Mixed Carbon Source Utilization of Meat-Spoiling *Pseudomonas fragi* 72 in Relation to Oxygen Limitation and Carbon Dioxide Inhibition

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The growth of meat-spoiling *Pseudomonas fragi* 72 was studied on a defined salt medium supplemented with L-aspartate, citrate, creatine, creatinine, D-glucose, l-glutamate, and l-lactate. The utilization of the different carbon sources was followed in batch and continuous culture and under the influence of oxygen limitation and carbon dioxide inhibition (50% CO₂ in air). Under nonrestricted atmospheric conditions in batch culture, the organism showed a preference in the utilization of the carbon sources in the order glucose > lactate > citrate > aspartate-glutamate > creatine > creatinine. The first five sources were utilized simultaneously. The order of preference was changed in continuous culture to lactate-citrate-glutamate-aspartate > glucose > creatine > creatinine. All carbon sources were utilized at lower dilution rates, but as the rate was increased the concentration of the carbon sources started to increase in the effluent and the preference could be seen. Under conditions of oxygen limitation the preference for glucose was weakened, but for lactate it was slightly enhanced (batch and continuous culture). Under conditions of CO₂ inhibition, the preference for glucose was enhanced. However, lactate and amino acids were still preferred to glucose in the continuous culture. The utilization of creatine and creatinine was blocked by CO₂ in batch culture, and only a slight utilization of creatine was noticed in a chemostat at lower dilution rates.

When beef, pork, lamb, poultry, and fish stored aerobically under refrigeration become spoiled it is generally considered to be due to the activity of pseudomonads (4, 13, 15, 16, 20, 27). The most common *Pseudomonas* species found in beef, pork, lamb, and poultry meat appears to be *Pseudomonas fragi* (2, 4, 25, 31).

According to Gill (13), the spoilage flora growing on a meat surface utilizes compounds such as glucose, free amino acids, and lactate. These carbon sources are present in adequate amounts to support growth until spoilage has occurred; i.e., even if the concentration is decreasing in the uppermost layer, the compounds diffuse from below. Proteolytic activity and penetration of bacteria down in the tissue does not occur before the meat is already spoiled. Furthermore, Gill (13) has claimed that the *Pseudomonas* flora has a preference for glucose, and it is not until glucose is depleted that the organisms attack the free amino acids (the amino acids are consumed before lactate [14]), and at this stage the meat soon gives off an offensive odor because of the production of volatile by-products from the amino acid catabolism. In fact, Gill (13) has suggested that this is the explanation for the short shelf life of meat with a high pH (meat deficient in glucose [DFD meat]), i.e., the *Pseudomonas* flora utilize free amino acids from the start; hence, the meat spoils quickly.

An important environmental factor capable of strongly restricting aerobic growth is the gas phase. It has been suggested that the maximum aerobic cell density on the meat surface is not determined by substrate limitation but by oxygen limitation (13). Moreover, Newton and Rigg (26) have claimed that *Pseudomonas* cells growing at submaximal rates under oxygen limitation would not be subjected to catabolite repression and so could degrade amino acids in the presence of glucose, indicating that under the free availability of oxygen the cells would be under catabolite repression of glucose.

However, the growth of *Pseudomonas* spp. is affected not only by the partial pressure of oxygen, but also by the pressure of carbon dioxide (7, 8, 21). As soon as the meat is packed in plastic film, the carbon dioxide content of the gas phase increases to a point at which it influences the growth rate of *Pseudomonas* spp. The growth rate of *P.fragi* 72 was reduced by 17 to 19% with only 4% carbon dioxide in the gas phase (defined medium at 25°C [21]). No data have been found in the literature indicating whether the inhibitory effect of carbon dioxide could influence the choice of carbon source by the organisms. A great deal of data, however, are available on the beneficial effects of carbon dioxide on the shelf life of different types of meat (3, 10, 11, 24).

The purpose of this study was to illuminate the carbon source utilization of *P. fragi* growing on a mixture of compounds that may be available on the surface of meat and the effect of oxygen limitation or carbon dioxide inhibition on this utilization. *P. fragi* 72, which originates from spoiled, refrigerated beef, has been found previously to be a typical meat-spoiling strain (25). The organism was grown in batch and continuous cultures on a mixture of L-aspartate–citrate–creatine–creatine–D-glucose–L-glutamate–L-lactate.

