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Competition for L-Glutamate between Specialised and Versatile *Clostridium* Species

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Abstract. *Clostridium cochlearium* could be reproducibly enriched in an L-aspartate- and L-glutamate-limited, anaerobic chemostat inoculated with anaerobic sludge. L-glutamate, L-glutamine and L-histidine were the only fermentable substrates. Less specialised clostridia of the *C. tetanomorphum* type could only be isolated from batch enrichments with L-glutamate and L-aspartate as energy sources. Competition experiments with *C. cochlearium* and *C. tetanomorphum* in a L-glutamate-limited chemostat resulted in the selective elimination of the latter species. Addition of glucose to the medium resulted in coexistence of both species. The molar growth yields for L-glutamate at different dilution rates at 30° C were determined for both species. The maximum specific growth rates on L-glutamate were 0.55 h^{-1} for *C. cochlearium* and 0.35 h^{-1} for *C. tetanomorphum*.

Key words: *Clostridium cochlearium* – *Clostridium tetanomorphum* – Glutamate fermentation – Anaerobic mixed cultures.

As reported previously (Laanbroek et al., 1977) the result of an enrichment experiment with an anaerobic energy-limited chemostat in which L-aspartate and L-glutamate were applied as energy sources was the dominance of two different bacterial species. One of these, a *Campylobacter* spec., fermented L-aspartate and its characteristics have been described (Laanbroek et al., 1977). The other bacterium was a *Clostridium* species and its identification will be presented in this paper. Its fermentative abilities appeared to be restricted to L-glutamate, L-glutamine and L-histidine. The enrichment of this species in an anaerobic L-glutamate-limited chemostat, inoculated with anaerobic sludge, was reproducible. However, batch enrichments with L-glutamate, inoculated with sludge from the same source, showed the occurrence of L-glutamate-fermenting clostridia which were more versatile with respect to other fermentable substrates. Hence competition experiments were made to determine the ecological niche of both types of organisms. The results of these experiments will be described below and comparative data of the energetics of L-glutamate fermentation of both types of bacteria will be presented.

Materials and Methods

Cultures Used

Clostridium cochlearium and *Clostridium tetanomorphum* (ATCC 15920) were used in the experiments described. *C. cochlearium* was isolated from an anaerobic, L-aspartate and L-glutamate-limited chemostat inoculated with sludge from an anaerobic digester, which was fed with amino acid-rich waste water (Laanbroek et al., 1977). *C. tetanomorphum* was obtained from Professor G. Gottschalk, University of Göttingen, Federal Republic of Germany.

Growth Conditions

The anaerobic growth medium contained the following compounds per litre of demineralised water: $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.02 g; K_2HPO_4, 0.75 g; $NaH_2PO_4 \cdot H_2O$, 0.25 g; cysteine. HCl, 0.5 g; resazurin, 1 mg; 2 ml trace elements solution of Vishniac and Santer (1957) in a 5 fold dilution, together with a carbon- and energy source. The pH was adjusted to 7.1 before autoclaving. The type and concentration of the carbon- and energy source was dependent upon the experiment made. Substrate utilization tests were made at 30° C in anaerobic agar shake tubes, which contained the mineral medium plus 0.5 % (w/v) carbon- und energy source, 0.1 % (w/v) yeast extract and 1.0 % (w/v) agar. The carbon- and energy source was omitted in the blank.

The maximum specific growth rate on L-glutamate was determined in 3 litre conical flasks, which contained 100 ml cell suspension under a nitrogen atmosphere. Turbidity was measured in a tube, which had been connected to the conical flask and which also fitted into a Vitatron colorimeter.

Differential Tests

All differential tests were made according to Holdeman et al. (1977) at 30° C in 20 ml anaerobic Hungate tubes, which contained 10 ml of test medium under a gas atmosphere of oxygen-free nitrogen.

