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The Effect of 2-Oxoglutarate and Biotin in the Release of Amino Acids by *Citrobacter intermedius* C3

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Excretion of amino acids by *Citrobacter intermedius* C3 was assayed in a mineral medium with glucose as carbon source. Glutamic acid is the main amino acid excreted in liquid medium and it is also detected at the colonial level in solid medium. Mutants with different behaviour with respect to the excretion of amino acids are studied. The presence of 2-oxoglutarate in the medium induced excretion in all strains. On the other hand when biotin was added to the culture media amino acid excretion was partially reduced.

The release of amino acids by bacteria depends on the genotype, growth phase and environment (3, 8). Strain C3 of *Citrobacter intermedius* actively excretes amino acids, mainly glutamic acid, when it is grown at 30° C in a mineral medium with glucose as the only carbon source. Wild type C3 presents a reversible dissociation of excreting and non excreting colonies, as detected by using *Leuconostoc mesenteroides* as indicator strain of glutamic acid excretion, such property being associated with the presence of extrachromosomal DNA (6). The availability of several mutants with different behaviour with respect to the excretion of glutamic acid at the colonial level induced us to initiate the present study in order to quantify the excretion of glutamic acid and other amino acids in liquid medium and to correlate the excretion of amino acids to the growth phase. At the same time there has been studied the effect of 2-oxoglutarate and biotin on the excretion of amino acids by *Citrobacter intermedius* C3.

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Materials and Methods

Bacterial strains. Wild type *Citrobacter intermedius* C3 has been previously described; it presents a reversible dissociation with respect to glutamic acid excretion at the colonial level (1, 6). Mutant CBC315 obtained by treatment with nitrosoguanidine is auxotrophic for proline and does not excrete glutamic acid detectable at the colonial level. Strain CBC356* is a Pro$^-$ His$^-$ mutant obtained by two succesive treatment of wild type C3 with NTG; moreover, at the colonial level it does not present the characteristic dissociation of wild type, excreting all the colonies. *Leuconostoc mesenteroides* P60 was used to test glutamic acid excretion at the colonial level.

Media and growth conditions. Cells were grown in M1 and M1α media which were previously described (1, 4). Briefly M1 is a mineral medium with glucose as the only carbon source; in M1α glucose is partially substituted by 2-oxoglutarate. When auxotrophic mutants were grown media were supplemented with 20 mg l^{-1} of the amino acids for which the strains are auxotrophic. Biotin was added at a final concentration of 100 μg l^{-1}.

To study the amino acids excretion in liquid media bacteria were grown in 500 ml flasks with 200 ml of the corresponding medium. After inoculation with 5 ml containing 1×10^8 cells ml^{-1} from a culture at the end of the exponential growth phase, flasks were incubated at 30° C and shaken at 125 strokes min^{-1}. Growth was measured by optical density at 500 nm.

Assay of amino acids. To determine the released amino acids, cells were separated from the medium by centrifuging at 7,500 x*g* for 15 min at 4° C. Supernatants were filtered with a Millipore equipment through a filter of 0.22 μm of pore size, and their content in amino acids was analyzed in an amino acid autoanalizer Beckman model 119-C by using resins AA20 (Beckman). Elution buffers of sodium citrate 0.2 N were used at pH 3.25, 4.12 and 6.40 at a flow rate of 35 ml h^{-1}. The amount of excreted amino acids is expressed as mg l^{-1} of the initial medium of growth. Samples were analyzed at the times indicated in the results section. Glutamic acid excretion at the colonial level was assayed by pouring a suspension of *Leuconostoc mesenteroides* P60 in Glutamic Acid Assay Broth (Difco) with 7.5 g l^{-1} of agar onto plates with colonies previously grown for 24 h at 30° C. After 30 h of incubation at 37° C a halo of *L. mesenteroides* growth surrounded those colonies that released glutamic acid (2).

Results

Excretion of amino acids in liquid M1 medium. Amino acids begin to be released during the logarithmic growth phase by wild type C3 and mutant strain CBC356. However, the highest amount of excreted amino acids is reached long after the end of the exponential growth phase. In all this period glutamic acid is the most abundant amino acid released (table I), following in amount alanine, valine, tyrosine and serine. Mutant CBC315 does not yield significant amounts of amino acids, besides tyrosine; in this strain glutamic acid excretion is not detected during the first 48 hours. The maximum amount of free amino acids excreted by wild type C3 in M1 is around 297 mg l^{-1} and is reached at 48 h of incubation; glutamic acid represents 95 % of the total amount. At the same age of the culture, strain CBC356 gives a maximum value for total amino acids of 157 mg l^{-1}, of which glutamic acid represents 74 %. The only amino

* Strains CBC315 and CBC356 were named 3MA2 and 1230 respectively in a previous paper.

