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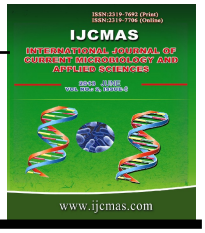

Original Research Article

Identification of genetic elements from *A. orientalis* contributing to triphenylmethane decolorization

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Keywords

Crystal violet; *Amycolatopsis* sp; decolorization; *Mycobacterim* sp; *Rhodococcus* sp.

A B S T R A C T

An *Amycolatopsis* species isolate was found to break down two structurally distinct dyes, one of which is crystal violet. This study aimed to identify the genes involved in triphenylmethane dye biodegradation. A library was constructed and four genes were isolated and identified as3-deoxy-7-phosphoheptulonate synthase, N5, N10- methylenetetrahydro methanopterin reductase, polycystic kidney domain I and glucose/sorbosone dehydrogenase. Gene expression was conducted in the host species *Streptomyces lividans*. The synergistic action of the segenes led to complete crystal violet decolorization. Additionally, the activity of these genes was tested in two other bacterial species, namely *Mycobacterium* sp. and *Rhodococcus* sp. The range of dye classes decolorized was extended in both species, showing that the segenes adopted novel functional potentials within these hosts.

Introduction

Crystal violet is a triphenylmethane dye which is commonly used in the clothing industry to dye wool, silk and cotton (Kim *et al*., 2005).While knowledge of the genes and enzymes involved in the biodegradation of this dye is restricted; the mechanistic pathway of its break down is well understood. Yatome and colleagues (1993) were the first to elucidate the degradation of crystal violet by *Nocardia* sp., identifying the main product as Michler's ketone. Chen and coworkers (2007) were able to elucidate the biodegradation pathway of crystal violet by *Pseudomonas putida* and concluded that the dye is broken down by demethylation. Similarly, Kumar and colleagues (2011) found that *Aspergillus* sp. decolorized methyl violet using the same mechanism.Jang and colleagues (2005) were the first to report on the cloning of a triphenylmethane reductase gene (TMR) isolated from the Gram negative *Citrobacter* sp. KCTC 18061P.The sequence did not show similarity to proteins of known functions hence they proposed that

the enzyme is a novel class of the short-chain dehydrogenase reductase family. Kim and coworkers (2008) predicted the mechanism of action of the reductase on malachite green through crystallization experiments linked to structure-function analysis studies. These researchers were able to workout which amino acids and atoms are involved at each stage of the decolorization process. Similarly, only one study documenting a gene responsible for triphenylmethane dye mineralization in a Gram positive species was recently conducted. The *fbi C* gene from *M. smegmatis,* which encodes a7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) synthase involved in the biosynthesis of electron carrier coenzyme F_{420} was linked to this activity (Guerra-Lopez *et al*., 2007).

Since dye effluent normally contains an array of dye classes (and thus structures) the effective treatment of this waste water would need to include microbes with the ability to degrade numerous structurally diverse dyes. In light of this we isolated a potent crystal violet degrader, identified the genes involved and through heterologous expression of these genes demonstrated that it is possible to increase the range of substrate degradation not present in the original host.

Materials and Methods

Chemicals

The dyes used in this study include eriochrome black T, orange II, congo red, crystal violet, amido black, fast green, biebrich scarlet, ponceau S, janus green, bismarck brown Y, brilliant green, tartrazine, amaranth, basic fuchsin and malachite green purchased from Saarchem, South Africa.

Bacterial strains and vectors

The bacterial strains and vectors used in this study are shown in Table 1 and Table 2.

Isolation of dye decolorizing organisms

Decolorization was detected on minimal media agar plates containing (L): stock III (x10) 100ml; NH_4Cl_2 1g; agar 15g supplemented with relevant dye (10 μg/ml) and glucose (0.25%). Bacteria were spotted onto the plates using a replicator and incubated at 30^{o}C. The plates were monitored continuously over a period of 7 days and viewed above a white light illuminator. The detection of clear zones surrounding the colonies was recorded as decolorization.

