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Full Length Research Paper

Expression of melanin and insecticidal protein from *Rhodotorula glutinis* in *Escherichia coli*

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Both the salmon/red melanin and the insecticidal producing genes of *Rhodotorula glutinis* was successfully expressed in *Escherichia coli* using plasmid pZErO -1. This work suggests that in *Rhodotorula* species melanin and insecticidal toxin are co-expressed and therefore possibly co-evolved.

Key words: *Rhodotorula glutinis*, *Escherichia coli*, Melanin, Insecticidal Protein.

INTRODUCTION

It has been shown that *Rhodotorula glutinins* produces salmon/red melanin and proteinaceous crystal toxic to insect just like *Bacillus thuringienses* (Oloke and Glick, 2005). Various strains of *Bacillus thuringiensis* produce proteinaceous crystals toxic to different insect larvae (Bulla et al., 1977; Holmes and Monro, 1965; Lawbaw, 1964), making these organisms candidates for use on a large scale as biological insecticides. Unfortunately, although numerous formulations of *B. thuringiensis* have been used as a bioinsecticide (Armstrong et al., 1985; Goldberg and Margalit, 1977; Viseser et al., 1986), these preparations generally have low efficacies (Beeker et al., 1992; Mulla, 1985; Mulligan et al., 1980) . One of the major factors affecting the stability and thus the efficacy of *B. thuringiensis* is photoinactivation (Ignoffo et al., 1977) although other factors such as heat, dessication and pH may also play some role (Leong et al., 1980).

Several attempts to achieve photoprotection of the *B. thuringiensis* insecticidal toxin have included encapsulation (Dunkle and Shasha 1988), granular formulation (Ahmed et al., 1973), and the addition of a variety of UV absorbing compounds (Morris 1983; Margulies et al., 1985; Cohen et al., 1991). Although synthetic UV chemical photostabilizers offer *B. thuringiensis* some protection, their use in the environment may introduce ecological problems related to soil and water pollution. The black melanin isolated from another soil bacterium has been found to provide photoprotection to the *B. thuringiensis* toxin (Liu et al., 1993). In this paper, the isolation of *E. coli* transformants capable of producing both the *Rhodotorula glutunis* insecticidal toxin and salmon/red pigment are described.

MATERIALS AND METHODS

Plasmid DNA was prepared from *Rhodotorula glutinis* by the procedure of Krostad et al. (1983). For preferential isolation of plasmids 50 megadaltons (mD) in size and smaller cultures were grown in 500 mL of L broth (Difco Laboratories, Detroit, MI) supplemented with glucose to give a final concentration of 0.1% in a 2.8 L Fernback flask with shaking at 37^{o}C; cells were harvested at an optical density at 600 nM of 0.8. For the preferential isolation of plasmids 30 mD and larger, cells were grown separately in two types of media: in 500 mL coconut water (Oloke and Glick, 2005) supplemented with glucose and in 500 mL of SPY medium (Spizizen medium; 20) supplemented with 0.1% yeast extract and 0.1% glucose in a 2.8 L Fernback flask with shaking at 37^{o}C; cells were harvested at an optical density at 600 nM of 0.7.

Total cell DNA was prepared from *Rhodotorula glutinis* as described by Kronstad et al. (1983). Cells grown in coconut water medium were harvested by centrifugation, washed with a solution containing 100 mM NaCl, 10 mM Tris (pH 7.9), and 10 mM EDTA (pH 7.9 before being lysed by the addition of lysozyme. The isolated DNA was dissolved in 10 nM Tris pH 7.9.

Restriction enzymes were used as recommended by the supplier (New England Biolabs), and recombinant DNA procedures were carried out as described by Sambrook et al., 1989. A genomic DNA library of *Rhodotorula glutinis* was prepared in plasmid pZErO-1 (Invitrogen Corp., San Diego, CA) using the conditions suggested by the manufacturer.

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Two L aliquots of the ligation mixture were used to transform *E. coli* TOP10F cells (Invitrogen Corp., San Diego, CA). The transformants were plated on Low Salt LB-Zeocin-IPTG medium (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 1 mM IPTG and 500 g/L Zeocin.).