Table I. *Release of free amino acid in liquid media by* Citrobacter intermedius *C3.*
Biotin is used at 100 μg/l. Results are expressed as mg of amino acid per liter of the liquid medium. ND = not detected.

Media	Age culture h	Wild strain C3					CBC356					CBC315				
		ser	glu	ala	val	tyr	ser	glu	ala	val	tyr	ser	glu	ala	val	tyr
M1	8	ND	5.6	0.8	ND	18.6	ND	1.6	1	ND	20.2	12	ND	0.4	ND	21
	24	ND	99.4	ND	ND	12.5	12.8	117	ND	21	6.8	ND	ND	ND	ND	9.6
	48	4	272.8	ND	3.4	7.4	12.8	116	ND	21	7	ND	ND	0.6	ND	5.8
	72	2.8	208	1	3	22	11.4	88.2	0.3	4.8	19.8	ND	ND	3.2	0.4	21.8
M1α	8	ND	8.6	1.8	ND	16	1	1.6	0.8	6.4	16.8	1	1.2	2.8	ND	18
	24	ND	114.2	77.6	9	10.9	ND	124	34.2	144.4	9.4	ND	78.4	36.4	5.8	14
	48	ND	229.2	82.8	1.2	15	ND	706	30	51.6	12.4	ND	36	0.4	ND	10
	72	ND	212.6	79.1	2.4	10.4	31.6	352.2	22	ND	ND	ND	24	ND	ND	4.6
M1-biotin	24	ND	114	8	2	20	ND	84	18	18	26	ND	ND	ND	ND	20
	48	ND	256	6	4	30	ND	182	12	20	26	ND	ND	ND	ND	22
M1α-biotin	24	ND	12	ND	1.5	22	ND	54	ND	ND	28	ND	ND	ND	ND	26
	48	ND	132	16	1	16	ND	606	ND	ND	25	ND	ND	ND	ND	24

acid that is progressively accumulated in wild type C3 and strain CBC356 is glutamic acid. The values for alanine, valine and serine do not show any repetitive pattern of distribution through the passing of time, and the amount of detected tyrosine is more or less constant in all the strains during all the phases of growth.

Effect of 2-oxoglutarate. a) *Liquid medium.* Addition of 2-oxoglutarate into liquid M1 medium mincreases only slightly the growth rate and the maximum growth of wild type C3 and mutant CBC356. However, the total growth of strain CBC315 in M1α increases around 2.5 fold with respect to the one obtained in M1 (fig. 1). On the other hand the amount of released amino acids is greater in M1α than in M1 in wild type C3 and mutant strain CBC356. Mainly in this last one the augmentation of total excreted amino acids in the presence of 2-oxoglutarate may be 5 fold as high, being glutamic acid 88 % of the total yield of amino acids. In strain CBC315, which does not excrete detectable amounts of glutamic acid and releases only very low amounts of other amino acids in M1, the addition of 2-oxoglutarate clearly induces the excretion of glutamic acid and also of alanine and valine (table I).

b) *Solid medium.* As it has been previously described, when C3 grows on M1 agar it presents a dissociation between excreting and non excreting colonies (6). This dissociation was completely suppressed on M1α agar, on which all the colonies excreted glutamic acid. Mutant CBC315, which gives 100 % of non excreting colonies growing on M1, presented 100 % of excreting colonies on M1α. On the other hand all the colonies of strain CBC356 excreted glutamic acid either on M1 agar or on M1α agar (table II).

Effect of biotin. a) *Liquid media.* The addition of 100 $\mu g\ l^{-1}$ of biotin into liquid M1 and mainly M1α, greatly reduced the amount of amino acid excretion by wild type and CBC356 at 24 h and 48 h. This phenomenon was observed for all amino