Decolorization assay

Decolorization experiments in liquid culture were carried out in test tubes containing 5 ml of stock III liquid (x1) containing (g/L): K_2HPO_4. $3H_2O$ 9.17g; $KH_2PO_4$2.68g; $MgSO_4$0.1g in addition to the appropriate dye/s (10 μg/ml) and carbon source (0.25%). The inoculum was prepared by growing the strains in complex media till an OD_{600} of 2 was obtained. This was washed three times in sterile distilled water and an equal volume added to each of the test tubes and incubated. Decolorization was monitored spectrophotometrically by reading the decrease in absorbance of the supernatant at the absorbance maxima for each dye. Culture media was used to zero the spectrophotometer. 1 ml of the culture was microfuged to remove the bacterial pellet and the supernatant added to an absorbance cuvette (A). Uninoculated controls were included to determine the initial absorbance (A_0). All experiments were carried out in inter experimental duplication and an

Table.1 Bacterial strains used in this study

Species	Strain	Characteristics	Source
Escherichia coli	MM294-4	*endA1, hsd R17, gyr A*, highly transformable	Genetics culture collection
Escherichia coli	λ lysogen	λ lysogen of MM294-4	Genetics culture collection
Streptomyces lividans	TK23	Highly transformable streptomycete host, non-crystal violet decolorizer	John Innes Institute
Rhodococcuserythropolis	SQ1	Highly transformable, crystal violet decolorizer	Dabbs *et al.,* 1995
Rhodococcusopacus	HLPA1	Crystal violet decolorizer	Genetics culture collection
Rhodococcusrhodochrous	RI8	Crystal violet decolorizer	Genetics culture collection
Mycobacterium smegmatis	mc^2 155	Highly transformable, crystal violet decolorizer	Genetics culture collection
Gordoniarubripertincta	25593	Crystal violet decolorizer	Genetics culture collection
Amycolatopsisorientalis	SY6	Crystal violet decolorizer	Soil

Table.2 Vectors and associated characteristics

Vector	Characteristics	Source
pLR591	*bla*resistance marker for Gram negatives and tsr resistance marker for Streptomycetes, *Eco*RIendonuclease gene expression regulated by λ promoter, pIJ101 Streptomycete replicon , pMB1 replicon	Hill *et al.,* 1989 and this study
pDA71	*Rhodococcus* sp. replicon, *Eco*RIendonuclease gene expression regulated by λ promoter	Dabbs *et al*., 1995
pNV18	pAL5000ori, pMB1 replicon, blue-white selection, multiple cloning site	(Chiba *et al*., 2007)
pNV19	Same as pNV18 except the multiple cloning site is in the opposite orientation	(Chiba *et al*., 2007)
pUC18	High copy number, *bla*resistance marker, blue-white selection, pMB1 replicon	Fermentas

average used for the calculation. The percentage of decolorization was calculated as by Yang*et al.,* (2003):

$$\% \text{ decolorization} = \frac{A_0 - A}{A0} \times 100$$

where, A_0 = initial absorbance and A = absorbance following bacterial inoculation at time t. Bacteria were not preconditioned prior to inoculation for decolorization experiments.

DNA manipulations

Restriction enzymes and T4 DNA ligase were purchased from MBI Fermentas and used according to the manufacturer's protocol.

Genomic DNA isolation

Genomic DNA was isolated using the method of Pospiech and Neumann (1995).

Streptomyces sp. PEG mediated transformation

The procedure of Dabbs and colleagues (1990) was followed with the following modifications: *S. lividans* TK23 was grown in 10 ml LBSG containing (g/L): tryptone 10 g, yeast extract 5 g, sodium chloride 5 g, glycine 5 g, sucrose 100 g for 36-40 h. Cells were incubated in 1 mg/ml lysozyme for 30 min. Protoplasts were regenerated on R2YE plates (g/L): tryptone 12 g, yeast extract 6 g, sodium chloride 3.6 g, sucrose 123.6 g, glucose 4 g, magnesium chloride 4 g, agar 22 g, TES (5.73 %) 40 ml, mono potassium phosphate (0.5 %) 12 ml, calcium chloride 24 ml and incubated for 18 h before performing a thiostrepton (30 µg/ml) antibiotic overlay.

Heterologous expression of genes in *Rhodococcus* sp.

The crystal violet decolorizing gene fragments obtained from the genomic library were ligated into the unique *Bgl*II site of the *Rhodococcus* sp. replicon vector pDA71. The recombinant vector was transformed into *R. erythropolis* SQ1 using the method of Dabbs *et al.,* (1990).

Heterologous expression of genes in *Mycobacterium* sp.

The crystal violet decolorizing gene fragments obtained from the genomic library were ligated into the unique *Bam*HI site of the *Mycobacterium* sp. replicon vectors, pNV18 and pNV19 and transformed according to the method of Hatfull and Jacobs (2000) into *M. smegmatis* mc^2 155.

Construction of Streptomycete - *E.coli* shuttle vector

The method of Hill *et al.,* (1989) was followed to reconstruct pLR591.