Transmission electron microscopy was carried out as described by Bechtel and Bulla, 1976. *E. coli* transformants were suspended in 4% glutaraldehyde in 0.01 M phosphate-buffered saline at pH 7.2 for 5 min at 4^{o}C then pelleted and suspended in 2% agar at 55^{o}C. The agar was immediately cooled to 4 ^{o}C, cut into 1 mm cubes, and placed into fresh cold glutaraldehyde for 1 h. Samples were washed four times in cold 0.01 M phosphate buffer for a total of 80 min. After washing, the yeasts were postfixed in 1% OsO_4 at 4 ^{o}C for 1 h. Samples were rinsed in double-distilled water for 30 min and then stained overnight in 0.5% aqueous uranyl acetate. The yeasts were dehydrated by passing them through a graded acetone series and then embedded in Epon 812. Samples were cut with a diamond knife on a Porter-Blum MT -2b ultramicrotome, stained with lead citrate, and examined in a Philips EM 201 electron microscope operated at 60 kV. Serial sections, 150 nM thick were placed on slotted grids previously coated with Formvar and carbon.

Scanning electron microscopy was carried out as described by Calabrese et al., 1976. A drop of each culture suspension (~106 cells/mL) was placed on a Formvar coated grid and excess liquid drawn off with Whatman No. 50 filter paper. The dried grid was then shadowed with a 63.5 mm Pt-Pd wire at 20 A for 20 sec in the tungsten basket of a Varian PS10E vacumn evaporator.

The pigments isolated from the transformants were purified as described by Liu et al., 1993 and characterized as described by Fuqua et al., 1991. The photoprotective effect of the pigment was assessed as described by Liu et al., 1993.

The crystals of the transformant were obtained by sucrose gradient centrifugation (Aronson et al., 1991) and solubilized in 0.3 M Na_2CO_3- - mercaptoethnol, pH 9.7 (Calabrese and Nickerson, 1980). The solubilized crystals were dialyzed at 4^{o}C against several changes of 0.03 M $NaHCO_3$, pH 8.5. Inclusions were suspended in the latter buffer and used for bioassay against fourth instar larvae of *Aedes aegypti* as described (Aronson et al., 1991; Ingle et al., 1993), and the LC_{50} was calculated.

RESULTS AND DISCUSSION

Agarose gel electrophoresis of plasmid DNA extracted from *Rhodotorula glutinis* indicates the presence of a single large plasmid whose size is equal to or greater than about 50 kb (Figure 1). Since it was not known whether the gene encoding the biosynthesis of the salmon/red melanin was presented within the plasmid or the chromosomal DNA, total cellular DNA was used to construct a clone bank of *Rhodotorula glutinis* DNA in the *E. coli* plasmid pZErO-1.

The crystal protein gene is located on the chromosome as well as on a plasmid in a strain of *Bacillus thuringiensis* subspecies *kurstaki* (Khawalled et al., 1990) and in subspecies *thuringiensis* strain berliner 1715 (Held et al., 1982). *E. coli* cells transformed with this clone bank were plated on low salt LB medium containing zeocin as the selective antibiotic and colonies that produced a red or yellow colour following five days of growth at 37^{o}C were isolated and characterized. Of the five thousand colonies that were plated on this medium, three were selected for further study. One of these three colonies was coloured

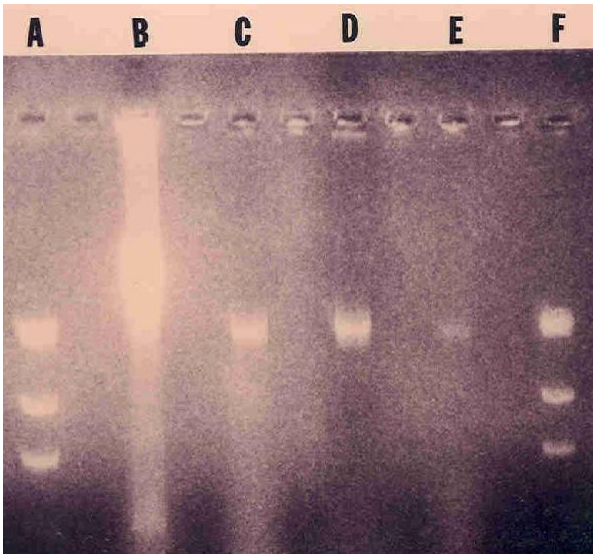

Figure 1. Horizontal agarose gel electrophoresis of the genomic and plasmid DNA of *Rhodotorula glutinis*. A and F = HindIII-digested DNA; B= *R. glutinis* DNA; C-E = plasmid DNA isolated from *R. glutinis* cultured on coconut water, SPY medium and Luria broth, respectively.

