CHARACTERIZATION OF AZO DYE REDUCTION

IN *ENTEROCOCCUS FAECALIS*

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ABBREVIATIONS

ATP – Adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

C - Celsius

FMN – Flavin mononucleotide

HPLC – High Performance Liquid Chromatography

kDa – Kilo Dalton

K_m – Michaelis-Menten constant

NADH – Nicotinamide adenine dinucleotide (reduced form)

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced form)

NAD(P)H - - Nicotinamide adenine dinucleotide (phosphate) [reduced form]

rpm – Revolutions per minute

UDP – Uridine diphosphate

V_{max} – Maximum velocity of an enzyme catalyzed reaction

CHAPTER I

INTRODUCTION

Living organisms have the ability to adapt to their surroundings. This important strategy adopted by them is the basis for survival and propagation in an often highly competitive environment in which limited nutrient resources are available. Selective pressure from the environment forces only the fittest organisms to thrive, as this is the basis for Darwin's theory of evolution. To this end, the presence of certain genes in an organism whose products are used to metabolize unique nutrients may be explained by their environment. Prokaryotes living in complex communities are able to obtain unique genes from each other through lateral gene transfer. This process leads to tremendous genetic diversity and hence prokaryotes are known to possess an enormous metabolic capacity.

There is interest in knowing how organisms have evolved to metabolize xenobiotics, compounds that are not naturally encountered by the organism. Evolutionary adaptability to the environment takes millions of years; however, bacteria are known to adapt to these compounds after only a few years. Hence, the ability of bacteria to degrade and utilize xenobiotic compounds is of particular interest because it will provide better insight into the processes of molecular evolution and environmental adaptability.

CHAPTER II

REVIEW OF LITERATURE

General Introduction

The majority of life forms on earth are microscopic. Although they are invisible to the naked eye, they have a tremendous impact in nature. It is hard to imagine places that are not inhabited by microbes. The reason for their successful colonization in various habitats is due to their diversity and adaptability. The vast majority of microorganisms fall into the Bacteria and Archaea Domain of life or the Monera kingdom. These prokaryotic cells are the major contributors of the planet's total biomass and are estimated to be about 5×10^{30} cells (Madigan M.T., 2003). Most of these microorganisms can not be cultured and maintained in an artificial laboratory environment. Hence, before the advancements of ribotyping and genetic analysis, our knowledge of the diversity of microbes in a given habitat was limited.

Archaea are considered to be the most ancient life forms and live in extreme environments. Bacteria also thrive in various conditions depending on their physiology and nutritional requirements. These organisms live in complex communities, particularly biofilms. Microbes interact with the environment and consume the surrounding nutrients. Some of the metabolic waste products produced by one species of bacteria are used as

nutrient sources for other species. Bacteria can have various electron sources which include organic compounds (chemoorganotrophs) like glucose as well as simple inorganic compounds (chemolithotrophs) like molecular hydrogen. Similarly, their terminal electron acceptors may include oxygen (aerobic respiration), inorganic compounds other than oxygen (anaerobic respiration) and endogenous organic compounds (fermentation). Within the Bacteria Domain, cyanobacteria undergo photosynthesis which involves capturing energy from sunlight and converting it to chemical energy (ATP).

Due to their myriad metabolic systems and pathways, bacteria play an important role in their respective ecological niches. Environmentally, they are major contributors to the generation and maintenance of biogeochemical cycles. In a process called bioremediation, the unique metabolic capacity of certain microbes can be used to clean up environmental pollution that is otherwise recalcitrant and hazardous to humans and the environment. The process of fermentation by microbes has been exploited for many years in the food and beverage industries. Nitrogen-fixing bacteria have a huge economic impact on legume crops in agriculture. Finally, microbes play an important role in the treatment of waste water to remove dissolved organic compounds.

Over the past few decades, bacteria and yeast have been used in biotechnology and genetic engineering to produce various products of commercial interest and their genetic manipulation has revolutionized fundamental research at the molecular level.

Role of Microbes in Disease

The causative agent of many plant and animal diseases are microbes and the diagnosis, prevention and treatment of these diseases can have a major impact on human and animal health as well as agricultural industries. Although most of the application of biotechnology is for beneficial purposes, there is always the danger of bioterrorism.

The importance of microorganisms in human health and disease can not be over stated. Besides understanding their role in pathogenesis, it is also equally important to understand the non-pathogenic association of bacteria with humans.

Human Microbiota

Although pre-natal exposure to bacteria does not occur, infants first encounter bacteria in the birth canal of the mother. Eventually, all parts of the body are directly exposed to the environment i.e., skin and mucous membranes of the conjunctiva in the eyes, ears, upper respiratory tract, gastrointestinal (GI) tract and urogenital tract (females) become colonized by bacteria. These bacteria outnumber microbial eukaryotes and archaea and their colonization is a dynamic process which varies with age, sex, genetics, diet and tissue (Todar, 2007)**.**

The tissue tropism of bacteria is dependant upon the milieu offered by the host. The temperature, pH and available nutrients vary within different regions of the human body and certain species of bacteria acquire a selective advantage over others based on their specific nutritional and metabolic requirements. For example, *Staphylococcus epidermidis* is predominantly present on the surface of the skin where the pH is between 4 and 6 (Madigan M.T., 2003), while *Enterococcus faecalis* and *Escherichia coli* are

present in the colon where the pH is closer to neutral. Besides the host environment, the colonization of bacteria is also dependent on the attachment of a bacterial ligand to a host tissue receptor, such as the attachment of *Streptococcus pyogenes* to the amino terminus of fibronectin in the pharyngeal epithelium via its surface protein F (Todar, 2007)**.** Most of the microbes are benign, as only a few are opportunistic pathogens which can cause infection under certain conditions that include a weakened immune system. There are many benefits associated with the microbiota as they prevent the colonization of pathogens by either sequestering nutrients from them or by producing antimicrobials, i.e., bacteriocins that inhibit growth or kill the cells. Other benefits include the stimulation of the immune system and their contribution to the development of certain organs like the caecum and Peyer's patches of the human intestine (Todar, 2007) .

Normal Flora of the GI tract

The largest numbers of bacteria that inhabit the human body reside in the GI tract. The numbers in the adult GI tract are estimated to be 10^{14} cells, which is an order of magnitude greater than all the cells in the human body (Savage, 1977). The initial estimated number of species in the GI tract was about 400-500 (Simon, 1984). The colonization of bacteria within the GI tract depends upon several factors. These include, but are not limited to, the process of birth (i.e. whether natural or caesarean section) and mode of nutrition, eg. breast-fed or bottle-fed (Long and Swenson, 1977). Throughout the life of the individual, the number and predominant species of bacteria can vary depending upon diet, age and health (Moore and Moore, 1995).

A recent metagenomic approach by scientists at The Institute of Genomic Research (TIGR) has estimated more than one thousand species of microbes in the gut (Gill et al., 2006). Thus, the collective number of microbial genes is more than one hundred times the number of genes in the human genome and hence exhibit tremendous species and sub-species diversity. However, the superkingdom diversity is low, showing only 8 of the 55 bacterial divisions (Backhed et al., 2005). Most of the bacteria are present in the colon, whose bacterial cell density is about as high as those found under optimal growth conditions in laboratory nutrient medium (Levison, 1990); however, their generation time is slower *in situ* than *in vitro* (Mitsuoka, 1978). The conditions in the upper GI tract are not conducive for stable colonization of bacteria because the gastric, biliary and pancreatic secretions kill most of them (Guarner and Malagelada, 2003). The colon may be similar to a chemoststat because digested food material enters through the caecum at one end and faeces are excreated at the other end. The ratio of anaerobes to aerobes is 100-1000 to 1 (Sears, 2005). Within this complex environment there is a high degree of interaction between the host and microbes. This communication is enhanced because the bacteria are anatomically separated from the internal blood vessels and lymphatic system by a single layer of epithelial cells (O'Hara and Shanahan, 2006). Using *Bacteroides thetaiotaomicron* to colonize gnotobiotic mice (Hooper et al., 2001), the authors showed that this resulting colonization altered the expression of several host genes associated with many metabolic and developmental functions. In terms of microbial impact, the metabolic activity of the microorganisms within the GI tract is so high that it is considered an organ within the GI itself (Bocci, 1992).

There are several complex metabolic functions associated with intestinal bacteria. The components of the diet that could not be digested in the upper GI tract are fermented in the colon, where most of the bacteria with the highest metabolic diversity are present (Roberfroid et al., 1995). This process thus maximizes the acquisition of energy available from the diet as well as provides the required nutrients to sustain the microbial population. Humans lack many of the enzymes that are needed to digest plant carbohydrates like, xylan and pectin (Gill et al., 2006), although complex carbohydrates, oligosaccharides and proteins are fermented to generate short-chain fatty acids. These are not only good sources of energy but also play a vital role in the maturation and differentiation of epithelial cells (Guarner and Malagelada, 2003). There are other compounds metabolized by the gut flora including, nitrogen, glucuronides, cholesterol, antibiotic, intestinal mucin, steroidal and non-steroidal compounds and bile acids. Associated with metabolism are enzyme reactions involving the hydrolysis of sulphate esters and amide compounds as well as aromatization, dehydroxylation and decarboxylation (Drasar, 1974). Finally, vitamin synthesis, especially Vitamin K and the absorbance of important ions is an important contribution to the host by the intestinal flora.

Enterococcus faecalis

These organisms are Gram-positive cocci, non-spore forming and facultative anaerobes. They are classified as Firmicutes due to their low G+C content and are one of the major inhabitants of the GI tract of mammals. This opportunistic pathogen is associated with many diseases including, endocarditis and nosocomial infections. The

recent emergence of vancomycin-resistant *E. faecalis* has provided the impetus for extensive research on this microorganism.

Xenobiotic Metabolism

Xenobiotics are chemical compounds that are foreign to the host. Most of these compounds are widely used in the food and drug industry. Since the microflora have diverse and unique metabolic capabilities, they play an important role in the metabolism of these compounds. Xenobiotics are sometimes toxic and mutagenic and hence their chemical modification by enzymatic processes of the liver and intestinal flora is necessary to reduce or eliminate their deleterious effect. The metabolic activities of the gut microflora are associated with reductive and hydrolytic processes (Drasar, 1974). There are two reaction Phases (I & II) which are involved directly towards the detoxification/modification of xenobiotics.

Phase I Reactions

Cytochrome P450 is a heme-protein that plays a very important role in this phase. This family of mixed-function mono-oxygenases is present in many tissues, but they are most diverse in the liver microsomes. The oxidative process utilizes two protons and two electrons from NADPH (Roland et al., 1993)**.** A second important enzyme is the flavin-monooxygenase or a mixed-function amine oxidase. Both these enzymes modify the xenobiotic by the addition of a hydroxyl moiety (Gandolfi, 1986). The major chemical reactions in this phase include hydrolysis, esterification and amidation to expose preexisting functional groups. Once exposed, these functional groups are cleaved to

produce other functional groups for further Phase II biotransformation. Other reactions in this Phase are oxidation and reduction processes. The chemical modification of the xenobiotics makes them more hydrophilic to enhance their elimination (Roland et al., 1993).

Phase II Reactions

The xenobiotics that are modified in Phase I undergo further biotransformation by a different set of enzymes to make them more hydrophilic. The main family of enzymes involved in these processes are glutathione-S-transferase and UDP-glucuronosyltransferases. The biotransformation process involves the conjugation of glutathione and glucuronic acid to the Phase I products (Roland et al., 1993). Other enzymes involved in Phase II reactions include acetyltransferases and sulfotransferases (Airoldi et al., 2004). These biosynthetic pathways require the activation of cofactors or a substrate and utilize ATP for energy (Gandolfi, 1986). Additionally, transferases include epoxide hydrolases. The hydrophilic metabolites are excreted through bile and eliminated in urine. Since the biliary contents are excreted into the intestines via the enterohepatic pathway (cycling), the metabolites can be further modified by the intestinal bacteria.

Role of Intestinal Flora in Xenobiotic Metabolism and Carcinogenesis

The intestinal microflora have a very high metabolic capacity. They can modify xenobiotics directly or they could biotransform metabolites generated from microsomal cytochrome P450. Several enzymes are associated with the xenobiotic-metabolizing potential of intestinal flora.

Demethylases are involved in the demethylation of methylmecury which is associated with its detoxification since the demethylated product can not be absorbed by the intestine and is easily excreated (Rowland et al., 1983). An important class of enzymes produced by intestinal microflora is the β-glycosidases which includes, β-glucosidase, β-galactosidase and β-glucoronidase. These enzymes hydrolyze the glycosidic bond that connects a sugar molecule to a non-sugar molecule. These enzymes can generate toxic or carcinogenic compounds**,** for example, the deconjugating enzyme β-glucoronidase can activate azoxymethane, a dimethylhydrazine carcinogenic derivative to induce intestinal cancer (Roland et al., 1993).

The toxicity of heterocyclic aromatic amines (HAA) is attributed to the formation of DNA binding adducts such as N-hydroxyl derivatives which are formed in the liver by the cytochrome P450 family of enzymes (CYP), especially CYP1A2 (Airoldi et al., 2004). Intestinal bacteria can also oxidize HAAs to form N-hydroxyl derivatives. Some of the HAA can bind directly to the cell wall of the intestinal bacteria as well. There are two paths that the N-hydroxyl derivatives can undergo in the intestine. First, they may be transported to the liver from the intestine via the hepatic portal system to form glucuronide conjugates by UDP glucuronosyl transferases. The N-glucuronides are then transported back to the intestine where the enzyme, -glucuronidase hydrolyses them to N-hydroxyl arylamines again (Kadlubar, 1985; Knasmuller et al., 2001). The second pathway involves the action of Phase II enzymes, acetyltransferases and sulfotransferases to convert the N-hydroxyl arylamines to O-esters such as N-acetoxy arylamines and N-sulfonyloxy arylamines (Airoldi et al., 2004). These O-esterified derivatives are much more electrophilic than the N-hydroxyl derivatives and are considered ultimate carcinogens (Kadlubar et al., 1977; Kadlubar, 1985; Airoldi et al., 2004). The increased

electrophilic properties of these O-esters is due to the formation of a stable nitrenium intermediate, formed by heterolytic cleavage of the N-O bond which readily binds to DNA and subsequently damages it (Kadlubar, 1985; Borosky, 2007).

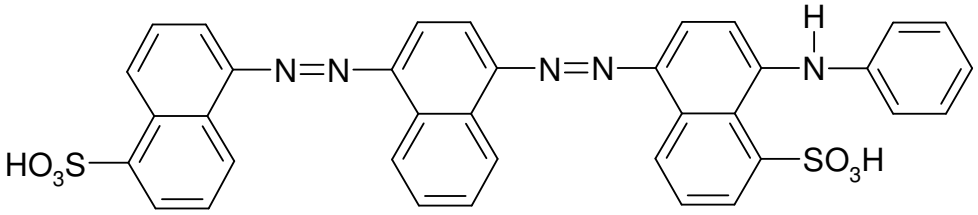
Dyes

Humans have been using natural dyes like inorganic pigments and plant aromatic compounds for centuries (Zee, 2002). However, in 1856, W.H Perkin, an English chemist was the first to produce and patent the synthetic dye mauveine which subsequently led to research and development of other synthetic dyes (Welham, 2000). Dyes are aromatic compounds that absorb light in the visible electromagnetic spectrum (350-700 nm). The color of dyes is dependent on the chromophore and auxochrome, the former being chemical bonds like -N=N-, -C=C- and –C=N- where delocalization of electrons in the conjugated double bond system occurs and the latter being molecules like ammonia, carboxylic acid and hydroxyl groups which donate or accept electrons (Zee, 2002). Synthetic dyes are classified on the basis of their chromophore. The dyes can be broadly classified into cationic (basic dyes), anionic (direct, reactive, acid dyes) and uncharged (disperse dyes).

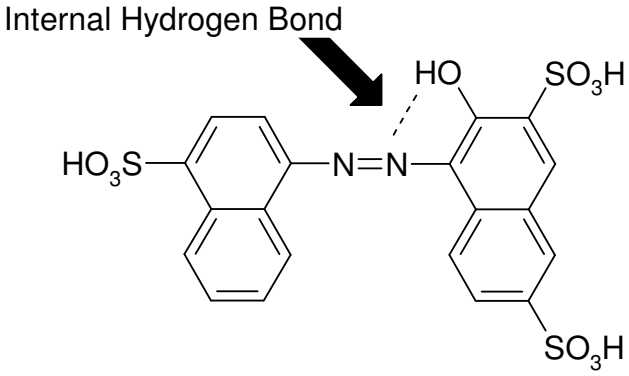
Azo Dyes

Azo dyes are characterized by a specific –N=N- chromophore group or azo bond. Depending upon the number of azo bonds present, these dyes are classified into monoazo, diazo and polyazo groups. They are one of the most important dyes used in the textile, pharmaceutical and food industries (Chung et al., 1992; Chen, 2006). Interestingly, the only known natural compound that contains an azo group is 4,4’-

dihydroxyazobenzene (Gill, 1984). Some azo dyes presently used contain potentially carcinogenic compounds (Platzek et al., 1999). These dyes are cleaved to form toxic aromatic amines by enzymes in the liver and intestinal flora (Nakayama et al., 1983; Chung et al., 1992; Chen, 2006). The susceptibility for azo bond cleavage depends upon the surrounding auxochrome groups. Electron withdrawing groups in the *ortho* position relative to the azo bond decrease the electron density around the azo bond, thus making it more amenable to reduction. This may explain why extracts from *Sphingomonas* sp. strain BN6 reduced Acid Black 24 with much lower activity than Amaranth (Fig. 1) (Russ et al., 2000).



