Substrates Used by *Brochothrix thermosphacta* When Growing on Meat

FREDERICK H. GRAU

CSIRO Division of Food Research, Meat Research Laboratory, Cannon Hill, Queensland 4170, Australia

(Received for publication November 5, 1987)

**ABSTRACT**

Substrates used by *Brochothrix thermosphacta* when growing aerobically on meat include glucose, ribose, glycero1, glycerol-3-phosphate and inosine. Glycogen and inosine-monophosphate are not used. Of these substrates, only glucose and ribose are metabolized during anaerobic growth. Ribose is probably the major energy source for anaerobic growth on high pH meat.

*Brochothrix thermosphacta* commonly constitutes a significant proportion of the final bacterial flora found on stored red meat (8). It has been reported that, on meat, glucose and glutamate are both used for aerobic growth and only glucose for anaerobic growth (9,18). However, in laboratory media neither glutamate nor amino acids sustain aerobic growth (10,15). Furthermore, high pH (>6.0) meat, which has a low glucose content (3,17), is able to support a high population of both aerobically and anaerobically growing *B. thermosphacta* (5). It is unclear which substrates are used by this organism for growth on meat.

A strain of *Enterobacter* sp. has been reported to inhibit the growth of *B. thermosphacta* on meat under anaerobic conditions by competing more successfully for glucose (18). On the other hand, the production of an inhibitory substance by an isolate of *Lactobacillus* sp. was suggested since, although this organism apparently inhibited *B. thermosphacta*, it competed less successfully for glucose than did *B. thermosphacta*. If substrates additional to glucose are used by *B. thermosphacta*, then competition for glucose may not be of direct importance in explanations of microbial interactions and inhibitions. What may appear to be the production of inhibitory substances may simply be competition for another substrate. A knowledge of the substrates used by organisms growing on meat may allow a more rational analysis of apparent microbial interactions and aid in the selection of possible organisms to limit the growth of spoilage bacteria such as *B. thermosphacta*.

The work reported in this paper shows that, during aerobic growth on beef and lamb, *B. thermosphacta* metabolizes glucose, ribose, glycero1, glycerol-3-phosphate and inosine and, during anaerobic growth, utilizes glucose and ribose. On high pH muscle, ribose is probably the major energy source for anaerobic growth of *B. thermosphacta*.

**MATERIALS AND METHODS**

**Muscle and muscle extracts**

Pieces of muscle were aseptically obtained (5) from post-rigor beef (M. semitendinosus) or lamb (M. longissimus dorsi) tissue. Aqueous muscle extracts were prepared by blending muscle pieces with an equal mass of distilled water and adjusting the pH to 5.5 with 1 N HCl. After centrifugation (10,000 x g for 20 min), the supernatant was heated to 80°C and held at this temperature for 3 min, then chilled. The extract was again centrifuged and the pH of the supernatant adjusted to pH 6.5 with 1 N NaOH. This heated, aqueous extract was filter sterilized.

**Inocula**

*B. thermosphacta* ATCC 11509 was grown aerobically at 5°C in nutrient broth no. 2 (Oxoid) supplemented with 0.3% yeast extract (Difco) and 0.2% glucose (NBYG). Cells were harvested (3000 x g for 10 min) during exponential growth, washed and then suspended in distilled water. Strips of muscle (ca. 6x1x1 cm) were dipped into the cell suspension, blotted dry, and comminuted in a blender to give an inoculated mince containing about 10⁷ colony forming units (CFU)/g.

Muscle-extracts were also inoculated with washed cells which had been grown at 25°C either aerobically or anaerobically in NBYG.

For studies into potential growth substrates for *B. thermosphacta*, inocula were grown at 25°C either in NBYG or in a casamino acids-yeast extract-vitamins medium (CYV) containing 0.1% glucose. CYV medium was a minimal growth medium (10) supplemented with 0.1% vitamin-free casamino acids (Difco) and 0.05% yeast extract. Aerobically or anaerobically grown cells were washed and suspended in half-strength mineral salts 56 (5) to provide the inocula.

