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Degradation of azo dyes containing aminonaphthol by *Sphingomonas* sp strain 1CX

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Sphingomonas **sp strain 1CX was isolated from a wastewater treatment plant and is capable of aerobically degrading a suite of azo dyes, using them as a sole source of carbon and nitrogen. All azo dyes known to be decolorized by strain 1CX (Orange II, Acid Orange 8, Acid Orange 10, Acid Red 4, and Acid Red 88) have in their structure either 1-amino-2-naphthol or 2-amino-1-naphthol. In addition, an analysis of the structures of the dyes degraded suggests that there are certain positions and types of substituents on the azo dye which determine if degradation will occur. Growth and dye decolorization occurs only aerobically and does not occur under fermentative or denitrification conditions. The mechanism by which 1CX decolorizes azo dyes appears to be through reductive cleavage of the azo bond. In the case of Orange II, the initial degradation products were sulfanilic acid and 1-amino-2-naphthol. Sulfanilic acid, however, was not used by 1CX as a growth substrate. The addition of glucose or inorganic nitrogen inhibited growth and decoloration of azo dyes by 1CX. Attempts to grow the organism on chemically defined media containing several different amino acids and sugars as sources of nitrogen and carbon were not successful. Phylogenetic analysis of** ***Sphingomonas*** **sp strain 1CX shows it to be related to, but distinct from, other azo dye-decolorizing** ***Sphingomonas*** **spp strains isolated previously from the same wastewater treatment facility.**

Keywords: azo dyes; *Sphingomonas*; biodegradation; sulfanilic acid

Introduction

Azo dyes constitute the most frequently used type of dye for coloring both textiles and foods. Each year an estimated 7×10^5 tons of azo dyes are manufactured [29], with much of these dyes eventually discharged into the environment in waste water [2]. Unfortunately, azo dyes present in wastewater streams are generally unaffected by activated sludge processes [19]. The azo bond, and the sulfonate moiety common to many azo dyes, appear to be xenobiotic and often render azo dyes recalcitrant to aerobic biodegradation [15]. In contrast, the azo bond may be reduced non-specifically by flavin molecules under anaerobic conditions [27]. Sulfonated, aromatic amines resulting from the cleavage of sulfonated azo dyes, however, require oxygen for further biodegradation. Biodegradation of aromatic amines is important because these compounds are often mutagenic [23]. 1-amino-2-naphthol, for example, is a potential degradation product of many azo dyes and is a known carcinogen [8].

A combination of aerobic and anaerobic processes, resulting in non-toxic products, is a potential approach to bioremediate azo dye-contaminated wastewater. One example of such an approach would be to use an anaerobic digester followed by an activated sludge process [7]. A second example is the use of mixed cultures containing both aerobic and anaerobic degraders, such as the reported mineralization of the sulfonated azo dye Mordant Yellow 3 by a bacterial consortium [12,13]. This consortium, however, could only degrade a narrow spectrum of azo dyes.

A simpler approach would be to select for individual aerobic organisms that can degrade azo dyes to non-toxic products. Several strains of aerobic, azo dye-decolorizing bacteria were previously isolated from the Mill Creek Sewage Treatment Plant in Cincinnati, Ohio [4]. However, none of these strains grew on azo dyes as the sole source of carbon and nitrogen. The objective of the current study was to isolate and characterize a bacterial strain capable of aerobic degradation of azo dyes as sole carbon and nitrogen source. The eventual goal is to use such an organism in bioreactors for the treatment of azo dye-containing waste waters.

Materials and methods

Isolation and initial characterization of strain 1CX

A 1-L sample of waste liquor was collected from the Mill Creek Sewage Treatment Plant. The sample was introduced into a Rotating Drum Bioreactor (RDBR) containing mineral salts and Orange II (10 mg L^{-1}) as the only source of carbon and nitrogen. Design and operating conditions of the RDBR are described in Zhang *et al* [28]. Strain 1CX was routinely cultured on R2A medium [24] supplemented with azo dyes. Utilization of various carbon sources was evaluated using BIOLOG GN microplates (BIOLOG, Hayward, CA, USA) according to the manufacturer's instructions.

Dyes

Orange II, Acid Orange 6 (AO6), Acid Orange 8 (AO8), Acid Orange 10 (AO10), Acid Red 4 (AR4), Acid Red 88

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(AR88), Sunset Yellow, Acid Alizurin Violet, Acid Red 44 (AR44), Acid Red 14 (AR14), Amaranth, Calmagite, and Erichrome Black T were obtained from Aldrich Chemical Co, Milwaukee, WI, USA. Orange I was obtained from Pfaltz and Bauer, Waterbury, CT, USA. All other dyes were purified preparations of commercial dyes provided by MW Tabor (Department of Environmental Health, University of Cincinnati).