Constructionand screeningof genomic libraries

Genomic DNA was partially digested with *Bgl*II and the fragments ligated to pLR591 cleaved at the unique *Bgl*II site. These were transformed into *S.lividans* TK23 using PEG-mediated transformation. Transformants were patched onto minimal media plates supplemented with crystal violet (10 µg/ml) and glucose (0.25%). Plates were incubated for 2 weeks and routinely analyzed for zones of clearing.

Sequencing of inserts

Gene fragments were excised from pLR591 and ligated into the *Bam*HI site of pUC18 and sequenced using a Spectromedix LCC sequencer in conjunction with an ABI BigDye Terminator v3.1 sequencing kit.

Detoxification analysis

The supernatant, decolorized following incubation of the dye with *A. orientalis*, was transferred to Eppendorf tubes and microfuged in order to pellet the cells. The supernatant was then collected and passed through a sterile 0.25 µm filter. Five ml of this was transferred to a test tube and 10 µl of a stationary phase (OD_{600} = 1) *E. coli* culture added. The *E. coli* inoculum was prepared by growing the strain in Luria-broth overnight and washed prior to inoculation. The controls included the use of non-supplemented minimal media and minimal media to which glucose (0.25%) was added. An appropriate volume of *E. coli* cells was added to each test tube and all were incubated for 2 h on a rotating wheel at 37^{o}C. Following the incubation, a serial dilution was carried out and plated onto LA media (g/L): tryptone 10 g; yeast extract 5 g; sodium chloride 5 g; agar 15 g. This was incubated overnight and the colony forming units per ml determined.

Thin layer chromatography (TLC)

TLC was conducted as described by Jang *et al*., (2005). This was used to visually detect the break down of crystal violet.

Nucleotide and protein sequence matches

Protein and DNA sequences were matched using the blastx and nucleotide blast facilities of NCBI EntrezPubmed. Default parameters were used in both cases.

Result and Discussion

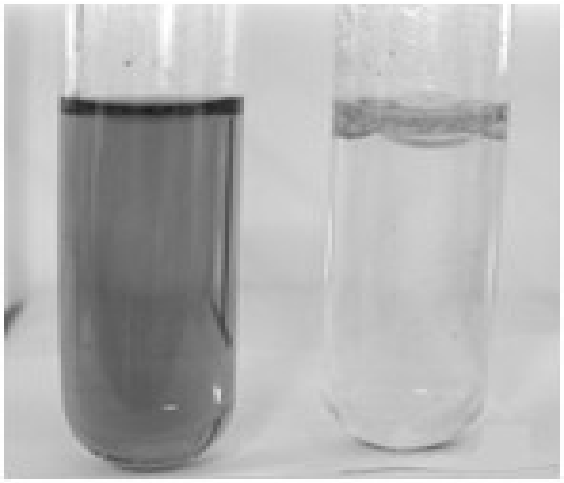
Dye decolorization

A. orientalis SY6 was added to minimal media containing crystal violet as the sole carbon source. This strain demonstrated a potent decolorizing ability and clearing of the dye was visible just five minutes after incubation with the isolate (Figure.1).Acclimatization of SY6 showed that the strain could improve its decolorization capacity up to 30µg/ml of the dye, however even prolonged incubation showed no ability to decolorize beyond this concentration. Results were monitored on thin layer chromatography in 5, 10, 20, 30 and 60 min. intervals (Table 3). At5 min. a dramatic decrease in intensity was observed; its monitoring on TLC against untreated crystal violet confirmed this extreme degradation. Four main bands were observed from 5-20min.The lowest band was no longer visible at 30 min. and both lower bands were not detected at 60 min.

Table.3 Rf values calculated from TLC of decolorized *A. orientalis* SY6 supernatant

Time (min.)	Rf values
5	0.79;0.68;0.57 ; 0.40
10	0.79;0.68;0.57 ; 0.40
20	0.79;0.68;0.57 ; 0.40
30	0.79;0.68;0.57
60	0.79;0.68

Figure.1 Mineralization of crystal violet (10µg/ml) by strainSY6. Tube on the left represents the dye only control and the tube on the right was inoculated with strainSY6, following 20 min. incubation.



The possibility of extracellular enzymatic reduction was eliminated by adding dye to the cell free extracellular supernatant and the cofactors NADH and NAD(P)H (if the enzyme is cofactor dependent) and analyzing the decolorization percentage during prolonged incubation; an in significant decrease was indicative of cellular related mineralization.