**MATERIALS AND METHODS**

Organism and inoculation procedures. The organism used was *P. fragi* 72, which was originally isolated from refrigerated, spoiled beef. *P. fragi* 72 has been characterized previously (25). Stock cultures were freeze-dried and stored at 4°C. Before inoculation *P. fragi* was subcultured first in nutrient broth for 8 h (25°C, pH 6.0; Difco Laboratories, Detroit, Mich.) and then in the salt medium described below supplemented with L-aspartic acid–citric acid–creatine–D-glucose–L-glutamic acid–L-lactic acid (at the concentrations given below) and incubated for 15.5 h (25°C and pH 6.0). The fermentor described below was then inoculated with 80 ml of the growth suspension. The inoculate gave an *A*₂₅₀ to the
the fermentor medium of about 0.2. The viable count in the fermentor was about 10^6 CFU/ml (Tryptone-glucose-extract agar [Difco]; 25°C for 3 days).

Substrate. The growth medium of the fermentor had the following composition (in grams per liter unless otherwise indicated): Na_2HPO_4 · 2H_2O (0.40); KH_2PO_4 (0.37); MgSO_4 (0.50); (NH_4)_2SO_4 (0.25); CaCl_2 (0.10); MnSO_4 · H_2O (0.05); FeSO_4 · 7H_2O (0.01); ZnSO_4 · 7H_2O (0.005); CuSO_4 · 5H_2O (0.005); silicone antifoaming agent (30% [wt/vol]; BDH Chemicals Ltd.; 0.1 ml/liter); L-aspartic acid (3702; BDH; 0.61); citric acid-H_2O (244; Merck & Co., Inc.; Rahway, N.J.; 1:12); creatine-H_2O (C-3630; Sigma Chemical Co., St. Louis, Mo.; 0.79); d-glucose (10117; BDH; 0.95); l-glutamic acid (G-1251; Sigma; 0.78); L-lactic acid (L-1750; Sigma; 0.48). The glucose was sterilized separately by heating at 121°C for 20 min at a concentration of 9.5% (wt/vol), whereas the medium, adjusted to pH 4.0, was heat sterilized in the fermentor at 121°C for 30 min.

The medium described above was used in the batch cultures while the concentrations of the carbon sources were decreased in the medium used for the continuous cultures, i.e., the following amounts were applied (in grams per liter): L-aspartic acid (0.29); citric acid (0.53); creatine-H_2O (0.37); d-glucose (0.45); L-glutamic acid (0.37); L-lactic acid (0.22).

This medium was used to heat sterilized at 121°C for 1 h in 20-liter glass bottles. The glucose was heated separately in 18% (wt/vol) solution and was added to the medium after heating.

In one series of experiments the carbon sources of the medium were supplemented with proportions which were closely resembling those of meat. The composition was as follows (in grams per liter): L-aspartic acid (0.50); citric acid (0.50); creatine (5.0); d-glucose (1.0); L-glutamic acid (1.0); L-lactic acid (8.0).

Fermentor equipment and culture control. The culture vessel was a fermentor with a working volume of 1.0 liter (Chemoferm AB, Hägersten, Sweden). The medium was continuously fed into the fermentor with a membrane pump (precision dosage pump FE 211; Braun, Melsungen, Federal Republic of Germany), and continuous outflow was achieved through an overflow outlet and a tube pump (MHRE MK 3 flow inducer; Watson-Marlow, Falmouth, United Kingdom). The fermentor and equipment have been described previously (21).

The pH was kept constant at 6.0 by automatic titration with 0.6 M HCl or when necessary with 1.0 M NaOH (pH meter, PHM 62; titrator, TTT 60; Radiometer, Copenhagen, Denmark). The temperature was kept at 25°C and controlled with a heating bath which circulated water around an outer shell of the fermentor. The stirrer speed was 1,200 rpm.