Acid Black 24



Amaranth

Figure 1. Structure of Azo dyes Acid Black 24 and Amaranth

Metabolism of Azo Dyes

Azo dyes are capable of being metabolized by oxidative and reductive processes. These dyes, especially those that are lipophilic in nature, can be oxidized by cytochrome P450 at the carbon or nitrogen atoms of the molecule keeping the azo bond intact (Hunger, 1994). Besides oxidation by microsomal enzymes, some azo dyes can also be oxidized by an extracellular peroxidase from *Streptomyces chromofuscus* A11 (Pasti-Grigsby et al., 1996). The S5 strain, which is a mutant of the S1 strain of *Hydrogenophaga intermedia* is able to grow on 4-carboxy-42-sulfoazobenzene, a sulfonated azo compound, as the sole carbon and energy source (Blumel et al., 1998). Azo dye reduction is an important first step in their generation of carcinogenic amines which is catalyzed primarily by intestinal flora (Brown, 1993) and also by enzymes in the mammalian liver. Azo reduction is a Phase I process that can occur in the liver microsome by cytochrome P450 as well as intestinal bacteria.

Azo Dye Reduction by Intestinal Bacteria

The azo bridge (N=N) can be reduced by azoreductases to produce colorless aromatic amines (Chung et al., 1992). One of the first studies on the metabolism of azo dyes by bacteria showed that *Bacillus pyocyaneus* and *Proteus* species reduced 90 of 102 azo dyes tested (Dieckhues, 1960). Azo dye reduction may occur within the cell (endogenously) or external to the cell (exogenously). The redox potential of azo dyes is much lower than oxygen and hence in the presence of oxygen (aerobic environment) these dyes are not reduced. It has been shown that an anaerobic environment with a redox potential between -100 and -300 mV promotes optimal azo dye reduction in the intestine (Schroder and Johansson, 1973). Other studies have shown that electron carriers in the external environment facilitate azo reduction (Brown, 1981; Semde´ R, 1998). It has been

suggested that some azo dyes are reduced by a non-enzymatic process extracellularly due to the presence of electron shuttles (Dubin and Wright, 1975). Studies on the reduction of the azo dye, Red 2G by cell extracts of *E. faecalis* have shown that FMN and reduced pyridine nucleotides were essential (Gingell and Walker, 1971). Other studies have shown that the reduction of azo dyes by anaerobic cell suspensions showed zero-order kinetics where the reduction rate was independent of azo dye concentration but dependent on the bacterial cell suspension only (Dubin and Wright, 1975). Studies by Rafii et al. (Rafii et al., 1990) showed that some anaerobic intestinal bacteria had the ability to reduce Direct Blue 15. All strains reduced the dyes under anaerobic conditions, but some required flavin compounds for reduction. According to the authors, the azoreductases from all the strains were produced constitutively and the dye reduction occurred extracellularly. Recently, it was shown that *Shewanella decolorationis* could grow with an azo compound as the sole electron acceptor and that quinones, dehydrogenases and cytochromes from the electron transport chain play an important role in the pathway involved in azo reduction under anaerobic conditions (Hong et al., 2007).

Azoreductase

One of the first true azoreductase enzymes to be purified and characterized was from *Pseudomonas* KF46 (Zimmermann et al., 1982). This enzyme was a monomer capable of reducing Orange II and carboxy-Orange II and could utilize NADH as well as NADPH as electron donors. Its molecular weight was 30 kDa. Subsequently, an Orange I azoreductase was isolated from *Pseudomonas* K24 (Zimmermann et al., 1984). Although it shared common features with the Orange II azoreductase from strain KF46 in terms of

its monomeric structure, substrate and co-substrate specificities, the Orange I azoreductase was 21 kDa and did not have any antigenic cross reactivity with the Orange II azoreductase. Neither of these azoreductases required externally added flavins for activity. No N-terminal sequencing was performed on these azoreductases, so no direct comparison of the proteins at the primary structure level was available. Later, in other independent studies, the azoreductase from *Xenophilus azovorans* KF46F (earlier called *Pseudomonas* KF46) (Blumel et al., 2002) and *Pigmentiphaga kullae* K24 (earlier called *Pseudomonas* K24) (Blumel and Stolz, 2003) were purified and their N-terminal residues was sequenced. These two azoreductases did not share any homology which would explain the lack of antigenic cross reactivity, however, both the sequences possessed a NAD(P)H binding motif.

The first flavin-dependent azoreductases from an intestinal bacterium were isolated from *Shigella dysenteriae* type 1 and these enzymes could utilize NADH as well as NADPH for activity. Azoreductase I was a dimer and had a molecular weight of 28 kDa, while azoreductase II was a monomer and had a molecular weight of 11 kDa. Azoreductase II was not able to reduce Amaranth, but the dye was reduced by azoreductase I (Ghosh et al., 1992), which shows different substrate specificities. Similarly, two azoreductases from *E. coli* K12 were purified, one being a monomer (12 kDa) and the other a dimer (28 kDa) (Ghosh et al., 1993). Although their substrate specificities were slightly different, both azoreductases could utilize NAD(P)H as electron donors. However, since there was no genetic information available for these azoreductases, they could not be compared.

The azoreductase from *Bacillus* sp. OY1-2 was the first to be heterologously expressed and purified from *E. coli* (Suzuki et al., 2001). The purified recombinant protein was shown to reduce several azo dyes using NADPH as the electron donor, however no flavins were required for the reaction. A ping-pong mechanism was used for dye reduction by the azoreductase from *Rhodobacter sphaeroides* AS1.1737 which shares 97% identity with *Bacillus* sp. OY1-2 (Bin et al., 2004).

The first human intestinal azoreductase in which the molecular and functional aspect of the enzyme was characterized came from *E. coli*. The *acpD* gene product (AcpD) which was thought to be an acyl carrier protein (ACP) phosphodiesterase was shown to possess azoreductase but no phosphodiesterase activity in *E. coli* JM109 (Nakanishi et al., 2001). The azoreductase from *Pseudomonas aeruginosa* which was also identified as the product of the *acpD* gene, like the *E. coli* AzoR, did not possess phosphodiesterase activity (Wang et al., 2007). The AcpD enzyme from *E. coli* and *P. aeruginosa* were later renamed AzoR and paAzoR1 respectively. AzoR did not have an FMN moiety bound to it and externally added FMN was essential for activity. This enzyme could utilize only NADH as an electron donor and not NADPH. The mechanism involved a ping pong Bi-Bi process where 2 mol of NADH reduced 1 mol of Methyl Red via FMN. On comparison of AzoR with other azoreductases whose sequence was determined, there was only moderate homology with the N-terminal domain of NAD(P)H: quinone oxidoreductase (NQO1) from rat. The hydrogen bonding residues in the N-terminal region of NQO1 which contains the FMN and nicotinamide ribose of $NADP^+$ binding sites are well conserved in AzoR.

Subsequently, the *acpD* (*azoR*) gene sequence from *E. coli* was used as a query to BLAST search the genome of *E. faecalis*. This search led to the identification of a gene that was annotated as a putative ACP which shared only 34% sequence identity with AzoR (Chen et al., 2004). The enzyme (AzoA) was expressed and purified from *E. coli.* It is a dimer of 43 kDa with an FMN molecule bound to each monomer tightly, but non-covalently. The enzyme could utilize NADH only as an electron donor to reduce Methyl Red. Using a similar approach to identify an azoreductase, the azoreductase from *Staphylococcus aureus* was identified using the gene sequence of the *Bacillus* sp. OY1-2 azoreductase as a query against the *S. aureus* database (Chen et al., 2005). This enzyme, designated Azo1, was a flavoprotein as well; however it was a tetramer in its native state and could only utilize NADPH for activity. This enzyme is constitutively expressed in *S. aureus* and its gene shows 32% identity with the azoreductase gene from *Bacillus* sp. OY1-2.

Crystal structures of Azoreductase

The first azoreductase from a human intestinal bacterium to be crystallized was also the AzoR from *E. coli* (PDB code: 1V4B) (Ito et al., 2006). The FMN-enzyme complex was shown to be a homodimeric protein with both monomers contributing to the catalytic site. Each monomer contained a flavodoxin-like fold with FMN at the interface of the dimers. The monomers contain a typical Rossmann NAD(P) -binding fold with five parallel β-sheets in the center flanked by six α-helices. The NADH binding site in AzoR is unique as it does not contain the conserved NADH binding motif (G*X*G*XX*G).

Conformational changes around the active site have been attributed to the binding of several substrates.

Following the crystallization of AzoR, the crystal structure of AzoA from *E. faecalis* was also determined complexed with FMN (PDB code: 2HPV) (Liu et al., 2007). The crystal structure was very similar to AzoR and superimposition of the two structures revealed a difference around loop L 12 of AzoR which is close to the catalytic site and may play a role in the binding of NAD(P)H. The conserved phenylalanine, which is present in the active site of AzoR and rat quinone reductase is absent in the active site of AzoA and Leucine is present instead. However, like AzoR, Loop 9 and Loop 13 in AzoA are associated with the regulation of the active site.

The azoreductase from *P. aeruginosa* paAzoR1 (PDB code: 2v9c) was crystallized with FMN and Methyl Red (Wang et al., 2007). The Methyl Red moiety is positioned on the *Si* face of the isoalloxazine ring of FMN and π-stacking interactions also contribute to its stability in the hydrophobic pocket. Although paAzoR1 is a tetramer (dimer of dimers), its overall structure is very similar to AzoR and AzoA which are dimers i.e., they all contain a flavodoxin-like fold. The active site of these enzymes is formed with both monomers and is highly conserved (Liu et al., 2007; Wang et al., 2007) with the FMN molecule oriented in the same position i.e., the ribityl tail is embedded in the protein and the *Si* face of the isoalloxazine ring exposed to the substrate binding site. Figure 2 compares the crystal structures of AzoA, AzoR and paAzoR monomers with the FMN moiety bound and shows the high architectural similarity between them.

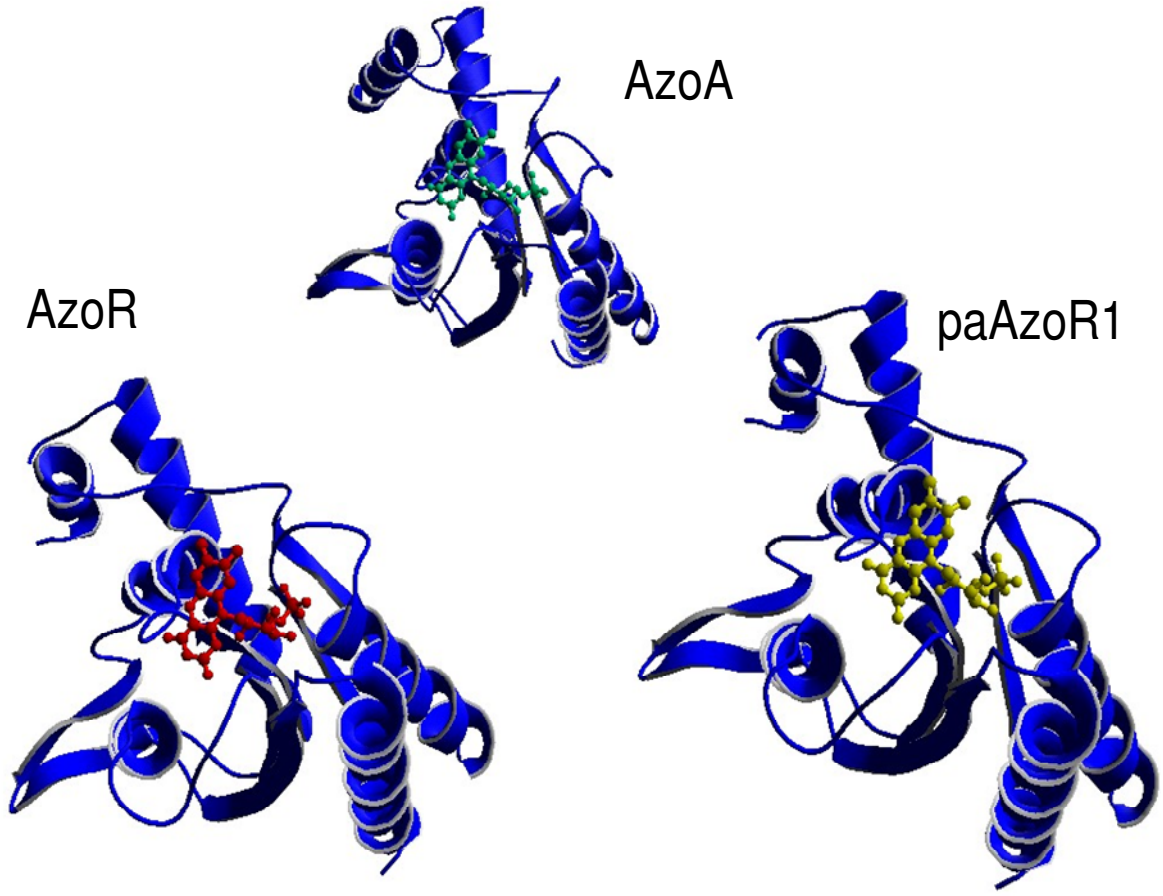


Fig. 2. Crystal structures of azoreductases. Monomers from *E. faecalis* AzoA, *E. coli* AzoR and *P. aeruginosa* paAzoR1. The FMN moiety is represented as ball-and-stick. DeepView (Swiss-PDB) was used to generate the images.

The crystal structure of the reduced form of AzoR has also been crystallized (Ito et al., 2008). In the reduced state, there is a twisted-butterfly bend of the FMN isoalloxazine ring with the solvent molecules being rearranged. The hydrophobic region - around the isoalloxazine ring in the binding pocket is important for substrate binding. Also, by replacing Arginine 59 with Alanine, the substrate specificity could be expanded to include p-Methyl Red. This is because of the absence of an electrostatic interaction between Arg-59 and the carboxy group around Methyl Red which is formed in the wild-type enzyme.

Conclusion

Azo dyes are anthropogenic compounds that are widely used in several industries including the food and drug industries. Research in azo dyes is of importance not only because of their contribution to the economies of the world but these dyes are also recalcitrant to degradation and are environmental pollutants. From the human health perspective, some of these dyes can be metabolized to carcinogenic compounds by azoreductases from intestinal microbiota and cytochrome P450.

Most of the earlier studies have focused on the metabolism of these dyes by liver microsomal enzymes. Although, it is now known that a majority of the hydrophilic azo dyes are metabolized by intestinal flora. Important information gained from azoreductase studies includes the varied enzyme structure and function. For example, it is difficult to identify the enzymes based on a common motif. In addition, some classification schemes have been created based on cofactor requirements. Information on the crystal structures of azoreductases has given important clues towards substrate binding and specificity.

However, there is still a lack of information available on the physiological effects of these dyes on intestinal flora, the cellular environment in which the azoreductase gene is expressed, and the evolutionary nature of the enzyme. A robust classification of these enzymes will be important in determining the evolution of these enzymes and may allude to a physiological function for these enzymes which have not been identified to date.

CHAPTER III

PHYSIOLOGICAL CHARACTERIZATION OF *ENTEROCOCCUS FAECALIS* DURING AZOREDUCTASE ACTIVITY

Abstract

Azo dyes are widely used in the food, pharmaceutical, paper and textile industries. Some azo dyes are known to produce carcinogenic compounds upon reductive cleavage of the azo bond (N=N) by intestinal flora. There is not much information available on the effect of these dyes on the physiology of the gut microflora or the kinetics of reduction in different environments. The azoreductase activity of *E. faecalis,* an important opportunistic intestinal pathogen, was tested using the azo dyes Methyl Red, Direct Blue 15 and Tartrazine under the following conditions: a complex supplemented Brain Heart Infusion (BHIS) medium under aerobic and anaerobic conditions, a simple phosphate-buffered saline glucose (PBSG) medium under anaerobic conditions and cell extracts using NADH and NADPH as electron donors under aerobic conditions. The kinetics of reduction in all these conditions was determined. The effect of high and low concentrations of each dye on the growth of *E. faecalis* was also tested. The results show that the sulfonated azo dye Tartrazine was completely reduced extracellularly in BHIS under aerobic and anaerobic conditions while

the other sulfonated azo dye, Direct Blue 15 was completely reduced only under anaerobic conditions. The non-polar azo dye, Methyl Red was also completely reduced with the highest rate of reduction in both conditions. The generation time of *E. faecalis* in the presence of a high concentration of Tartrazine was significantly lower than the control while the generation time was unaffected in the presence of low and high concentrations of Methyl Red, Direct Blue 15 and 20 μM Tartrazine. Our results suggest that the metabolism of different azo dyes by *E. faecalis* depends on the physio-chemical properties of the azo dyes and the conditions of the surrounding environment in the presence of actively dividing cells.

The following chapter has been published in "Microbial Ecology in Health and Disease', June, 2008 and appears here with the journal's permission. Figure 4 does not appear in the published article.

Introduction

Human beings are constantly exposed to various xenobiotics from the environment. The main route of entry for these xenobiotics is the gastrointestinal (GI) tract (Hanninen et al., 1987). The metabolic complexity of gut microflora has played an important role in drug (Mikov, 1994) and azo dye (Chung et al., 1992; Chen, 2006) metabolism.

Azo dyes are widely used in the paper, textile, food, cosmetic and pharmaceutical industries and the reduction of the azo bond (N=N) is observed by decolorization of the dye (Rafii et al., 1990). The distal region of the GI tract is mainly anaerobic and under these conditions, most of the azo compounds are biologically reduced (Stolz, 2001). Azo dye metabolism is of interest from the human health perspective because several intestinal microorganisms possess azoreductase activity and some of these dyes are known to produce mutagenic and carcinogenic compounds upon reduction (Chung, 1983). Besides their role in carcinogenesis, the biological role of azo reduction and the impact from these types of diverse azo dyes on the intestinal microbial ecology and overall human health is poorly understood. To this end, very little information is available regarding the physiological effect of azo dyes on the gut flora.