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Enrichment Techniques

Enrichments of L-glutamate-fermenting clostridia were made at 30° C in both batch and continuous culture; the latter at 4 different dilution rates, which varied from 0.02–0.20 h^{-1}. Batch culture enrichments were made in screw-cap bottles completely filled wich mineral medium supplemented with 0.35% (w/v) L-aspartate, 0.35% (w/v) L-glutamate and 0.1% (w/v) yeast extract. Continuous culture enrichments were made in a chemostat described previously (Laanbroek et al., 1977) and fed with the same medium. Anaerobic sludge from a digester fed with waste water rich in L-aspartate, L-glutamate and their amides, was used as inoculum in every case. After 3 or 4 days incubation in batch culture, or after 5 volume changes in continuous culture, samples from the cultures were pasteurized and agar shakes were made. These cultures contained the mineral salts medium supplemented with 0.5% (w/v) L-aspartate, 0.5% (w/v) L-glutamate, 0.1% (w/v) yeast extract and 1.0% (w/v) agar. After 4 days incubation at 30° C single colonies were tested for growth on L-aspartate, L-glutamate and glucose.

Cell Yield Studies

Cell yield studies of *C. cochlearium* and *C. tetanomorphum* were made in L-glutamate-limited chemostats. Steady-state cell carbon was determined as follows: the culture was centrifuged for 20 min at 10.000 × *g* in a Sorvall refrigerated superspeed RC2-B centrifuge at 4° C. The pellet was washed first with 3.2 mM and then with 1.6 mM phosphate buffer (pH 7.0). Finally the sediment was resuspended in CO_2-free water and the content of organic carbon determined with a Beckman 915A carbon analyzer, connected to a Beckman 865 infrared analyzer. The molar growth yield was calculated from the following equation:

$$Y = x/(S_R\text{-}s)$$

where x is the cell carbon determined and S_R-s is the amount of L-glutamate used.

Competition Experiments

Competition experiments were made in an anaerobic energy-limited chemostat. The mineral medium was supplemented with 0.35% (w/v) L-glutamate and 0.1% (w/v) yeast extract. In one case 0.35% (w/v) glucose was also added.

Total cell number was determined from a dilution series of anaerobic agar shakes in Hungate tubes under oxygen-free nitrogen at 30° C. The number of *C. tetanomorphum* cells were similarly determined at 45° C, since *C. cochlearium* was unable to grow at this temperature. The agar shake tubes contained the mineral medium supplemented with 1.0% (w/v) L-glutamate and 0.1% (w/v) yeast extract. The organic cell carbon was determined as described above. The percentage number of spores in the culture was estimated by direct counting.

Chemical Analyses

L-glutamate was determined according to the method of Bernt and Bergmeyer (1970). Volatile fatty acids, carbon dioxide and ammonia were estimated as described previously (Laanbroek et al., 1977). Hydrogen was determined by the method of Laanbroek et al. (1978).

Results

Isolation of Clostridium cochlearium and Its Differentiation from Clostridium tetanomorphum

In the first enrichment made (Laanbroek et al., 1977) an anaerobic chemostat was fed with L-aspartate and L-glutamate as growth-limiting substrates. The L-glutamate-fermenting *Clostridium* spec. that became dominant was isolated in pure culture by making successive dilution series in anaerobic agar shake cultures, applying a mineral medium with yeast extract, L-aspartate and L-glutamate.

The characteristics of this bacterium were as follows. Cells were rod-shaped (0.8 μm wide and 4.0–10.0 μm long) and were motile by means of peritrichous flagella. They were able to form terminal ovoid endospores. The swollen end of the spore-bearing cells gave them a spoon-like appearance. Colonies in agar were cream-coloured and lenticular. The bacterium was a strict anaerobe and the only fermentable substrates were L-glutamate, L-glutamine and L-histidine. Other substrates tested with a negative result were: glucose, fructose, pyruvate, L-lactate, citrate, α-oxoglutarate, succinate, fumarate, DL-malate, oxaloacetate, L-alanine, L-arginine, L-aspartate, L-asparagine, L-cysteine, glycine, L-isoleucine, DL-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-tryptophan, L-tyrosine, L-threonine, L-serine, L-valine and xanthine. Little growth was obtained unless yeast extract was included in the medium. No further analysis was made of the specific growth factor requirements. The stoichiometry of L-glutamate fermentation was as follows:

$$1.00 \text{ glutamate} \rightarrow 1.30 \text{ acetate} + 0.35 \text{ butyrate} + 0.95\ CO_2 + 0.25\ H_2 + 0.96\ NH_3$$

The maximum specific growth rate at 30° C in a medium containing L-glutamate plus 0.1% yeast extract was 0.55 h^{-1}. The characteristics as described above are similar to those given for *C. cochlearium* (Smith and Hobbs, 1974) in Bergey's Manual.

