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Research Paper

Quantitative relationship between maximum growth rates and the intracellular pattern of α-esterase and β-esterase activity of leguminous infecting bacteria

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To the memory of Dr. Edsel Brussa.

Sixteen strains belonging to three families of the *Rhizobiales* order (*Bradyrhizobiaceae*, *Phyllobacteriaceae and Rhizobiaceae*) were evaluated according their specific growth rates (μ) and the activity of intracellular α-esterase and β-esterase isoenzymes. The average esterase activity of 48 isoenzymes assayed belonging to five strains with low (μ_{max} = 0.08–0.12 h^{-1}), four medium (μ_{max} = 0.13–0.22 h^{-1}) and seven high (μ_{max} = 0.24–0.28 h^{-1}) growth rate values were 22.1 ± 4.3; 8.7 ± 2.2 and 3.9 ± 1.7 U g^{-1} respectively. An inversely proportional relationship between the activity of the whole pattern of esterases and μ_{max} was found. Our results illustrate a feature of intracellular esterases, ascribable in a variety of cellular functions, which might be related to characteristics μ_{max} of legume infecting bacteria.

Introduction

The order *Rhizobiales* is a prokaryotic order belonging to the α-proteobacteria subdivision; it comprises both plant symbionts and plant and animal pathogens such as *Rhizobium*, *Agrobacterium* and *Brucella*, respectively. Some rhizobia (*Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium*) species are intensively studied for their nitrogen-fixing ability when in symbiosis with leguminous plants [1]. Inoculants are commercial formulations containing rhizobia that can be applied to the seed or the soil during planting (reviewed in [2]). Owing to significant differences in the maximal growth rate values of rhizobia genera and the fact that the manufacture of inoculants involves a large-scale fermentation step, one of the important parameters governing the cost of biomass production is the relationship between the bacterial growth rates and the timesaving variable of the process [3]. Nowadays, soybean (*Glycine max)* has become an important agricultural commodity, with a steady increase in worldwide annual production (http://www.fao.org). This situation has enhanced the demand for strains that specifically infect soybean roots (*Bradyrhizobium* spp.), and which are known to have the lower μ_{max} values and, therefore, longer fermentation times among rhizobia [3–5].

Changes in the specific growth rate of a given microbial strain growing under different conditions might be ascribable to several phenomena, for example, the modulation of the stringent response regulating the concentration of guanosine tetra-(ppGpp) and pentaphosphates (pppGpp) [6]. Gram-negative members of *Proteobacteria* employ acylated homoserine lactones (acyl-HSLs) as quorum-sensing diffusible signal molecules for monitoring the density of populations, which is quite a prevalent phenomenon among *Rhizobiales* [7,8].

The metabolic events driving the microbial specific growth rates define an interesting subject of scientific exploration among bacteria [9]. However, there is very limited knowledge about the molecular and biochemical mechanisms driving the large μ_{max} differences of microorganisms and no reports concerning the different growing rates among rhizobia genera [3,9,10]. In this study we have examined the relationship between the maximum growth rates and the entire pool of intracellular esterase isoenzymes activities of strains belonging to the *Sinorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium* genera.

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Materials and methods

Microbial strains

The strains *Bradyrhizobium japonicum* E109, *Rhizobium leguminosarum* D70, *Mesorhizobium loti* LL30, LL32 and CH38, *Sinorhizobium meliloti* B36, B58 and B399 were purchased from Instituto Nacional de Tecnología Agropecuaria (INTA Castelar, Argentina—http://www.inta.gov.ar/imyza/index.htm). *Sinorhizobium meliloti* U137 and *Mesorhizobium loti* U510 from Ministerio de Ganadería Agricultura y Pesca (MGAP Montevideo, Uruguay—http://www.mgap.gub.uy/Renare/default.htm). *Bradyrhizobium elkanii* SEMIA 587, SEMIA 5019, *Bradyrhizobium japonicum* SEMIA 5080 and SEMIA 5979 from FEPAGRO/MIRCEN *Rhizobium* Culture Collection (Porto Alegre, Brasil (http://wdcm.nig.ac.jp/CCINFO/CCINFO.xml?443). *Sinorhizobium meliloti* Lq22 and Lq51, were isolated from regionally cultivated alfalfa in La Pampa, Argentina. Stock cultures were kept at −18 °C in 20% (w/v) glycerol and agar slants at 4 °C.