E. faecalis is an important organism and opportunistic pathogen of the gut due to its role in food fermentation, nosocomial infections and resistance to multiple antibiotics (McBride et al., 2007). The biochemical and molecular biology of the bacterium has been well characterized. The azoreductase activity of the cellular extracts has been shown for Red 2G as a model azo dye (Walker et al., 1971). In addition, an aerobic FMN-dependant azoreductase gene from *E. faecalis* has been isolated (Chen et al., 2004) and

the protein crystallized (Liu et al., 2007). Unfortunately, there is not much information on the growth phase response and the corresponding azoreductase activity rates for *E. faecalis* with different sulfonated and nonsulfonated azo dyes, which can provide important biological information and impact measurements for this important intestinal bacterium.

This study investigates the effect of azo dyes on the growth of an *E. faecalis* strain isolated from the human intestine. The kinetics of azo dye reduction in the lag and log phase of growth under both aerobic and anaerobic conditions in complex and simple media as well as in the cell extracts of this strain was studied. Methyl Red, a mono, nonsulfonated azo dye and pH indicator is a model azo dye used in several studies. Tartrazine is also a mono, sulfonated azo dye and is currently used in the food industry. Direct Blue 15 is a bis, sulfonated azo dye which upon reduction, produces 3',3'-dimethoxybenzidine, a known carcinogenic compound (Cerniglia et al., 1982).

Materials and methods

Bacterial strain and media

Enterococcus faecalis ATCC 27274 was grown in Brain Heart Infusion medium (Difco Laboratories, Sparks, MD, USA) supplemented per liter with: 10 g Yeast extract: 0.075 mg K_2HPO_4: 0.175 mg KH_2PO_4: 0.450 mg $(NH_4)_2SO_4$: 0.045 mg NaCl: 0.09 mg $MgSO_4$: 0.015 mg $CaCl_2$: 0.002 mg Hemin: 400 mg Na_2CO_3 and 600 mg Cysteine-HCl: pH adjusted to 7.0. This supplemented Brain Heart Infusion medium (BHIS) was prepared anaerobically in an ethanol-sterilized anaerobic glove box (Forma Scientific, Model 1024, Marietta, OH, USA) after sterilization in an autoclave at 15 psi for 20 min at 121°C. Modified phosphate buffered saline-glucose (PBSG): 137 mM NaCl: 2.7 mM KCl: 10 mM Na_2PO_4: 2 mM KH_2PO_4: 600 mg Cysteine-HCl: 6 mM glucose: pH adjusted to 7.4 was used as the growth-arrest medium. To prepare the glycerol stocks, 200 µL of sterile 80% glycerol was mixed with 800 µL *E. faecalis* culture (grown in BHIS) and stored at -80°C.

Azo dyes

Azo dyes used in this study were, Methyl Red (MCB Chemicals, Norwood, Ohio, USA; molecular weight, 269.31, $2[4\text{-}(CH_3)_2NC_6H_4N{:}N]C_6H_4CO_2H$, CAS registry 63451-28-5), Direct Blue 15 (MP Biomedicals, Solon, OH, USA, molecular weight 992.8, $C_{34}H_{24}N_6O_{16}S_4Na_4$, CAS registry 2429-74-5) and Tartrazine (Sigma, St. Louis, MO, USA, molecular weight, 534.4, $C_{16}H_9N_4Na_3O_9S_2$, CAS registry 1934-21-0).

The assay for each dye under all culture and cell extract experiments was carried out using three independent replicates and the data is presented as the mean of the three data points with standard deviations.

Anaerobic azoreductase assay for whole cells

Nine mL of BHIS containing 20 μM of each azo dye was dispensed into separate screw-capped (with a rubber septum) test tubes in an anaerobic glove box with an atmosphere of nitrogen. A loop-full of *E. faecalis* from a frozen glycerol stock was aseptically transferred to a sterile BHIS tube (containing no dye) and incubated at 37°C without shaking. After 12 h incubation, 5 mL of this culture was transferred to a 250 mL bottle containing 100 mL of BHIS and incubated again at 37°C. The culture was allowed to grow to mid log-phase (optical density$_{600}$ ~ 0.4) and immediately 1 mL was transferred to the 9 mL BHIS test tubes containing the respective azo dyes using a sterile 1 mL syringe and needle (26 G1/2) (BD, Franklin Lakes, NJ, USA) in the anaerobic glove box. For the control experiment, 1 mL of sterile BHIS was added to the test tubes instead of the *E. faecalis* culture. The tubes were inverted twice to mix and incubated at 37°C. After 5 min time intervals for Methyl Red and 10 min time intervals for Tartrazine and Direct Blue 15, the set of three test tubes (for each dye) were removed from the incubator and 1 mL from each tube was transferred to a 1.5 mL microfuge tube. The samples were centrifuged at 14,000 x g for 1 min and the supernatant was transferred to a plastic cuvette (Spectrocell, Oreland, PA, USA). Azoreductase activity was determined by a decrease in the absorbance maxima wavelength peak area for the azo dyes using a UV-visible spectrophotometer (UV-1601PC, Shimadzu, Columbia, MD, USA). The peak area

for Direct Blue 15 was determined between wavelengths 500-700 nm, Tartrazine between 400-500 nm and Methyl Red between 420-500 nm. The concentration of the dyes was determined from the dye calibration graph using known concentrations of the azo dyes dissolved in BHIS. The pellet from the centrifuged sample was resuspended in distilled water and the optical density (OD) at 600 nm was used to determine growth.

PBSG was used as the growth-arrest medium to determine azoreductase activity. One hundred mL of mid-log phase *E. faecalis* culture in BHIS was centrifuged at 6,000 x g for 10 min. The supernatant was discarded and the pellet was washed and resuspended in 100 mL of sterile oxygen-free PBSG. One mL of this sample was subsequently transferred to 9 mL PBSG containing the azo dye and incubated at 37°C. The amount of each dye used in the assay was the same and azoreductase activity was determined as above.

Aerobic azoreductase activity by whole cells

Ten mL mid-log phase *E. faecalis* culture was added to 40 mL BHIS containing 20 μM of the azo dyes in separate 500 mL Erlenmeyer flasks. The flasks were incubated at 37°C. To assure oxygen saturation, the flasks were incubated on a shaker at 200 rpm. At 1 h time intervals, 1 mL samples were removed aseptically and the azoreductase activity was determined as above. Ten mL of sterile BHIS was added instead of the *E. faecalis* culture for the control experiments.

Preparation of cell extracts

E. faecalis was grown for 24 h in 2 L BHIS at 37°C. The cells were harvested at 6000 x g for 10 min at 4°C. The pellet was resuspended in 30 mL lysis buffer (50 mM K_2HPO_4- KH_2PO_4 buffer, pH 8.0, 300 mM NaCl and 10% glycerol). Lysozyme was added to a final concentration of 1 mg/mL and the sample was incubated on ice for 1 h. The sample was sonicated for 2 min on ice using an intermediate probe of a Sonic 300 Dismembrator (Artek systems Corp., Farmingdale, NY, USA). The homogenate was centrifuged at 20,000 x g for 20 min at 4°C and the supernatant was collected and stored at -20°C.

Azoreductase assay of the cell extract

The assay was performed in a final volume of 1 mL and contained 50 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.2, 20 μM azo dye, 20 μM FMN, 0.5mM NADH or NADPH [NAD(P)H] and an appropriate amount of cell extract. The solution was made anaerobic by bubbling with oxygen-free nitrogen for 20 min. The sample was pre-incubated at 37°C for 5 min and the reaction was initiated by the addition of NADH or NADPH [NAD(P)H]. Azoreductase activity was determined by an absorbance reduction in the first 2 min at 430 nm for Methyl Red, 428 nm for Tartrazine and 604 nm for Direct Blue 15 using a UV-Visible spectrophotometer (UV-1601PC, Shimadzu, Columbia, MD, USA). The concentrations of the respective dyes were determined from a calibration graph using known concentrations of the azo dyes dissolved in the assay buffer.

Protein determination

The Lowry method (Lowry O.H, 1951) was used to determine the protein content from whole cells (prepared by alkaline lysis) for the experiments in BHIS and PBSG using bovine serum albumin (BSA) as a standard. The protein content in the cell extracts was determined by applying the formula: mg/mL = $1.55A_{280} - 0.76A_{260}$ (Groves et al., 1968)

Results

Reduction of azo dyes by whole cells

Methyl Red, Tartrazine and Direct Blue 15 were completely reduced in 20 min, 40 min and 50 min respectively when *E. faecalis* was grown in BHIS anaerobically (Fig. 3a). The dye controls remained stable over the time course for the experiments (data not shown). Due to an increase in biomass with every subsequent time interval, each dye concentration data point was divided by the whole cell total protein amount (determined by the Lowry method) for that particular time interval to normalize the data for biomass content. When *E. faecalis* was boiled and added to BHIS with the azo dyes and incubated for 12 h, there was no reduction observed (data not shown). To determine dye stability in the presence of actively growing cells, *Bacteroides stercoris* was grown anaerobically in BHIS with 25 μM Direct Blue 15. After 6 h, *B. stercoris* had reached an optical density $(OD)_{600}$ = 0.9 but only 20% of the dye was reduced and within 1 h, the concentration of Direct Blue 15 remained relatively constant (Fig. 4). This shows that the reduction of Direct Blue 15 in BHIS by *E. faecalis* is specific to the organism and not the medium.

Under aerobic conditions, it took three times longer to completely reduce Methyl Red and Tartrazine compared to anaerobic conditions (Fig. 3b). Similarly, Direct Blue 15 was not completely reduced under aerobic conditions and only 75% of the dye was reduced in 4 h. The incomplete reduction of Direct Blue 15 produced a non-linear reduction rate that was asymptotic to time.

To address the influence of bacterial growth phase and the contribution of a complex medium on azo dye reduction, the reduction of these dyes was tested in a simple buffered medium, PBSG, anaerobically. Glucose was added to the PBS buffer as a source

of electrons for azo dye reduction. The cells, which had grown to log phase in BHIS, were washed with the buffer prior to their introduction in to the PBSG medium. Although there was no growth in this medium (OD_{600} remained constant over time), the cells were still viable because cell growth resumed when the cells, after 24 h incubation in PBSG, were transferred to BHIS (data not shown). Hence, the cells are in a prolonged lag phase in the PBSG medium. Figure 3c shows that Direct Blue 15 was not reduced. The 90% Methyl Red reduction, which occurred in 20 min (Fig. 3c), was similar to the value obtained in the anaerobic BHIS condition (Fig 3a). Tartrazine was reduced by 80% in 28 h (Fig. 3c) which was significantly longer than its complete reduction in anaerobic BHIS which took 40 min (Fig. 3a). The overall pattern of reduction for the azo dyes in PBSG was the same as BHIS, i.e. the reduction rate for Methyl Red was the highest followed by Tartrazine and Direct Blue 15. The data suggests that the rate of azo dye reduction is influenced by the growth phase of the cells and possibly contribution from BHIS as well.

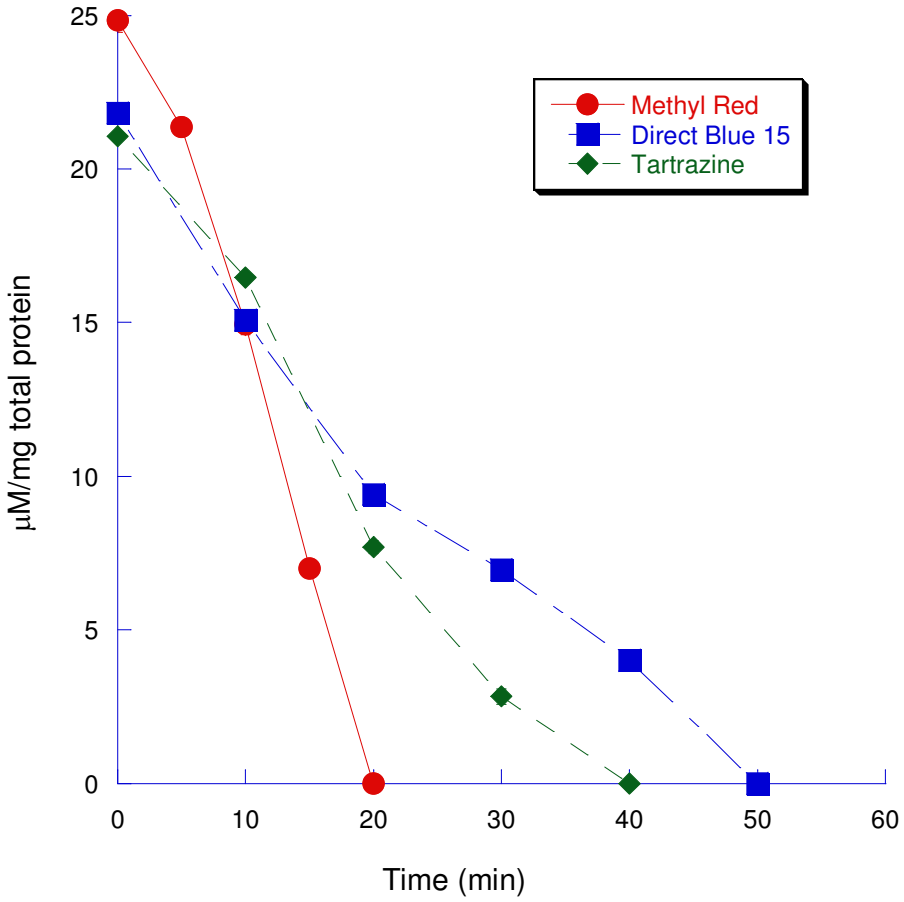


Fig. 3a. Anaerobic reduction by *E. faecalis* whole cells. Direct Blue 15 (blue), Methyl Red (red) and Tartrazine (green). The units on the y-axis are expressed as dye concentration (µM) per milligram of total protein. The 10 mL culture inoculated with mid-log phase cells was incubated without shaking at 37°C. The controls contained dye only in BHIS. Each data point is a mean of three independent experiments.

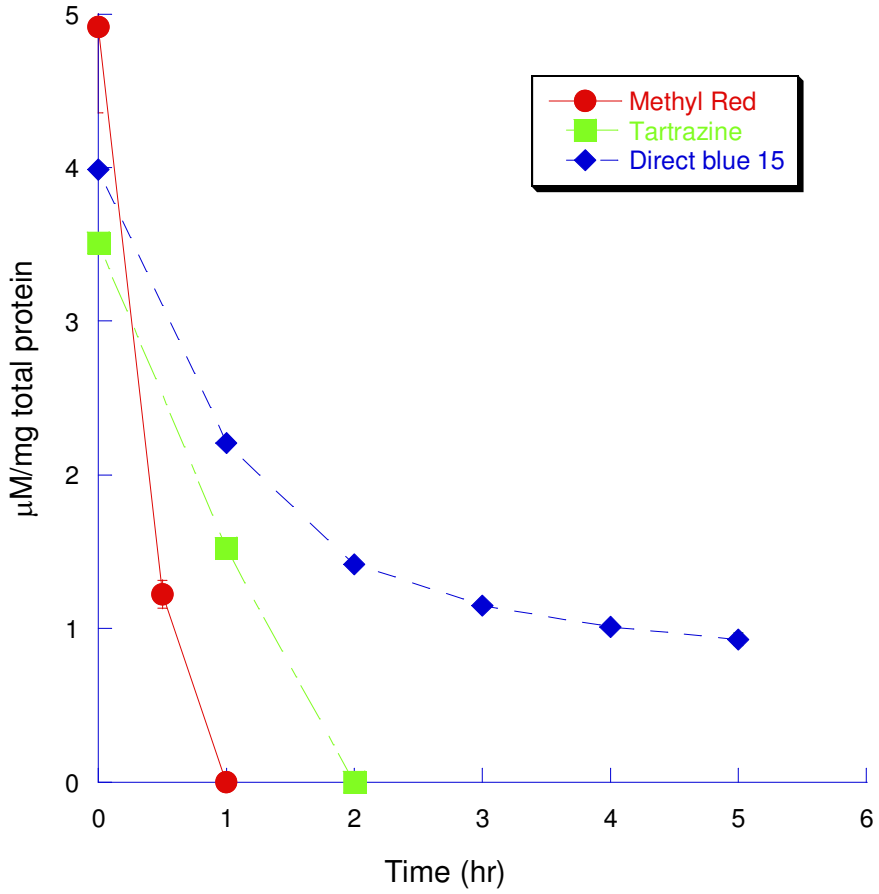


Fig. 3b. Aerobic reduction by *E. faecalis* whole cells. Direct Blue 15 (blue), Methyl Red (red) and Tartrazine (green). The 500 mL flasks containing 50 mL culture were incubated at 37°C and 200 rpm. Note, due to the high biomass, the ratio of dye concentration to amount to total protein on the y-axis is lower compared to the anaerobic assay. The controls contained dye only in BHIS. Each data point is the mean of triplicates.