Screening for azo dye decolorization

Azo dyes were added individually to R2A agar at 20 mg L^{-1} and the plates streaked with strain 1CX. The plates were incubated for 1 week at 28°C and then inspected for zones of decoloration around colonies. Azo dyes with mono azo and multiple azo bonds, and with various degrees and positions of sulfonation, were evaluated. If an azo dye was decolorized on R2A by strain 1CX, it was further evaluated as described below.

Determination of growth on azo dyes

The ability of a dye to act as the sole source of carbon and nitrogen for strain 1CX was determined by growth in a liquid mineral medium containing reagent grade chemicals of the following composition: $MgSO_4 \cdot 7H_2O$, 3.12 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 2.02 g L^{-1}, $Na_2HPO_4 \cdot 7H_2O$, 2.26 g L^{-1}; K_2HPO_4, 1.51 g L^{-1}; KH_2PO_4, 2.92 g L^{-1}; and $FeCl_3 \cdot 6H_2O$, 20 μg L^{-1}. Aqueous solutions of azo dyes (0.1%) were sterilized by filtration through 0.45-μm pore size membranes and added individually to the mineral medium to attain a final dye concentration of 50 mg L^{-1}, except where noted. The inoculum (added at 1:1000) was a stationary phase culture of strain 1CX that had been previously grown to a cell density of approximately 1.25×10^8 CFU ml^{-1} in the mineral medium supplemented with 500 mg L^{-1} yeast extract.

Decolorization was measured as a function of decreasing absorbance measured at the maximum absorbance of the dye in a Perkin Elmer Lambda 2 UV/VIS dual beam spectrophotometer. The wavelength of maximum absorbance for each dye was as follows: Orange II, 483 nm; AO8, 490 nm; AO10, 475 nm; AR88, 505 nm; and AR4, 508 nm. Suspensions of strain 1CX were centrifuged at $10\,000 \times g$ for 5 min to pellet the cells, allowing color determination of the supernatant. Color loss was based on a standard curve comparing dye concentration to absorbance as a percent of uninoculated control flasks. Cell growth was calculated by plating dilutions of the culture on R2A medium.

The presence of sulfanilic acid was determined on a Dionex™ ion chromatograph model 4000i (Dionex Corporation, Sunnyvale, CA, USA) using a standard Dionex anion method with a 4-mm format anion self-regenerating suppression system. The eluent consisted of 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$ (pH 9.7) at a flow of 2.0 ml min^{-1} through an IonPac AS4A analytical column.

Ribosomal DNA sequencing and phylogenetic analysis

Strain 1CX was grown to approximately 10^9 CFU ml^{-1} in R2A broth. A 25-ml aliquot of cells was centrifuged for 15 min at $10\,000 \times g$. The resulting cell pellet was resuspended in 5 ml of 10 mM Tris buffer pH 8.0 containing 1.0 mM EDTA and incubated for 20 min at 37°C. Thereafter, 2.5 mg of Proteinase K and 50 mg of SDS were added and incubated for 2 h at 37°C. Genomic DNA was collected by phenol extraction and ethanol precipitation of DNA. The crude DNA was purified using a Wizard™ Clean-Up System kit (Promega, Madison, WI, USA) and then cut with the restriction enzyme *Pst*I in NEB Buffer 3 (New England Biolabs, Beverly, MA, USA) for 24 h at 37°C. Amplification of the nearly entire 16S rRNA gene was done by PCR using the following reaction mixture in a final volume of 50 μl: 5.0 μl of 10× *Taq* polymerase buffer A, 3 μl of each 2.5 mM dNTP base, 2.5 μl of 20 mM 8F primer, 2.5 μl of 20 mM 1492R primer, 15 μl of double distilled water, 1 μl of 1.0% bovine serum albumin fraction 5, 5.0 μl of 10 mM $MgSO_4$, and 3.0 μl sample DNA. The primers have been described previously [16]. PCR was conducted in a Perkin Elmer thermocycler under the following conditions: hot start at 95°C for 5 min before addition of *Taq* polymerase (2.5 units made up to 4 μl with 1× *Taq* Buffer A). Twenty-five cycles were used, each consisting of a denaturing stage (50 s at 95°C), an annealing stage (50 s at 45°C), and an extension stage (100 s at 72°C).

