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Role of Tyramine Synthesis by Food-Borne *Enterococcus durans* in Adaptation to the Gastrointestinal Tract Environment[▽]

Pilar Fernández de Palencia,[1] Maria Fernández,[2] Maria Luz Mohedano,[1] Victor Ladero,[2] Cristina Quevedo,[1] Miguel A. Alvarez,[2] and Paloma López[1]*

Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain,[1] and Instituto de Productos Lácteos de Asturias, CSIC, Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain[2]

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Biogenic amines in food constitute a human health risk. Here we report that tyramine-producing *Enterococcus durans* strain IPLA655 (from cheese) was able to produce tyramine under conditions simulating transit through the gastrointestinal tract. Activation of the tyramine biosynthetic pathway contributed to binding and immunomodulation of enterocytes.

Biogenic amines (BAs) are formed by the decarboxylation of amino acids and are involved in important biological functions in the human body, such as nervous transmission, gastric acid secretion, the immune response, and cell growth and differentiation. Alterations of physiological concentrations of BAs have been correlated with several disorders, such as allergies, Parkinson's syndrome, and migraines (15). The levels of BAs in the body are in most cases the sum of endogenous synthesis and exogenous contribution. However, in the case of tyramine, the source is only exogenous, mainly by the ingestion of foodstuffs in which BAs have accumulated by the action of decarboxylating bacteria, though synthesis by the gastrointestinal tract (GIT) microbiota should not be dismissed. The consumption of food containing high concentrations of tyramine can induce adverse reactions such as nausea, headaches, and blood pressure alterations, especially in combination with the use of monoamine oxidase inhibitors as antidepressants (8). The contribution of GIT microbiota to the biosynthesis of polyamine, a particular type of BA, has been quantified (13); however, there is no information regarding the contribution of endogenous microbiota to the biosynthesis of tyramine or the role of food-borne BA-producing microorganisms once they reach the GIT.

Certain species of *Enterococcus* and *Lactobacillus* are the main organisms responsible for tyramine accumulation in fermented foods (10). Tyramine-producing enterococci have been isolated from human feces (7). In order to contribute to the BA pool, a food-borne tyramine-producing strain must survive passage through the GIT and produce tyramine under such conditions. The contribution is enhanced if the microorganism is able to persist in the intestine. To test the possibilities of survival and persistence under these conditions, *Enterococcus durans* IPLA655, a strain isolated from cheese that is able to synthesize tyramine via tyrosine decarboxylation (2), was selected for this study.

To monitor the survival and tyramine production capabilities of *E. durans* IPLA655 under GIT conditions, a model system previously validated with lactic acid bacteria (LAB) and *Bifidobacterium* strains of food origin was used (3, 4). This model simulates the normal physiological conditions of the GIT, including the presence of lysozyme (saliva) and gastric (G) stress provoked by pepsin at gradually decreasing pH values (from 5.0 to 1.8). After G stress at pH 5.0 or pH 4.1, stress in the small intestine was also assayed (by determining the presence of bile salts and pancreatin at a GI pH of 6.5). Bacteria were grown to early stationary phase in ESTY medium (0.5% beef extract, 0.5% tryptone, 0.25% yeast extract, 0.5% ascorbic acid, 1.9% disodium glycerophosphate, 0.025% magnesium sulfate) (Pronadisa, Madrid, Spain), which contains a basal tyrosine concentration of about 26 μM, supplemented with 0.5% glucose in the absence or presence of 10 mM tyrosine. After sedimentation and resuspension in fresh medium, the bacteria were exposed to the various stresses.