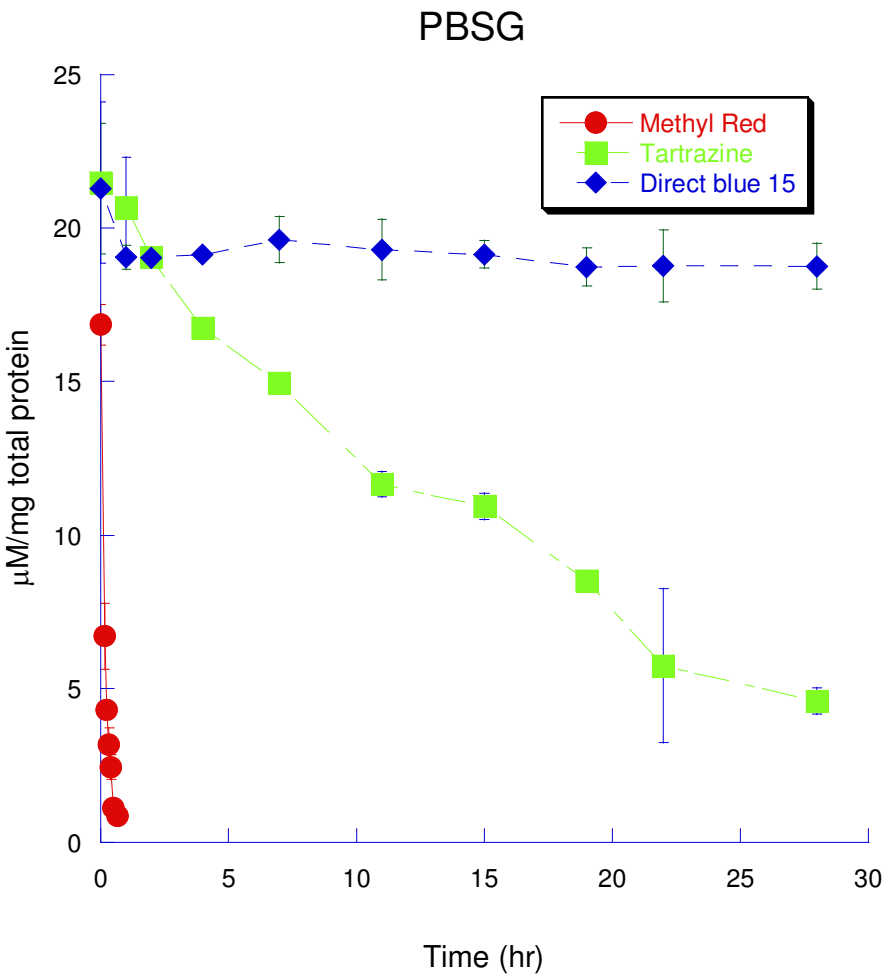


Fig. 3c. Reduction in PBSG by *E. faecalis* whole cells. Direct Blue 15 (Blue), Methyl Red (red) and Tartrazine (green). The 10 mL culture inoculated with mid-log phase cells (washed in PBSG buffer) was incubated without shaking at 37°C. The controls contained dye only PBSG. Each data point is the mean of triplicates.

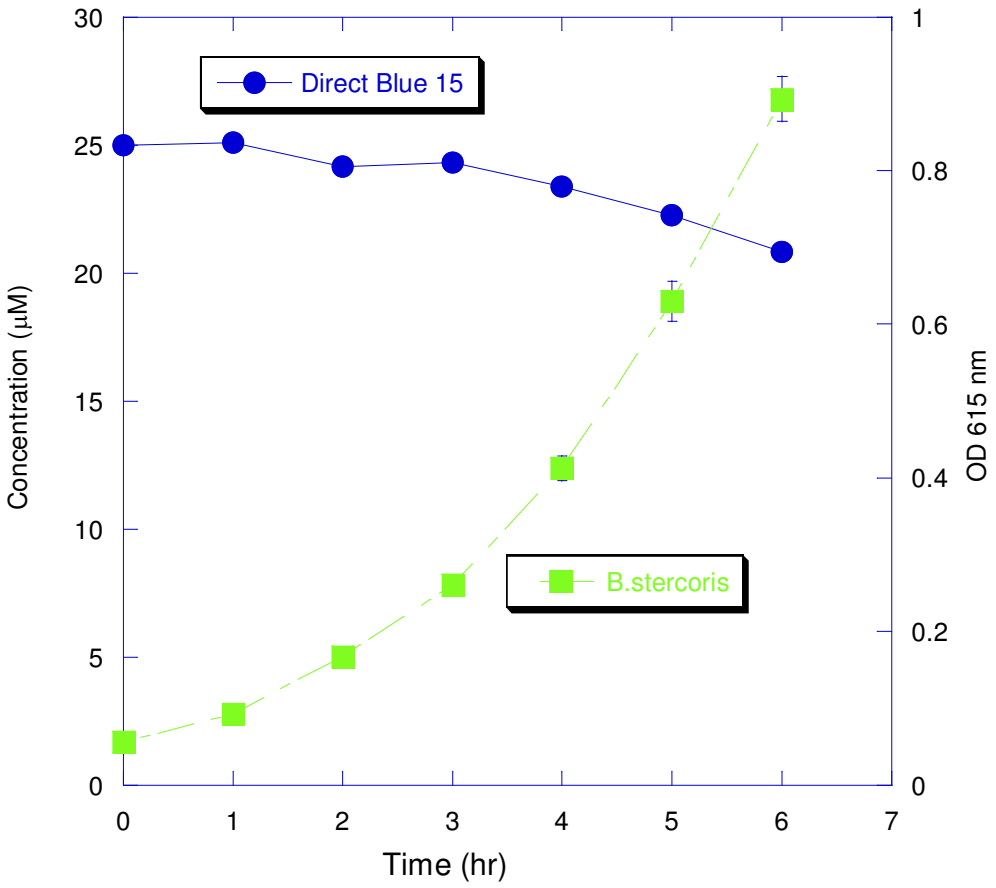


Fig. 4. Azo dye reduction by *B. stercoris.* Direct Blue 15 concentration in µM (blue), optical density (OD) (green). The data is a mean of three independent experiments.

Kinetics of azo dye reduction

The quantitative rates of azo dye reduction under various conditions are shown in Table I. The kinetic rate values were calculated from the linear portion of the curves. All the data are compared to the reduction rate kinetics of whole cells in BHIS under anaerobic conditions since these rates were highest for all three dyes. The reduction rate for Methyl Red was consistently high in BHIS under anaerobic conditions, PBSG medium and the cell extract using NADH as the electron source. However, the reduction of Methyl Red decreased when cells were incubated aerobically and the kinetic rate for NADPH was less than NADH.

The reduction rate kinetics for Tartrazine reduced by more than half in the cell extract assay using NADH and a further decrease was observed in activity under aerobic growth conditions and with cell extract using NADPH. However, in the PBSG medium, there was a sixty-fold decrease in reduction kinetics. Direct Blue 15 showed a four-fold decrease in reduction kinetics under aerobic conditions and very low activity in the cell extract assays. There was no detectable reduction for Direct Blue 15 in the PBSG medium. Even when the biomass was increased almost ten-fold for the experiment in PBSG, the reduction rate kinetics for Tartrazine and Direct Blue 15 was still lower than the values in BHIS under anaerobic conditions (data not shown). The decrease in azoreductase activity in the cell extract compared to the whole cells for Tartrazine and Direct Blue 15 suggests that most of the reduction of these dyes occurs outside the cell or is associated with actively growing cells. The reduction kinetics of Direct Blue 15 is more than 90% lower than Tartrazine in the cell extract assays while it is only 25% lower in the anaerobic BHIS condition (Table I). The reduction rate of Methyl Red was the

Table I.

Condition	**nmol/min/mg**		
	Methyl Red	**Direct Blue 15**	**Tartrazine**
	mean (± SD)	mean (± SD)	mean (± SD)
Whole cell[† ‡] (anaerobic)	9.83 (± 1.42)	4.83 (± 0.11)	6.43 (± 0.02)
Whole cell [‡] (aerobic)	6.17 (± 0.38)	1.07 (± 0.08)	1.65 (± 0)
PBSG [†] (lag phase) (anaerobic)	8.61 (± 0.57)	ND[*]	0.11 (± 0.01)
Cell extract (NADH) (anaerobic)	9.86 (± 1.91)	0.21 (± 0.04)	2.68 (± 0.03)
Cell extract (NADPH) (anaerobic)	1.97 (± 1.17)	0.06 (± 0.01)	1.3 (± 0.45)

Table I. Kinetics of azo dye reduction by *E. faecalis* whole cells and cell extracts.

***** ND, not detected

† The amount of cells in the starting culture ~10^7 cfu/ml.

‡ BHIS medium

The azo dye concentration used for all experiments was 20 μM. The whole cell protein content was determined by the Lowry method using BSA as the standard and the protein content for the cell extract was determined at 280 nm. All the experiments were done at 37°C. The data is the mean of triplicates. SD – standard deviation

same in the whole cell and cell extract assay using NADH as the electron donor, which suggests that due to its small size (molecular weight 269.31) and hydrophobic nature, the dye may diffuse into the cell.

Effect of azo dyes on cell growth

The effect of the three azo dyes, at concentrations of 20 μM and 200 μM, on the generation time of *E. faecalis* when grown anaerobically in the presence of BHIS is shown in Table II. There was no significant difference between the generation times of *E. faecalis* grown in the presence of Methyl Red compared to the control. However, the generation time and biomass in 200 μM of Methyl Red was significantly higher than 20 μM (Fig. 5c). There was a significant decrease in the generation times of *E. faecalis* when grown in the presence of Tartrazine as compared to the control. In addition, the final biomass increased when the cells were grown in the presence of 200 μM Tartrazine

compared to 20 μM (Fig. 5a). The results suggest that Tartrazine stimulates growth of cells in BHIS. When *E. faecalis* was grown in mineral salt medium with 20 μM Tartrazine as the only carbon source and monitored over a four week period, there was no increase in biomass, although there was approximately 50% reduction in dye concentration compared to the control (data not shown). Hence the stimulation of growth by Tartrazine in BHI is not due to its role as an additional carbon source. There was no significant difference in the generation time and biomass of cells grown in 200 μM Direct Blue 15 compared to the 20 μM Direct Blue 15 (Fig. 5b) .

The growth profiles of *E. faecalis* in the presence of both concentrations of Methyl Red and Tartrazine was the same, but there was a difference in the growth profile when the cells were grown in the presence of 200 μM Direct Blue 15 compared to 20 μM. The cells grown in the presence of 200 μM Direct Blue 15 reached log phase after 2 h while the cells grown in 20 μM reached log phase after 30 min (Fig. 5b). However, after 3 h of growth in the presence of both concentrations of Direct Blue 15 the overall biomass was the same. The initial 200 μM concentration of Direct Blue 15 may have an inhibitory effect on the growth rate because the dye to cell ratio is high. This was observed by the presence of a blue pellet when the sample was prepared (see Material and Methods). The blue pellet may signify that the reduction rate is saturated and the non-reduced dye molecules have attached to the cell wall. Since the dye is a large molecule (molecular weight: 992.8), its presence on the cell wall may prevent the transport of essential nutrients from the medium to the cell. When the dye to cell ratio is reduced, maximum growth rate resumes. This was observed by the disappearance of the blue color in the pellet during later sample processing.

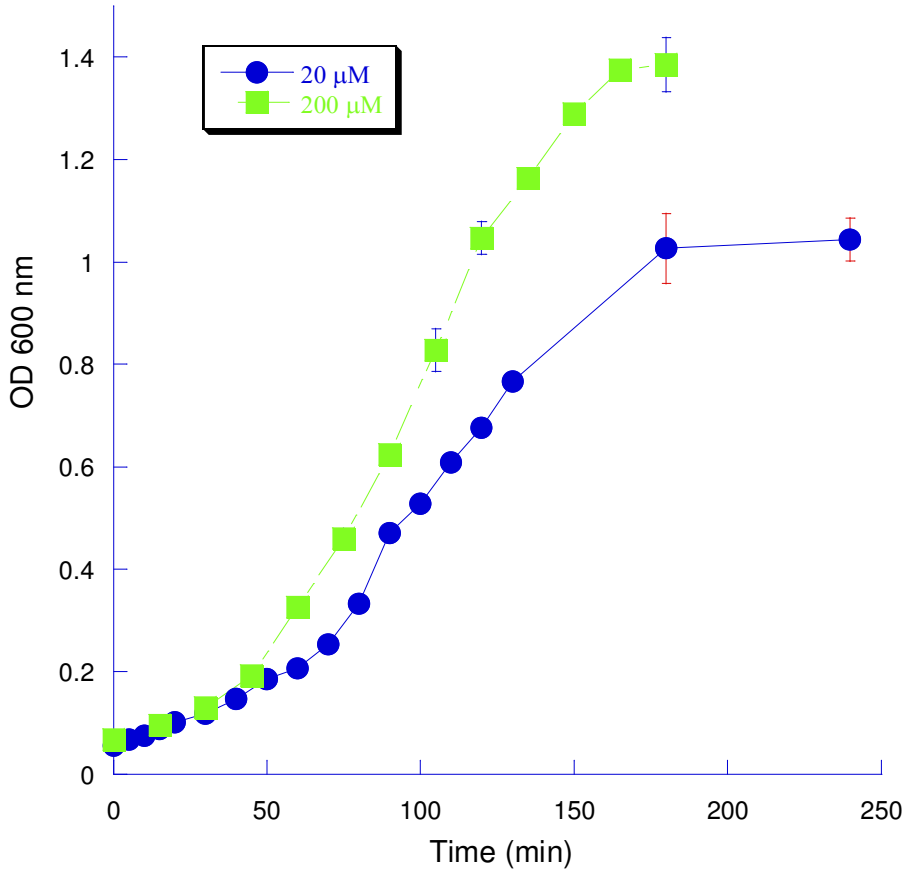


Fig. 5a. Growth effect with Tartrazine. 20 µM (blue), 200 µM (green). The data is a mean of three independent experiments.

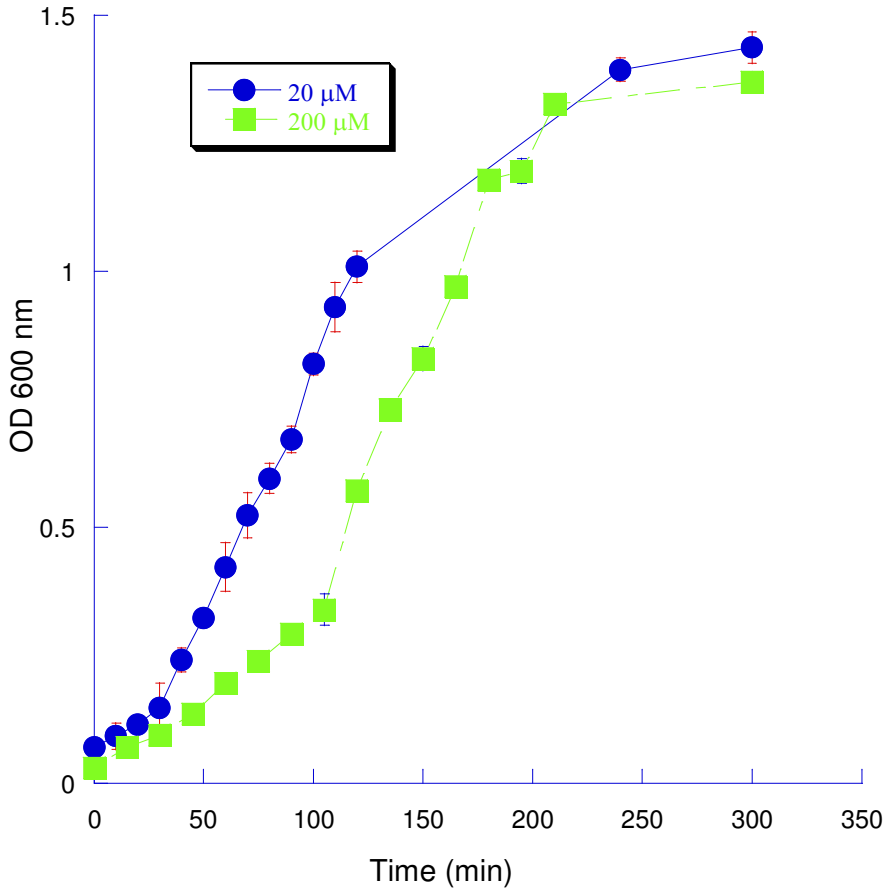


Fig. 5b. Growth effect with Direct Blue 15. 20 µM (blue), 200 µM (green). The data is a mean of three independent experiments.

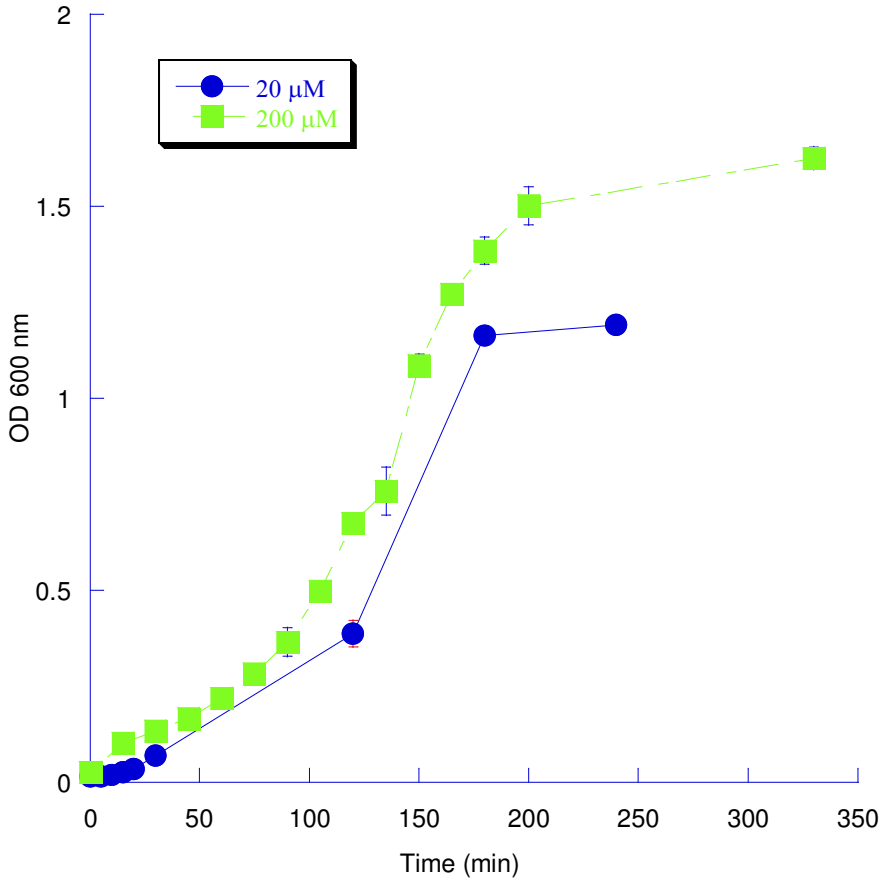


Fig 5c. Growth effect with Methyl Red. 20 µM (blue), 200 µM (green). The data is a mean of three independent experiments.