Culture media

The culture medium for specific growth rate calculation for *S. meliloti*, *R. leguminosarum*, *M. loti* strains was g L^{-1}: Sucrose 10; yeast extract 4; KNO_3 0.8; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; $MnSO_4$ 0.001; $FeCl_3$ 0.001 and for *B. japonicum and B. elkanii* g L^{-1}: Glycerol 10, yeast extract 4; KNO_3 0.8; $(NH_4)_2PO_4$ 0.3; K_2HPO_4 0.5; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; $MnSO_4$ 0.001; $FeCl_3$ 0.001. Culture medium for zymographic assay g L^{-1}: Tryptone 5.0; yeast extract 3.0; $CaCl_2$ 0.87. The pH of both media was adjusted to 6.8.

Culture conditions

Hundred mL medium was inoculated with 10% (v/v) inocula of 24 h for fast-growers and 36 h for medium and slow-growing strains, rendering an initial cell concentration of $\approx 10^8$ c.f.u. mL^{-1}. 1. The flasks were incubated in an orbital shaker (250 rev min^{-1}) at 28 °C. Biomass concentration was monitored by measuring cell optical density (600 nm). For final biomass, dry weight and colony forming units (c.f.u. mL^{-1}) were determined according to Lorda *et al.* [11].

Intracellular protein extracts

Bacterial cultures (10 mL) were centrifuged at 4 °C for 20 min at 1500 × *g*, washed twice with 1 mL of 0.9 M NaCl, and suspended in 0.5 mL of a solution containing g L^{-1}: $MgCl_2$ 0.48, β-mercaptoethanol 1.0, glycerol 150 and lysozyme 24×10^6 units L^{-1} in 0.1 M Tris–HCl pH 7.2. Then, they were incubated for 10 min at 25 °C, centrifuged (6200 × *g*, 10 min) and suspended in buffer 0.1 M Tris–HCl pH 8.8 containing 150 g L^{-1} glycerol.

The cells were sonicated 6 times of 30 seconds on ice using a Kontes Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ, USA) and then centrifuged at 6200 × *g* for 10 min at 4 °C. The supernatants (intracellular protein extracts) were stored at −20 °C until processing.

Non-denaturing PAGE and zymographic staining

Samples underwent electrophoresis on vertical slab gels using a MiniProteanII electrophoresis apparatus (Biorad, Laboratories). The solution 300:8 g L^{-1} (w/w) acrylamide:bisacrylamide, was used at final concentration of 100 g L^{-1} for resolving gel and 50 g L^{-1} for stacking gel, containing 0.37 M Tris–HCl (pH 8.8) and 0.12 M Tris–HCl (pH 6.8) respectively. The mixture acrylamide:bisacrylamide was polymerized by adding 1.75 mM ammonium persulphate and 10.3 mM TEMED (N,N,N′,N′Tetra-,N′Tetramethylethylenediamine) at room temperature. The electrode buffer was 25 mM Tris and 192 mM Glycine (pH 8.3). Samples containing 25 μg of protein were loaded into the gel. The electrophoresis was performed at constant voltage (200 V) at room temperature.

Zymographic staining was developed with Fast Blue RR and, α and β naphthyl acetate as substrates [12]. The gels were incubated at 25 °C in a freshly made 1:1 mixture of solutions A and B. Solution A contained g L^{-1}: 0.6 α and 0.4 β naphthyl acetate (Sigma Chemical Co.) dissolved in 10 mL acetone and added to 900 mL of 0.1 M Tris–HCl buffer pH 6.2. Solution B consisted of g L^{-1}: 1.0 Fast Blue RR (Aldrich Chemical Co.) in 0.1 M Tris–HCl buffer (pH 6.2). Finally the reaction was stopped by washing and immersing the gels in 5:30:75 acetic acid, ethanol, water. Reproducibility of profiles obtained on non-denaturing PAGE was estimated by comparing duplicated extracts and performing duplicate runs of a single extract on separated gels.

Quantification of esterase activity

Esterase assay was performed according to Sohaskey and Barbour [13]. Briefly, 30 μL of enzyme solution were added to 1.0 mL of 100 mM potassium phosphate (pH 6.2) buffer and, after 10 min, 100 μL of 100 mM *p*-nitrophenylacetate in cold methanol was added. The reactions proceeded at 25 °C and the absorbance was continuously determined by means of an Ocean Optics USB4000 spectrometer at 420 nm (ε_{420} 11.614 $mM^{-1}cm^{-1}$). One enzymatic activity unit (U) was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute.