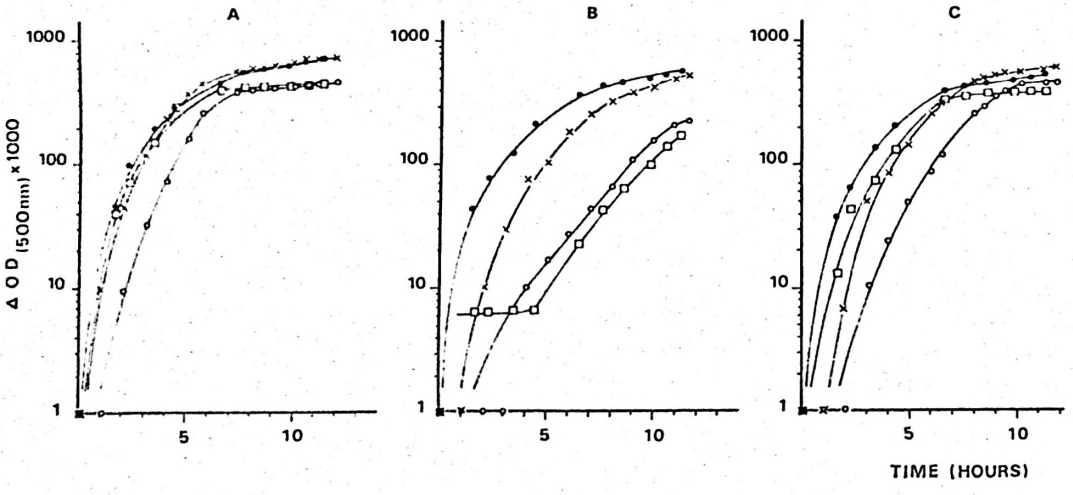

Fig. 1. *Growth of* Citrobacter intermedius *C3 and related strains in M1 (—○—○—); M1α (—×—×—); M1-bio (—□—□—); M1α-bio (—●—●—).*
A) wild strain C3; *B*) CBC315; *C*) CBC356.

Table II. *Excretion of glutamic acid at colonial level.*

Strain	Media			
	M1	M1α	M1-biotin	M1α-biotin
Wild type C3	65±5	100	42±5	100
CBC356	100	100	55±5	100
CBC315	0	100	0	100

Numeric data represent the percentage of glutamic acid excreting colonies according the criterium previously described (8).

acids expressed in table I except for tyrosine which is not affected by biotin. In strain CBC315 the effect of biotin is even more dramatic, since glutamic acid is not detected in the extracellular pools (table I). However biotin does not affect either growth rate or total growth of any of the strains (figure 1).

b) *Solid media.* When biotin was added into M1 agar at the concentration above indicated a change in the percentage of glutamic acid excreting colonies was observed in wild type C3 and in strain CBC356. In wild type the percentage of excreting colonies decreased from around 65 % to approximately 42 %. In strain CBC356, the reduction of the percentage of excreting colonies was from 100 % to around 60 % (table II). On M1 strain CBC315 did not excrete glutamic acid in the presence of biotin, either. The addition of biotin to M1α agar did not change the percentage of excreting colonies in any of the three strains, the totality of colonies beeing excretors (table II), although halos of colonies of strain CBC315 were clearly smaller than those obtained in M1α. In fact the addition of biotin in M1α might have had a similar effect as in M1, but since glutamic acid excretion is greater than in M1, the decrease in the levels of excreted glutamic acid may not change the pattern of excreting colonies because there may still be enough glutamic acid around the colonies to allow *L. mesenteroides* to grow.

Discussion

Glutamic acid, tyrosine, valine, alanine and serine represent over 99 % of the total amount of amino acids excreted by *Citrobacter intermedius* C3. Among them glutamic acid is the most abundant one. Excretion begins in the logarithmic growth phase, but the maximum level of excreted amino acids is obtained long after the stationary phase has been reached. Therefore strain C3 behaves like *Escherichia coli* (7).

Addition of 2-oxoglutarate to the media causes an increase, both in the level of extracellular amino acids in liquid medium, and in the number of glutamic acid excreting colonies on solid medium. Since 2-oxoglutarate does not affect bacterial permeability, we can conclude that it induces the synthesis of glutamic acid and of all those amino acids that are biosynthesized by transamination from glutamic acid.

The yield of amino acids, mainly glutamic acid, is reduced when the herein studied strains are grown in liquid media supplemented with 100 $\mu g\ l^{-1}$ of biotin. Moreover, the addition of the same concentration of biotin to solid media clearly reduces the percentage of excreting colonies. It is known that biotin deficiency causes in the cell membrane abnormalities that promote excretion of glutamic acid in several groups of glutamic acid producing bacteria (5). Since biotin impairs the excretion of amino acids by *C. intermedius* C3, it can be deduced that the excretion of amino acids in *C. intermedius* C3 may be partially due to some deficiency in biotin.

A similar pattern of excretion is observed in both solid and liquid media, though it is impossible to establish a direct correlation, because of the difficulty on determining when cells in both media are in the same growth phase and physiological state. Moreover, the addition of 2-oxoglutarate and biotin causes the same

effects in the excretion pattern in both liquid and solid media.

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Resumen

Se estudia la excreción de aminoácidos por *Citrobacter intermedius* C3 cuando crece en medio mineral con glucosa. El aminoácido mayoritariamente excretado es el ácido glutámico. Su excreción se detecta en medio líquido y también a nivel colonial en medio sólido. Se estudia la excreción en diversos mutantes que tienen un comportamiento diferencial con respecto a la excreción de aminoácidos. La adición de 2-oxoglutarato en el medio induce un aumento de la excreción en todas las cepas. En cambio, la presencia de biotina reduce parcialmente la excreción.

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