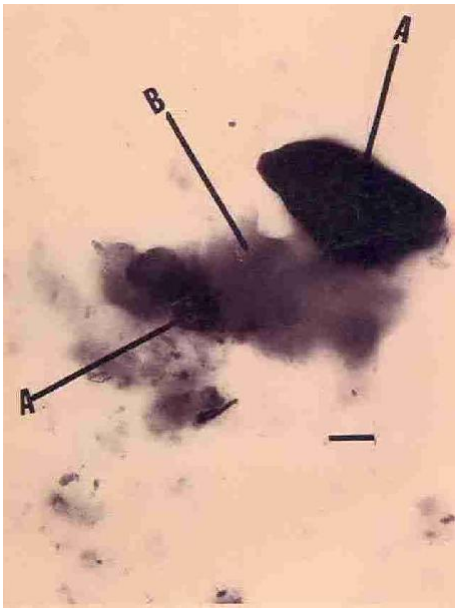

Figure 2. Transmision electron micrograph of *Rhdotorula glutinis* A = inclusion bodies; B = cellular debris. Bar = 0.1 m.

salmon/red while the other two were yellow.

Colonies of the salmon/red-colour transformant are similar in colour to the parent *Rhodotorula* strain and also appear to produce visible inclusion bodies (Figures 2 and 3).However, this transformant grows very poorly, producing
Only small colonies, in comparison

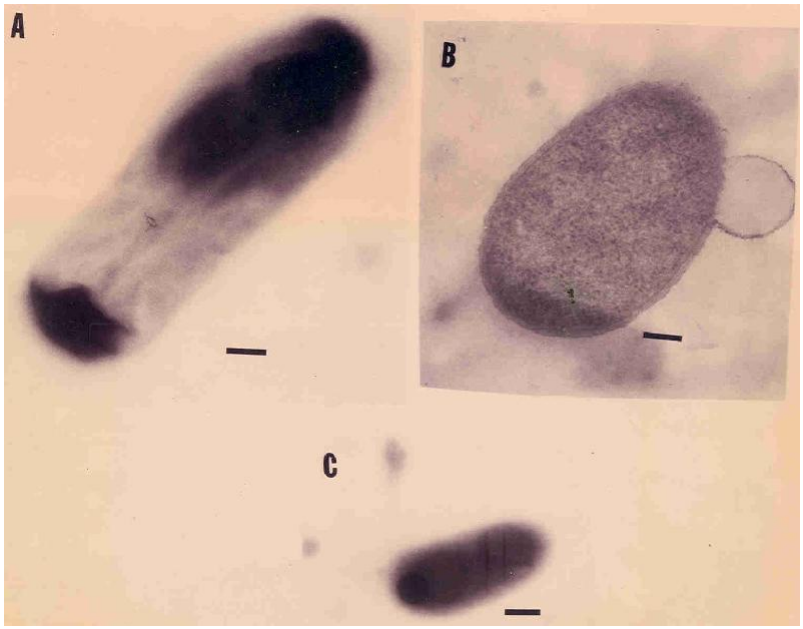

Figure 3. Transmission electron micrograph of *E. Coli* transformants carrying DNA from *Rhodotorula glutinis* A = red transformant; B = large yellow transformant; C = small yellow transformant; Bar = 0.1 m.