The resulting 16S rRNA fragment was purified with a Wizard PCR Preps DNA Purification System kit (Promega) and sequenced using an Applied Biosystems automated sequencer Model 373A using 8F and 704F primers [16]. The sequence was submitted to the CHECK—CHIMERA program of the Ribosomal Database Project (RDP) at the University of Illinois to check for possible chimeric artifacts [17]. A preliminary analysis of the 16S rRNA gene sequence was obtained using the SIMILARITY—RANK program of the RDP. Manual sequence alignment was performed on the basis of conserved features of primary and secondary structures using sequences from closely related strains obtained from the RDP and GenBank. Phylogenetic analysis was carried out using maximum likelihood and neighbor joining as implemented by PHYLIP [6], and maximum parsimony as implemented by PAUP [26]. The genus *Caulobacter* was used as the outgroup.

Results and discussion

Isolation of strain 1CX

After 7 days, decolorization of the medium and formation of a biofilm occurred in the RDBR inoculated with waste liquor. Effluent from the RDBR was plated onto R2A medium supplemented with 500 mg L^{-1} Orange II. After 2 weeks incubation at 28°C, a zone of decoloration surrounded several mucoid colonies. Attempts to subculture isolated colonies that retained their ability to decolorize Orange II were at first unsuccessful. However, repeated subculturing from areas of confluent growth consistently yielded colonies that retained the ability to decolorize Orange II. It was therefore suspected that mixed populations of azo and non-azo dye decolorizers were present in the mucoid colonies. Sulfanilic acid, a common by-product of Orange II degradation [5], was added to R2A plates (400 mg L^{-1} sulfanilic acid, neutralized to pH 7.0) containing Orange II (20 mg L^{-1}). This modification resulted in isolated colonies of non-mucoid, azo dye-decolorizing bacteria. Subculturing of these bacterial colonies consistently

resulted in the Orange II decolorizing trait. One such strain was selected and designated strain 1CX.

Decolorization of azo dyes by strain 1CX

All azo dyes decolorized by strain 1CX, as shown in Figure 1, have present in their structure either 1-amino-2-naphthol (Orange II, AO8, AO10, AR88) or 2-amino-1-naphthol (AR4) that has been coupled via the azo bond to a phenyl or naphthyl moiety. Similar dyes, such as AO6 or Orange I, which lack the 1-amino-2-naphthol or 2-amino-1-naphthol structure, were not decolorized. This type of azo dye specificity has been reported for other bacteria [14,22]. Moreover, strain 1CX is able to decolorize dyes that contain one or two polar sulfonate groups. The position of the sulfonate group appears to be important. None of the azo dyes decolorized by strain 1CX have sulfonate groups on both sides of the azo bond. Sunset Yellow, for example, is not decolorized (Figure 1). This may be related to transport of the dye into the cell since sulfonate groups inhibit transport of certain azo dyes across the cell membrane [18]. We found no evidence for extracellular secretion of an azo reductase by strain 1CX, as filter-sterilized solutions of decolorized growth media were unable to decolorize freshly introduced dye (data not shown).

The position of sulfonate groups may also prevent access to the azo bond, and subsequent dye decolorization. The proximity of the large and highly dissociated sulfonate group at the 8 position on AR44 may cause the opposing naphthalene structure to be repelled, distorting the azo reductase active site. Azo bond reduction is typically the first step in bacterial biodegradation of an azo dye [27]. Unlike AR44, AR88 does not possess sulfonate groups that affect access to the azo bond, and it is decolorized, despite the presence of the opposing napthalene group. Similar to AR44, the decolorized dye Acid Orange 10 contains a sulfonate group at the 8 position, but it does not contain an opposing napthalene group as does AR44.

Calmagite is used as a color indicator for water hardness since its color changes depending upon the presence or absence of Ca^{2+} in solution [9]. The hydroxyl groups at the 2 and 2′ positions complex Ca^{2+}, perhaps affecting access of the azo reductase to the azo bond. Similarly, Acid Aliz-