The chosen gas compositions were supplied to the culture at the following flow rates (in liters per hour): air (71); 50% air–50% CO_2 (81); 4% air–95% N_2 (74). The air was mixed with CO_2 (Alfax AB, Malmo, Sweden; purity, 99.998%) or nitrogen (Alfax AB; 99.999%) aided by flow meters (F-150; A; Porter Instrument Co. Ltd., Hatfield, United Kingdom). The oxygen content of the gas flow was measured with a paramagnetic oxygen analyzer (Servomex, OA 540; Taylor Instrument Analytic Ltd., Crawborough, United Kingdom).

Growth experiments. Each culture experiment was started as a batch culture for 7 to 10 h. The continuous culture was then started at the lowest dilution rate and increased in stages until the biomass was washed out. After each variation in the dilution rate (D) and before samples for analytical measurements were taken the culture was run for 136 h at D = 0.03, 64 h at D = 0.05 to 0.08, 40 h at D = 0.1 to 0.15, and 23 h at D > 0.2. In batch culture the samples were taken at time intervals coincident with growth. All cultures were run at pH 6.0 and 25°C.

The biomass was estimated by measuring the A_620 of the fermentor medium (Turner spectrophotometer; 330; the same 1-cm cuvette was used for all measurements) and the dry weight, which was estimated by filtration through a membrane filter (type GS Millipore Corp., Bedford, Mass.) with a pore diameter of 0.22 μm; the filter was dried for 1 h at 105°C before it was weighed. The dry weights increased proportionally to the A_620 values. However, the latter were generally more reproducible, and the biomass is reported as absorbance. Viable count was done on Tryptone-glucose-extract agar (Difco) for 3 days at 25°C.

Samples for carbon source analyses were cooled on ice for 5 min, centrifuged at 6,000 rpm, frozen, and stored at −50°C until the analyses were performed. The concentration of L-aspartic acid, citric acid, creatine, creatinine, d-glucose, d-glucronic acid, d-glucono-δ-lactone, l-glutamic acid, and L-lactic acid were determined enzymatically. Test reagents were purchased from Boehringer Mannheim Scandinavia AB (Bromma, Sweden) as complete test kits and tests were performed in accordance with the test instructions from the manufacturer (1).

The sensitivities of the enzymatic methods at a carbon source concentration of 1.0 g/liter were as follows (in grams per liter): L-aspartic acid (±0.01); citric acid (±0.01); creatine (±0.04); d-glucose (±0.01); d-gluconic acid (±0.02); d-glucono-δ-lactone (±0.02); l-glutamic acid (±0.05); l-lactic acid (±0.02). The measured carbon source in the fermentor medium before inoculation had a maximum deviation from the theoretical value as follows (in grams per liter): L-aspartic acid (±0.02); citric acid (±0.05); creatine and creatinine (±0.10); d-glucose (±0.03); l-glutamic acid (±0.07); l-lactic acid (±0.02). The lowest limit for the analyses for all the carbon sources was about 0.01 g/liter.

All experiments were run twice, and the results were similar. However, all reported data are from the second series of experiments.

Calculations. The maximum specific growth rate (μ_max) in the batch cultures was based on the absorbance measurements and were calculated by the method of Pirt (29). The growth yield (Y) was calculated as grams (dry weight) of biomass per gram of consumed carbon source.

RESULTS

Batch cultures. The utilization of different compounds in air by P. fragi 72 is shown in Fig. 1.

Glucose and the amino acids were the first compounds to be depleted. However, the utilization of the different carbon sources (with the exception of creatine and creatinine) apparently began simultaneously. The utilization of creatine and creatinine began when glucose and nearly all of the amino acids were depleted and the biomass had reached a fairly high value. The viable count at this point (7 h: A_620 = 2.40) was 10^8 CFU/ml, whereas it was 1.4 × 10^5 CFU/ml at the beginning of the experiment.

Figure 2 shows the utilization of the different compounds when, in contrast to the previous experiment, they were present initially at the same concentration. This utilization was studied under different atmospheric conditions.

Under a nonrestricted atmosphere (air), the organism showed a preference in utilizing the different carbon sources in the order glucose > lactate > aspartate-glutamate > creatine-creatinine (Fig. 2a).