Table II

Azo dyes	**Generation time (min)**	
	20µM	**200µM**
	Mean (± SD)	Mean (± SD)
Control (no dye)	56.3 (± 4.7)	
Methyl Red	56.9 (± 0.4)	60.1 (± 1.2)
Tartrazine	48.7 (± 1.1)	40.1 (± 0.6)
Direct Blue 15	47.1 (± 2.6)	51.7 (± 4.0)

Table II. Generation time of *E faecalis* in the presence of various azo dyes.

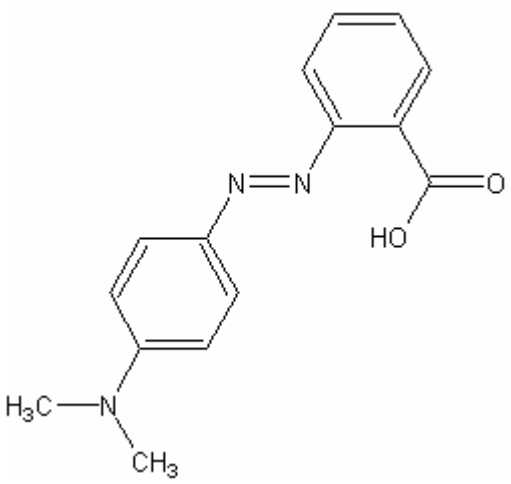
The cells were grown in BHIS anaerobically at 37°C. The control had cell growth in BHI without dye. The data is a mean of triplicates. SD – standard deviation

Discussion

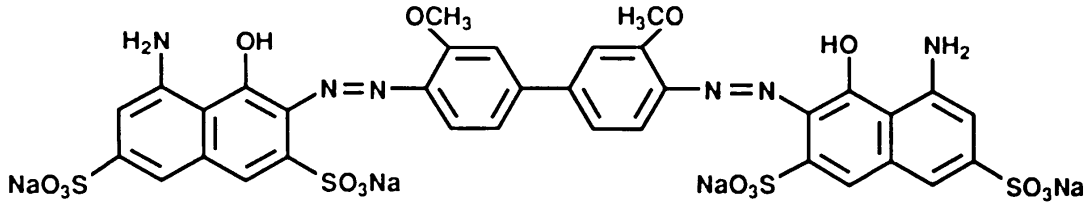
The role of intestinal microflora in health and disease is not fully understood and currently there is very little information available on the physiological effect of these azo dyes on them. This is the first study to show the kinetics of azo dye reduction at different physiological growth phases as well as the effect of these azo dyes on the growth rate of *E. faecalis.*

The milieu of the GI tract is rich in nutrients and the metabolic activity of the microflora is very high. *E. faecalis* is an important and major resident of the distal region of GI tract. Hence, in order to simulate a nutrient-rich and complex environment to study the azoreductase activity, BHIS was chosen. The colon, where *E. faecalis* resides, is similar to a chemostat since the flow of nutrients in a healthy adult is continuous and the microflora rarely encounters a nutrient poor situation in order to reach stationary phase (Gibson, 1994). Since the microflora are constantly dividing and are metabolically very active, it was reasoned that azoreductase activity would be highest in the log phase. The current study demonstrated, via kinetic analysis, that the reduction was highest when cells were actively growing in BHIS under anaerobic conditions (Table I)

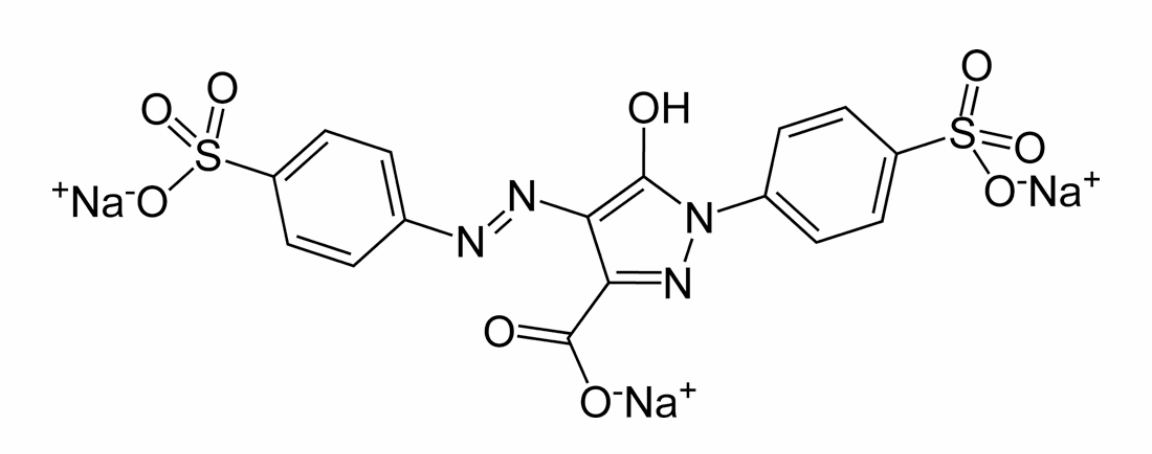
The three azo dyes used in this study were chosen because of their unique properties (Fig. 6). Methyl Red is a non-polar, mono azo dye, Tartrazine is a polar, sulfonated, mono azo dye and Direct Blue 15 is a polar, sulfonated, bis azo dye. It has been shown that the functional group in the vicinity of the azo bond can affect the rate of azo reduction (Walker and Ryan, 1971; Russ et al., 2000). In Methyl Red, the hydrogen of the hydroxyl group from the carboxylic acid in the *ortho* position can form a



Methyl Red



Direct Blue 15



Tartrazine

Fig.6 Structure of Methyl Red, Direct Blue 15 and Tartrazine.

hydrogen bond with the nitrogen from the azo bond, thus reducing the electron density around it. The reduced electron density around the azo bond may make it more amenable to reductive cleavage. Tartrazine and Direct Blue 15 have multiple sulfonic acid groups making them very hydrophilic. The close proximity of the sulfonic group and the methyl ester group in Direct Blue 15, both of which are *ortho* to the azo bond, may cause steric hindrance for azo bond cleavage. In Tartrazine, as compared to Direct Blue 15, the lack of bulky groups around the azo bond may lower steric hindrance, providing more access for reductive cleavage. A greater amount of Tartrazine may be entering the cell compared to Direct Blue 15, possibly due to Tartrazine (molecular weight: 534.4) being a smaller compound than Direct Blue 15 (molecular weight: 992.8). Since the redox potential of the three azo dyes is lower than the redox potential of oxygen; the data suggests that the presence of oxygen may sequester the electrons from the azo bond thereby reducing the rate of reduction. On the other hand, BHIS medium, which is complex and supports rapid growth of *E. faecalis*, may contribute indirectly by acting in concert with extracellular by-products of the metabolically active *E. faecalis* to reduce the azo dyes or the dyes may be supplemental electron acceptors. Therefore the physiological state of rapidly growing *E. faecalis* in BHIS may also contribute to the rate of azo dye reduction because metabolism and transport in the log phase is high. Currently, there is no information available on the effect of the bacterial growth phase on azo dye reduction and our results are the first to demonstrate this effect.

Further, the non-polar Methyl Red may enter the cell through simple or facilitated diffusion while the entry of the polar dyes Tartrazine and Direct Blue 15 are more difficult because the cell membrane is non-polar. In addition, their entry may be

further limited in the lag phase (PBSG medium) where metabolism and transport is low. The non-detectable and low azoreductase activity of Direct Blue 15 and Tartrazine respectively, in PBSG and very low reduction level in cell extracts may indicate that the high reduction rate in the complex BHIS medium in an anaerobic environment occurs by a non-enzymatic mechanism, since it is known that reduced inorganic compounds from microbial metabolism increase the reduction rate of azo dyes (Stolz, 2001). Rafii (Rafii et al., 1990) showed that azoreductase activity in *Clostridium perfringens* and other intestinal bacteria was irreversibly inactivated in the presence of oxygen, but our data shows that azoreductase activity in the presence of oxygen was only reduced but not inhibited (Fig. 3b, Table I). BHIS alone does not contribute to azo dye reduction, as observed in the control experiments which contained dye only and no cells.

Majority of the current research in this field has focused on identifying and isolating the azoreductase gene from the organisms. The azoreductase flavoprotein that was isolated from *E. faecalis* was specific for NADH and not NADPH (Chen et al., 2004). However, our results with cell extract show that azoreductase activity was observed with NADPH as well (Table I). This result is in agreement with an earlier study (Walker et al., 1971). It strongly suggests that there are other azoreductases present within a cell. However, the higher extracellular azoreductase activity observed for the sulfonated dyes indicates that the role of intracellular azoreductases in the reduction of these dyes is minimal.

The results have shown that the azo dyes tested did not inhibit growth and Tartrazine had the most effect on growth as it decreased generation time (Table II). Methyl Red had the highest overall kinetic rate of reduction under all conditions; except

in the cell extract using NADPH. Azoreductase activity for all three dyes was higher under anaerobic compared to aerobic conditions in BHIS. The reduction kinetics for sulfonated azo dyes, Direct Blue 15 and Tartrazine was lowest in PBSG; which may be due to the lack of actively dividing cells in that medium. Thus, the physiological state of the *E. faecalis* cells in PBSG (lag-phase) may prevent the sulfonated azo dyes from entering the cells.

The azoreductase activity of *E. faecalis* is a complex process and can not be generalized for all azo dyes. In addition, there maybe multiple enzymes and metabolic by-products that are involved in this process. Future work will involve the identification of an NADPH-dependent azoreductase.

CHAPTER IV

CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF A PUTATIVE OXIDOREDUCTASE FROM *ENTEROCOCCUS FAECALIS*

Abstract

Azoreductases reduce the azo bond (N=N) in azo compounds. They do not share significant homology at the primary structure level which makes identification and classification difficult. Based on domain homology, a putative oxidoreductase was identified from the proteome of *E. faecalis* with potential azoreductase activity. The gene coding for the putative oxidoreductase was cloned, expressed and purified from *E. coli.* No azoreductase activity was detected and possible reasons for inactivity are discussed.

Introduction

The microorganisms in the human intestine have the ability to metabolize a variety of xenobiotics, including azo dyes. Azo dyes are utilized in the textile, pharmaceutical, cosmetic and food industry. Since thousands of tons of azo dyes are being used daily, a majority of the studies on the microbial degradation of these dyes are focused on bioremediation. However, the exposure of these dyes to humans is also a concern as these dyes can be mutagenic and carcinogenic (Chung et al., 1992; Rafii et al., 1999).

Studies have shown that azo dyes can be reduced by rat liver microsomal cytochrome P450 (Zbaida and Levine, 1990), producing aromatic amines that are activated by human cytochrome P450 such as CYP1A2 (Guengerich and Shimada, 1998). Azo dyes can also be reduced by microbes under both aerobic and anaerobic conditions. Initial studies on azo dye reduction focused on the identification of microbes that reduce azo compounds. However, with the advances in whole genome sequencing, the focus has shifted towards identifying the gene that codes for the azoreductase. In addition, it is possible to identify potential candidate genes and predict their product's function in several organisms using sophisticated bioinformatics tools. To gain insight into the evolution of this enzyme, a number of azoreductases have been identified in order to obtain homologs which support a common evolutionary origin.

Enterococcus faecalis is a Gram-positive facultative anaerobe present in the intestine. An azoreductase from *E. faecalis*, Azo A, has been cloned in *E. coli* and characterized (Chen et al., 2004). Azo A was shown to be an aerobic azoreductase that

was dependent on NADH only. However, a previous study has shown that the crude extracts of *E. faecalis* can utilize NAD(P)H to reduce Red 2G (Gingell and Walker, 1971). Hence, other azoreductases may be present that utilize NADPH for activity.

Our interest was to identify an NADPH-dependent azoreductase. Based on common structural motifs found in several azoreductases, a putative azoreductase was identified. The putative azoreductase was heterologously expressed and tested for activity.

Materials and Methods

*Bacteria and media***.**

Enterococcus faecalis ATCC 27274 was grown in Brain Heart Infusion (Difco) broth at 37°C under anaerobic conditions.

Identification of protein domains

The UniProtKB/TrEMBL database was used to select biochemically characterized azoreductases. Protein domains were identified from the Pfam database which contains multiple sequence alignments and profile-Hidden Markov Models (HMM) for a variety of protein domains. The proteins containing the domains of interest were identified from the species distribution list in the Pfam database.

Cloning of azoB gene

Genomic DNA from *E. faecalis* was isolated as described previously (Pospiech and Neumann, 1995). Polymerase chain reaction (PCR) was performed using genomic DNA as template. The forward primer 5'- cagccatatgaaaaaaattatcggc-3' contains an *Nde*I site upstream of the start codon. The reverse primer 5'-gatggatccgcgaatcagcgtttgatccgac-3' contains a *Bam*HI site after the stop codon of *azoB*. Thirty cycles were carried out for the PCR with the following conditions: melting temperature, 94°C for 1 min.; annealing temperature, 50°C for 1 min.; and extension temperature, 72°C for 1 min using *Taq* DNA polymerase. A single cycle of the final extension at 72°C was carried out for 7 min. The amplified product was observed on 0.8% agarose gel.

This amplified product was cloned into the pCR2.1-TOPO vector and transformed into the TOP10 competent cells provided with the TOPO TA cloning kit (Invitrogen). The cloned fragment was sequenced. The plasmid containing the insert was digested with *Nde*I (Invitrogen) and *Bam*HI (Invitrogen) to release the fragment, which was then subcloned into pET15b, an expression vector. The pET15b vector has a hexa-Histidine tag which allows purification of the expressed protein. The positive recombinant pET15-b (pAzoB) clone was screened and identified using colony PCR. In order to express the protein, the pAzoB plasmid was isolated using the Qiagen miniprep kit (Qiagen) and transformed into NovaBlue DE3 expression cells (Novagen). The positive transformed cloned was identified using amplicillin as the selection factor and frozen at -80°C.

Expression and purification of AzoB

The -80°C frozen NovaBlue DE3 expression cells containing pAzoB and the same expression cells without plasmid (control) were grown in 5 mL Luria-Bertani (LB) broth containing the appropriate antibiotic selection for 10 h at 37°C. These cultures were subsequently transferred to 1 L LB broth. The flasks were incubated on a shaker at 37°C and 300 rpm until the $OD_{600} = 0.6$. Isopropyl-1-thio-β-galactopyranoside (IPTG) was added (1 mM final concentration) to each flask and incubated under the same conditions for 3 h. The cells were then harvested at 4000 x g for 20 min at 4°C. The spent medium was discarded and the pellet was frozen at -20°C.

The pellet was thawed and resuspended in 10 mL of lysis buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750 mM sodium chloride, 20 mM Imidazole in

double-distilled water, pH 8.0). Lysozyme was added to the tube containing the resuspended cells to a final concentration of 1 mg/mL and the tube was incubated on ice for 30 min. These cells were transferred to a prechilled 50 mL glass beaker and sonicated using an intermediate tip at 60% output (Artek systems corporation). Five second pulses were repeated ten times with two minute incubation time on ice between each pulse. The sonicated sample was centrifuged at 4°C for 30 min at 10,000 x g. The supernatant was collected and subsequently purified. Expression of the protein was determined on a 12.5% SDS-PAGE gel.

All the purification steps were carried out at 4°C. Two mL of 50% (in lysis buffer) Ni-NTA superflow resin was used for every 8 mL of supernatant in a 15 mL tube. Binding of the His-tagged protein to the resin occurred by gently mixing at 4°C for 1 h. The content was centrifuged for 2 min at 3,900 rpm using a clinical centrifuge. The supernatant was collected and the resin was washed twice with 8 mL lysis buffer and each time the contents were centrifuged under the same condition as above. The protein was eluted with 2 mL elution buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750 mM sodium chloride, 250 mM Imidazole in double-distilled water, pH 8.0) four times and each time the centrifugation steps were as above.

All the elution samples were pooled and dialyzed for 6 h in 2 L of dialysis buffer (50 mM potassium phosphate buffer, pH 7.0, 10 mM magnesium chloride, 10 mM sodium chloride and 10% glycerol) and then in fresh 2 L dialysis buffer overnight. The protein concentration was estimated to be 0.13 μg/ μL using BSA as the protein standard.