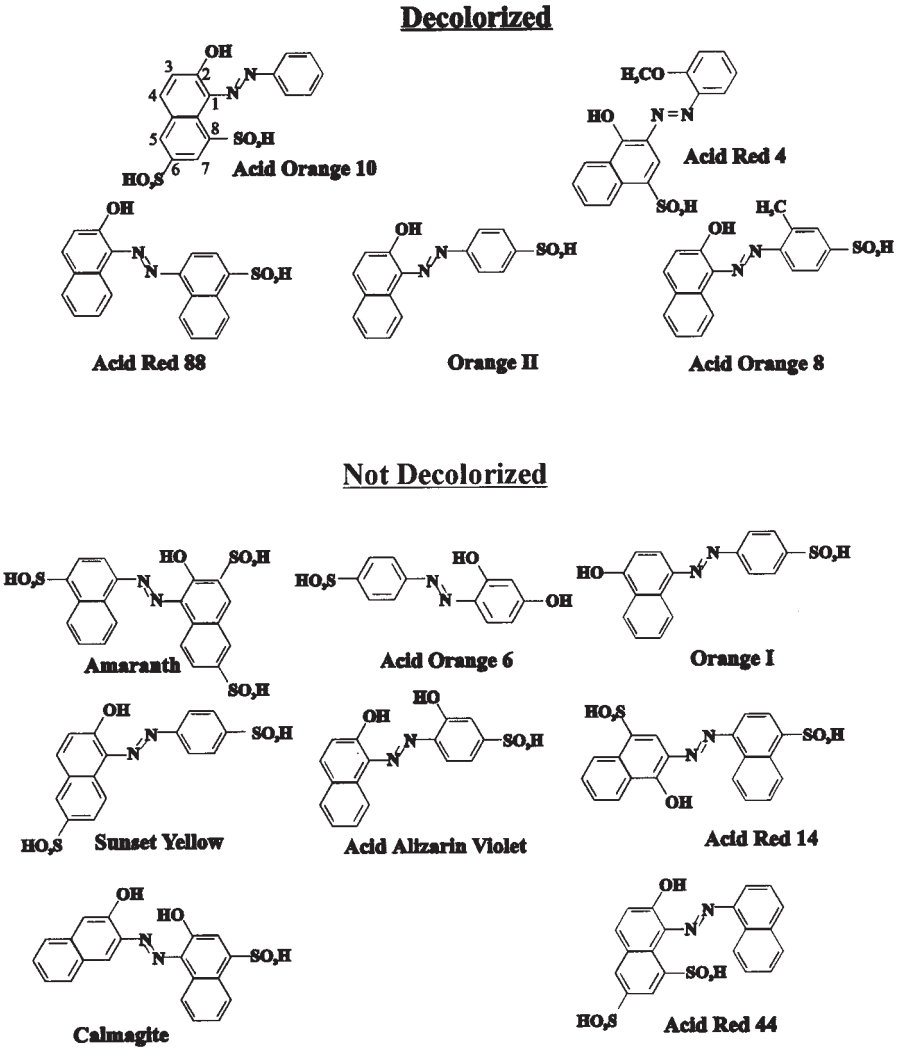

Figure 1 Azo dye structures that were evaluated for decolorization by strain 1CX in R2A agar. An example of the numbering system used for substituent groups is shown for Acid Orange 10.

arin Violet also possesses hydroxyl groups in the 2 and 2′ positions and is not decolorized by strain 1CX.

Growth of strain 1CX on azo dyes

The rapid decolorization of AR88 appeared to be due to rapid precipitation of this dye, which cleared the medium within 24 h (Table 1). This precipitation also occurred in non-inoculated controls. Most of the dyes decolorized by strain 1CX also served as a sole carbon and energy source when present in mineral medium. The one exception is AR4, which is decolorized in R2A medium (Figure 1), but not in mineral medium (Table 1). Interestingly, AR4 is the one azo dye which is decolorized by strain 1CX that would result in the production of 3-amino-4-hydroxynaphthalene-1-sulfonate, as opposed to 1-amino-2-naphthol, suggesting that 1CX can only use 1-amino-2-naphthol (and the related 8-amino-7-hydroxynapthalene-1,3-disulfonate produced from AO10) as a growth substrate. In contrast to the other dyes, however, the guaranteed purity of AR4 is only 50%. Therefore, an alternative explanation is that unreacted 1-amino-2-methoxybenzene used in the synthesis of AR4 [11] may be toxic to strain 1CX. Aniline, which is used in the synthesis of AO10, and is structurally similar to 1-amino-2-oxymethylbenzene, is toxic to strain 1CX (data not shown). This alternative explanation is unlikely, however, since strain 1CX grew on R2A medium supplemented with AR4, with concurrent decolorization of the dye. Strain 1CX was not able to decolorize AR4 in mineral medium over a wide range of dye concentrations ranging from 5 to 500 mg L^{-1} (data not shown).