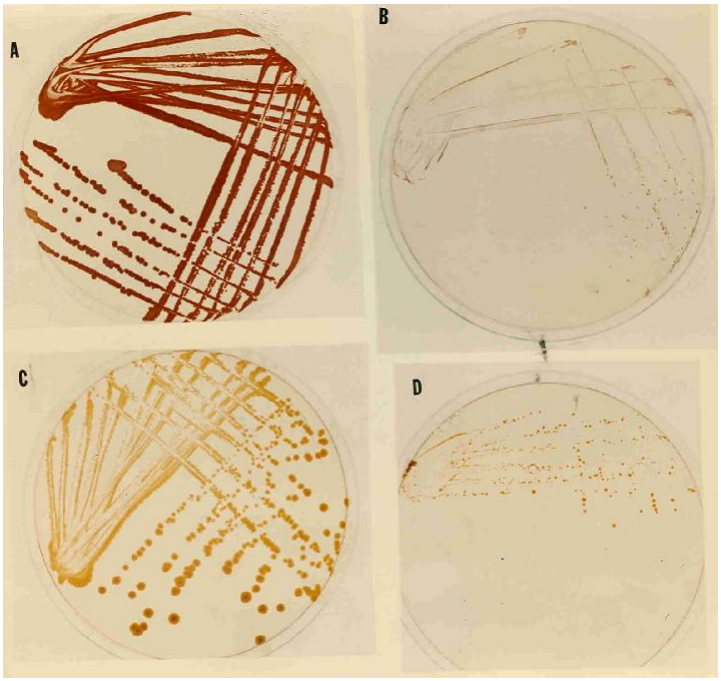

Figure 4. Colony morphology of *Rhodotorula glutinis* and *E. coli* transformats. A = *Rhodotorula glutinis*, B = red transformant; C = large yellow transformant; D = small yellow transformant.

with the parent *Rhodotorula* strain which produces large colonies on rich media (Figure 4). The differences between the parent *Rhodotorula* strain and the salmon/red *E. coli* transformant are best seen when these two are examined by scanning electron microscopy (Figure 5). Cells from the parent *Rhodotorula* strain are approximately 5.2 by 2.2 m

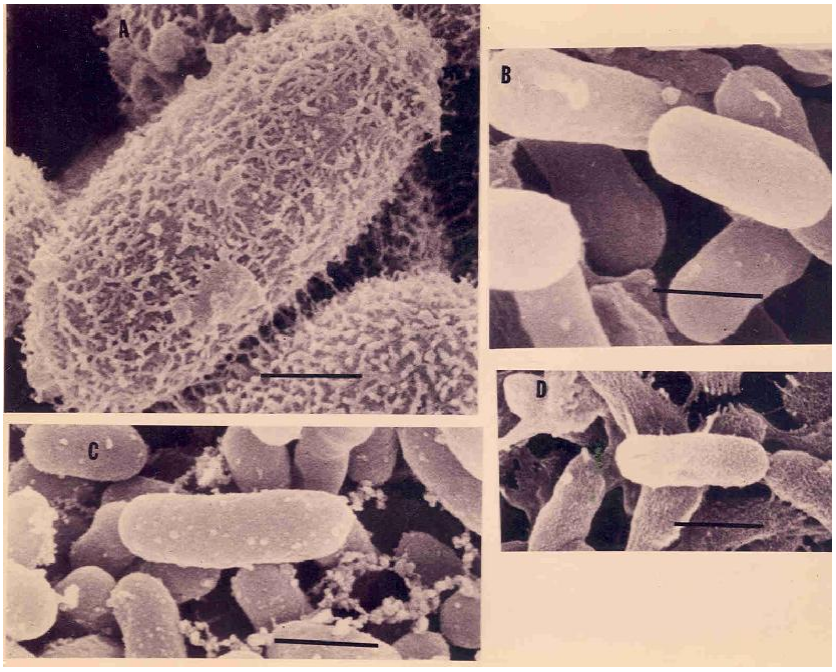

Figure 5. Scanning electron micrograph of *Rhodotorula glutinis* and *E. coli* transformants. A = *Rhodotorula glutinis* B = red transformant; C = large yellow transformant; D = small yellow transormant, Bar = 1 m.

in size while the *E. coli* transformant cells are approximately 1.8 by 0.8 m in size (Figure 5). In addition, the surface of the parent *Rhodotorula* cells appears coarse and fibrous while the surface of the salmon/red *E. coli* transformant cells is smooth (Figure 5).