For zymogram imaging analysis, production of α or β-naphthol was monitored by coupling to Fast Blue RR and photographs of the gel were taken at different time intervals using a digital camera fixed at 0.6 m (Sony, 6 mega-pixels resolution). The 32-bit colour images were split into red, green and blue (RGB) components using the software ImageJ (National Institutes of Health, USA: http://rsb.info.nih.gov/ij/). Images corresponding to the green component were chosen for esterase quantification owing to the highest signal to noise ratio. The naphthol-Fast Blue RR complex produced for each activity band was quantified as integrated optical density units (IOD) and normalized dividing them by IOD_{max} to make the signal independent of the band size in the gel. The change in the signal per minute was standardized to enzyme units by measuring esterase activity both in solution and by zymogram imaging analysis of porcine liver esterase (Sigma Aldrich, USA).

Results

This study includes 16 *Rhizobiales* strains that are able to establish symbiotic relationships with legume crops. Previously, the zymographic pattern of α-esterase and β-esterase isoenzymes was an approach to study the specificity of legume–*Rhizobiales* interactions as well as the biodiversity of legume infecting bacteria [14,15]. Moreover, zymographic esterase patterns amplify the

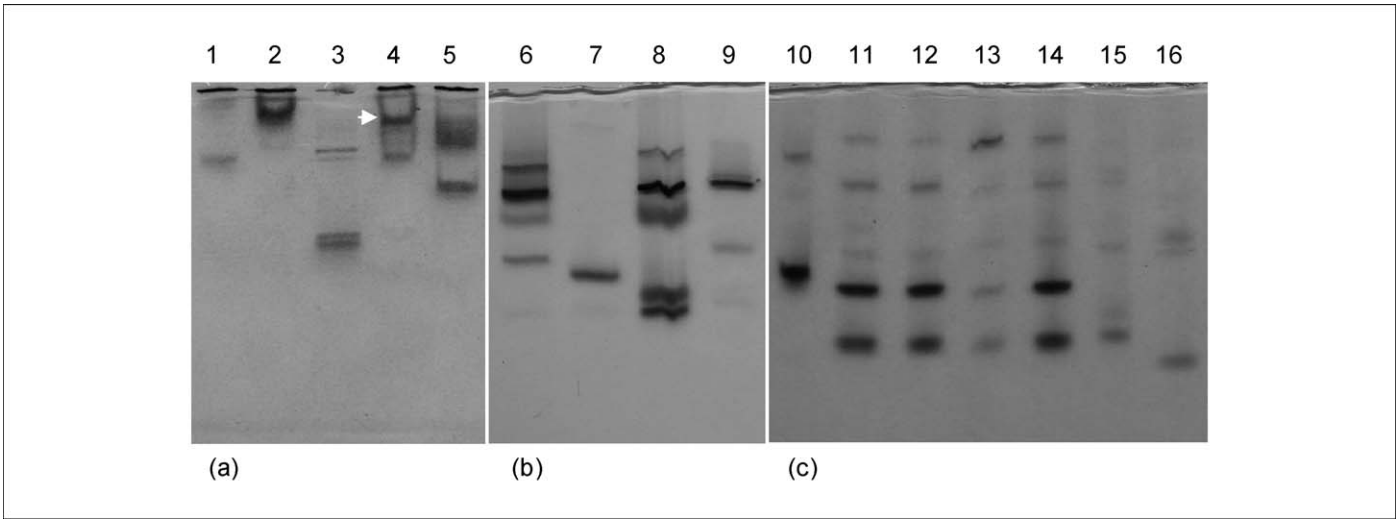

FIGURE 1

Zymographic analysis on non-denaturing PAGE of α-esterase and β-esterases of 16 strains of leguminous infecting bacteria. A: Low growth rate strains: lane 1, *Bradyrhizobium japonicum* E109; 2, *B. japonicum* S5080; 3, *B. japonicum* S5079; 4, *B. elkanii* S587; 5, *B. elkanii* S5019. B: Medium growth rate strains: lane 6, *Mesorhizobium loti* U510; 7, *M. loti* LL30; 8, *M. loti* LL32; 9, *M. loti* CH38. C: High growth rate strains: lane 10, *Rhizobium leguminosarum* D70; 11, *Sinorhizobium meliloti* Lq51; 12, *S. meliloti* Lq22; 13, *S. meliloti* B36; 14, *S. meliloti* B58; 15, *S. meliloti* B399; 16, *S. meliloti* U137. The white arrow marks the band that showed the maximum activity value (27.9 U g^{-1}) obtained with *B. japonicum* S5079.

microbial characterization allowing the detection of differences at strain level (e.g. Figure 1; [14]).