**Aerobic and anaerobic growth**

Cultures (8 ml) were grown aerobically in 125-ml side-arm flasks in a Gyrotory water-bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Anaerobic cultures were grown in Thunberg tubes which had been evacuated and flushed four times with high purity nitrogen. Growth was monitored by turbidity (Klett-Summerson colorimeter, no. 66 filter), or by sur-
face plating appropriate dilutions in 0.1% peptone (Oxoid) on tryptone soya agar (Oxoid) supplemented with 0.5% yeast extract and 0.2% glucose and on the medium of Gardner (7). Plates were incubated at 25°C and colonies counted after 2 and 3 d.

Chemical analysis
Bacterial cells were removed from heated aqueous extracts used in growth experiments by centrifugation at 10,000 x g for 10 min, and the supernatants used to estimate glycogen and to prepare perchlorate deproteinized extracts. Mince was homogenized in a Buhler homogenizer (E. Buhler, Tubingen, West Germany) with an equal mass of sterile distilled water at 5°C. After samples were taken for the determination of viable count and pH, samples (0.3-0.4 g) were used for the estimation of glycogen (14). These samples were chilled, perchloric acid (9.2 M) added to a final concentration of 0.25 M, and the pH adjusted to ca. 5 with 30% KOH. Then 1 ml of amylglucosidase solution (1 mg protein/ml 0.2 M acetate, pH 4.8) was added, and the samples incubated at 40°C for 2 h.

The remainder of the mince homogenate or aqueous extract was deproteinized in 0.25 M perchloric acid, the pH adjusted to pH 6.5 with 30% KOH, and the precipitated perchlorate removed by centrifugation.

The deproteinized supernatants were used in the following assays: glucose and glucose-6-phosphate (2) using hexokinase and glucose-6-phosphate dehydrogenase; glyceraldehyde-3-phosphate dehydrogenase; inosine and hypoxanthine (6) using nucleoside phosphorylase and xanthine oxidase. All enzymes were from Boehringer-Mannheim (West Germany). An estimate of inosine-monophosphate (milli-molar extinction coefficient of 12.3) was obtained in equimolar amounts as inosine disappeared. There was no change in the amount of glycogen (1.9 mM glucose equivalents), glucose-6-phosphate (0.48 mM), or inosine-monophosphate, inosine and hypoxanthine, the substrates used and their order of disappearance was similar. Figure 1 shows the utilization of some growth substrates during aerobic growth in an extract from muscle of pH 5.8. Glucose (1.9 mM) was used first, followed by glyceraldehyde (0.79 mM), and glyceraldehyde-3-phosphate (0.25 mM) and inosine (0.3 mM consumed). Hypoxanthine accumulated in equimolar amounts as inosine disappeared. There was no change in the amount of glycogen (1.9 mM glucose equivalents), glucose-6-phosphate (0.48 mM), or inosine-monophosphate (2.2 mM) during growth. In uninoculated

RESULTS

Anaerobic growth on muscle of high pH
When minced beef muscle of pH 6.8, inoculated with B. thermosphacta, was stored anaerobically at 5°C in Thunberg tubes, the organism grew with a generation time of 12 h to give a final population of about 2 x 10^8 CFU/g. Glucose, glyceraldehyde-6-phosphate and glycogen were not present in detectable quantities in the mince (<0.04, <0.04 and <0.2 μmoles glucose equivalents/g wet weight respectively). During storage at 5°C the concentrations of both inosine and inosine-monophosphate declined (from 1.7 and 2.6 respectively to 0.2 and 0.9 μmoles/g) and the amount of hypoxanthine increased (from 3.0 to 6.0 μmoles/g), but there was no significant difference between inoculated and sterile mince. The concentration of free ribose increased from 0.45 to 0.8-0.85 μmoles/g during storage of both the inoculated and sterile mince. However, near the end of the growth of B. thermosphacta, the ribose content fell and, as growth ceased, the ribose content in the inoculated mince was less than 0.1 μmoles/g. During this time ribose remained in the range 0.8-0.85 μmoles/g in the sterile mince. This implies that ribose was being used by B. thermosphacta for growth.