Native molecular weight determination

Discontinuous polyacrylamide gel electrophoresis (PAGE) was used to determine the native molecular weight under non-denaturing conditions as described by Laemmli (Laemmli, 1970). The resolving mini-gels used were 6, 8, 10, 11 and 12% which were prepared in 1.5 M Tris-Cl, pH 8.8. Electrophoresis was carried out in a Mini-Protean II unit (Bio-Rad) at 4°C in Tris-Glycine buffer, pH 8.3. No sodium dodecyl sulfate (SDS) was added to any of the buffers. After electrophoresis, the gels were stained in Coomassie Blue R-250 and destained in Methanol:acetic acid:water (v/v/v) (2:3:15). The mobilities for standards and AzoB were determined. The standards used to determine the molecular weight were lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken albumin (45 kDa), bovine serum albumin monomer (66 kDa) and bovine serum albumin dimer (132 kDa). The Furgeson plot was constructed with mobility i.e., [100.log (*Rf* .100)] for each protein vs. % gel. The slopes of these were subsequently used to construct a plot against the known molecular weights of the standards to determine the native molecular weight of AzoB.

AzoB enzyme activity for methyl red

The assay was carried out in a quartz cuvette in a total reaction volume of 1 mL. The reaction was initiated by the addition of 0.5 mM NADPH. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 50 μM of Methyl Red, 10 μM FMN and enzyme. The extinction coefficient for Methyl Red at 430 nm was 23,360 $M^{-1}cm^{-1}$. The enzyme activity was measured by a decrease in absorbance at 430 nm over a 5

min period using a UV-visible spectrophotometer (UV-1601PC, Shimadzu). The negative control assay was performed under the same conditions as above except that Bovine serum albumin (BSA) was used in place of the enzyme. The azoreductase from *E. faecium* was used as the positive control. One unit (U) of enzyme activity was defined as the reduction of 1 μmol of methyl red per minute.

Results and Discussion

Identification of a putative NADPH-specific azoreductase in E. faecalis

There are no conserved regions in the primary sequence of azoreductases. However, domain motifs were used to identify commonalities between the bacterial azoreductases. Table III shows the domain and cofactor requirements for different azoreductases. A majority of the azoreductases are categorized into one of two domains, flavodoxin_2 domain (PF02525) and FMN_red domain (PF03358) as identified by the Pfam database. These two domains fall into the same clan of flavoproteins.

The AzoA in *E. faecalis* is the only protein with a flavodoxin_2 domain in its proteome. However, our analysis showed there are three other proteins with an FMN_red domain that have not been characterized yet which are EF1226, EF1698 and EF1227. Recently, EF1226 was suggested to be an ancestral azoreductase in *E. faecalis* based on a BLAST comparison with the azoreductase from *Geobacillus stearothermophilus* (Bafana and Chakrabarti, 2008). Therefore, EF1226 (*azoB*) was chosen as the candidate gene which may encode for the protein with NADPH-dependent azoreductase activity.

The genomic locus of *azoB* indicates that it is flanked by two genes. The open-reading frame (ORF) upstream of *azoB* is a putative thiamin biosynthesis lipoprotein, *apbE* (ORF1) and the ORF downstream is a hypothetical protein (ORF3). There are 27 nucleotides between the stop codon of the upstream ORF1 and the start codon of *azoB* and 19 nucleotides between the stop codon of *azoB* and the start of the downstream ORF3. The 27 nucleotides between ORF1 and *azoB* do not contain a conserved promoter binding site (TATA box) but contain a putative ribosomal binding site (AGGA).

However, there is a 'TATATA' sequence within ORF1 which is approximately 50 nucleotides upstream of the stop codon. The 19 nucleotide region between *azoB* and ORF3 does not contain a consensus Shine-Dalgarno or promoter sequence.

A BLAST search with the AzoB protein sequence did not produce any hits with an azoreductase using a threshold E value less than 0.1. All the matches were annotated as hypothetical or putative oxidoreductases.

Table III

Organism	**Domain**	**Cofactor Requirements**		
		Electron Donor		
		FMN	**NADH**	**NADPH**
E. faecalis (AzoA)	Flavodoxin_2	+	+	-
S. aureus (Azo1)	FMN_red	+	-	+
E. coli (AzoR)	Flavodoxin_2	+	+	-
P. aeruginosa (paAzoR1)	Flavodoxin_2	+	+	+
Bacillus OY 1-2	FMN_red	-	ND*	+
R. sphaeroides AS1.1737	FMN_red	-	+	ND*
E. agglomerans	NA[a]	-	+	ND*
X. azovorans	NmrA	-	+	+
P. kullae	COG2910/NmrA	-	+	+

Table III. Domain and cofactor requirements for azoreductases.

ND* not detected;

NA[a] not available

(+) required; (-) not required

Cloning and expression of AzoB in E.coli

The primers for EF1226 produced an amplicon of 664 base pairs and the PCR product was successfully cloned and subcloned into the TOPO TA and pET15-b cloning vectors. Figure 7 shows protein profiles including the expressed protein that was purified to apparent homogeneity.

The DNA sequence of the cloned *azoB* gene when compared to the genomic sequence was identical, however when the gene sequence of this strain was compared to sequence of strain V583 in the database, the translated sequence showed an amino acid residue change from glutamate (E) to aspartate (D) (Fig 8).

Native molecular weight determination of AzoB

The native molecular weight of AzoB was determined using 6, 8, 10, 11 and 12% non-denaturing resolving gels. The slopes for each protein standard as well as AzoB were determined from the Furgeson plot (Fig 9a) and subsequently plotted against molecular weight (Fig 9b). Based on these plots, the native molecular weight of AzoB was shown to be 27 kDa. The theoretical molecular weight is 23 kDa. Thus, the results suggest AzoB is a monomer. The slight increase in the empirically determined molecular weight is due to the hexa-histidine tag associated with the protein. AzoB seems to be similar to the azoreductase of *Enterobacter agglomerans* (Moutaouakkil et al., 2003) and *Xenophilus azovorans* KF46F (Blumel et al., 2002).

The spectrum of AzoB showed a single peak at 278 nm. This demonstrated that no flavin moiety was associated with the protein.

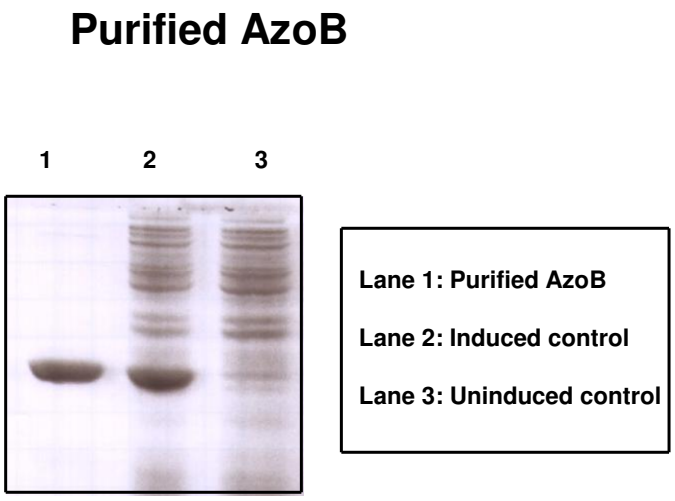


Fig. 7. AzoB purification profile.12.5% SDS-PAGE of heterologously expressed and purified AzoB from *E. coli*

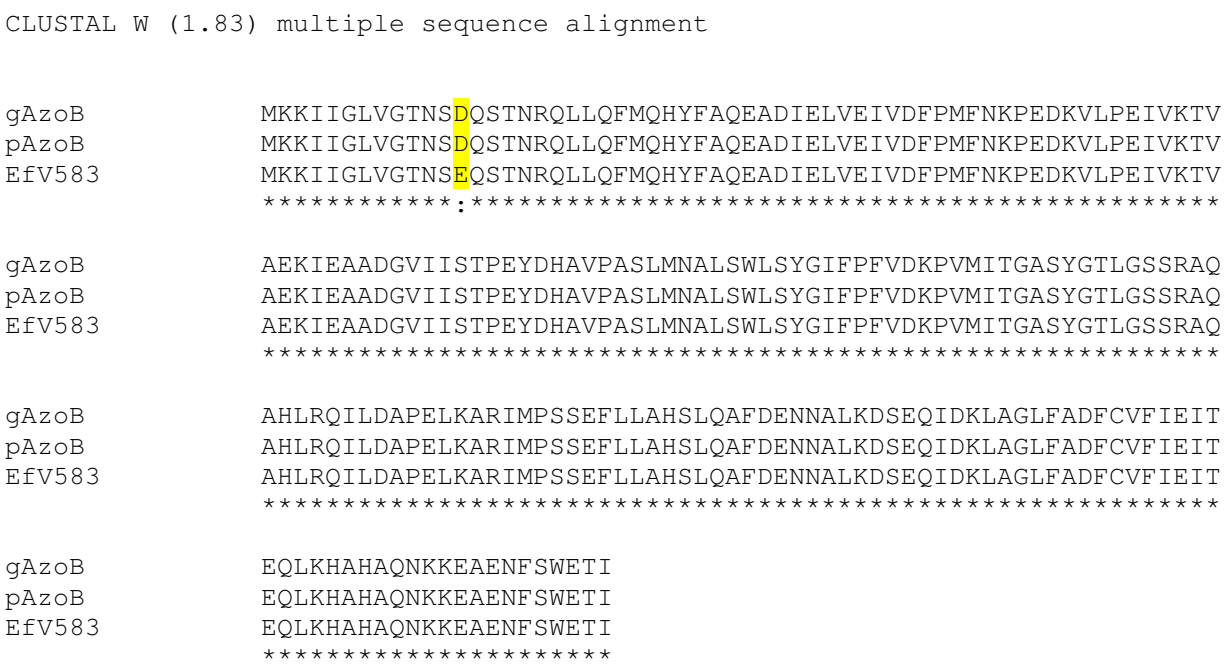


Fig. 8. Clustal W alignment of AzoB. Deduced AzoB protein sequence from genomic DNA (gAzoB), cloned gene (pAzoB) and the strain V583 present in the NCBI database (EfV583). The amino acid residue change is shown in yellow.

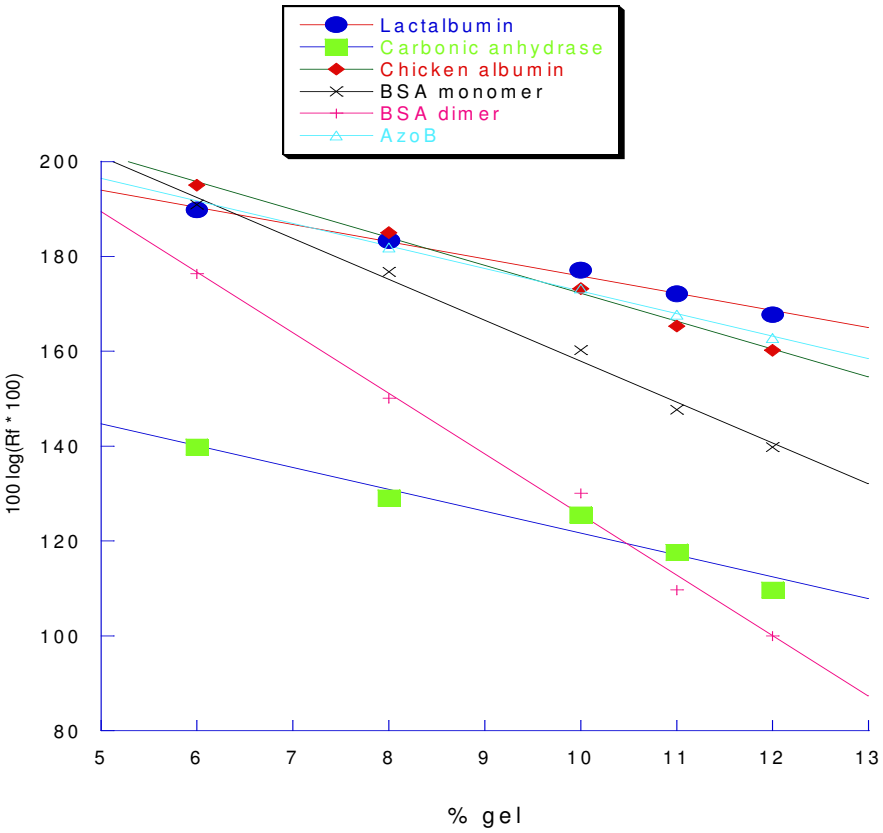

Lactalbumin
Carbonic anhydrase
Chicken albumin
BSA monomer
BSA dimer
AzoB
200
180
160
140
120
100
80
100 log(Rf * 100)
5
6
7
8
9
10
11
12
13
% gel

Fig. 9a

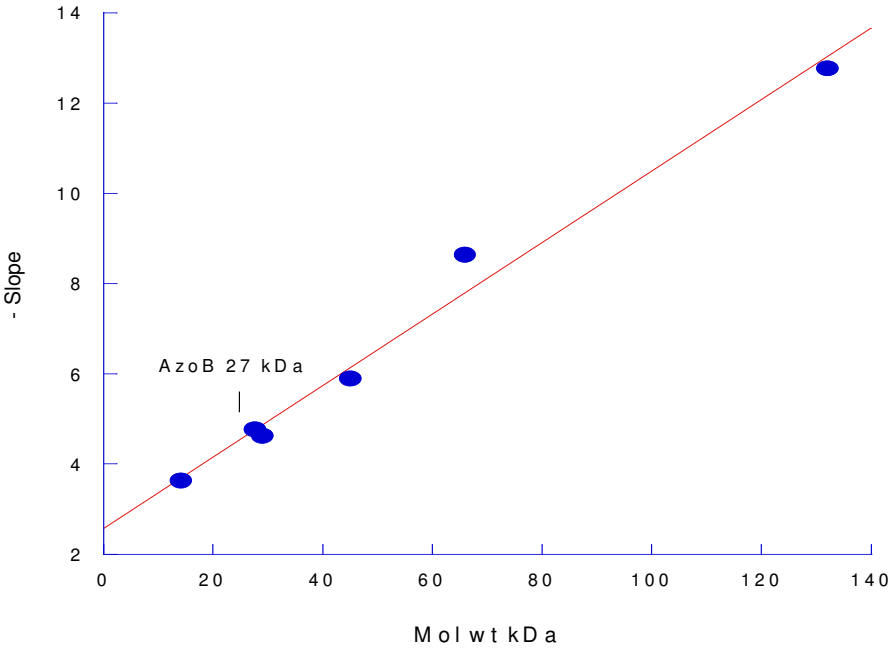

14
12
10
8
6
4
2
- Slope
AzoB 27 kDa
0
20
40
60
80
100
120
140
Mol wt kDa

Fig 9b

Fig. 9. a) Furgeson plot. Plot generated from the mobilities of AzoB and protein standards against resolving gel percentage. b) Native molecular weight determination of AzoB. Plot of slopes, determined from the Furgeson plot vs. molecular weight.

Enzyme activity

AzoB did not reduce Methyl Red, Direct Blue 15, Tartrazine and Acid Red 88 in the presence of FMN and NADPH for 45 min at 25 °C. The enzyme was then preincubated with each substrate and FMN for 5 min at 37 °C, followed by the addition of NAD(P)H to initiate the reaction, and no activity was detected. The overexpressed enzyme was purified using conditions which minimize denaturation/loss of activity (i.e, at pH 8.0 and 4 °C). A close analysis of the sequence of the cloned insert did not show any in frame-shift mutations. The same procedure and conditions proved successful for another azoreductase, *E. faecium,* which rules out technical issues that may contribute to the nonfunctioning AzoB enzyme. It was thus concluded that AzoB was ineffective as an azoreductase.

There may be several reasons that could contribute to the lack of azoreductase activity. First, it is not known whether the enzyme is expressed in the wild-type cell since the BLAST search did not identify any functional homologs. An *in silico* based annotation of a gene need not necessarily predict its correct functional status. Biochemical characterization of the annotated gene product is needed to confirm its function. Secondly, it is likely that the *apbE-azoB*-hypothetical gene is part of an operon and the putative oxidoreductase (AzoB) may require the presence of these other gene

products to be active, as this gene cluster (putative thiamin biosynthesis lipoprotein, *apbE,* putative oxidoreductase (AzoB in this study) and a hypothetical protein) is present in 11 different species of closely related bacteria which include *Streptococcus, Lactococcus and Lactobacillus*. Third, the presence of the His-tag at the N-terminal may have interfered with its function. Although the protein was overexpressed in *E. coli*, it may not have folded correctly since the cellular environment of the Gram-negative *E. coli* is different from its Gram-positive native host, *E. faecalis.* Lastly, the *azoB* gene product may have lost its functional ability through evolutionary changes.

In conclusion, EF1226 from *E. faecalis* was heterologously expressed and purified from *E. coli* but did not reduce any of the azo dyes tested thus far. However, using a similar strategy to identify azoreductases based on common domain matches, an azoreductase from *E. faecium* was successfully purified and characterized. Hence, the use of domain homology is an additional criterion to identify azoreductases but should not be considered as the only canon in this regard.

CHAPTER V

PURIFICATION AND IDENTIFICATION OF AN FMN-DEPENDENT NAD(P)H AZOREDUCTASE FROM *ENTEROCOCCUS FAECALIS*

Abstract

Azoreductases reduce the azo bond (N=N) in azo dyes to produce colorless amine products. The products produced upon reduction of azo dyes can become environmental pollutants and some may be carcinogenic as well. Azoreductases have been found in bacteria, fungi and mammals. However, their primary amino acid structures have very little homology, which makes classification difficult. The flavin-dependent azoreductases are grouped into those that utilize NADH, NADPH or both [NAD(P)H] as electron donors for azo dye reduction. A heterologously expressed azoreductase from *E. faecalis* (AzoA) in *E. coli* was previously shown to utilize NADH and not NADPH for azo dye reduction. However, crude cell extracts from *E. faecalis* have shown activity with NADPH. Thus, the native azoreductase that utilized NADPH was purified by hydrophobic, anion exchange and affinity chromatography. An assay was performed on a polyacrylamide gel after electrophoresis under native conditions and the protein that decolorized the azo dye Methyl Red with NAD(P)H was identified by mass spectrometry to be AzoA. Kinetic studies showed that AzoA had a preference for NADH over NADPH and its specific activity was more than 100-fold higher compared to the previous study using NADH as the electron donor.