Combined dyes decolorized by *A.orientalis* SY6

The range of decolorization by strainSY6extendedtoseveral triphenylmethane dyes other than crystal violet such as brilliant green, basic fuchsin and malachite green. Since the isolate was capable of degrading these dyes individually, it was investigated whether a combination of these colorants would also be easily decolorized (Figure. 2).The isolated is played a remarkable ability to decolorize the combined triphenylmethane dyes just as effectively as that of a single triphenylmethane dye. Since it could also decolorize amido black and janus green individually we tested whether the addition of these to the combination of triphenylmethane dyes could also be decolorized.

Comparison of strain SY6 dye degradation to *R. erythropolis* and *M. smegmatis* strains

The rapid degradation of crystal violet by *M. smegmatis* and *R. erythropolis* closely matched that of strain SY6.The decolorized supernatant of both strains was run on TLC and the Rf values calculated for the observed bands. Similar banding patterns and correspondingly Rfvalues were obtained for all three strains (Table 4).

Table.4 Rf values calculated from TLC

Strain	Rfvalues
*R.erythropolis*SQ1	0.78;0.66;0.56
*M.smegmatis*mc^{2}155	0.80;0.67;0.57
*A.orientalis*SY6	0.79;0.68;0.57

Detoxification of crystal violet by *A. orientalis* SY6

Strain SY6 decolorized the dye into a colorless derivative, however the toxicity of the by- products generated needed to be determined. This was done by monitoring the growth of an indicator strain, *E. coli* in the presence of the decolorized media. Inhibition of the strain was taken as a sign of toxicity. The colony forming units (CFU) per ml of *E. coli* in the decolorized supernatant closely matched that of the media supplemented with a carbon source

and was two times higher than non-supplemented media (Figure. 3). This reflects no inhibition of the strain and thus complete detoxification.

Creation of genomic library of *A. orientalis* SY6

In order to isolate the genes responsible for decolorizing crystal violet a genomic library was created. The partial digestion of *A. orientalis* SY6 DNA with BglII yielded an average DNA fragment size of approximately 3800 bp. Calculations estimated that less than 7000 clones were needed to ensure a high probability of containing the genes of interest. A positive selection library was created, meaning the transformable host *S. lividans* TK23 did not have the ability to grow on crystal violet at a low cell inoculum. Hence, any clones that were found to survive on the dye plates were screened. Following the patching of approximately 5000 clones, ten colonies displaying a resistance to the triphenylmethane dye were isolated.

Screening and isolation of clones capable of crystal violet decolorization

A plasmid DNA extraction was conducted on all ten clones, of which three carried inserts. These clones were named SlivCV2, SlivCV4 and SlivCV6 and the DNA carried referred to as pCV2, pCV4 and pCV6 respectively. *S. lividans* clones CV2, CV4 and CV6 carried ~ 600 bp, > 2000 bp and > 2000 bp gene fragments respectively.

Decolorization by combined SlivCV clones

The individual incubation of CV clones in crystal violet showed evidence of partial decolorization. Noticeably, the dye was removed from the media, however visually it was evident that it adhered to the cell wall of the bacteria. Unexpectedly, it was the combination of all three clones that led to the complete decolorization of the triphenylmethane dye. Two clones were combined in various combinations in order to assess if the eliminated clone was required for complete mineralization. The elimination of any of the clones resulted in partial decolorization.

Sequence identification of CV clones

Insert sequences were compared against the NCBI PubMed database, revealing the results shown in Table 5. Insert CV2 showed high sequence identity to a DAHP synthase. Insert CV4 carried two genes namely a methylmalonyl-CoA mutase and reductase related protein. In insert CV6 a glucose/sorbosone dehydrogenase and PKD domain occurred at the beginning of the fragment followed by five LGFP repeats located at the end of the fragment.

Range of dyes decolorized by combined clones

The decolorization activity of the clones was determined in the presence of other dyes. Other than crystal violet the SlivCV clones were able to decolorize three sulfonated azo dyes, namely amaranth, ponceau S and biebrich scarlet (Table 6). Although congo red and eriochrome T were removed from the media they remained tightly bound to the cell wall of the strains. The pCV2, pCV4 and pCV6 recombinant vectors were transformed into a *Rhodococcus* sp. host and referred to as SQ1CV2, SQ1CV4 and SQ1CV6

respectively. The host strain into which the DNA was transformed possessed the ability to decolorize four of the five triphenylmethane dyes, as did the clones, hence it was not possible to evaluate whether any contribution was made by the expressed genes. Instead dyes which the original host could not decolorize were concentrated on. The SQ1CV clones additionally decolorized the sulfonated dyes amido black, orange II and biebrich scarlet (Table 6). Notably, this was not similar to the pattern of decolorization when in the S. lividans host. The pCV2, pCV4 and pCV6 recombinant vectors were transformed into a *Mycobacterium* sp. host and referred to as 155CV2, 155CV4 and 155CV6 respectively. The combined 155CV clones were able to decolorize amido black, bismarck brown and janus green (Table 6). Again this was slightly different to the expression of the genes in the latter and former strains.

