Plasmid 66 (2011) 152–158

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Plasmid encoded antibiotics inhibit protozoan predation of *Escherichia coli* K12

Adnan Ahmetagic, Daniel S. Philip, Derek S. Sarovich, Daniel W. Kluver, John M. Pemberton *

School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia 4072, Australia

ARTICLE INFO

Article history:
Received 18 May 2011
Accepted 24 July 2011
Available online 3 August 2011
Communicated by Dr. J. Casadesus

Keywords:
Violacein
Streptomyces
Antibiotic
Protozoa
Escherichia coli
Expression

ABSTRACT

Bacterial plasmids and phages encode the synthesis of toxic molecules that inhibit protozoan predation. One such toxic molecule is violacein, a purple pigmented, anti-tumour antibiotic produced by the Gram-negative soil bacterium *Chromobacterium violaceum*. In the current experiments a range of *Escherichia coli* K12 strains were genetically engineered to produce violacein and a number of its coloured, biosynthetic intermediates. A bactivorous predatory protozoan isolate, *Colpoda sp.A4*, was isolated from soil and tested for its ability to 'graze' on various violacein producing strains of *E. coli* K12. A grazing assay was developed based on protozoan "plaque" formation. Using this assay, *E. coli* K12 strains producing violacein were highly resistant to protozoan predation. However *E. coli* K12 strains producing violacein intermediates, showed low or no resistance to predation. In separate experiments, when either erythromycin or pentachlorophenol were added to the plaque assay medium, protozoan predation of *E. coli* K12 was markedly reduced. The inhibitory effects of these two molecules were removed if *E. coli* K12 strains were genetically engineered to inactivate the toxic molecules. In the case of erythromycin, the *E. coli* K12 assay strain was engineered to produce an erythromycin inactivating esterase, PlpA. For pentachlorophenol, the *E. coli* K12 assay strain was engineered to produce a PCP inactivating enzyme pentachlorophenol-4-monooxygenase (PcpB). This study indicates that in environments containing large numbers of protozoa, bacteria which use efflux pumps to remove toxins unchanged from the cell may have an evolutionary advantage over bacteria which enzymatically inactivate toxins.

1. Introduction

Studies have revealed that survival and growth of bacteria in a range of environments is substantially affected by predatory protozoa. In response to this predation, bacteria have developed a number of survival strategies that range from size variation to the production of toxic molecules (Matz and Kjelleberg, 2005). Bacteria produce a range of anti-protozoan molecules which include the plasmid encoded antibiotics of *Streptomycetes* (Kinashi, 2011), the phage encoded Shiga toxins of strains of *Escherichia coli* O157:H7 (Steinberg and Levin, 2007; Lim et al., 2010) and the intensely purple-pigmented anti-cancer agent, violacein of *Chromobacterium violaceum* and *Janthinobacterium lividum*. Violacein is highly toxic to a range of parasites including the malarial parasite *Plasmodium falciparum*, the leishmaniasis pathogen *Leishmania* and *Trypanosoma cruzi*, which is responsible for Chagas disease (Duran et al., 2007). Recent studies in mice demonstrate that violacein not only protects against infection by the malarial parasite, *Plasmodium falciparum*, but can also cure mice already infected (Lopes et al., 2009). In addition, violacein producing strains of the bacterium *J. lividum* living symbiotically on the skin of certain frogs, reduces the severity of infection by the 'chytrid' fungus *Batrachochytrium dendrobatidis* thought to be a contributing factor to frog extinctions worldwide (Becker et al., 2009).

* Corresponding author. Fax: +61 7 33654620.
E-mail address: j.pemberton@uq.edu.au (J.M. Pemberton).

