Differences in the utilization of the glutamate family of amino acids by thermophilic proteolytic bacteria

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Three strains of thermophilic proteolytic bacteria which were supposed to belong to different taxonomic units were analysed with respect to the ability to utilize the glutamate family of amino acids. Strain N-3 could grow with all members of the glutamate family, including a mixture of them. Strains 3 and 36A could not utilize glutamic acid, glutamine, arginine and proline as the sole carbon sources, the mixture of these amino acids was also not suitable for growth. Glucose enabled the growth of all three strains tested in experiments with glutamic acid + glucose, glutamine + glucose, proline + glucose, the mixture of acids + glucose. Only arginine + glucose was not suitable for the growth, even for strain N-3 which utilized both arginine and glucose as the sole carbon source. The diauxic growth was established only for strain 3 in the medium containing glutamate + glucose. Therefore, the differences in the metabolism of amino acids were correlated with the differences in supposed taxonomic position of the strains tested.

Key words: amino acid, glutamate family, thermophilic bacteria

INTRODUCTION

Amino acids are very important for the normal functioning of all biological cells, including bacterial. They are the building blocks of proteins and provide materials for a variety of important cellular processes such as energy generation, carbon and nitrogen metabolism, cell wall synthesis, etc. [1].

Metabolism of amino acids has been widely studied in mesophilic bacilli, including *Bacillus subtilis, B. licheniformis, B. cereus* and *B. megaterium* [2, 3]. Far less attention was paid to thermophilic bacilli. To our knowledge, metabolism of amino acids by thermophilic bacilli has been studied only in *Geobacillus stearothermophilus, Bacillus caldolyticus* and *B. caldotenax* [1, 2, 4–7].

In order to gain further insight into the catabolism of the glutamate family of amino acids in thermophilic bacilli, we have investigated the ability of thermophilic proteolytic bacteria from a geothermal site in Lithuania to utilize the members of the family (glutamate, glutamine, arginine and proline) in mixture and as sole carbon sources. We also determined the possibility of carbon catabolite repression.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains N-3, 3 and 36A were isolated from a geothermal site in Lithuania. All the strains were thermophilic aerobic spore-formers. The strains differed in 16S rDNA RFLP profile and 16S-23S rDNA ITS-PCR pattern. They apparently belonged to different taxonomic units, although all of them were closely related to the thermophilic bacilli [8].

Media and culture conditions

Na_2HPO_4 was omitted from medium M9 to yield medium mM9. The medium was prepared in 50 mM Tris-HCl buffer (pH 7.8; +60 °C) [9]. The mM9V and mM9A media were mM9 supplemented with vitamins and amino acids, respectively. The mM9AV medium was mM9 supplemented both with vitamins and amino acids. The medium mM9V was used to determine bacterial growth with a sole source of carbon and in experiments with two carbon sources (amino acid + glucose).

Amino acids of the glutamate family (glutamate, glutamine, arginine and proline) at concentrations

0.4% were used as the sole sources of carbon. The two carbon source (amino acid + glucose) medium contained each amino acid at a concentration of 0.4% as the main carbon source and glucose at a concentration of 0.25% as the additional carbon source. The medium mM9GFV was mM9V supplemented with the mixture of four amino acids of the glutamate family, each at a concentration of 0.2%. The medium mM9AV-glutamate family was mM9AV with the glutamate family omitted.

Bacteria were cultivated in 250 ml Erlenmeyer flasks at a temperature of 60 °C without shaking. The volume of the medium was 100 ml. Inoculation (5% vol/vol) was done with late-exponential-phase cells from cultures grown on nutrient agar plates. The inoculum was prepared in sterile water to avoid carryover of amino acids. Growth was determined by measuring optical density at 600 nm. The initial optical density OD_{600} at the moment of inoculation was 0.07–0.08.

The specific growth rate, μ, was calculated from the equation $\ln(X/X_0) = \mu \times (t–t_0)$ [10]: t – time; X – OD at time t.

RESULTS AND DISCUSSION

The length of the lag phase, the OD_{max} and the specific growth rate were choosed as the criteria for comparison of the growth of different strains in the defined media. According to the literature data, OD_{450} = 0.2–0.25 is common in the medium with glutamate for thermophilic *Bacillus* spp. [5].

The strains 36A and 3 were not able to utilize any amino acid from the glutamate family as the sole source of carbon (Table).

Strain N-3 could use glutamic acid, glutamine, arginine and proline as a sole source of carbon (Table, Fig. 1). The specific growth rate of N-3 was greatest in the medium with glutamine.The lag phase was shorter and the OD_{max} was higher with glutamic acid than with any of the other three amino acids.