As will be described below, the sludge from which *C. cochlearium* was isolated also contained clostridia which could in addition to L-glutamate ferment glucose. These organisms were presumably similar or identical to *C. tetanomorphum*, a species which for unknown reasons was not included in the latest edition of Bergey's Manual (Smith and Hobbs, 1974). Anticipating a more detailed study of the ecological niches of both types of organisms, a comparison was made of several characteristics of the newly isolated *C. cochlearium* and *C. tetanomorphum* (ATCC 15920) which is summarised in Table 1. Also included are the data of Spray et al. (1957) for both species described in Bergey's Manual (1957). The ability of *C. tetanomorphum* to grow at 45° C was used to differentiate between both species in the competition studies to be described below. *C. tetanomorphum* had a maximum specific growth rate at 30° C in a medium which contained L-glutamate plus 0.1% yeast extract of 0.35 h^{-1}.

Table 1. Characteristics of the isolated *Clostridium* and of *C. tetanomorphum* (ATCC 15920), compared with data of *C. cochlearium* and *C. tetanomorphum* from Bergey's Manual (Spray et al., 1957)

	Newly isolated *Clostridium*	*C. tetanomorphum* (ATCC 15920)	Data from Bergey's Manual (1957)	
			C. cochlearium	*C. tetanomorphum*
Spore	Ovoid, terminal	Spherical, terminal	Ovoid, terminal	Spherical, terminal
Motility	+	+	+	+
Gram reaction	weakly +	+	weakly +	+
Gelatin hydrolysis	weakly +	−	−[a]	−
Growth on glucose	−	+	−	+
Milk digestion	−	−	−	−
Chopped meat digestion	−	−	−	−
Indole production	+	−	−	−
Growth at 45° C	−	+	N. M.[b]	N. M.[b]

[a] Weak to negative according to Holdeman et al. (1977)
[b] Not mentioned

Table 2. Ability of clostridia, isolated from batch or continuous cultures, to grow on L-glutamate, L-aspartate or glucose

	Number of enrichments	Number of isolates	Growth of isolates on		
			L-glutamate	L-aspartate	Glucose
Batch culture	2	7	7	0	2
Continuous culture D 0.02 h^{-1}	1	4	3	0	0
Continuous culture D 0.05 h^{-1}	1	3	3	0	0
Continuous culture D 0.1 h^{-1}	1	4	3	0	0
Continuous culture D 0.2 h^{-1}	1	4	4	0	0

Occurrence of L-Glutamate-Fermenting Clostridia other than C. cochlearium

The results described above raised to the following questions: 1) To what extent are enrichments in an anaerobic L-glutamate-limited chemostat that gave rise to the dominance of *C. cochlearium* reproducible and 2) Are less specialised clostridia also present in the sludge?

To answer these questions the enrichments in the chemostat were repeated at different dilution rates, and in addition some batch culture enrichments were made with L-aspartate and L-glutamate as energy sources. The results of these experiments are described in Table 2, and show that enrichments in a L-glutamate-limited chemostat yielded specialised clostridia whereas batch culture enrichments inoculated with the same sludge gave rise to clostridia some of which in addition to L-glutamate also fermented glucose.