Tyramine production under these conditions was quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) (6), which revealed that in the presence of 10 mM tyrosine, the bacterium was able to produce tyramine under the assay conditions (Fig. 1). Maximum production was observed after G stress at pHs 5.0 and 4.1, at which approximately 2×10^8 CFU ml^{-1} (determined by viable plate counting on ESTY solid medium) were able to synthesize and release to the culture supernatant a high concentration of tyramine (729 ± 25 μM) during the 20-min incubation. The higher level of tyramine production by *E. durans* observed at pH 5.0 could correlate with the detection at this pH of the maximum transcription levels of the *tyrP* and *tdcA* genes, which encode, respectively, the tyrosine/tyramine antiporter and the tyrosine decarboxylase, which catalyzes the synthesis of tyramine from tyrosine (9). The pH value of 5.0 is also close to the reported optimal pH (5.4) of tyrosine decarboxylase (11). Interestingly, significant concentrations of tyramine (270 μM) were also observed in the samples exposed to pH 1.8, even though only 8.6×10^1 CFU ml^{-1} were detected at the end of the assay, indicating that under gastric conditions, the tyrosine decarboxylase could catalyze tyramine biosynthesis either in nonviable cells and/or in culture supernatants.

In order to understand the role of tyramine biosynthesis in

* Corresponding author. Mailing address: Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: 34-918373112. Fax: 34-915360432. E-mail: plg@cib.csic.es.

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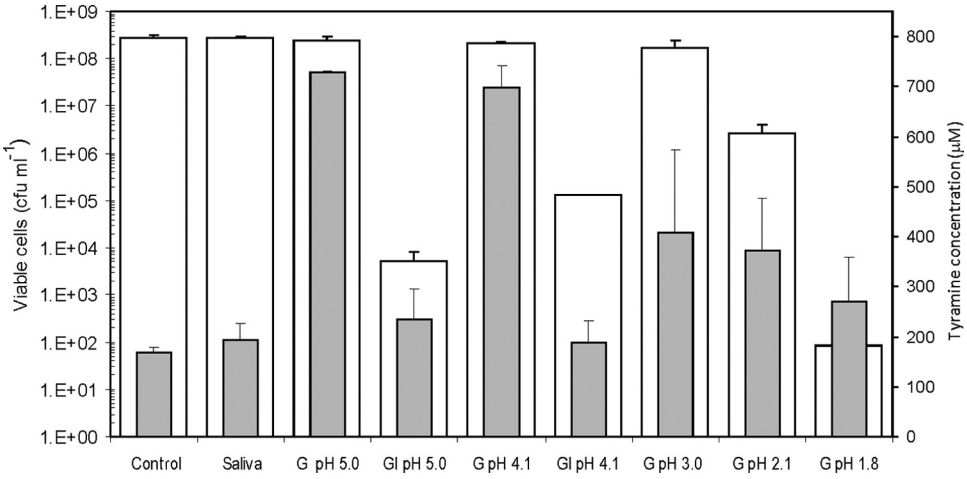

FIG. 1. Tyramine produced (gray bars) by *E. durans* IPLA655 under GIT stress. The numbers of cells in the cultures are expressed as log CFU ml^{-1} (white bars). Each value is the mean of the results of three independent experiments. The experiments were performed in the presence of 10 mM tyrosine.

cell survival under GIT conditions, the assays were performed in the presence or absence of 10 mM tyrosine. In addition, a knockout strain was constructed by replacing the *tdcA* gene with the chloramphenicol-resistance gene, using pMN20-CM, a suicide pUC19-derived plasmid harboring the 5′ and 3′ flanking regions of the *E. durans* IPLA655 *tdcA* gene. This plasmid was introduced by electroporation, and the double-crossover mutant genotype was confirmed by PCR and Southern hybridization (data not shown). The inability of the *E. durans* Δ*tdcA* mutant to produce tyramine was confirmed by RP-HPLC (data not shown).

Survival of the wild-type and mutant strains under GIT stress conditions was assessed by viable plate counting (Fig. 2) after they were grown to the early stationary phase in ESTY medium plus 0.5% glucose in either the presence or absence of tyrosine. Approximately 50% of both bacterial populations were able to survive under G stress at pH 3.0 in either the presence or absence of tyrosine. For most of the analyzed conditions, no differences were detected between the two strains. We detected a significant increase in cell survival only for the mutant strain under GI stress at pH 5.0. Possibly this was due to utilization of the tyrosine for protein synthesis, since we detected a marked reduction in tyrosine levels (from 10 mM to 306 μM) without concomitant tyramine production in samples of the mutant strain supernatant (data not shown). In addition, under G stress at pH 2.1, a marked increase in cell survival, from 6.46×10^4 to 2.69×10^6 CFU ml^{-1}, was detected in the presence of tyrosine for the wild-type strain but not for the mutant strain. This accords with the finding that the tyramine biosynthetic pathway conserves the viability of *Enterococcus faecium* E17 cells in a medium buffered at pH 2.5 (14). However, the GIT challenge involves not only acidic