When P. fragi was oxygen limited (Fig. 2b), the order of
preference was generally the same as under nonlimited conditions. However, the difference in preference for glucose and lactate nearly vanished.

In 50% CO₂ (no oxygen limitation), the preference for glucose was enhanced as compared with the situation in air (Fig. 2c). The utilization of creatine and creatinine was blocked for at least 24 h (Fig. 2c).

The maximum specific growth rate (μmax) of P. fragi 72 was 0.5 h⁻¹ in air and 0.2 h⁻¹ in 50% CO₂-air. The final growth yield was 0.3 g (dry weight) per g of consumed carbon source in all tested gas atmospheres.

The utilization of glucose, creatine, and creatinine is more closely demonstrated in Fig. 3 (same experiment as that shown in Fig. 2a). A large proportion of glucose was converted to gluconate, and a considerably smaller proportion was converted to gluconolactone before catabolism. Creatine was utilized at a slightly higher rate than creatinine (Fig. 3). The viable count was 1.5 × 10⁸ CFU/ml at the start (time zero) and 10⁹ CFU/ml after 7 h (end of the biomass curve).

Continuous cultures. The utilization of the different carbon sources by P. fragi 72 under different gas atmospheres in continuous culture is shown in Fig. 4. The cultures can be characterized as a carbon source-limited chemostat at the lower dilution rates. However, as the dilution rate increased, the concentration of the different carbon sources in the effluent started to increase, and the order of preference for a certain carbon source could be established.

In air, the preference was very low for creatine or creatinine and slightly lower for glucose than for the rest of the carbon sources (Fig. 4a).
When the culture was oxygen limited, the organism preferred the carbon sources in the order lactate > glutamate > citrate-aspartate > glucose > creatine-creatinine (Fig. 4b).

Under CO₂ inhibition, the utilization of creatine or creatinine was very weak, whereas the order of preference for the remainder was lactate > aspartate-glutamate > glucose-citrate (Fig. 4c).

**DISCUSSION**

The utilization of a mixed carbon source by *P. fragi* 72 was examined in batch and continuous cultures. With regard to the continuous culture, it should be noted that a biofilm was established at the higher dilution rates. The establishment of such a biofilm at dilution rates near the maximum specific growth rate (μ_max) and the effect of such a biofilm on the growth characteristics of a chemostat with *Pseudomonas* spp. has been reported previously (22, 23). However, in the present study it was not considered crucial whether the organism was represented by freely suspended cells or attached ones. In fact, the microbiological activity on meat takes place in a biofilm on the meat surface.

In general, batch and continuous cultures gave similar results with regard to the organism’s preferred carbon source. However, one important exception was the preference for glucose; i.e., the organism started to utilize glucose in the batch culture (Fig. 1), whereas glucose was released at a relatively early stage in the continuous culture (Fig. 4). This may be due to the establishment of a biofilm in the continuous culture; i.e., the recorded difference in glucose preference may be the difference between immobilized and suspended cells.

However, there are other explanations that should be considered. Microorganisms have different uptake systems for glucose. For example, *Pseudomonas aeruginosa* alternatively can take up glucose directly or convert it to gluconate and 2-oxoglucuronate on the outside of the cell membrane before uptake (5). Furthermore, the glucose concentration has a controlling effect on the choice of an uptake system by an organism (17, 19). The glucose concentration of the microenvironment in the present study differed between the two culture systems.

Hence, the balance be-
tween the systems may have changed. This might, in turn, alter the maximum uptake capacity of the organism.

However, an indication for the difference between batch and continuous cultures may be that the maximum uptake potential that the organism can obtain for a certain carbon source differs between different sources. Höfe (18) has shown that the uptake potential for glucose increases in time in a glucose-limited chemostat of Cytophaga johnsonae. Similar results have been reported for Escherichia coli growing on tryptophan (28).