0147-619X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2011.07.006

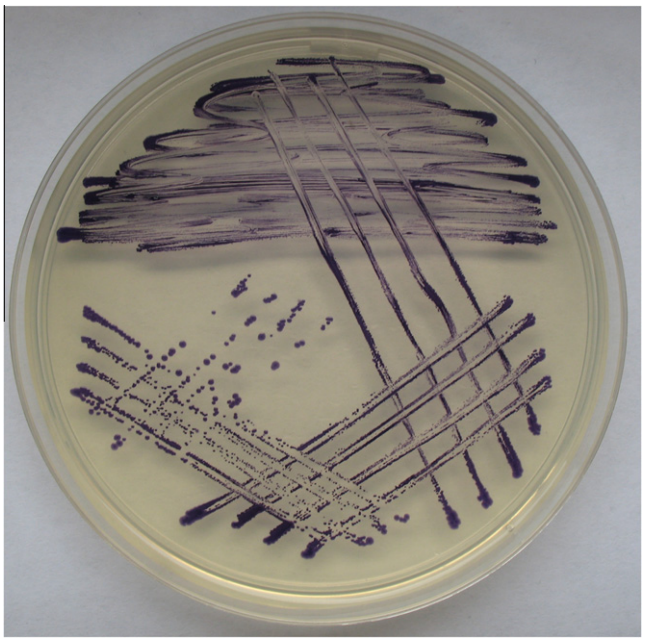

Fig. 1. *E. coli* K12 producing violacein. JM109 pPSX-*vioABCDE* was 16 streaked onto a PYE agar plate and incubated for 48 h at 35 °C then photographed.

The violacein gene cluster from *C. violaceum* was the first antibiotic biosynthesis pathway to be cloned and expressed in *E. coli* K12 (Pemberton et al., 1991) producing a purple-pigmented strain of this bacterium (Fig. 1).

The violacein pathway consists of five enzymes that convert tryptophan and oxygen into violacein (August et al., 2000; Balibar and Walsh, 2006; Nishizawa et al., 2006). The indolocarbazole synthase (VioB) alone catalyses the formation of the violacein core molecule (Ryan et al., 2008; Ryan and Drennan, 2009). However, the monooxygenase VioA dramatically increases core formation. Null mutations in either *vio*A or *vio*B abolish the formation of violacein or any of the coloured pathway intermediates synthesised by *E. coli* K12 and such strains remain white. Null mutations in *vio*C result in the accumulation of proviolacein, and *E. coli* K12 turns green. While null mutations in *vio*D result in the accumulation of deoxyviolacein and *E. coli* K12 turns blue. The exact role of VioE remains unclear.

Violacein belongs to a group of anti-tumour antibiotics familiar to medical science, the indolocarbazoles. *Streptomycetes* and related soil bacteria synthesise most indolocarbazoles. An indolocarbazole well known to medical science is staurosporine, the most potent apoptotic agent known to science. Staurosporine acts against protein kinases, most notably protein kinase C (PKC) (Tamaoki et al., 1986). Initial sequencing of the staurosporine gene cluster from *Streptomyces* sp. TP-A0274 revealed that it encoded a homolog of VioB and that staurosporine core formation occurs by the same mechanism as in violacein synthesis (Hyun et al., 2003; Howard-Jones and Walsh, 2005). All known indolocarbazole synthesizing bacteria encode homologs of VioB (Ryan and Drennan, 2009). A recent study of anti-protozoan molecules from Mediterranean marine sponges revealed that one was staurosporine indicating that other indolocarbazoles may have antiparasitic activities (Pimentel-Elardo et al., 2010). Genome sequencing of *Streptomyces clavuligerus* ATCC 27064, revealed that it contained a staurosporine gene cluster located on a giant linear plasmid of 1.8 Mb in length (Medema et al., 2010). The presence of indolocarbazole gene clusters on mobile genetic elements may play a significant role in the evolution of bacterial populations that resist predation by protozoa.

The development of the cosmid/BAC cloning vector pPSX allowed the stable cloning and low-level expression of the violacein gene cluster in *E. coli* K12 DH5α (Sarovich and Pemberton, 2007). Recent research demonstrated that stable over-production and hyper production of violacein in *E. coli* K12 can be achieved using a combination of the vector pPSX, the bacterial host *E. coli* K12 (JM109 or antibiotic resistant mutants) and a promoter mutation (*opv-1*) upstream of the violacein cluster (Ahmetagic and Pemberton, 2010, 2011). The availability of these strains provided the opportunity to test protozoan predation of *E. coli* K12 strains synthesising violacein and intermediates in the violacein pathway. The techniques developed in these studies were also used to examine protozoan predation of *E. coli* K12 in the presence of the macrolide antibiotic erythromycin and the persistent organochlorine pesticide pentachlorophenol.

2. Methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown on PYEA media consisting of 5 g/L NaCl, 5 g/L yeast extract, 3 g/L peptone and