The K_m for NADPH was more than 150-fold greater than the Km for NADH. This is the first study to show that AzoA can utilize NAD(P)H to reduce Methyl Red.

Introduction

Azoreductases are enzymes that reduce the azo bond (N=N) in azo dyes to produce colorless amine products (Stolz, 2001). These dyes are widely used in the paper, textile, food, cosmetic and pharmaceutical industries, and are regarded as environmental pollutants which danger human health due to their role in carcinogenesis (Chung and Cerniglia, 1992; Stolz, 2001; Chen, 2006).

Although azo dyes are anthropogenic, azo dye reduction is observed in many organisms by enzymes which include rat liver enzyme cytochrome P450 (Zbaida and Levine, 1990), rabbit liver aldehyde oxidase (Stoddart and Levine, 1992) and azoreductases from the intestinal microbiota, environmental bacteria and fungi (Chen, 2006) as well as halotolerant and halophilic organisms (Asad et al., 2007).

Bacterial azoreductases from various organisms are very diverse and variation exists even among these enzymes within the same organism (Ghosh et al., 1992). For example, very little homology at the primary amino acid level exist. However, a classification scheme based on the secondary and tertiary amino acid analysis has been developed (Abraham KJ, 2007). Based on function, another classification scheme has been developed in which azoreductases are categorized as either flavin-dependant azoreductases (Nakanishi et al., 2001; Chen et al., 2004; Chen et al., 2005) or flavin-independent azoreductases (Blumel et al., 2002; Blumel and Stolz, 2003).The flavin-dependent azoreductases are further organized into three groups; NADH only (Nakanishi et al., 2001; Chen et al., 2004), NADPH only (Chen et al., 2005) or both (Ghosh et al., 1992; Liu et al., 2007; Wang et al., 2007) as these coenzymes serve as electron donors.

E. faecalis is a Gram-positive opportunistic pathogen present in the intestine of mammals. The heterologously expressed and purified azoreductase, AzoA has been characterized (Chen et al., 2004) and recently the enzyme was crystallized (Liu et al., 2007). According to Chen *et al.*, AzoA is an FMN-dependent azoreductase that can use only NADH as an electron donor. However, our studies on crude cell extracts from wild-type as well as previous studies (Walker and Ryan, 1971) have shown that azoreductase activity involves NADPH as well as the electron donor. This observed activity could be due to the presence of other NADPH-specific azoreductases or altered activity caused by the heterologously expressed enzyme.

The current study investigated the observed NADPH activity from a cytoplasmic fraction obtained from *E. faecalis.* The results describes the purification and identification of a native azoreductase (AzoA) from *E. faecalis* and its pseudo-first order kinetic rates using NADH, NADPH, FMN and the azo dye Methyl Red as substrates.

Materials and Methods

E. faecalis ATCC 27274 was grown in Brain Heart Infusion (BHI) medium (Difco Laboratories, Spark, MD, USA). For long term storage, 16% glycerol stocks of the organism were prepared and stored at -80°C. Methyl Red (MCB Chemicals, Norwood, Ohio, USA; molecular weight, 269.31, $2[4\text{-}(CH_3)_2NC_6H_4N{:}N]C_6H_4CO_2H$, CAS registry 63451-28-5) was used as the azo dye under investigation. NAD(P)H (> 99% purity) was from Calbiochem (San Diego, CA, USA) and FMN was from Sigma (St. Louis, MO, USA). Chromatography resins used were Octyl Sepharose™ 4 Fast flow from Amersham Biosciences (Piscataway, NJ, USA) and DEAE Bio-Gel® agarose 100-200 mesh and Affi-Gel® Blue Gel 100-200 mesh from Bio-Rad (Hercules, CA, USA). The chromatography columns used for hydrophobic interaction chromatography and ion exchange chromatography were Flex-Column® from Kimble/Kontes (Vineland, NJ, USA)

Purification of azoreductase

Preparation of cell extract: A single colony of *E. faecalis* grown on a BHI plate was used to inoculate 5 mL of BHI broth. The culture was grown at 37°C for 12 h and 1 mL was transferred to 20 L of BHI broth. The 20 L culture was incubated at 37°C untill an $OD_{600} = 0.9$ (late log phase) was reached. The cells were harvested at 6000 x g for 10 min at 4°C. The bacterial pellet was resuspended in buffer A (50 mM potassium phosphate buffer, pH 7).

Lysozyme and DNase I were added at final concentrations of 1 mg/mL and 10 μg/mL respectively, and the sample was incubated at 37°C for 20 min. The sample was sonicated on ice at 60% output using a Sonic 300 Dismembrator (Artek systems corporation, farmindale, NY, USA) for a total of 6 min with 20 second pulses and phenylmethanesulphonylfluoride (PMSF, 1 mM final concentration) was immediately added. The lysate was clarified by centrifugation at 100,000 x g for 30 min at 4°C and the pellet was discarded. Ammonium sulfate was added at 4°C to the clarified cell extract to a final concentration of 0.5 M and EDTA was added to a final concentration of 0.5 mM. Note: All subsequent purifications steps were carried out at 4°C.

Hydrophobic Interaction Chromatography (HIC) – The sample was applied at a linear flow rate of 8.5 cm/h to an octyl sepharose 4 flast flow column (2.5 x 15.5 cm) that was equilibrated with buffer B (50 mM potassium phosphate buffer, pH 7; 0.5 mM EDTA; 0.5 M ammonium sulfate). After applying the sample, the column was washed with two volumes of 25 mM potassium phosphate buffer, pH 7 and the bound protein was eluted with 30% isopropanol. The eluted protein was concentrated to 20 mL and diafiltrated with 200 mL of buffer C (10 mM Tris-Cl, pH 8, the pH of Tris-Cl was adjusted at 4°C with HCl) in an ultrafiltration cell (Amicon Inc., Beverly, MA, USA) using a 10,000 NMWL membrane (Millipore Corporation, Bedford, MA, USA).

Ion Exchange Chromatography (IEX) – The sample was diluted with buffer C to obtain a final protein concentration of 7.5 mg/mL and applied at a linear flow rate of 7 cm/h to a DEAE Bio-gel agarose column (2.5 x 10.5 cm) which was previously equilibrated with buffer C. The column was washed with 300 mL of buffer C. The

protein was eluted with 300 mL of buffer C containing a linear gradient of NaCl from 0-200 mM. The fractions (6 mL each) that had the highest azoreductase activity were pooled together and concentrated to 11 mL.

Affinity Chromatography – The pooled and concentrated sample was applied at a linear flow rate of 9 cm/h to Affi-gel Blue gel (2 x 1.5 cm) that was equilibrated with buffer C. The column was washed with 12 mL of buffer C containing 1 M NaCl. The bound protein was eluted with 12 mL of 30% isopropanol containing 3 M NaCl. The sample was concentrated and diafiltrated with 100 mL of buffer A. The enzyme fraction was aliquoted and stored at – 20°C in 50% glycerol.

Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard.

Azoreductase activity assay

Qualitative assay – A preparative mini 5-12.5% gradient resolving polyacrylamide gel was prepared according to the method by Laemmli (Laemmli, 1970), without the addition of sodium dodecyl sulfate (SDS) using a Mini-PROTEAN® II apparatus (Bio-Rad Hercules, CA, USA). The protein sample from the affinity chromatography fraction (0.3 mg/mL) was loaded in the single preparative reference well. Electrophoresis was performed at 4°C in Tris-glycine buffer, pH 8.3 at 6 milliamps constant current. After electrophoresis, the gel was split down the middle and each half was placed in two shallow trays. To one tray, 5 mL buffer DH (Tris-Cl, pH 7.5; 0.5 mM Methyl Red; 10 μM FMN; 1 mM NADH) was added and to the other tray, 5mL buffer

DPH (Tris-Cl, pH 7.5; 0.5 mM Methyl Red; 10 μM FMN; 2 mM NADPH) was added. Each tray was incubated for 10 min at room temperature with gentle shaking to assure that solutions were evenly distributed across the gels. After the zones of clearing were observed, the center and the periphery of the clear zone on both gels were marked by a notch and the mobility of the clearing zone calculated. The gels were subsequently directly stained by Coomassie Blue R-250 and using the notch as the reference point, the mobility of the visible protein band was determined. Mobility was calculated as the ratio of the distance of center of clearing zone (protein) to the distance traveled by the tracking dye.

Quantitative assay - The assay was carried out in 1 mL cuvettes and contained 50 mM phosphate buffer, (pH 7.2); 20 μM Methyl Red; 20 μM FMN; 0.5 mM NADPH and an appropriate amount of enzyme (1-800 μg). The reaction was initiated by adding NADPH. The enzyme activity was determined by an absorbance reduction in the first 2 min at 430 nm for Methyl Red using a UV-Visible spectrophotometer (UV-1601PC, Shimadzu, Columbia, MD, USA). The extinction coefficient for Methyl Red used was 23,360 M^{-1} cm^{-1}. A unit (U) of azoreductase activity was defined as the amount of enzyme required to reduce 1 μmol per min of Methyl Red.

Protein Identification

Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF): The protein band corresponding to the zone of clearing seen in Fig. 10B was excised and washed with 50% acetonitrile/25mM ammonium bicarbonate (pH 8.0). The sample was

dehydrated with 100% acetonitrile and subsequently digested with a solution of 15 µg/ml sequencing-grade trypsin in 25 mM bicarbonate buffer. The peptides were extracted with 0.5% trifluoroacetic acid (TFA) mixed with α-cyano-4-hydroxycinnamic acid and analyzed in reflectron mode using a Voyager-DE™ PRO Biospectrometry Workstation (PerSeptive Biosystems, Inc., USA). Spectra were deisotoped using a detection threshold that was manually adjusted to exclude spectral noise, and the result peak list was used to search the MSDB database (3239079 sequences) using the Mascot search engine (Matrix Science, London, UK). Search parameters included a statement of 100 ppm mass accuracy and the amino acid modifications: Met oxidation, pyroglutamate cyclization of glutamine, and acrylamide adducts of Cysteine.

Capillary HPLC-electrospray ionization tandem mass spectra (HPLC-ESI-MS/MS): The HPLC-ESI-MS/MS data for the trypsin digested sample were acquired on a Thermo Fisher LTQ linear ion trap mass spectrometer fitted with a New Objective PicoView 550 nanospray interface. On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC micro HPLC: column, PicoFrit™ (New Objective, Woburn, MA, USA; 75 µm i.d.) packed to 10 cm with C18 adsorbent (Vydac, Grace, Deerfield, IL, USA; 218MS 5 µm, 300 Å); mobile phase A, 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA); mobile phase B, 90% acetonitrile/0.5% HAc/0.005% TFA; gradient 2 to 42% B in 30 min; flow rate, 0.4 µl/min. MS conditions were: ESI voltage, 2.9 kV; isolation window for MS/MS, 3; relative collision energy, 35%; scan strategy, survey scan followed by acquisition of data dependent collision-induced dissociation (CID) spectra of the seven most intense ions in the survey scan

above a set threshold. The uninterpreted CID spectra were searched against the NCBInr database [20080310 (6298708 sequences; 2152697077 residues)] by means of Mascot (Matrix Science). Variable modifications considered for the searches included: oxidation of His, Met, and Trp; Trp conversion to kynurenin. Determination of protein identity probabilities were accomplished by Scaffold™ (Proteome Software, Portland, Oregon, USA). Scaffold verifies peptide identifications assigned by SEQUEST and Mascot using the X!Tandem database searching program (Craig and Beavis, 2003). Scaffold then probabilistically validates these peptide identifications using PeptideProphet (Keller et al., 2002) and derives corresponding protein probabilities using ProteinProphet (Nesvizhskii et al., 2003).

Pseudo-first order kinetics

To determine the apparent pseudo-kinetic parameters, the concentration of one substrate was varied and the other substrates' remained constant. The constant concentration for Methyl Red was 20 μM and the varying concentrations were 2 - 20 μM. For NADH, the constant was 1 mM and varying concentrations were 10 - 80 μM. For FMN, constant concentration was 20 μM and varying concentrations were 0.5 - 5 μM. For NADPH the varying concentrations were 1.5 - 10 mM. The parameters were determined from the double reciprocal plot using the KaleidaGraph software (Synergy software).

Results

Enzyme purification

Methyl Red, NADPH and FMN were used for the azoreductase assays during all the purification steps. The initial specific azoreductase activity in the crude cytoplasmic extracts was 0.003 U/mg. A combination of hydrophobic interaction, ion-exchange and affinity chromatography was used to purify over 200 – fold of the native azoreductase with a recovery of 10% (Table IV). The protein concentration throughout the purification was determined by the Bradford method.

Native PAGE assay for azoreductase

The affinity chromatography purified fraction was subjected to electrophoresis at 4°C on a discontinuous native PAGE gel. The gel was divided in half and each half was incubated separately at room temp with 1 mM NADH and 2 mM NADPH to determine which reduced pyridine nucleotide could be utilized as an electron donor. A clearing zone, indicating Methyl Red reduction, was observed in both gels (Fig. 10 Aa and Ab). The mobility of the clear zones and the Coomassie Blue stained bands on both gels were the same which suggested that the same enzyme could utilize NAD(P)H as electron donors to reduce Methyl Red (Fig 10 A and B). However, the zone of clearing was larger when NADH was used as the electron donor (Fig. 10 Aa) as compared to when NADPH was used as the electron donor (Fig. 10 Ab). This was attributed to a much greater affinity for NADH by the enzyme as the electron donor as compared to NADPH. These

Table IV

Step	Total protein (mg)	Total activity U/ mg total protein	Specific activity U/mg	Yield (%)	Purification (fold increase)
Extract	2080	6.2	0.003	100	1
Octyl-sepharose 4 fast flow	203	2.8	0.014	45	5
DEAE Bio-gel	10	2.2	0.224	35	75
Affi-gel	1	0.63	0.626	10	209

Table IV. Summary of azoreductase purification

Note: All enzyme assays were performed with 0.5 mM NADPH, 10 μM FMN, 20 μM Methyl Red in 50 mM Potassium Phosphate buffer, pH 7.0 at 25°C.

One unit of enzyme activity is defined as the amount of enzyme required to reduce 1μmol of Methyl Red per minute.

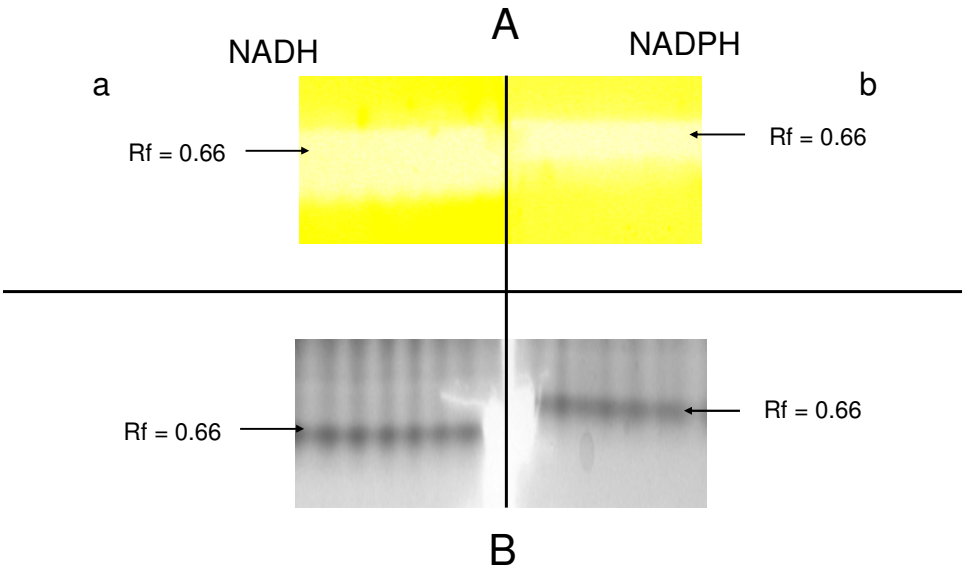


Fig 10. Native gel assay: A) 5-12.5% gradient native PAGE incubated with 0.5mM Methyl Red + 10 µM FMN, a - 1mM NADH, b - 2mM NADPH. B) Gel Aa and Ab stained with Coomassie Blue

Note: Gel with single large well was divided in half and incubated in separate conditions

Table V

Azo Dye	Specific Activity (U/mg)		Substrate selectivity ratio
	NADH 0.1 mM	NADPH 0.1 mM	$\left[\frac{\text{specific activity of NADH}}{\text{specific activity of NADPH}}\right]$
Methyl Red	14.3 (± 0.6)	0.2 (± 0.03)	71.5

Table V. Comparison of Methyl Red reduction between NADPH vs NADH as electron donors.

The results are means of three independent experiments (± standard deviation)

Note: - The affinity purified fraction was used in the assay

results were further corroborated by the 70 – fold higher specific activity of the enzyme in the presence of 0.1mM NADH as compared to 0.1 mM NADPH (Table V).

Identification of the azoreductase

The protein band corresponding to the zone of clearing on the native PAGE assay (Fig 10B) was analyzed by trypsonolysis, MALDI-TOF mass spectrometry, and database searching. Using this approach, the predominant protein in the band was identified as a putative acyl carrier protein from *E. faecalis* V583 (AAO82310 from NCBI), yielding a Mascot score of 116 versus the statistical significance threshold of 78 ($p<0.05$). An analysis of this hypothetical protein sequence revealed that it was the same azoreductase (AzoA) identified previously by Chen *et al.* (Chen et al., 2004). All other candidate identifications had scores less than 78, and were therefore regarded as statistically insignificant. The identification of azoreductase was based on matching 9 of 16 experimental ion masses, representing 49% of the azoreductase sequence (Fig. 11) and these were assigned from the most intense peaks observed.