Competition for L-Glutamate between C. cochlearium and C. tetanomorphum

As described above, sludge from the anaerobic digester contained both specialised and more versatile clostridia. However, the specialist outgrew the more versatile clostridium in a L-glutamate-limited chemostat without other energy sources. Thus it would appear that coexistence of both types of clostridia is only possible when in addition to L-glutamate an other substrate such as glucose is present. To examine this hypothesis, equal numbers of *C. cochlearium* and *C. tetanomorphum* were inoculated into L-glutamate-limited chemostats and the dilution rates set at 0.04 h^{-1} and 0.33 h^{-1}. In both cases *C. tetanomorphum* was competitively eliminated. Addition of glucose to the medium, however, resulted in coexistence, although washout of *C. tetanomorphum* again occurred when

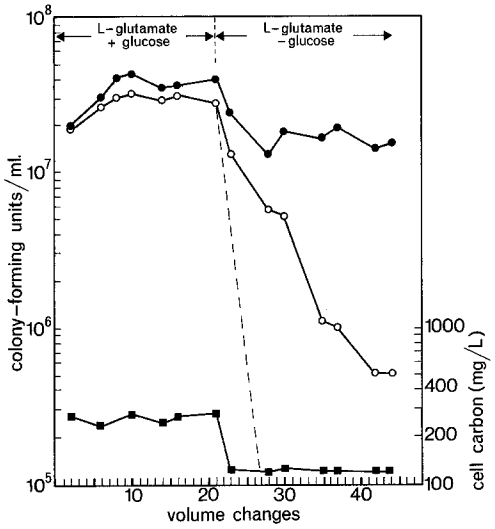

Fig. 1. Competition for L-glutamate between *Clostridium cochlearium* and *Clostridium tetanomorphum* in an energy-limited chemostat at dilution rate 0.04 h^{-1}, at 30° C and pH 7.0 ●——● total count of colonies; ○——○ count of colonies of *C. tetanomorphum*; ■——■ amount of cell carbon; ---- theoretical washout rate. During the first 21 volume changes the chemostat was fed with medium, which contained 18.7 mM L-glutamate and 17.7 mM glucose. Afterwards the glucose was omitted

this substrate was subsequently omitted (Fig. 1). Washout of *C. tetanomorphum* was accompanied by an increase in the number of spherical spores of *C. tetanomorphum* in the culture: from 0–4% of the vegetative cells.

Cell Yield Study of C. cochlearium and of C. tetanomorphum

To determine the true molar growth yield for L-glutamate, *C. cochlearium* was grown anaerobically in a L-glutamate-limited chemostat. The influent medium contained 37.4 mM L-glutamate plus 0.1% yeast extract. Up to a dilution rate of 0.20 h^{-1}, 2.5 mM L-glutamate was present in the culture. Above this dilution rate the concentration of L-glutamate increased progressively to 7.5 mM at a dilution rate of 0.50 h^{-1}. Acetate, butyrate, carbon dioxide, hydrogen and ammonia were the only detectable fermentation products. The fermentation pattern varied with the dilution rate; more acetate and hydrogen but less butyrate were produced at higher growth rates, as is shown in Table 3.

Table 3. Material balances of the fermentation of L-glutamate of *Clostridium cochlearium* grown in a L-glutamate-limited chemostat at 30° C. The growth medium contained 37.4 mM L-glutamate and 0.1% yeast extract. Fermentation products are given in mo per mo of glutamate consumed

Dilution rate h^{-1}	mM L-glutamate consumed	Acetate	Butyrate	CO_2	H_2	NH_3	Y[a] glut	C-recovery (%)	Redox balance
0.02	34.9	1.02	0.53	0.92	0.04	1.06	2.1	102	1.15
0.06	34.9	1.04	0.52	1.12	0.17	1.07	3.6	106	1.03
0.08	34.9	1.00	0.50	1.13	0.17	1.06	3.9	103	1.00
0.20	34.9	1.27	0.40	0.89	0.31	0.88	4.2	101	1.07
0.30	33.1	1.34	0.37	0.86	0.27	0.89	4.4	100	1.06
0.40	31.9	1.29	0.36	0.85	0.21	0.94	4.6	97	1.06
0.50	29.9	1.26	0.30	0.72	0.26	0.77	4.0	89	1.04

[a] mg cell carbon/mmol glutamate

Table 4. Material balances of L-glutamate-fermentation by *Clostridium tetanomorphum* grown in a L-glutamate-limited chemostat at 30° C. Growth medium contained 37.4 mM L-glutamate and 0.1% yeast extract. Fermentation products are given in mo per mo glutamate consumed