In the United States, three classes of dyes are classified as harmful, these include triphenylmethane, azo and anthraquinone dyes (Guerra-Lopez *et al*., 2007). This study focused on the triphenylmethane class of dyes. Prior to this just two Gram positive species were linked with the ability to degrade crystal violet, hence it was assumed that this was a rare trait (Guerra-Lopez *et al*., 2007; Sani and Banerjee, 1999). However, when screening known isolates from our laboratory genetics culture collection it was surprising to find that this ability is in fact common among *Rhodococcus* sp., *Mycobacterium* sp. and *Gordonia* sp. The strains displaying a potent crystal violet decolorization capacity include *R. rhodochrous* RI8, *R. erythropolis* SQ1, *R. opacus* HLPA1, *M. smegmatis* mc2 155, *G. rubripertincta* 25593 and *A. orientalis* SY6. Of these strains SY6 was chosen for supplementary genetic screening.

Table. 5 Closest protein matches to isolated sequences

Clone	Description	Species	Maximum	Evalue
2	3-deoxy-7-phosphoheptulonate synthase(DAHPsynthase)	*Amycolatopsis mediterranei* U32	95	1e-100
4	Methylmalonyl-CoAmutaseand N5,N10-methylene-tetrahydromethanopterin	*Amycolatopsis mediterranei*U32	85;82	2e-86; 2e-56
6	LGFPrepeat-containingprotein, glucose/ sorbosone dehydrogenase, polycystic kidney disease I (PKD) domain	*Amycolatopsis mediterranei* U32	61 ; 75 ; 72	7e-54 ; 8e-52 ; 2e-03

Figure.2 Monitoringofthedecolorizationofmultipledyes(10µg/ml). AB – amido black, JG – janus green, CV – crystal violet, BG – brilliant green, BF – basic fuchsin, MG – malachite green. Error bars represent the standard deviation between two replicate assays.

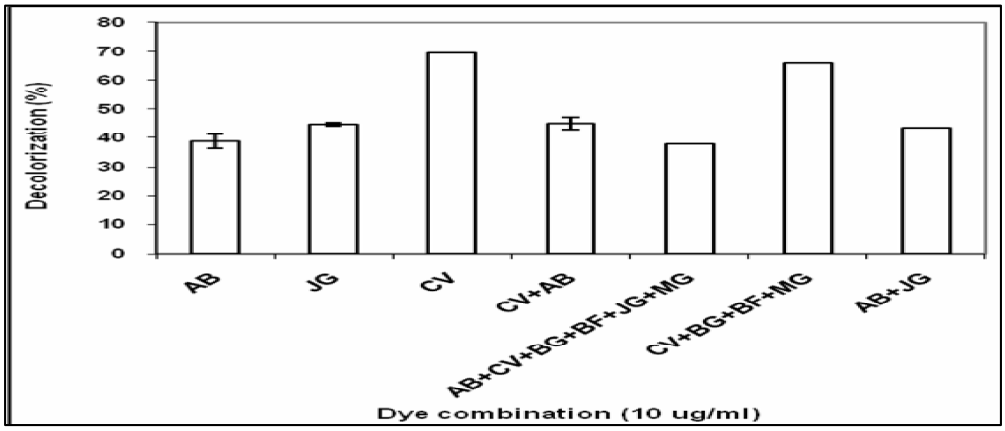


Figure.3 CFU/mlofindicatorstrainin (A) minimalmedia, (B) minimalmediasupplemented with glucose(0.25%) and (C) decolorized crystal violet (10µg/ml) supernatant of *A. orientalis*SY6. Error bars represent the standard deviation between two replicate assays.

