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Short communication

Antibiosis by *Bacillus amyloliquefaciens* ribonuclease barnase expressed in *Escherichia coli* against symbiotic and endophytic nitrogen-fixing bacteria

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Abstract

A modified antibiosis assay was used to evaluate growth inhibition of symbiotic and endophytic bacteria by *E. coli* strains producing *Bacillus amyloliquefaciens* ribonuclease, barnase. Inhibition zones were only observed when the assays were performed in minimal medium agar. However, bacterial growth inhibition was not detected when using rich medium or susceptible strains expressing the ribonuclease inhibitor protein, barstar. Our results suggest that barnase may act as a broad range bacteriocin. The ecological significance of these results is discussed.

Keywords: Barnase; Antibiosis; Symbiotic and endophytic bacteria

Barnase is a potent ribonuclease, an enzyme that breaks down RNA indiscriminately. The *barnase* gene was isolated from the soil bacterium, *Bacillus amyloliquefaciens*, which also produces the barstar protein, the specific inhibitor of barnase (Paddon and Hartley, 1985; Hartley, 1988). Barstar binds to barnase and inactivates the enzyme. Barnase has been widely used as a lethal gene in biological research and as a molecular tool in genetic engineering because it degrades RNA non-specifically. Several regulatory systems have been developed to control ribonuclease expression and activity in prokaryotic and eukaryotic cells (Beals and Goldberg, 1997; De Block et al., 1997; Kuvshinov et al., 2001; Bi et al., 2001; Leuchtenberger et al., 2001; Ramos et al., 2005). These systems include conditional expression of the *barnase* gene by the use of a tightly regulated promoter or the regulated co-expression of the *barstar* and *barnase* genes. When the barstar protein is present, it binds tightly to barnase and inhibits the intracellular ribonuclease activity. Disproportion-

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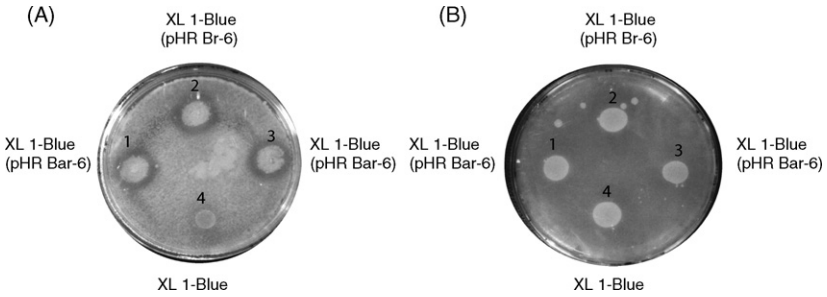

Fig. 1. *B*arnase antibiosis against *R. tropici* PRF81 grown on YM (A) and TY (B) agar plates. A clear zone shows growth inhibition around the *E. coli* XL1-Blue colonies expressing the barnase gene by the plasmid pHRBar-6. No growth inhibition was observed by with the host XL1-Blue *E. coli* strain, a strain which does not produce barnase.

ate expression of the *barstar* and *barnase* genes or the onset of barnase expression unaccompanied by its specific inhibitor barstar causes rapid cell death. Because of this activity, the barnase is actually being exploited as a conditional 'suicide gene' in therapeutic cell treatments to cause death of mammalian cells (Leuchtenberger et al., 2001), including in human systems (Prior et al., 1991) and in transgenic plant cells (Mariani et al., 1990; Beals and Goldberg, 1997; De Block et al., 1997; Kuvshinov et al., 2001).

In the regulatory system to control barnase cytotoxicity and expression proposed by Ramos et al. (2005) the *barstar* and *lacI* genes are under control of the NifA σ^{54}-dependent *Sinorhizobium meliloti nifH* promoter and the barnase gene is under control of the *ptac* promoter. The plasmids pHRBar-6 and pHRbar-13 (Ramos et al., 2005), used in this system, contain the structural barnase gene, cloned from *B. amyloliquefaciens*, downstream from the *phoA* signal sequence of *Escherichia coli* and the synthetic *tac* promoter (Hartley, 1988). Thus, the active and mature barnase is exported to the periplasmic space in *E. coli* strains harboring these plasmids. Here we show that the toxic protein leaks into the environment and kills other bacteria.

A modification of the trifolitoxin production and resistance bioassay (Breil et al., 1993) was used as an antibiosis assay to evaluate the barnase inhibitory effect against bacterial cells that do not express the intracellular inhibitor. Three 10 μL volumes of a saturated culture of an *E. coli* strain harboring the plasmid expressing the barnase gene were placed on yeast-mannitol (YM) or tryptone-yeast (TY) agar plates to permit bacterial growth, barnase production and diffusion around the colony. After 2 days at 30 °C, the strains to be tested were spread over the agar plates in a fine layer using a sterile velvet pad on a cylindrical replicator, as follows: The velvet was slightly moistened in a dish containing a turbid suspension (optical density at 600 nm of 0.1) of the strain to be tested for barnase sensitivity or resistance. After obtaining a uniform absorption of the velvet, the excess of culture was removed by pressing against a sterile Petri dish, and the replicator then was pressed softly against the agar containing the barnase-producing colonies. After 3 days at 30 °C, the plates were examined for the presence of zones of growth inhibition of the spread strains (Fig. 1). The *E. coli* strains that did not produce barnase and/or harbored the pHR28A10 plasmid (Ramos et al., 2005), which expressed the inhibitor barstar, were also included in the bioassays as controls (Table 1).

E. coli strains expressing barnase inhibited the growth of the symbiotic bacterial strains, including *Rhizobium tropici* PRF81, *Rhizobium etli* CNF 42, *Rhizobium leguminosarum* bv. *Phaseoli* CNPAF 512, *Bradyrhizobium japonicum* strains SEMIA 5079 and SEMIA 5080, and *B. elkanii* SEMIA 5019. Growth inhibition was observed against two endophytic bacteria, *Herbaspirillum seropedicae* SMR1 and *Azospirillum brasilense* FP2, and two *E. coli* strains DH10B and XL1-Blue (not shown). Assays with the *R. tropici* PRF81 were on YM and TY agar plates (Somasegaran and Hoben, 1994) and, with *E. coli* strains on NFDM (Cannon, 1984) and Luria Bertani (LB) agar plates. Inhibition zones were not observed when the assays were performed using the rich media TY or LB agar, possibly because both *R. tropici* and *E. coli* grew faster than on YM and NFDM medium, respectively. Proba-