Because enzymatic changes in the content of potential growth substrates in mince made it difficult to interpret which compounds were being used by B. thermosphacta, heated-aqueous extracts of beef and lamb muscle were used for further studies. Such extracts had the additional advantage that muscles of differing pH values could be used and the extracts adjusted to a constant pH.

Growth in extracts from muscle of pH <5.9
In heated, aqueous extracts, prepared from either beef or lamb muscle of pH 5.6-5.8 and adjusted to pH 6.5, B. thermosphacta grew aerobically at 25°C to a population of at least 3 x 10^8 CFU/ml. Though in extracts prepared from different muscle samples the absolute quantities of potential growth substrates varied (particularly glycogen, inosine-monophosphate, inosine and hypoxanthine), the substrates used and their order of disappearance was similar. Figure 1 shows the utilization of some growth substrates during aerobic growth in an extract from muscle of pH 5.8. Glucose (1.9 mM) was used first, followed by glycerol (0.79 mM), and glyceraldehyde-3-phosphate (0.25 mM) and inosine (0.3 mM consumed). Hypoxanthine accumulated in equimolar amounts as inosine disappeared. There was no change in the amount of glycogen (1.9 mM glucose equivalents), glyceraldehyde-6-phosphate (0.48 mM), or inosine-monophosphate (2.2 mM) during growth. In uninoculated
extracts there were no changes in the concentrations of any of these compounds during the 10 h incubation period at 25°C.

When extracts made from muscle of pH 5.6-5.8 were incubated anaerobically at 25°C, *B. thermosphacta* grew from ca. 10³ CFU/ml to ca. 10⁹/ml in 42-48 h. During growth there were no changes in the concentrations of glycogen, glucose-6-phosphate, glycerol-3-phosphate, glycerol, inosine-monophosphate, inosine, or hypoxanthine in the extracts. The glucose content, on the other hand, decreased during anaerobic growth and was undetectable (<0.01 mM) when growth ceased.

**Growth in extracts from muscle of pH 6.6-6.8**

In heated, aqueous extracts prepared from beef or lamb muscle of pH 6.6-6.8, no glycogen, glucose-6-phosphate or glucose was detected.

When *B. thermosphacta* was grown aerobically at 25°C in such extracts adjusted to pH 6.5, there was an initial phase of rapid growth followed by a phase of much slower growth (Fig. 2). The concentration of inosine-monophosphate (1.6 mM) did not change. During the faster period of growth, glycerol (0.91 mM) and ribose (0.15 mM) disappeared from the extract. During the slower period of growth, both glycerol-3-phosphate (0.66 mM) and inosine (0.64 mM) were used and hypoxanthine accumulated (0.64 mM increase). The addition of 3.7 mM glucose to such extracts not only increased the cell yield but also extended the phase of rapid growth. It appears that either the accumulation of hypoxanthine, or the use of inosine or glycerol-3-phosphate as energy sources was the cause of the slower secondary growth phase.

Anaerobically, *B. thermosphacta* grew at 25°C from inocula of 10³ CFU/ml to reach only ca. 2 x 10⁷/ml in 32-48 h. There was no change in the concentrations of glucose-3-phosphate, glycerol, inosine-monophosphate, inosine or hypoxanthine in the extracts. Ribose, initially present at ca. 0.2 mM, disappeared by the time growth ceased. The addition of 3.7 mM glucose to extracts increased the final population of *B. thermosphacta* to about 3 x 10⁸ CFU/ml, indicating that, in these extracts of high pH lean, growth was limited by the amount of utilizable carbohydrate. Inclusion of the material precipitated during the heating step in the preparation of the extract did not increase the final population.