To ensure that there were no other proteins in the sample that may have contributed to the azoreductase activity; the trypsin-digested sample was subjected to a more sensitive analysis using nano-bore chromatography in conjunction with electrospray tandem mass spectrometry (HPLC-ESI-MS/MS). Tandem mass spectrometry and database searching again identified the putative acyl carrier protein from *E. faecalis* V583

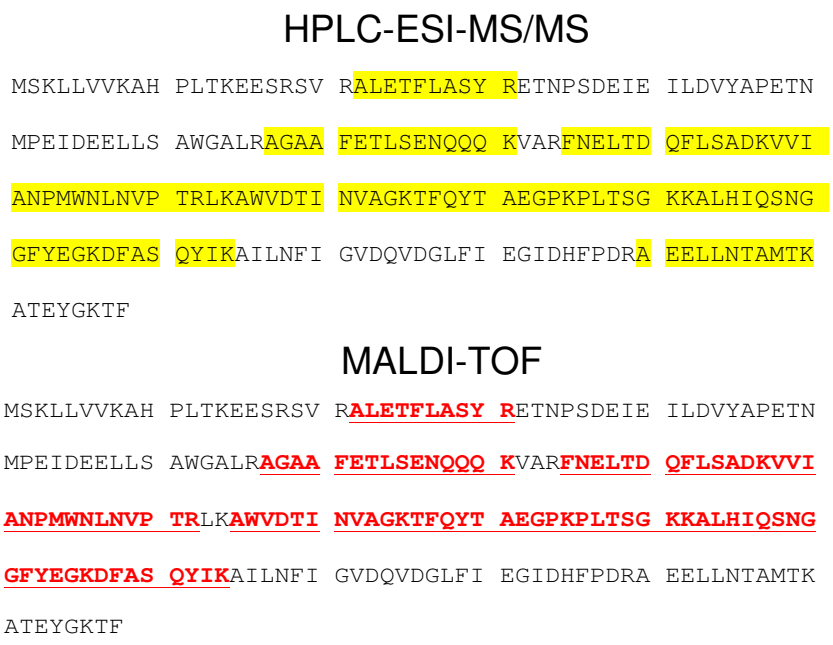


Fig 11. Sequence coverage of AzoA by mass spectrometry. LC-MS/MS (shaded) and MALDI-TOF (underlined).

(AAO82310 from NCBI) with search metrics supporting this identification to 100% probability for the protein and 95% probability for each peptide (17 unique spectra representing 11 unique peptides which covered 56% of AzoA sequence) (Fig. 11). LC-MS/MS and a database search at 99.9% minimum protein and 95% minimum peptide statistical thresholds identified 16 proteins based on a minimum of 2 peptides, including low-levels of keratin contamination. The spectral count indicated that AzoA was the most abundant protein in the sample i.e., 48 AzoA-derived spectra in contrast to 22 spectra from hypoxanthine-guanine phosphoribosyl transferase and 12 spectra from human keratin which were the next most abundant proteins. No other oxidoreductases were detected in the band of clearing, even when the stringency for identification was relaxed to include proteins associated with/identified by only one peptide, a minimum protein identification threshold of 95% and a minimum peptide identification threshold of 90%.

Pseudo-first order kinetics

In order to reduce Methyl Red, the enzyme required FMN and NAD(P)H. Table VI indicates the kinetic parameters for the substrates which were determined from the double reciprocal plot (Fig. 12a and 12b) . The V_{max} values for all substrates were almost the same, but the apparent K_m value for NADPH was more than 180 – fold higher than NADH. Also, the apparent K_m values for Methyl Red and NADH were lower than those described previously (Chen et al., 2004). FMN had the lowest apparent K_m value of 3 μM. No azoreductase activity was observed when the sulfonated azo dyes, Direct Blue 15 and Tartrazine, were used as substrate which is consistent with the previous study.

Table VI

Substrate	apparent K_m	apparent V_{max} (U/mg)
Methyl Red	11 μM (± 3)	29 (± 5)
FMN	3 μM (± 1)	31 (± 5)
NADH	82 μM (±17)	26 (± 4)
NADPH	15 mM (± 3)	32 (± 5)

Table VI. Apparent kinetic parameters for AzoA

The results are means of three independent experiments (± standard deviation)

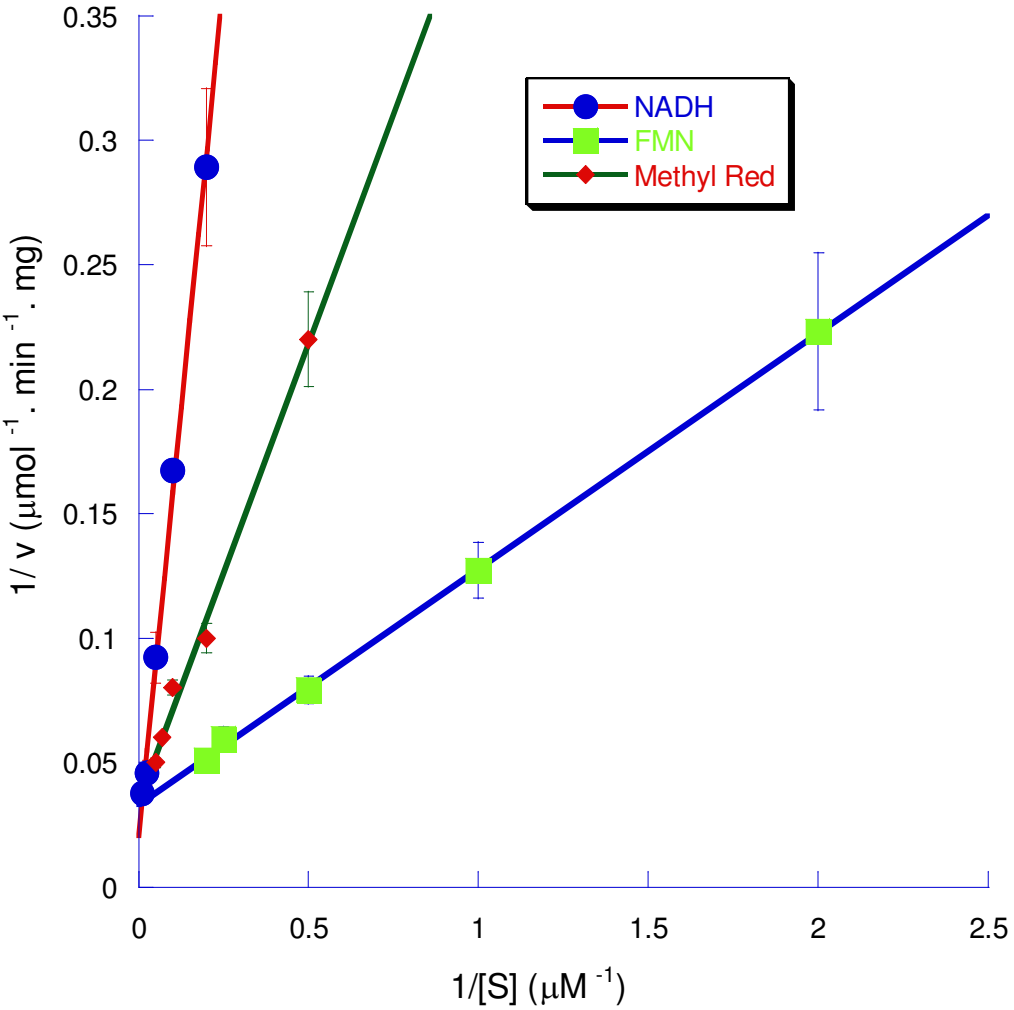


Fig.12a. Kinetic plot for Methyl Red, FMN and NADH. Double reciprocal plot of initial velocity (v) versus substrate concentration of NADH, FMN and Methyl Red in µM. To determine pseudo-first order kinetic parameters for each substrate, the other two substrates concentrations were kept constant (See Material and Methods)

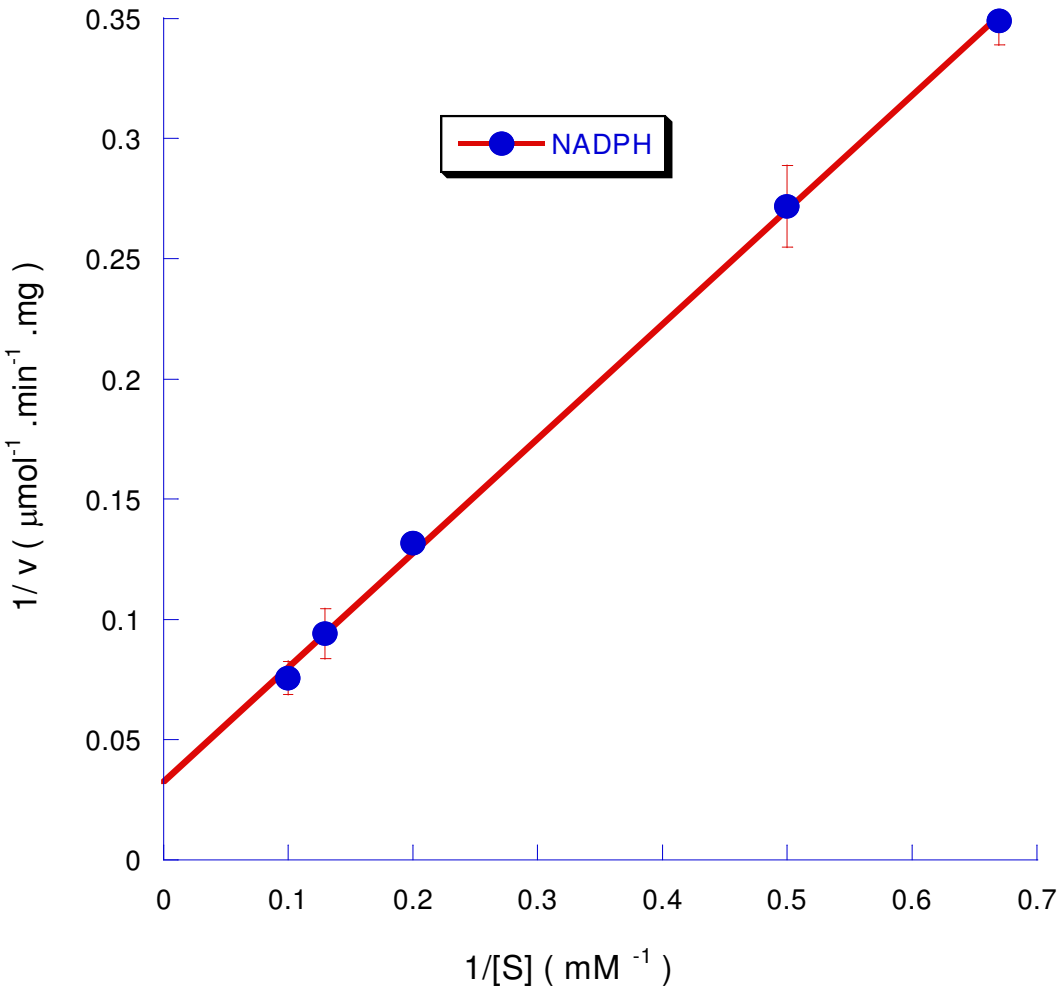


Fig.12b. Kinetic plot for NADPH. Double reciprocal plot of initial velocity (v) versus NADPH substrate concentration in mM. The concentrations of Methyl Red and FMN were kept constant

It is important to note that the aforementioned definition of specific activity is μmol/min/mg while the specific activity as defined by the earlier study (Chen et al., 2004) was μM/min/mg. Thus, when redefining the V_{max} of AzoA from the earlier study, the recalculated value is more than 100-fold lower compared to this study.

Discussion

Azoreductases are ubiquitous enzymes present in a number of organisms with a variety of azo dye substrate specificities. It is important to gather information from several azoreductases which are isolated from different organisms in order to gain better insight into the enzymes structure and function. Since there is very low homology at the enzymes' primary structure level, it is difficult to cluster the azoreductases based on this feature alone. Presently, flavin-dependent azoreductases can be divided into three categories based on their preference for an electron donor; the first group can utilize NADH only, the second group can utilize NADPH only and the third group can utilize both.

Previous work (Walker and Ryan, 1971) demonstrated that *E. faecalis* cell extracts could utilize both NAD(P)H as electron donors for azoreductase activity, although activity was much greater for NADH compared to NADPH . Our work also supported the activity of both NADH and NADPH. When compared to a previous study in which the heterologously expressed AzoA enzyme from *E. faecalis* was shown to utilize only NADH for azo dye reduction, it was reasoned that there may be other azoreductases present in wild-type *E. faecalis*.

Analysis of the *E. faecalis* proteome revealed two domains as determined from the Pfam database, the flavodoxin_2 (PF02525) domain and the FMN_red domain (PF03358). An *in silico* analysis of azoreductases has also confirmed the presence of these commonalities (Bafana and Chakrabarti, 2008). AzoA was the only protein in the *E. faecalis* proteome that has a flavodoxin_2 domain. However, three proteins possess an FMN_red domain in the *E. faecalis* proteome. It was reasoned that these proteins may

also possess azoreductase activity as the azoreductase from *Staphylococcus aureus* (Chen et al., 2005) and Bacillus sp. OY1-2 (Suzuki et al., 2001) have an FMN_red domain and are specific for NADPH. Using a molecular approach, we showed that the putative oxidoreductase EF 1226 (Gen-Bank: AE016830) was not active based on heterologous expression (Chapter IV). Interestingly, this protein has been speculated to be an ancestral azoreductase in *E. faecalis* (Bafana and Chakrabarti, 2008).

Hence, using a biochemical and analytical approach, we showed that AzoA was able to use both NADH and NADPH for activity. Therefore, we conclude that AzoA is an NAD(P)H dependent enzyme and can be reclassified as an NADH and NADPH dependent azoreductase. It is likely that the NADPH activity from the previous study was not observed due to the low NADH activity of the enzyme from the heterologous system.

Our characterization of the native purified AzoA supported the association of the FMN and NAD(P)H binding pockets i.e., the reduction of the dye occurs by the transfer of electrons from NAD(P)H via FMN (Nakanishi et al., 2001; Chen et al., 2004). It was determined that externally added FMN was essential for activity of AzoA, most likely due to the FMN moieties being disassociated from the holoenzyme during the protein purification step related to the hydrophobic column. Our finding of FMN association is also supported by the crystal structure information of AzoA, AzoR and paAzoR1 (Ito et al., 2006; Wang et al., 2007).

Kinetic studies on AzoA showed the utilization of both pyridine nucleotide coenzymes as electron donors (Table VI). Analysis of the active sites, based on the crystal structures for *E. coli* AzoR (PDB code: 1V4B) (Ito et al., 2006), *P. aeruginosa* paAzoR1 (PDB code: 2v9c) (Wang et al., 2007), human FAD-dependent

NAD(P)H:quinine oxidoreductase NQO1 (PDB code: 1DXO) (Faig et al., 2000) and *E. faecalis* AzoA (PDB code: 2HPV) (Liu et al., 2007) showed they are highly conserved which supports the presence of a NAD(P)H binding pocket. In addition, the active site of NQO1, reveals that the NH in the main chain of Phe^{232} forms a hydrogen bond with an oxygen of the phosphate moiety in NADPH while this bond is not formed in the presence of NADH (Li et al., 1995). Finally, it has also been suggested that there maybe conformational flexibility in the active site which could lead to binding of both NADH as well as NADPH (Wang et al., 2007).

In conclusion, the azoreductase, AzoA isolated from *E. faecalis* under native conditions can utilize NAD(P)H as an electron donor but its preference is for NADH. The specific activity is more than a 100-fold higher when NADH is used as the electron donor as compared to its activity when expressed heterologously in *E. coli*. To this end, the azoreductase activity in *E. faecalis,* seems to possess a much higher activity compared to heterologously expressed protein, thereby providing more direct activity analysis of xenobiotic degradation within the intestine.

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VITA

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Major Field: Microbiology, Cell and Molecular Biology

Scope and Method of Study:

Majority of the dyes used in the paper, textile, food and pharmaceutical industries are azo dyes. Intestinal bacteria play an important role in the reduction of these dyes. Azoreductase, produced by intestinal microbiota, cleave the azo bond (N=N) in these dyes to produce colorless compounds, some of which are carcinogenic. *Enterococcus faecalis,* an intestinal bacterium is known to reduce a variety of azo dyes. The current study focuses on the physiological effects of three azo dyes, Methyl Red, Tartrazine and Direct Blue 15 on *E. faecalis* as well as the biochemical purification and characterization of a native azoreductase from this organism. Domain homology was also used as a criterion to identify and classify azoreductases.

Findings and Conclusions:

The results indicate that azo dyes are reduced at the highest rate under anaerobic conditions in actively dividing cells. Majority of the water-soluble azo dyes, Tartrazine and Direct Blue 15 were reduced externally but Methyl Red was reduced to an equal extent in the cytoplasmic fraction as well. NAD(P)H can serve as an electron donor for dye reduction. Most of the azoreductases contain either an FMN_red or flavodoxin_2 domain as defined by the Pfam database. These domains can serve as an additional criterion for identifying and classifying azoreductases. The enzyme activity of the native azoreductase (AzoA) from *E. faecalis* is a 100-fold more active than the heterologously expressed enzyme in *E. coli* and hence data from heterologously expressed enzymes must be interpreted with caution. AzoA can utilize NAD(P)H as electron donors for Methyl Red reduction, although it has a greater affinity for NADH over NADPH.

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