In general, the nature of bacterial growth with glucose (data not shown) or with amino acids was similar: OD_{max} was 0.15 or lower, the specific growth rate was low. Therefore, neither glucose nor amino acids of the glutamate family alone are good carbon sources for the strains tested.

Unexpected results were obtained with arginine + glucose (Table). In spite of the fact that N-3 used both arginine and glucose as sole carbon sources (see above), the medium containing these two carbon sources was not suitable for its growth (Fig. 3). Strains 36A and 3 did not grow in this medium, even though these two strains demonstrated the abi-

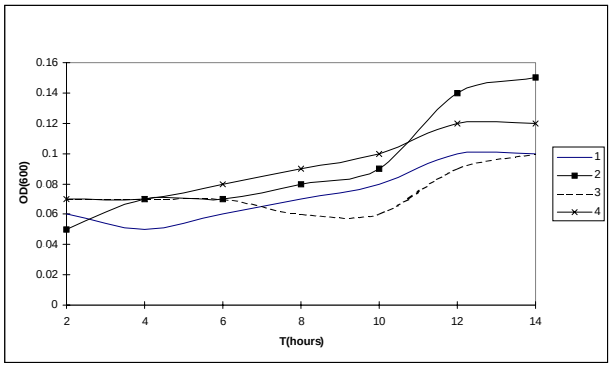

Fig. 1. Dynamics of the growth of strain N-3 in a medium mM9V containing 0.4% amino acid: 1 – arginine, 2 – glutamic acid, 3 – glutamine, 4 – proline

Table. **Utilization of the carbon sources**

Carbon source	3			N-3			36A		
	Lag, h	μ, h^{-1}	OD_{max}	Lag, h	μ, h^{-1}	OD_{max}	Lag, h	μ, h^{-1}	OD_{max}
Arginine		NG		4	0.09	0.1		NG	
Glutamic acid		NG		2	0.09	0.15		NG	
Glutamine		NG		10	0.12	0.1		NG	
Proline		NG		4	0.06	0.12		NG	
Arginine + glucose		NG			NG			NG	
Glutamic acid + glucose		Diauxie		10	0.8	0.5	10	0.8	0.5
Glutamine + glucose	6	0.09	0.16	10	0.4	0.2	4	0.22	0.3
Proline + glucose	4	0.17	0.2	4	0.09	0.17	4	0.17	0.32
Glutamate family		NG		6	0.11	0.16		NG	
Glutamate family + glucose	10	0.85	0.44	10	0.7	0,32	10	1.0	0.6
AA-glutamate family		NG		2	0.1	0.2		NG	
AA-glutamate family + glucose	4	0.25	0.28	2	0.09	0.25	4	0.24	0.34

NG, no growth; AA-glutamate family, amino acids in mM9AV with glutamate family of amino acids omitted.

lity to grow with glucose but not arginine as the sole carbon source (Fig. 2, Table).

The medium containing glutamic acid + glucose was suitable for the growth of all the strains tested (Table). The growth curves were identical for strains N-3 (Fig. 3) and 36A (data not shown). No diauxic growth was observed. The growth of strain 3 differed from that of the other two strains mentioned above (Fig. 2). Cells of strain 3 entered the first exponential phase 6 hours after inoculation, reaching the stationary phase the 10th hour after inoculation. In the 16th hour the growth entered the second exponential phase. Hence, diauxic growth was observed for strain 3 in the medium containing glutamic acid + glucose.

The media containing glutamine+glucose or proline + glucose were suitable for the growth of all three strains. For strain 3, proline + glucose were the better carbon source according to criteria mentioned previously. However, for strain N-3 the lag phase was shorter with proline + glucose, but OD_{max} and the specific growth rate were higher with glutamine + glucose. For strain 36A, the specific growth rate was greater with glutamine + glucose and both the lag phase and OD_{max} were similar, independent of the amino acid used. The diauxic growth was not observed (Figs. 2, 3).

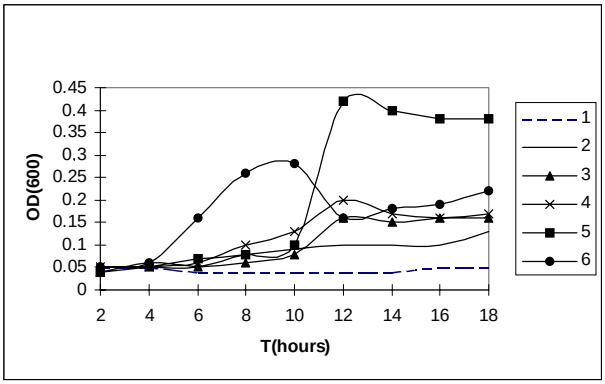

Fig. 2. Dynamics of the growth of strain 3 in a medium mM9V containing 0.25% glucose. 1–4, 0.4% amino acid: 1 – arginine, 2 – glutamic acid, 3 – glutamine, 4 – proline; 5 – glutamate family (0.2% of each amino acid); 6 – mM9AV with the glutamate family of amino acids omitted (mM9AV-glutamate family)