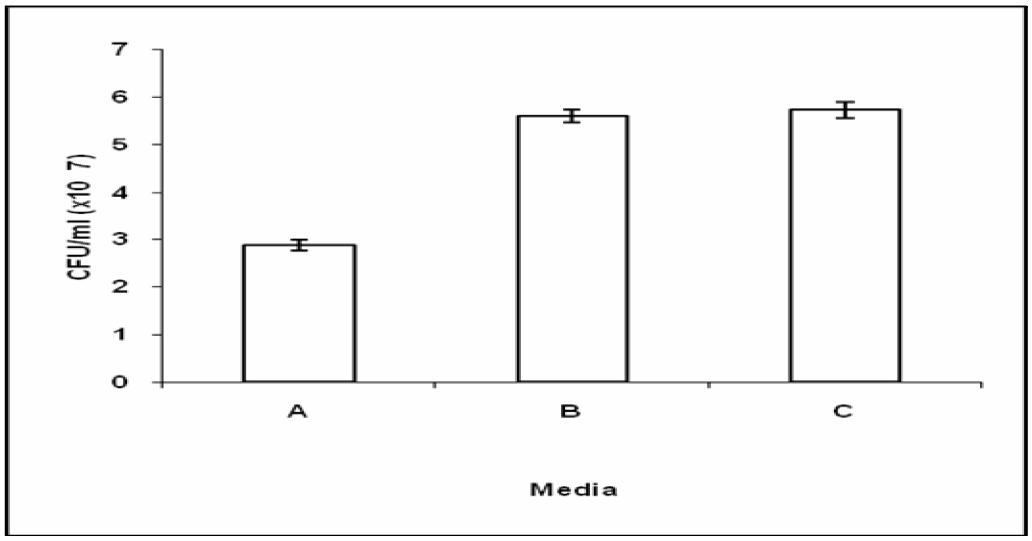


Table.6 Dyes decolorized by strains carrying pCV2, pCV4 and pCV6

Dyes	*S.lividans*TK23	SlivCV2+SlivCV4 + SlivCV6	*R.erythropolis*SQ1	SQ1CV2+ SQ1CV4+ SQ1CV6	*M.smegmatis*mc^{2}155	155CV2 + 155CV4+ 155CV6
BG	+	+	+	+	+	+
T	-	-	-	-	-	-
BF	-	-	+	+	+	+
CV	-	+	+	+	+	+
AB	+	+	-	+	-	+
OII	-	-	-	+	-	-
JG	+	+	+	+	-	+
MG	+	+	+	+	+	+
PS	-	+	-	-	-	-
BS	-	+	-	+	-	-
I	-	-	-	-	-	-
BB	-	-	-	-	-	+
CR	-	-*	-	-*	-	-*
EB	-	-*	-	-*	-	-*
A	-	+	-	-	-	-
FG	-	-	-	-	-	-
CV+BG+MG+B	ND	ND	-	+	ND	ND

BG-brilliant green, T-tartrazine, BF-basic fuchsin, CV-crystal violet, AB-amido black, OII-Orange II, JG-janus green, MG-malachite green, PS-ponceau S, BS-biebrich scarlet, I-Indigo, BB-bismarck brown, CR-congo red, EB-eriochrome black T, A-amaranth, FG-fast green; ND – not determined; *-dyeattachedto cellwall

In general, it was noticed that bacteria capable of decolorizing crystal violet were also able to mineralize other triphenylmethane dyes such as malachite green, brilliant green and basic fuchsin. This allows for the speculation that the enzymes responsible for triphenylmethane class degradation are either more lenient in their substrate interactions or the dye structural differences are minor and hence easier to accommodate. These results can be paralleled to those of the TMR (triphenylmethane reductase) enzyme isolated from *Citrobacter* sp.; malachite green was found to fit most favorably into the catalytic region of TMR, however the additional dimethylamino group in crystal violet produced a less favorable interaction while the positively charged amino groups in basic fuschin were problematic (Kim *et al*., 2008).

Strain SY6 was able to decolorize the azo dyes amido black, janus green and several triphenylmethane dyes effectively. Results confirmed that the combination of four triphenylmenthane dyes were efficiently decolorized to colorless derivatives. This suggests that the same enzyme/s could be involved in the reduction of all these dyes. The addition of the azo dyes amido black and janus green did not affect the decolorization ability. Notably, the decolorization percentages represented on the graph give the impression that the addition of the azo dyes reduced the overall decolorization. However, amido black and janus green are not reduced to colorless derivatives. Amido black is decolorized to a violet colored compound and janus green is reduced to diethylsafranine which remains pink in suspension (Lazarow and Cooperstein, 1953). Since the decolorization percentage corresponds to that of the individually reduced azo dyes this showed that the multiple dyes were effectively broken down. Similarly, Sani and Banerjee (1999) demonstrated the potent ability of *Kurthia* sp. to completely decolorize five 2µM triphenylmethane dyes in just 30 minutes.