Growth on Orange II

Orange II is a particularly suitable dye to study azo dye degradation because the degradation product sulfanilic acid is easily detected by ion chromatography. The 1-amino-2-naphthol which is also generated during reductive cleavage of the azo bond, however, is a transient species as it undergoes rapid autooxidation [3]. There is a concurrent appearance of sulfanilic acid and growth of strain 1CX as Orange II is decolorized (Figure 2), indicating that reduction of the azo bond has occurred. In contrast, several fungi and actinomycetes have been demonstrated to decolorize azo dyes by oxidative mechanisms, rather than through reduction of the azo bond [21].

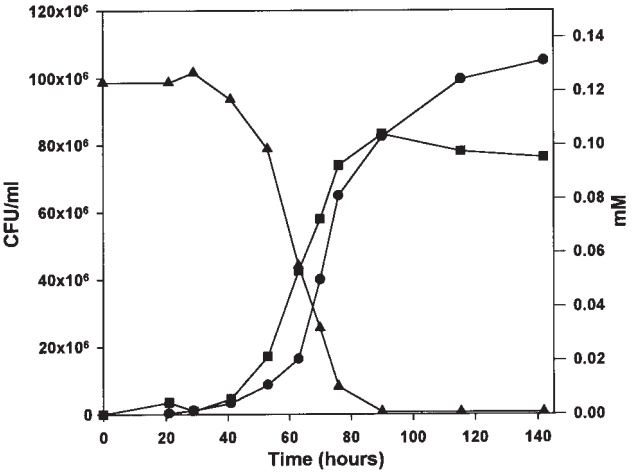

Figure 2 Degradative loss of Orange II with corresponding growth of strain 1CX. Symbols: ●, CFU ml^{-1}; ▲, mM Orange II; ■, mM sulfanilic acid.

Figure 2 also indicates that strain 1CX is unable to utilize sulfanilic acid, produced as a by-product during growth on Orange II, as a sole carbon and energy source. This was confirmed by studies in mineral medium containing sulfanilic acid with or without nitrate, which did not support growth of strain 1CX (data not shown). Strain 1CX must be able to desulfonate azo dyes to some extent, as it was able to grow in chemically defined media lacking any source of sulfur other than Orange II. Measurable levels of sulfate were not detected, however.

Characterization of strain 1CX

Strain 1CX is a Gram-negative obligate aerobe. Growth is inhibited at 37°C and cell death occurs at 40°C. Strain 1CX grows well in mineral medium containing yeast extract at 500 mg L^{-1}, but not at 5000 mg L^{-1}. Although growth of this strain occurred on R2A medium, which contains yeast extract, none of the single amino acids, carbohydrates, or organic acids present in the BIOLOG GN plate was utilized. In addition, the presence of nitrate, nitrite, ammonium, or glucose all inhibited dye degradation (data not shown).

Phylogenetically, strain 1CX is placed within the *Sphingomonas* group of α-Proteobacteria, but is distinct from all

Table 1 Azo dye degradation and growth by strain 1CX

Hours	Orange II		A08		AO10		AR88		AR4	
	% Decolorized	CFU ml^{-1}	% Decolorized	CFU ml^{-1}	% Decolorized	CFU ml^{-1}	% Decolorized	CFU ml^{-1}	% Decolorized	CFU ml^{-1}
0[a]	0	1.30×10^6	0	1.30×10^6	0	1.30×10^6	64	1.30×10^6	0	1.30×10^6
24	6	1.79×10^6	4	1.01×10^6	4	9.75×10^5	ND[b]	6.40×10^5	0	8.00×10^3
48	53	3.20×10^7	55	2.55×10^7	64	3.00×10^7	ND	2.80×10^6	0	ND
62	97	7.55×10^7	99	7.15×10^7	99	6.05×10^7	ND	ND	0	ND
96	99	6.95×10^7	100	7.00×10^7	99	7.65×10^7	ND	1.50×10^7	0	$<10^3$
120	99	9.30×10^7	99	4.95×10^7	99	6.65×10^7	ND	2.12×10^7	0	$<10^3$

[a]CFU values at 0 h refer to the initial inoculum. The inoculum was a stationary phase culture that had been previously grown to a cell density of approximately 1.2×10^8 CFU ml^{-1} in the mineral medium supplemented with 500 mg L^{-1} yeast extract.
[b]ND: not determined.

