Azo and triphenylmethane dye degradation in *Mycobacterium smegmatis*

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Introduction. Azo and triphenylmethane dyes are used extensively in the coloration of paper, clothing, food, cosmetics and pharmaceuticals. These dyes and their degradation by-products have been deemed hazardous by the Environmental Protection Agency as they may be toxic and/or mutagenic to living organisms. Bioremediation is an environmentally friendly and inexpensive alternative to chemical decomposition of these toxic products and many microorganisms capable of decolorizing and degrading azo and triphenylmethane dyes have been isolated. In the case of triphenylmethane dyes, a triphenylmethane reductase is responsible for the decolorization of the triphenylmethane dye, malachite green in Gram-negative bacteria, such as *Citrobacter* sp. Azoreductases that cleave the N=N bond in such dyes as methyl red have been characterized from a number of species, including *Staphylococcus aureus (Chen et al., 2005).* We have previously demonstrated that *Mycobacterium smegmatis*, a saprophyte, is able to decolorize and degrade both malachite green and methyl red. Malachite green is decolorized through a Coenzyme F420 dependent process as mutants lacking this cofactor are unable to decolorize malachite green (Guerra-Lopez *et al*., 2007). The enzyme that uses this cofactor has not been identified. In addition, a gene encoding a potential azoreductase has been identified in *M. smegmatis*. We present data on the recombinant azoreductase and describe the characterization of a *M. smegmatis* mutant disrupted in *azoR.*

Methods. To identify mycobacterial genes involved triphenylmethane dye decolorization, a random transposon mutant library of *M. smegmatis* was constructed and screened for mutants unable to decolorize malachite green. The site of transposon insertion was identified as by inverse PCR and arbitrary PCR as described in Guerra-Lopez *et al*. (2007) and the sequences obtained were BLAST searched against the *M. smegmatis* genome to identify the disrupted gene.

To validate that *Mycobacterium smegmatis* annotated gene MSMEG_3381 codes for an azoreductase, it was cloned into the recombinant protein expression vector, pet22, expressed and purified using affinity chromatography. An unmarked mutant disrupted in this gene was constructed using the two step method described by Parish et al (Parish & Stoker, 2000). Azoreductase assays were performed and the mutant was characterized with respect to growth on a range of azo dyes, quinone compounds, and oxidants (Liu *et al*., 2009).

Results. Over 20 000 *M. smegmatis* transposon mutants have been screened and approximately fifty mutants that are unable to decolorize malachite green have been identified. Aside from mutants disrupted in genes involved in coenzyme F420 biosynthesis, malachite green sensitive mutants are disrupted in genes that code for proteins that range in function from membrane transporters to genes involved in multiple cellular processes such as DNA repair. However, none of the genes identified showed any sequence homology to a reductase that would be able to reduce malachite green in a coenzyme F420 dependent manner.

A mutant disrupted in MSMEG_3381 does not have a severe growth defect and is able to grow as well as the wild-type strain under normal lab conditions. It is unable to decolorize azo dyes, such as methyl red and amido black. Since the *E. coli* azo reductase is also able to reduce quinone, the sensitivity to these electrophilic agents, such as catechol and menadione, was also tested and the mutant was found to be more sensitive as compared to wild-type. As menadione is also a redox cycling agent, sensitivity to other oxidants, such as diamide, hydrogen peroxide, and cumene hydroperoxide, was also tested.

Conclusions. In *M. smegmatis,* the enzymes responsible for the degradation of triphenylmethane dyes remain still to be identified; however, an azoreductase is involved in the degradation of azo dyes in *M. smegmatis*.

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