Dilution rate h^{-1}	mM L-glutamate consumed	Acetate	Butyrate	CO_2	H_2	NH_3	Y[a] glut	C-recovery (%)	Redox balance
0.02	37.3	1.10	0.40	1.03	0.03	0.97	3.5	97	0.89
0.05	37.3	1.26	0.35	1.02	0.17	0.97	4.5	99	0.92
0.10	37.0	1.46	0.36	–	0.31	1.00	4.9	–	–
0.20	37.2	1.29	0.37	0.73	0.13	0.94	4.6	96	1.16
0.30	25.4	1.34	0.39	0.85	0.22	0.94	4.3	102	1.10

[a] mg cell carbon/mmol glutamate

C. tetanomorphum was also grown anaerobically in a L-glutamatelimited chemostat. The medium contained 37.4 mM L-glutamate plus 0.1% yeast extract. Up to a dilution rate of 0.20 h^{-1}, the concentration of L-glutamate in the culture was 0.5 mM. This concentration increased progressively to 12. 0 mM at a dilution rate of 0.30 h^{-1}. The fermentation products were the same as in *C. cochlearium:* acetate, butyrate, carbon dioxide, hydrogen and ammonia (cf. Table 4).

Discussion

The occurrence of both specialised and more versatile bacteria in the same habitat has been repeatedly shown. One example was given by Barker (1937, 1939) who isolated both *Clostridium cochlearium* and *C. tetanomorphum* from batch enrichments with L-glutamate as energy source and which had been inoculated with soil samples.

Similar observations were made in the present study: The isolated *C. cochlearium* could only ferment L-glutamate, L-glutamine and L-histidine, whereas the more versatile *C. tetanomorphum* could in addition ferment a variety of other organic compounds, among which is glucose (Barker, 1961). In the agar dilution series inoculated from batch enrichments with L-glutamate as energy source colonies of both organisms were present in the highest dilutions, indicating that they occurred in approximately the same numbers in the enrichment. Since the maximum specific growth rate of *C. cochlearium* on L-glutamate is considerably higher than that of *C. tetanomorphum*, this may indicate that in the sludge used as inoculum the latter bacterium was present in greater numbers.

However, in the L-glutamate-limited chemostat enrichments highly specialised clostridia invariably became dominant. This was confirmed by competition experiments with mixtures of pure cultures of both clostridia. Even at a dilution rate of 0.04 h^{-1} where the growth yield on L-glutamate of *C. tetanomorphum* was higher than that of *C. cochlearium*, the former organism was selectively eliminated. Hence, in the presence of one growth-limiting substrate it is growth rate rather than growth yield that determines the outcome of competition.

When in an energy-limited chemostat both L-glutamate and glucose were presented, both clostridia could coexist. The washout rate of *C. tetanomorphum* when the glucose supply suddenly ceased indicated that it was still growing and hence consuming L-glutamate. While the number of colony-forming units of *C. tetanomorphum* progressively decreased, the total number of clostridia and the total amount of cell carbon became constant shortly after omission of glucose from the feed. This indicates that the population density of *C. cochlearium* increased, which can only have been due to an increase in the concentration of L-glutamate. In the presence of both glucose and L-glutamate *C. tetanomorphum* obviously fermented both substrates simultaneously. As *C. tetanomorphum* can ferment both L-glutamate and glucose simultaneously, an increase of the glucose concentration in the feed at constant dilution rate should eventually result in the elimination of *C. cochlearium*. This hypothesis is currently studied. In this particular case the growth yield of *C. tetanomorphum* on glucose would be of importance. The higher this is, the denser the population will be and the earlier the L-glutamate supply for *C. cochlearium* will reach a critical value.

In summary, the present study confirms the occurrence of a highly specialised bacterium, *C. cochlearium*, in an environment which cannot be considered as extreme. Its selective advantage is best expressed when the substrates it can utilize are the only organic compounds present. In that case, it outgrows its more versatile competitors. However, its population density becomes relatively low when a large variety of organic substrates is present, enabling versatile organisms to utilize different substrates simultaneously and thus lowering the food supply for the specialist.

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