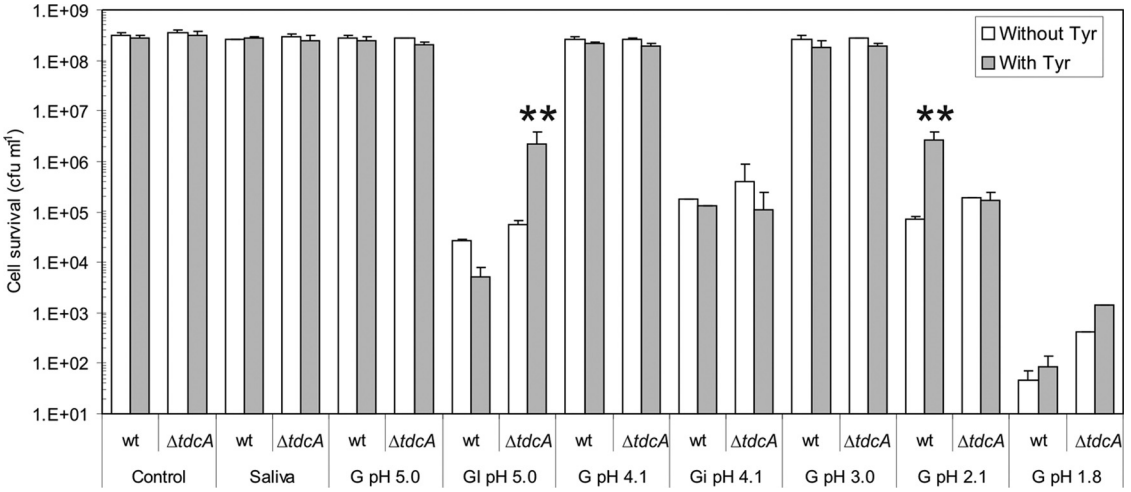

FIG. 2. Cell survival after gastric and gastrointestinal stresses. *E. durans* strain IPLA655 (wt) and the IPLA655 Δ*tdcA* mutant strain were subjected to various G or GI stresses as described in the text in the presence (gray bars) or absence (white bars) of 10 mM tyrosine (Tyr). Each value is the mean of the results of three independent experiments. Differences in survival in the presence or absence of tyrosine were tested by the two-tailed Student *t* test. **, $P < 0.01$.

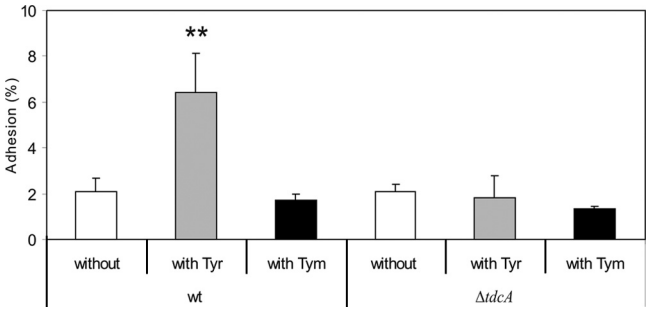

FIG. 3. Adhesion of *E. durans* strain IPLA655 and the IPLA655 Δ*tdcA* mutant strain to Caco-2 cells. Adhesion levels are expressed as percentages of the total number of bacteria (adhered plus unadhered) detected after exposure to Caco-2 cells for 1 h in the presence of either 10 mM tyrosine (Tyr; gray bars) or 140 μM tyramine (Tym; black bars) or in the absence of both compounds (white bars). Each adhesion assay was conducted in triplicate. Each value is the mean of the results of three independent experiments for which three independent determinations were performed. Differences in adhesion in the presence or absence of tyrosine were tested by the two-tailed Student *t* test. **, $P < 0.01$.

stress but also exposure to lysozyme, proteolytic enzymes, and bile salts, and the *Enterococcus* genus seems to be well adapted to intestinal conditions (5). This could explain the lack of a clear effect of tyrosine decarboxylation at pH values other than 2.1 for *E. durans* IPLA655.

