from low concentrations (1 to 8 $\mu\text{mol/ml}$) of glucose, but the fermentation was essentially homolactic if 0.5 % (w/v) sodium acetate was added to the medium (Table 1). We assume the added acetate prevented formation of acetate from pyruvate by resulting in the formation of acyl esters and thus decreasing the availability of CoA (Hespell, Joseph & Mortlock, 1969). These results explain why the Y_{glucose} values of 30 to 35 determined by Moustafa & Collins (1968) with *Streptococcus faecalis* 6783 are considerably higher than the value of 21 found for *S. faecalis* 10C1 by Smalley, Jahrling & Van Demark (1968). Each of these studies was with low concentrations of glucose (1 to 8 $\mu\text{mol/ml}$), but the medium used by Smalley *et al.* contained 0.5 % (w/v) sodium acetate. Furthermore, our results with *S. faecalis* 6783 (Table 1) show that Moustafa & Collins (1968) were incorrect in assuming that *S. faecalis* 6783 under strictly anaerobic conditions metabolizes low concentrations of glucose homolactically. In addition to emphasizing the importance of constituents of the medium, present results also show that the pH value of the medium is very important in determining $Y_{\text{substrate}}$ values. At pH 5.3 the Y_{glucose} value for *S. faecalis* 10C1 was 21, and it is apparent that the organism fermented the low concentrations of glucose homolactically.

Streptococcus faecalis 10C1 used the lipoic acid-dependent dehydrogenase system (O'Kane & Gunsalus, 1948; O'Kane, 1950; Gunsalus *et al.* 1952*a*) rather than the phosphoclastic mechanism (Gunsalus, 1953; Sokatch & Gunsalus, 1957) in metabolizing anaerobically to acetate approximately half of the pyruvate produced from the low concentrations of glucose, since only trace amounts of formate were produced simultaneously with the production of acetate, and since the Y_{glucose} value was decreased by addition of arsenite, a specific inhibitor of lipoic acid (Losada & Arnon, 1963). Similar to findings of Hempfling, Mainzer & Van Demark (1969), our results do not account for reoxidation of all the NADH produced in glycolysis and pyruvate oxidation. Production of lactate and ethanol by 10C1 at pH 6.3 without added acetate in BEO, BEV and modified Gunsalus media accounted for reoxidation of 46 to 65 % of the NADH. The remainder possibly was reoxidized by constituents of the media and in biosynthetic processes.

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