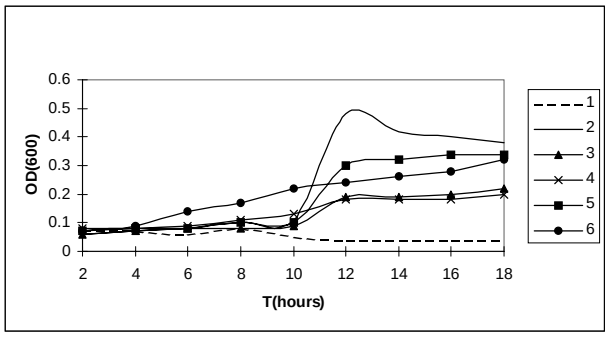

Fig. 3. Dynamics of the growth of the strain N-3 in a medium mM9V containing 0.25% glucose. 1–4, 0.4% amino acid: 1 – arginine, 2 – glutamic acid, 3 – glutamine, 4 – proline; 5 – glutamate family (0.2% each amino acid); 6 – mM9AV with the glutamate family of amino acids omitted (mM9AV-glutamate family)

To clarify utilization of the mixture of amino acids, the character of the growth with glutamate family of amino acids in mixture (0.2% each) was examined (Table).

As expected, all these amino acids in mixture did not support the growth of strains 3 and 36A in the medium mM9GFV. On the other hand, the medium mM9AV-glutamate family did not support the growth of these strains, either. These results raised a question about the interrelationships between the catabolism of glutamate family and the catabolism of the remaining amino acids in thermophilic bacilli.

It is noteworthy that glucose at a concentration of 0.25% (additional carbon source) allowed the growth of strains 3 (Fig. 2) and 36A (Table) both in mM9GFV and in mM9AV-glutamate family. The character of the growth of strains 3 and 36A was similar. The diauxic growth was not established.

Completely different growth curves were obtained for strain N-3 (Fig. 3). The medium mM9GFV allowed the growth of strain N-3. Supplementation of glucose at a concentration of 0.25% ensured the better growth: $OD_{+glucose}/OD_{-glucose} = 2$. No such effect of additional glucose was observed in mM9AV-glutamate family (Table). The diauxic growth was not established in either of the media.

In conclusion, in spite of the different manner of utilization of the glutamate family, the strains were similar in respect of the growth in the media mM9GFV with glucose and mM9AV-glutamate family with glucose. Although the lag phase was longer, OD_{max} and the specific growth rate were higher in the medium with glutamate family + glucose (mM9GFV + glucose) than in the mM9AV-glutamate family + glucose. The long lag phase is understandable, because the cells for the inoculum were grown on a rich medium. Therefore time for the metabolism to change is required. The glutamate family is not essential for all three strains in the media with glucose, although the growth in the medium without glutamate family was half as low.

Strains N-3, 3 and 36A were supposed to belong to different taxonomic units [8]. Consequently, the different manner of the utilization of carbon sources was not surprising.

While strain N-3 could utilize amino acids of the glutamate family, inability to grow with glutamic acid and glutamine was established for strains 3 and 36A.

Belitsky and Sonenshein mentioned that *Bacillus subtilis* strains of 168 lineage cannot grow with glutamic acid or glutamine as the sole carbon source. This is associated with the intrinsically inactive glutamate dehydrogenase [3]. The thermophilic *Geobacillus stearothermophilus* has active glutamate dehydrogenase [2]. It is not known whether glutamate dehydrogenase activity is present in other thermophilic bacilli or whether this property is common only for *G. stearothermophilus*.

Arginine and proline are known to be a good source of glutamic acid. Furthermore, arginine- and proline-degrading enzymes are induced by these amino acids [11, 12]. No apparent induction of these enzymes occured in experiments with strains 3 and 36A, whereas strain N-3 could grew with arginine and proline.

Inability to utilize arginine as the sole carbon source is mentioned for *Escherichia coli* [13]. But this property was not established for bacilli, either mesophilic or thermophilic. Hence, the reason for inability to grow with arginine is not clear.

A hierarchy for amino acid utilization is present in cells growing with mixtures of amino acids. Glutamic acid is depleted from the medium in the exponential phase, while arginine and proline are depleted during the late exponential growth [12]. The mechanism enabling such a hierarchy is known only for a few acids in *Bacillus subtilis.* In our experiment, strains 3 and 36A were not able to utilize glutamate family at all. Strain N-3 could utilize the glutamate family of amino acids, but no diauxic growth was observed. Apparently the hierarchy of amino acid utilization is present in thermophilic bacilli. One may speculate that some amino acids inhibit the transport of the others.