When they were plated on low salt LB Zeocin-IPTG medium, the colonies of one of the yellow transformants were significantly larger (colony diameter of approximately 3 mm) than the colonies of the salmon/red transformants (colony diameter < 1 mm) (Figure 4). The large yellow-coloured transformants colonies grow faster than either the salmon/red transformants or the other yellow-coloured *E. coli* transformant (colony diameter < 1 mm). The colony size of each of these *E. coli* transformants is inversely correlated with the size of the inclusions found within the transformed cells. That is, the cells from the large yellow-coloured transformant colonies have only one inclusion while the cells from the small yellow-coloured as well as the salmon/red-coloured transformant colonies have about three or four inclusions (Figure 3). Furthermore, scanning electron microscopic results suggest that individual cells from the large yellow-coloured transformant colonies are actually larger (2.3 by 0.7 m) than cells from the small yellow-coloured transformant colonies (1.6 by 0.6 m). Taken together, these data are consistent with the notion that transformed *E. coli* cells that produce the greatest amount of foreign protein (as indicated by the amount of inclusion body formation) are much more likely to be debilitated in their normal physiological functioning than are non-transformed cells (Klier et al., 1982). Based on observations with a number of different microorganisms, the sorts of physiological impairments that can result from a metabolic load being placed on transformed cells expressing high levels of foreign protein include alterations in cells size and growth rate (Glick 1995; Glick et al., 1985; Hong et al., 1995; Della-Gioppa et al., 1990).

Both the yellow and salmon/red pigments obtained from the transformants had chemical characteristics consistent with melanin including solubility in 1 M NaOH, precipitation in 1 M HCl and bleaching with 20% H_2O_2. The LC_{50} of the solubilized crystals from the red and yellow transformants were 93 and 75 ng/mL respectively; this is compared to 50 ng/L obtained for the parent *Rhodotorula glutinis* strain. Melanin isolated from the large yellow cells exhibits a photoprotective effect on pigmentless *B. thuringiensis* subsp. *kurstaki* cells similar to the effect observed for the salmon/red melanin from *Rhodotorulal* cells. For example, when *B. thuringiensis* subsp. *kurstaki* cells were exposed to 50 J/m^2 of 253 nm light only 0.0001% of the cells survived while 0.036% of the cells survived when the cells were mixed with 75 g/mL of yellow melanin (Figure 6). Although the percentage of cell survival obtained with the yellow melanin is less than that previously obtained with the salmon/red melanin from the parent cell, the yellow pigmented cells seems to better tolerate higher UV dose than the parent cells. Thus, no viable cells were obtained

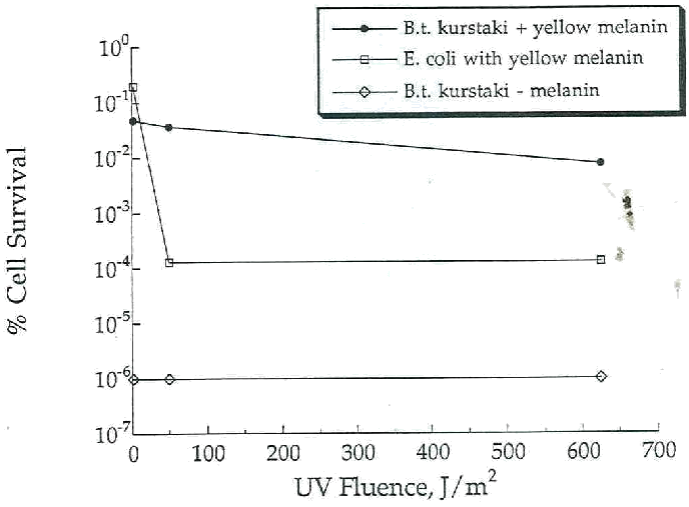

Figure 6. Survival of cells in the presence and absence of 75 g/mL of yellow melanin following irradiation with uv light.

when *Rhodotorula glutinis* cells were exposed to 1.23 x 10^2 J/m^2 of 25 nm light; however, 0.000036% of the yellow coloured cells survived.

It is somewhat surprising to find that all three transformants contains both the melanin pigment and the insecticidal toxin. However, this observation is consistent with the possibility that the genes encoding these functions are proximal to one another and may in fact be part of a single operon. If this is the case, it suggests that, at least for the *Rhdotorula spp*, melanin and insecticidal toxin are co-expressed and may have co-evolved. Moreover, melanin production may be an essential component of the insecticidal activity of *Rhodotorula glutinis*.

If, as previously reported (Glick et al., 1985) the synthesis of the melanin polymer requires the functioning of the products encoded by two separate genes for its production, the salmon /red-coloured transformant may be produced when both of these genes are functional while the yellow-coloured transformants (where the yellow pigments also exhibit properties consistent with melanin) may reflect the fact that the transformants are producing only the first enzyme in this pathway. Experiments designed to resolve this are currently underway in our laboratory.

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