The analysis of treated crystal violet by strains *A. orientalis* SY6, *R. erythr*opolis SQ1 and *M. smegmatis* mc2 155 on thin layer chromatography showed that similar band patterns occurred. Thus, it is probable that the same degradative pathway is present in all three strains. However, since the exact pathway has not been uncovered in any of these strains it is difficult to determine what end products are being produced. It was observed that following prolonged incubation, the two upper bands remained in the supernatant. From the literature we can deduce the possibility of one of these products being Michler's ketone which researchers found can remain stably in the media for 100 hours and in some instances can not be catabolized further (Chen *et al*., 2008; Yatome *et al*., 1993). The other product is likely to be leucocrystal violet which was reported in *Citrobacter* sp. as being the first product into which the dye was rapidly reduced (Jang *et al*., 2005). The lower bands which are only present in the early stages are likely to be derivatives of both or one of these compounds formed through reductive splitting or demethylation (Chen *et al*., 2008); its disappearance reflecting its metabolism by the isolate.

Results suggest that the decolorized triphenylmethane dye was fully detoxified by strain SY6. Chen and coauthors (2008) tested the decolorized supernatant of *Shewanella* sp. and found that it was not completely detoxified. However, these researchers used cell cultures which are more sensitive than the testing of bacterial resistance (Scribner *et al*., 1980).

Microbial decolorization can be attributed to either adsorption to the cell or biodegradation (Isik and Sponza, 2003). Investigation into the decolorization capacities of the individual SlivCV clones in liquid media was disappointing. Noticeably, the dye adsorbed onto the cell wall, indicative of incomplete biodegradation. Kalme and coauthors (2007) measured the activities of several enzymes during the degradation of direct blue- 6 by *Pseudomonas desmolyticum*. They found a significant increase in the oxidative enzymes, laccase and tyrosinase till complete decolorization at 96 hours, while reductase activities were only increased later after 48 hours. This inferred the synchronized working of collective genes involved in the biodegradative process. This is in contrast to other publications which have identified a single gene involved in dye breakdown (Jang *et al*., 2005; Guerra-Lopez *et al*., 2007). The attainment of unfavorably low decolorization rates using individual clones made us evaluate the possibility of several gene products working together to break down the dye. Correspondingly, the clones were combined and the extent of degradation monitored. By combining the strains it was noticed that the dye was completely removed from the media and the cells retained their original color, indicative of complete mineralization.

These clones were added in different combinations to establish the minimal number required for complete mineralization. It was evident that all three clones were required, however it was noticed that the contribution of each clone to the process of decolorization was unequal. A definite hierarchy was observed; the absence of SlivCV2 was the most profound, this was followed by SlivCV4 and SlivCV6 (data not shown).

The sequencing of the inserts from the recombinant vectors led to the identification of a DAHP synthase, methylmalonyl-CoA mutase, N5, N10 methylene-tetrahydromethanopterin reductase and a LGFP repeat containing protein with an associated dehydrogenase.

With no immediate relevant functionality observed with regard to the DAHP synthase, the pathway of the enzyme was looked at for an indirect mechanism of action resulting from the by-products or associated enzymes. The main involvement of DAHP synthase is the biosynthesis of phenylalanine, tyrosine and tryptophan through the shikimate pathway. This pathway is controlled by several enzymes with DAHP synthase catalyzing the first step through the condensation of D-erythrose 4-phosphate and phosphoenolpyruvate to form DAHP (Dosselaere and Vanderleyden, 2001). Through seven metabolic steps this is converted to chorismate which acts as the precursor for phenylalanine, tyrosine and tryptophan as well as many other diverse compounds, which include cofactors, coenzymes, phenazines and siderophores (Dosselaere and Vanderleyden, 2001). The DAHP synthase isolated in this study was most closely related to a class II synthase encoded by aroF in A. mediterranei. Hence, we looked closely at the reactions taking place along this pathway to the production of tyrosine and identified potential enzymes and thus biochemical reactions which could potentially interact with or influence the dye substrate. Three enzymes were identified, namely chorismate synthase which is catalyzed by a reduced flavin nucleotide cofactor supplied by an associated reductase (Herrmann, 1995), chorismate mutase which possesses dehydrogenase or dehydrolase activity and shikimate dehydrogenase which possesses the latter

activity as well (Qamra *et al*., 2006). Dehydrogenase activity has been identified in anaerobic bacteria and linked to the decolorization of azo dyes (Rafii and Cerniglia, 1995). A dehydrolase can possess an esterase activity (Akashi et al., 2005), the relevance to this is that Bafana and Chakrabarti (2008) by computational methods were able to identify acyl carrier protein phosphodiesterases as potential azo dye reductases.