In any case, the results revealed that the tyramine-producing *E. durans* strain IPLA655 is able to survive and to produce tyramine during passage through the GIT and therefore may contribute to tyramine content in the host. This contribution would be greater if such strains were able to colonize the gut and continue to synthesize tyramine. The ability to colonize the gut is related to the capacity to adhere to the intestinal epithelium. The difficulty of studying bacterial adhesion *in vivo* has led to the development of *in vitro* model systems that are based on adhesion to tissue culture cell lines such as Caco-2 cells, which, when differentiated, mimic mature enterocytes of the small intestine. Bacteria were exposed to Caco-2 cells under conditions previously described (4), and their adhesion was assessed by plate counting. In addition, the interactions of the strains with the Caco-2 cells were visualized by phase contrast and fluorescence microscopy (data not shown), since both the *E. durans* wild-type and mutant strains had been transformed with the plasmid pMV158GFP, which encodes the green fluorescent protein (GFP) (12). The ability to bind to Caco-2 cells was analyzed in the presence or absence of 10 mM tyrosine. For the adhesion assay, 1.25×10^5 epithelial cells were exposed to 1.25×10^7 bacterial cells in the presence of 1 ml of Dulbecco modified Eagle medium (DMEM) (Invitrogen, Barcelona, Spain) for 1 h at 37°C in a 5% CO_2 atmosphere as previously described (4). Interestingly, when tyrosine was present in the adhesion assay, a significant increase (approximately 3-fold) in the adherence of *E. durans* IPLA655 to Caco-2 cells was observed (Fig. 3). In contrast, the presence of tyrosine did not affect the binding of the mutant strain. Analysis of the supernatants from the adhesion samples by RP-HPLC revealed that in the presence of 10 mM tyrosine, the dairy (cheese) strain was able to synthesize tyramine ($1.4 \pm 0.2 \times 10^7$ bacteria produced 141 ± 15 nmol of tyramine in 1 h). Supplementing the assay with 140 μM tyramine did not affect the binding of either strain to Caco-2 cells (Fig. 3). These results suggest that activation of the tyramine biosynthetic pathway, rather than the actual production of tyramine, could be involved in this enhancement of adhesion.

Production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) by Caco-2 cells (1.25×10^5 cells) after 8 h of exposure to the *E. durans* wild-type and mutant strains (1.25×10^8 CFU) was quantified in sample supernatants as previously described (3) in the presence or absence of 10 mM tyrosine. In the absence of tyrosine, the presence of either strain did not significantly affect the levels of the cytokine produced and secreted by the Caco-2 cells (Table 1). In the control samples lacking bacteria, the presence of tyrosine resulted in a 2-fold decrease in TNF-α levels, which was accompanied by consumption of 83.5% of the tyrosine (Table 1). Significantly lower levels of this cytokine (8% and 3.8% of the levels with the mutant strain and the control, respectively) were detected in the presence of the wild-type strain than in the presence of the mutant or the control when tyrosine was included in the assay (Table 1). The production of tyramine was confirmed in the wild-type strain samples, in which it reached a concentration of 3.12 ± 19 mM in the presence of 10 mM tyrosine. The lack of a cytotoxic effect due to tyramine and bacteria was confirmed using cell proliferation kit II (XTT) (Roche Diagnostics, Mannheim, Germany) (data not shown). Moreover, a similar level of tyrosine (approximately 4.3 mM) was detected in samples exposed to both strains, indicating that the differences in cytokine levels provoked by the bacteria were not due to differences in the availability of tyrosine to the Caco-2 cells. Therefore, the reduction in the synthesis of TNF-α by the wild-type strain could be associated with the tyramine biosynthetic pathway.