It is generally considered that the spoilage bacteria of meat utilize low-molecular-weight, soluble compounds (4, 15). The mixture of carbon sources in the present study included glucose, amino acids (aspartate and glutamate), lactic acid, creatine, creatinine (in the medium some creatine spontaneously converted to creatinine), and citric acid (included in this study to prohibit precipitation in the medium). In most of the experiments the carbon sources were included in equimolar amounts. However, in one series of experiments the proportions were related more to the actual proportions of meat (Fig. 1 [15]). With respect to the amino acids, only aspartate and glutamate were included, and this was at a proportion that approximately corresponded to the total amount of free amino acids in the meat. However, glutamate and glutamine have been shown to represent a significant part of the total content of free amino acids in lean pork (35% [12]).

In published reports, considerable emphasis has been placed on the theory that glucose plays a key role in the mechanisms of aerobic meat spoilage (13, 15). Glucose is said to cause catabolite repression on the amino acid catabolism of Pseudomonas spp.; hence, these organisms only utilize glucose if there is any left after they start to utilize amino acids, thus spoiling the meat. It is assumed that the breakdown products from amino acids are much more deleterious to the meat quality than those from glucose. For example, Gill and Newton (14) have shown that fluorescent and nonfluorescent Pseudomonas spp. in meat juice medium utilize glucose better than amino acids and amino acids better than lactic acid.

The results of this study contradict the results described above on the following grounds: (i) P. fragi 72 utilized glucose, lactate, and the two tested amino acids simultaneously; (ii) P. fragi 72 preferred lactate before the amino acids (especially under conditions of O2 limitation).

In this context, it may be pointed out that the utilization rate of glucose could easily be misread because of the conversion of glucose to, for example, gluconate (Fig. 3), i.e., if the amounts of these converted products were not analyzed. Gill and Newton (14) have given no indication that they carried out tests to determine such possible intermediates.

The theory of the critical role of glucose has been extended further by Gill (13) in the context of beef with high pH (DFD-beef). Gill has suggested that the reason for the rapid spoilage of DFD-beef is not the higher pH but the lower amount of glucose in this meat; i.e., the Pseudomonas flora immediately attacks the amino acids, after which the meat becomes spoiled. However, there is a considerable amount of lactate in DFD-meat, i.e., about 40 μmol/g in beef with a pH above 6.2 (normal beef, about 90 μmol/g; S. Fabiansson, Ph.D. thesis, Swedish University of Agricultural Sciences, Uppsala, 1984). Considering that P. fragi 72, irrespective of the presence of glucose, utilized lactate simultaneously with the amino acids and even preferred lactate before the amino acids, the DFD-meat hypothesis of Gill is weak. Thus, the results of this study indicate that (i) the role of glucose in the spoilage of meat may have been overemphasized in the literature and (ii) lactate is of equal importance as glucose as a carbon source of meat-spoiling Pseudomonas.

Results of the present study demonstrate that P. fragi 72 is able to utilize creatine. This does not agree with results of the study of Gill and Newton (14), in which the Pseudomonas strains they tested were unable to utilize creatine. However, the capacity to utilize creatine is fairly common for the dominating Pseudomonas flora of meat (P. fragi [2, 31]).

It was shown that oxygen limitation does not change the preferred carbon source sequence order of the organism. This opposes the view that the preference of Pseudomonas flora for amino acids would increase under conditions of oxygen limitation (26, 27).

Finally, carbon dioxide was shown to change slightly the order of preference of the carbon sources. Thus, the particular preference for glucose, compared with that for amino acids, was enhanced in the presence of 50% CO2 (batch cultures; Fig. 1a and c). In continuous culture the same tendency for glucose utilization may be traced, but here, an increased tendency to prefer lactate was more obvious (Fig. 4a and c). One possible explanation for this could be that CO2 inhibited cell activity by interfering with the cell membrane (9, 30). Thus, test results similar to those of this study have been reported by Eklund (6) from studies on the inhibitory effect of p-hydroxybenzoic acids (parabens) on Pseudomonas. The inhibitor affected the cell membrane, and the uptake of amino acids through this affected membrane was strongly inhibited. At the same time, the passage of glucose was unaffected. Furthermore, Tan and Gill (32) have shown that CO2 exhibited a stronger inhibitory effect on the uptake of aspartate than of glucose in Pseudomonas fluorescens.

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LITERATURE CITED

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