**Growth in laboratory media**

The ability of *B. thermosphacta* to use some compounds present in meat as energy sources was tested in CYV medium. Aerobically the organism grew readily (generation time 1.7-2.2 h at 25°C) when glucose, ribose or glycerol (0.1% w/v) were added to CYV. There was no growth with glycogen, inosine-monophosphate or L-glutamate. With glucose-6-phosphate, growth occurred only after a long lag period (7-10 h). With glycerol-3-phosphate or inosine as substrates, the growth rate was slow (3.5-6 h/generation). As inosine was used and hypoxanthine accumulated growth slowed. The initial growth rate with 3.7 mM inosine was reduced about half by the addition of 11.1 mM hypoxanthine.

*B. thermosphacta* grew anaerobically in CYV medium when glucose or ribose was added, but there was no growth with glycogen, glucose-6-phosphate, glycerol-3-phosphate, glycerol or inosine-monophosphate.

**DISCUSSION**

From the results obtained with CYV medium and with heated aqueous extracts of meat, it appears that, of the potential growth substrates examined, glucose, ribose, and glycrol are the preferred substrates for aerobic growth by *B. thermosphacta* on meat. While glucose-3-phosphate and inosine are metabolized, they give only slow growth and are used only after glucose, ribose and glycrol. Furthermore, hypoxanthine derived from the breakdown of inosine appears to inhibit growth on inosine. The role of glucose-6-phosphate is less clear. In the experiments with meat extracts, glucose-6-phosphate was only found when glucose was also present and no utilization of glucose-6-phosphate was detected by the time growth ceased. Cells grown in NBYG or in glucose-CYV medium exhibited a long lag before growth took place in CYV with glucose-6-phosphate. If glucose-6-phosphate in meat is utilized for growth by *B. thermosphacta*, it will only be after glucose, ribose, glycrol, glycerol-3-phosphate and inosine have been used. Lactate and amino acids do not support significant growth in laboratory media (10,15).

The number of substrates from meat able to be used for anaerobic growth are more restricted and seem to be principally glucose and ribose.
The glucose content of muscle varies with its pH (3,17), and at pH values >6.4 there is little if any glucose available for growth. In the experiments with meat extracts derived from muscle of pH 6.6-6.8, growth was limited both aerobically and anaerobically by the concentration of available energy sources, since the addition of glucose increased the cell yield. Under anaerobic conditions, extracts from high pH muscle supported growth to only about 2 x 10^8 CFU/ml, whereas in mince, *B. thermosphacta* grew to around 2 x 10^6 CFU/g. The higher yield in mince is probably a reflection of the greater availability of ribose. In heated aqueous extracts, some ribose is presumably lost through Maillard-type browning reactions. More importantly, further production of ribose from the degradation of inosine-monophosphate and inosine is stopped. In muscle, the breakdown of these compounds continues for a considerable period during storage (20) with the rate being faster at higher pH values (19). Since muscle contains 6-8 μMoles of ATP + ADP + AMP/g (1,16), and there is virtually complete conversion of these nucleotides to hypoxanthine during storage, there is potentially 6-8 μMoles of ribose/g available for microbial growth. Not all of this ribose may be available for growth of *B. thermosphacta*. There are several pathways for inosine to break down to ribose and ribose phosphates, and the concentrations of free ribose and ribose phosphates found in stored muscle appear to be considerably less than the hypoxanthine produced (4,13).

Strom and Larsen (22) showed with aqueous extracts of herring that strains of *Enterobacter sp.*, *Proteus sp.* and *Aeromonas* sp. used ribose, inosine and inosine-monophosphate for anaerobic growth. The ability to ferment ribose appears to be common in lactic-acid bacteria isolated from meat (12,21). Thus, the post-mortem degradation of nucleotides provides energy sources that can be used by *B. thermosphacta*, lactic-acid bacteria, and fermentative gram-negative bacteria when these organisms are growing on high pH meat. Furthermore, competition for free or bound ribose may play a role in any interaction or inhibition of one organism by another.

ACKNOWLEDGMENTS

I am grateful to P. B. Vanderlinde for his skilled technical assistance. This work was supported by the Australian Meat Research Committee.

REFERENCES