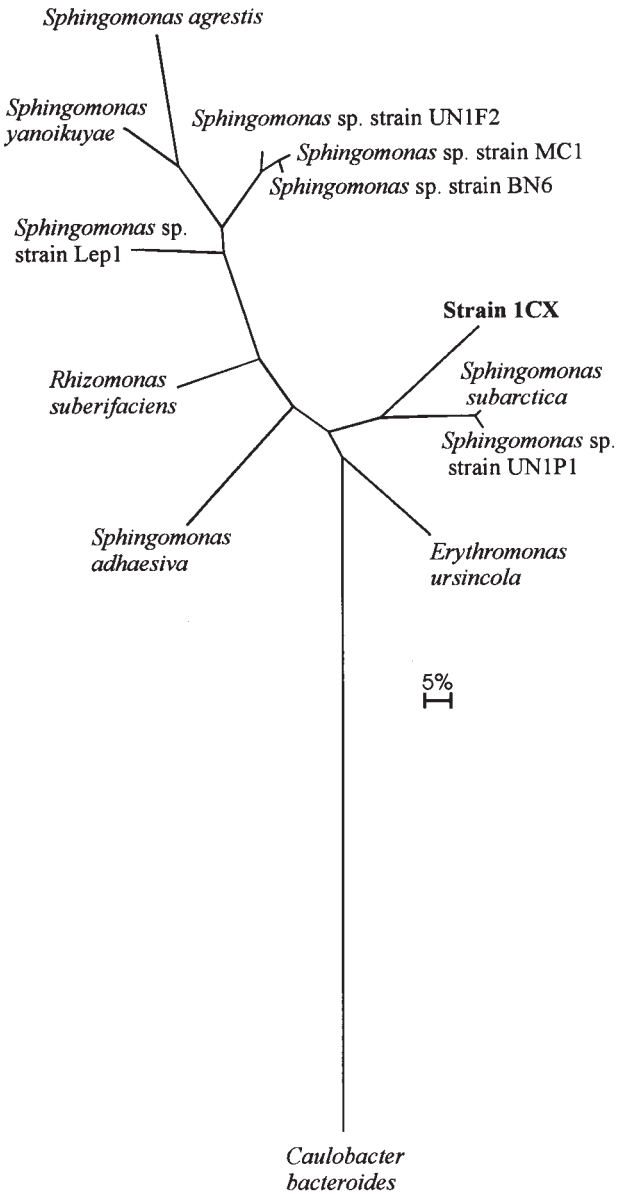

Figure 3 Maximum likelihood tree rooted with *Caulobacter*. The scale bar corresponds to 0.05 estimated nucleotide substitutions per nucleotide position.

other known species (Figure 3). The topologies of the maximum parsimony and the neighbor joining trees were both congruent to the maximum likelihood tree (data not shown), with all trees grouping strain 1CX within the *Sphingomonas* group. Interestingly, azo dye-decolorizing strains C7 and MC1 were obtained from the same sewage treatment plant as strain 1CX, are also *Sphingomonas* spp, and aerobically decolorize a common set of azo dyes: Orange II, AO8, and AR88 [4,10]. A pilot-scale activated sludge biological treatment system seeded with wastewater from this same sewage treatment plant only decolorized Orange II, A08, and AR88 from a set of 18 azo dyes which also included AO10 [25]. Unlike strain 1CX, strains MC1 and C7 are unable to utilize these dyes as carbon and nitrogen sources, and require glucose and ammonia for dye decolorization. A further difference is that, of the three strains, only 1CX is able to decolorize AO10.

Strain BN6, isolated from a different source, is an azo dye-degrading bacterial strain that is more closely related to C7 and MC1 than strain 1CX [20]. In contrast, strain BN6 is only able to reduce the azo bond under anaerobic conditions. Thereafter, it can use the cleavage product from Mordant Yellow 3 to support its aerobic growth. Strain S5, is an aerobic azo dye-degrading bacterium that was selected from a population of non-azo dye degraders after months of selective pressure [1]. This organism, however, has a very limited repertoire for azo dye degradation and is not related to the *Sphingomonas* group.

In summary, to our knowledge strain 1CX is the first bacterial strain shown to possess such a broad azo dye-degradation range under aerobic conditions. Further molecular studies will be necessary to determine if strain 1CX has a single azo reductase, which appears likely at this point. Current studies in our laboratory are examining the distribution of these related azo dye-degrading *Sphingomonas* spp strains in natural waters and waste treatment facilities, and examining their performance in bioreactors receiving dye-containing wastewaters.

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