On the basis of growth rate, nutritional requirement and enzymes, rhizobia can be divided in three groups: the fast-growing rhizobia, placed in the genera *Rhizobium and Sinorhizobium*, the slow-growing rhizobia represented by the genus *Bradyrhizobium* and there is one species of intermediate growth rate: *Mesorhizobium loti* [3]. The growing parameters of the strains, in the present study, are shown in Table 1. The fast-growing strains presented μ_{max} 0.24–0.28 h^{-1}, the slow-growing μ_{max} 0.08–0.12 h^{-1} and the ones of medium growth rate μ_{max} 0.13–0.22 h^{-1}. The average biomass yield (g L^{-1}) at the end of the fermentation was slightly lower for slow growing strains (3.5 ± 0.4) than medium and fast-growers (4.0 ± 0.1).

On the contrary, intracellular protein extracts of each strain underwent native-PAGE analysis developed for α-esterase and

TABLE 1

Growth parameters of leguminous infecting bacteria strains

Strain	Maximum growth rate	Biomass[a]	
	μ_{max} (h^{-1})	(g L^{-1})	(c.f.u. mL^{-1})
***Bradyrhizobium elkanii* S587**	0.08	3.12	1.0×10^{10}
***B. elkanii* S5019**	0.08	3.01	9.2×10^{9}
***B. japonicum* S5079**	0.10	3.91	3.7×10^{10}
***B. japonicum* S5080**	0.12	3.76	3.9×10^{10}
***B. japonicum* E109**	0.11	3.85	7.8×10^{10}
***Mesorhizobium loti* LL30**	0.14	4.10	1.08×10^{11}
***M. loti* LL32**	0.13	3.93	6.0×10^{10}
***M. loti* CH38**	0.15	4.12	1.1×10^{10}
***M. loti* U510**	0.22	4.20	2.0×10^{11}
***Sinorhizobium meliloti* B399**	0.25	4.14	3.1×10^{11}
***Rhizobium leguminosarum* D70**	0.28	3.89	4.0×10^{11}
***S. meliloti* U137**	0.27	4.01	9.6×10^{10}
***S. meliloti* Lq22**	0.26	4.18	1.2×10^{11}
***S. meliloti* Lq51**	0.26	4.22	1.8×10^{11}
***S. meliloti* B36**	0.24	3.96	9.1×10^{9}
***S. meliloti* B58**	0.26	4.20	2.6×10^{10}

[a] Biomass was measured at the end of the fermentation.

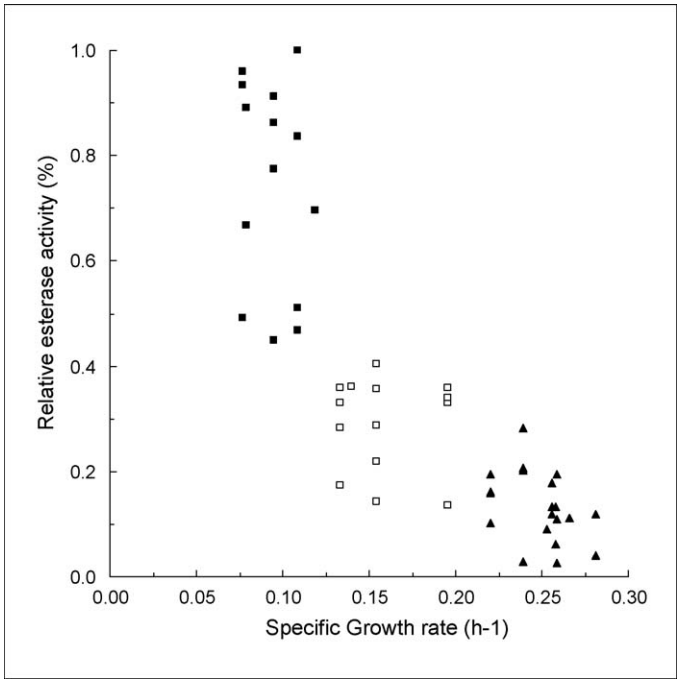

FIGURE 2

α-esterase and β-esterase activity of every intracellular isoenzyme of 16 strains of leguminous infecting bacteria plotted versus their μ_{max}. ■, Slow-growing strains (*Bradyrhizobium japonicum* E109, S5080; S5079; *B. elkanii* S587, S5019). □. Medium-growing strains (*Mesorhizobium loti* U510, LL30, LL32, CH38) and ▲. Fast-growing strains (*Rhizobium leguminosarum* D70; *Sinorhizobium meliloti* Lq51, Lq22, B36, B58, B399, U137). The 100% value corresponded to 27.9 U g^{-1}.