The presence of the uptake systems specific for L-arginine and acidic amino acids in *G. stearothermopilus* has been reported [6, 7]. To our knowledge, nothing is known about the uptake systems for glutamine and proline in thermophilic bacilli [1]. Further investigations are needed to clarify the relationship among the uptake systems for amino acids in thermophilic bacilli.

In *G. stearothermophilus* the glutamic acid carrier was reported to depend upon the external pH [6]. Glutamic acid uptake was most rapid at a low external pH (pH5.5 to 6.5) and decreased significantly with increasing it. Glutamic acid uptake depended on Na^+ and H^+. In our experiment the external pH was 7.8. This raises a possibility that the translocation process was ineffective. Furthermore, only trace amounts of Na^+ could be present in the culture medium. Although the glutamic acid carrier is highly specific for Na^+ [6, 7], the trace amount could be not enough for efficient transport. On the other hand, the arginine transport system is thought to be a uniport system. Hence, the trace quantity of Na^+ could not explain the inefficient transport of arginine.

Intriguing results were obtained using the media containing amino acid + glucose. The high specific growth rate and the culture yield cannot be explained only by the influence of glucose, especially in the case of strains 3 and 36A which did not utilize any amino acid of the glutamate family. Some amino acid degrading enzymes are known to be a subject of carbon catabolite repression [11]. In our experiment, only glutamic acid + glucose for strain 3 has showed diauxic growth as the outcome of carbon catabolite repression. In other cases no diauxy was observed. Our results suggest that amino acids of the glutamate family are not controlled by carbon catabolite in strains 3, 36A and N-3 (excluding the variant mentioned above). On the basis of our results we suggest that glucose and amino acids were metabolised simultaneously. Recently a simultaneous metabolism of glucose and amino acids has been reported for *Lactococcus lactis* grown under anaerobic conditions [14]. Previously, the same phenomenon has been determined for *Saccharomyces cerevisiae* [15]. With an excess of glutamic acid and glucose, there are two essentially non-overlapping flows of carbon under anaerobic conditions. Glutamic acid metabolism yields amino acids of its own family and some other products, while glucose metabolism yields the end products of fermentation. Hence, our results were in agreement with the literature data [14, 15]. The influence of the culture conditions (aerobic or anaerobic) on this phenomenon should be investigated in further experiments.

Thus, our investigations have shown that for our strains of thermophilic bacilli amino acids of the glutamate family are poor carbon sources and that supplemental glucose enables the utilization of amino acids. Carbon catabolite repression was observed only in one variant.

In conclusion, the differences in the metabolism of amino acids in glutamate family were in accordance with the analysis of 16S rDNA and 16S–23S rDNA internal transcription spacers [8]. The different taxonomic units demonstrated the different manner in the utilization of amino acids. Hence, the investigations of the metabolism of amino acids will proceed in our department.

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GLUTAMO RŪGŠTIES ŠEIMOS AMINO RŪGŠČIŲ ĮSISAVINIMO SKIRTUMAI TERMOFILINĖSE PROTEOLITINĖSE BAKTERIJOSE

S a n t r a u k a

Atlikta trijų sporas formuojančių proteolitinių termofilinių, artimų baciloms ir priklausančių skirtingiems taksonominiams vienetams bakterijų kamienų sugebėjimo įsisavinti glutamo rūgšties šeimai priklausančias amino rūgštis analizė. Parodyta, kad N-3 kamienas gali panaudoti kiekvieną šios šeimos amino rūgštį kaip vienintelį anglies šaltinį. Kartu šis kamienas auga terpėje, turinčioje minėtų amino rūgščių mišinį. Kiti du 3 ir 36A kamienai elgiasi priešingai: analogiškomis sąlygomis jie neaugo arba jų augimas buvo labai susilpnėjęs.

Terpėse su dviem anglies šaltiniais, kur pagrindiniu šaltiniu buvo viena iš amino rūgščių (glutamo rūgštis, glutaminas, prolinas), o papildomu anglies šaltiniu – gliukozė, stebėtas visų trijų kamienų augimas. Išimtį sudarė derinys argininas + gliukozė. Tokioje terpėje neaugo nei vienas kamienas. Pažymėtina, kad N-3 kamienas galėjo panaudoti tiek gliukozę, tiek argininą atskirai kaip vienintelius anglies šaltinius. Nors minėtų terpių sudėtyje buvo du anglies šaltiniai, diauksija nustatyta tik terpėje su glutamo rūgštimi ir gliukoze 3 kamieno atveju.

Gauti rezultatai rodo, kad skirtingiems taksonominiams vienetams priklausantys termofilinių bakterijų kamienai pasižymi įvairiu glutamo rūgšties šeimos amino rūgščių metabolizmu.