The overall results indicate that *E. durans* IPLA655, a tyramine-producing strain present in cheese, can survive in the intestinal environment and synthesize tyramine in the colon, using this ability as a survival and colonization mechanism that enhances adhesion to the intestinal epithelium and reduces Th1 activation of the immune system. Unfortunately for the host organism, the increased levels of tyramine can provoke

TABLE 1. Immunomodulation of Caco-2 cells by *E. durans* IPLA655 and the *E. durans* IPLA655 Δ*tdcA* strain[a]

Strain or control	TNF-α (pg ml^{-1})	Tyrosine (μM)	Tyramine (μM)
Strains			
IPLA655			
Without tyrosine	289.28 ± 39.22	15.4 ± 0.5	10.1 ± 2.1
With tyrosine	9.33 ± 1.04	4,196 ± 78	3,116 ± 189
IPLA655 Δ*tdcA*			
Without tyrosine	558.5 ± 83.75	20.03 ± 1.3	<0.05
With tyrosine	116 ± 13.75	4,512 ± 365	<0.05
Controls			
Without tyrosine	489.06 ± 76.94	<0.03	<0.05
With tyrosine	247.25 ± 54.17	1,654.13 ± 87.12	<0.05

[a] TNF-α produced by Caco-2 cells alone (control) or in response to wild-type or mutant strains after 8 h of incubation in the presence or absence of tyrosine or tyramine. Tyramine and tyrosine concentrations in the cell supernatants were also quantified by RP-HPLC. Each determination was performed in triplicate, and the mean value and standard deviation are indicated.

adverse reactions, especially in individuals with reduced detoxification systems (1). These results offer further evidence of the importance of eliminating the presence of BA-producing strains in order to manufacture safer foods.

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REFERENCES

1. **Bodmer, S., C. Imark, and M. Kneubühl.** 1999. Biogenic amines in foods: histamine and food processing. Inflamm. Res. **48:**296–300.
2. **Fernández, M., D. M. Linares, A. Rodríguez, and M. A. Alvarez.** 2007. Factors affecting tyramine production in *Enterococcus durans* IPLA655. Appl. Microbiol. Biotechnol. **73:**1400–1406.
3. **Fernández de Palencia, P., et al.** 2009. Probiotic properties of the 2-substituted (1,3)-β-D-glucan producing *Pediococcus parvulus* 2.6. Appl. Environ. Microbiol. **75:**4887–4891.
4. **Garai-Ibabe, G., et al.** 2010. Naturally occurring 2-substituted (1,3)-β-D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods. Bioresour. Technol. **101:**9254–9263.
5. **Klein, G.** 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. Int. J. Food Microbiol. **88:**123–131.
6. **Krause, I., A. Bockhardt, H. Neckermann, T. Henle, and H. Klostermeyer.** 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. J. Chromatog. A **715:**67–79.
7. **Ladero, V., M. Fernández, and M. A. Alvarez.** 2009. Isolation and identification of tyramine-producing enterococci from human fecal samples. Can. J. Microbiol. **55:**215–218.
8. **Ladero, V., M. Calles-Enríquez, M. Fernández, and M. A. Alvarez.** 2010. Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci. **6:**145–156.
9. **Linares, D. M., M. Fernández, M. C. Martín, and M. A. Alvarez.** 2009. Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration. Microb. Biotechnol. **2:**625–633.
10. **Linares, D. M., M. C. Martín, V. Ladero, M. A. Alvarez, and M. Fernández.** Biogenic amines in dairy products. Crit. Rev. Food Sci., in press.
11. **Moreno-Arribas, M. V., and A. Lonvaud-Funel.** 2001. Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis*. FEMS Microbiol. Lett. **195:**103–107.
12. **Nieto, C., and M. Espinosa.** 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid **49:**281–285.
13. **Noack, J., B. Kleessen, and M. Blaut.** 1999. Contribution of the intestinal microflora to polyamine formation in the gut. *In* S. Bardócz, J. Konikx, M. Grillo, and A. White (ed.), COST 917: biogenically active amines in food, vol. III. European Commission, Luxembourg.
14. **Pereira, C. I., D. Matos, M. V. San Romão, and M. T. Barreto Crespo.** 2009. Dual role for the tyrosine decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge and generation of a proton motive force. Appl. Environ. Microbiol. **75:**345–352.
15. **Premont, R. T., R. R. Gainetdinov, and M. G. Caron.** 2001. Following the trace of elusive amines. Proc. Natl. Acad. Sci. U. S. A. **98:**9474–9475.