Even though a reductase was present on pCV4, the vitamin B12 dependent methylmalonyl CoA mutase could not immediately be eliminated as playing a role in dye decomposition. The B12- dependent methylmalonyl-CoA mutase (mcm) catalyzes the conversion of succinyl-CoA into methylmalonyl-CoA (Gruber and Kratky, 2001). Once again the comprehensively studied enzyme structure and pathway were looked at to determine relevant reactions that could influence dye break down. The crystal structure of mcm revealed the presence of a TIM barrel domain, which differs from conventional barrel domains that are hydrophobic and large (Gruber and Kratky, 2001). The mcm barrel domain is hydrophilic and small; offering just enough space for the coenzyme end of the substrate to bind along the axis. From this it was conceded that the substrate specificity of mcm is rigid, therefore we propose no direct interaction of the mutase with crystal violet.

The N5, N10 - methylene-tetrahydromethanopterin reductase (mer) was originally isolated from *Methanobacterium thermoautotrophicum* and *Methanopyrus kandleri*. This gene plays a role in the pathway conversion of carbon dioxide to methane and is a F420 dependent reductase. From this we propose that the mer reductase is probably linked to crystal violet decolorization. This is further exemplified by the fact that a F 420 dependent enzyme was linked to malachite green mineralization in *M. smegmatis* (Guerra-Lopez *et al*., 2007).

LGFP repeat containing proteins are not well characterized; however one study suggests that they act as 'anchors' which attach the protein to the cell wall (Adindla *et al*., 2004). The PKDI domain has been identified in bacterial genomes in association with collagenases, chitinases and proteases. Orikoshi and coauthors (2005) set out to uncover the function of the PKD domain in relation to chitin degradation. Through site directed mutagenesis of key residues they demonstraed that the PKD associated domain within the chitinase A gene directly participates in the hydrolysis of chitin. This was a key article showing that the domain plays a direct role in the biodegradation process and does not merely act as a binding domain for substrates. Correspondingly, we hypothesize that the LGFP region allows for attachment to the cell wall, the PKD domain which occurs on the surface, binds to the substrate and via hydrolytic interaction with the dye is responsible for partial decolorization. Furthermore, the sorbosone dehydrogenase interacts with the dye via an oxidoreductive mechanism.

The response of the combined clones was analyzed in azo, sulfonated azo and other triphenylmethane dyes. It was interesting that the expression of these genes led to variable results, depending on the host strain into which it was introduced. A homolog of N5, N10-methylenetetrahydromethanopterin reductase occurs in *M. tuberculosis*, identified through the genome sequencing project, and through the KEGG pathway is

hypothesized to play a role in 1,1,1-Trichloro-2,2-bis (4- chlorophenyl) ethane (DDT) degradation (Kanehisa *et al*., 2004). This gene is proposed to oxidize 4-chlorobenzaldehyde to 4- chlorobenzoate, the last step in the biodegradative pathway (Kanehisa *et al.*, 2004). Thus we hypothesize that this oxidative activity is responsible for the decolorization of amido black, bismarck brown and janus green when pCV2 is introduced in *Mycobacterium* sp. In a *Rhodococcus* sp., enzymes closely related to N5, N10-methylenetetra-hydromethanopterin reductase were responsible for the biodegradation of 2,4,6-trinitrophenol (commonly found in pesticides, dyes and explosives) (Heiss *et al*., 2002). These enzymes took on the role of hydride transferases. Thus, it is possible that its function in the transferal of hydride ions allows this gene to contribute towards the decolorization of amido black, orange II and biebrich scarlet. This study underscores that the expression of genes in other species can lead to a broader range of activity. Moreover, there is an added advantage in expressing the genes in for instance, *Rhodococcus* species. These resilient strains have been implicated in the biodegradation of pollutants such as oil and pesticides and are commonly found in polluted wastewaters and toxic environments, making them a more practical choice for use when considering large scale bioremediation (Hoshima *et al*., 2006 ; Shao and Behki, 1996).

To our knowledge this is the first report of an *Amycolatopsis* sp. being implicated in crystal violet biodegradation and only the second report of the characterization of triphenylmethane decolorizing genes from a Gram positive bacterium. This isolate not only decolorized but detoxified the dye as well. This strains ability to rapidly breakdown a combination of triphenylmethane dyes within a short interval makes it a suitable candidate for further bioremediation work. The introduction of these genes into *Rhodococcus* sp. led to the creation of an improved consortium capable of decolorizing eight dyes. These results also indicate that it would be beneficial to start a trend in which genes are not only expressed in common species which are closely related but also tested in other species**.**

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