β-esterases activities (Figure 1). The polymorphism of the zymographic esterase pattern among *Rhizobiales* presents characteristic profiles that were correlated by Caballero-Mellado *et al.* [15] with the taxonomic classification based on biochemical tests as well as molecular analysis. The degree of inter-strain similarities related to esterase pattern is highly variable, fast-growing strains (*Rhizobiaceae* family) showed higher polymorphism homogeneity (Figure 1c), contrasting with the heterogeneity of the other two groups of legume infecting bacteria, families *Bradyrhizobiaceae* and *Phyllobacteriaceae* (Figure 1a and b).

Previously, the zymographic technique for bacterial characterizations was performed on the basis of electrophoretic mobilities of proteins and hydrolytic specificities toward different substrates of a given enzymatic activity [16,17]. Herein the zymogram analysis was kinetically analysed by measuring the intensity of the activity bands during time. It revealed that this group of bacteria showed a quantitative inverse relationship relating the μ_{max} of the strains and the activity of each intracellular esterase (Figure 2). The esterase average activities of 48 isoenzymes assayed were 22.1 ± 4.3; 8.7 ± 2.2 and 3.9 ± 1.7 U g^{-1} for slow, medium and fast-growers respectively (Table 1, Figure 2). We also found that this relationship was independent of the culture conditions of the strains (data not shown). Although 16 strains were used in this report, we described the observation of several years of the working group using the esterase zymographic analysis for characterizing legume infecting bacteria applied to a large number of strains [4,5], where slow-growers presented faster zymographic development (≈12 min) than fast-growers (≈60 min).

Discussion

There is evidence that growth rate affects cell physiology, size and cellular composition of microbial organisms [18]. The cellular RNA content has been shown to be strongly correlated with specific growth rates, although this relationship was not a straightforward approach for marine bacteria [19]. Additionally, enzymes of the central metabolism (phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthase) were identified as key steps that influence the growth rate of *E. coli* on gluconeogenic carbon sources [20].

Certainly, most characterization describing molecular events that govern microbial growth rate are descriptions of biochemical features occurring with a model microbial system growing in different culture conditions. But studies relating the differences of maximum growth rates values (μ_{max}) reached by different microbial species with metabolic events are scarce in the literature [3,10,21,22]. Shahab *et al.* [22] reported a regulatory mechanism that coordinates cell growth with the synthesis of essential macromolecules (protein, RNA and DNA) for *Streptomyces coelicolor* ($\mu_{max} \approx 0.35$) and *E. coli* ($\mu_{max} \approx 1.73$). But changes in the macromolecular content on both bacteria over a range of specific growing rates were basically similar. On the contrary, a quantitative relationship emerged between the amount of *rrn*A (*rrn*) operons per genome in *mycobacterial* species, where slow-growers carried a single copy of *rrn* operon and fast-growers had two copies [10]. In the same trend, an interesting report of Cox [3] established a theoretical comparison of the above two bacteria adding a third one as very slow-growing reference: *Mycobacterium* sp. ($\mu_{max} = 0.029$ h^{-1}), but the number of *rrn* operons plus the macromolecular compositions failed to predict the slow growing rates of *Mycobacterium* group.

In this work an inverse relationship between the μ_{max} of 16 rhizobia strains and the activity of the entire α-esterase and β-esterase pattern was demonstrated. Nevertheless, the physiological mechanisms underlying the phenomenon remain unknown, and owing to the promiscuity of esterases to find their natural substrate it would be a difficult assignment. Interestingly, NodB is an esterase (oligosaccharide deacetylase) required for the synthesis of nodulation factors by most rhizobia species [23]. However, it is induced in response to plant signalling, and since the cells for the present study were cultured in batch systems, it is suggested that esterases reported here did not include NodB. On the contrary, Giraud *et al.* [24] completely sequenced the genome of two *Bradyrhizobium* strains, which lacked of *nodB* genes, and identified several putative esterases that included carboxyl-esterases (3.1.1.1) and aryl-esterases (3.1.1.2), among others. Nevertheless, neither the transcription of such enzymes nor their function in the metabolism of the bacteria had been reported. This is the first description relating a biochemical feature (the activity of each intracellular esterase) with the μ_{max} of leguminous-infecting bacteria. Further studies involving esterase genes might lead to modified strains with shorter fermentations times and without affecting symbiotic efficiency.

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