J. Microbiol. Biotechnol. (2005), **15**(1), 221–225

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Decolorization of a Sulfonated Azo Dye, Congo Red, by *Staphylococcus* sp. EY-3

PARK, EUN-HEE[2], MOON-SUN JANG[1], IN-HO CHA[2], YONG-LARK CHOI[1], YOUNG-SU CHO[1], CHEORL-HO KIM[3], AND YOUNG-CHOON LEE[1]*

[1]*Faculty of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea*
[2]*Department of Microbiology, Busan Institute of Health and Environment, Busan 613-104, Korea*
[3]*Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Kyung-Pook 780-350, Korea*

Received: April 15, 2004
Accepted: May 17, 2004

Abstract A *Staphylococcus* sp. EY-3 with the capability of decolorizing Congo Red was isolated from soil at an effluent treatment plant of a textile and dyeing industry. This strain was able to almost completely decolorize a high concentration of Congo Red in 48 h under aerobic conditions. Optimal color removal (more than 96%) was achieved at 30–40°C, and no noticeable effects of different pH values (5.5–8.0) on decolorization were observed. This strain also exhibited a remarkable decolorization capability against azo dyes under aerobic conditions, even at a high concentration (dyes 1 g/l) of dye. The metabolic product of Congo Red degradation by this strain was identified by gas chromatography with mass selective detection (GC/MSD) to be an amine derivative benzidine.

Key words: Azo dye, biodegradation, Congo Red, decolorization, *Staphylococcus* sp.

Azo dyes constitute the largest class of synthetic dyes widely used in textile, paper, leather, cosmetics, and food industries [10]. Since most of them have mutagenic or carcinogenic properties [6], industrial effluent containing azo dyes must be treated before it is discharged into the environment. Although several physicochemical methods have been used to eliminate the colored effluents in wastewater [12, 13], they are generally expensive, of limited applicability, and produce a large amount of sludge. Therefore, great interest is focused on the microbial biodegradation of dyes as a better alternative. Azo dyes are generally considered to be xenobiotic compounds, which are rather recalcitrant against biodegradative processes in conventional sewage treatment systems [15]. Nevertheless, it has been demonstrated during the last few years that several microorganisms are able to transform azo dyes to noncolored products or even completely mineralize them under certain environmental conditions [2]. There are numerous reports which describe the biodecolorization of azo dyes under anaerobic conditions, however, only a few studies reported that azo dyes could be decolorized by bacteria under aerobic conditions [2–4, 7, 12, 16]. Depending on the structure of azo compounds, the biodegradability is reduced by the presence of substituents such as sulfonate groups [5]. Congo Red, a sulfonated diazo dye, has been reported to be the azo dye most resistant to microbial decolorization [8, 18]. Decolorization of Congo Red by bacteria is poorly understood so far, compared with other azo dyes.

In this paper, we report the isolation and characterization of a new potent bacterium, *Staphylococcus* sp. EY-3, capable of efficiently decolorizing azo dyes including Congo Red. To the best of our knowledge, this is the first report on azo dye decolorization by *Staphylococcus* sp.

Isolation and Characterization of Congo Red-Decolorizing Bacteria

Various soil samples were collected from the vicinity of an effluent treatment plant of a textile and dyeing industry, which is located in Saha-Gu, Busan, Korea, and screened for Congo Red-decolorizing microorganisms. The screening of the strains for dye decolorization was performed on MY agar plate (pH 7.0) containing (per liter): Na_2HPO_4 2 g, KH_2PO_4 1 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, NaCl 3 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $FeCl_3 \cdot 6H_2O$ 0.05 g, yeast extract 0.2 g, agar 15 g, and 140 μM Congo Red. Microorganisms were selected on the basis of clear zone on agar plate. The strains growing on the plates and decolorizing the dye were selected and grown in the same liquid medium. The

*Corresponding author
Phone: 82-51-200-7591; Fax: 82-51-200-6536;
E-mail: yclee@daunet.donga.ac.kr

Table 1. Microbial characteristics of the isolated strain EY-3.

Characteristics	Strain EY-3
Morphology	Sphere
Cell size (μm)	0.5-1.0
Motility	-
Gram staining	+
Spore formation	-
Aerobic growth	+
Growth in 10% NaCl	+
Cytochrome oxidase	-
Catalase	-
Urease	-
Coagulase	-
Novobiocin resistance	+
Nitrate reduction	-
β-Galactosidase	+
β-Glucosidase	-
Alkaline phosphatase	-
Acid (aerobically) from	
glucose	+
arabinose	-
xylose	-
mannose	+
mannitol	+
raffinose	-
cellobiose	+
saccharose	+
trehalose	+

Symbols: +, positive; -, negative.

general characteristics of the strain used for decolorization studies were determined according to *Bergey's Manual of Systematic Bacteriology* [14] and other physiological tests, including carbon-source utilization, by using the API 20E system (BioMerieux, France), API 50 CHE (BioMerieux), and BIOLOG GN test kit (MicroLog™ System, Biolog, CA, U.S.A.). Analysis of the 16S rDNA sequence of the isolated strain was performed in MicroID Co. Ltd., Seoul, Korea. One strain, No. EY-3, which had a high decolorization ability against Congo Red, was isolated. This isolate was Gram-positive, a sphere, nonmotile, aerobic, oxidase-negative, catalase-positive, novobiocin-resistant, and could grow well in the presence of 10% NaCl (Table 1). The 16S rDNA sequence of the isolate was highly identical to that of *Staphylococcus cohnii* (similarity, 97.0%). On the basis of the above results, the isolate EY-3 was identified as a member of the genus *Staphylococcus* and designated as *Staphylococcus* sp. EY-3.

Characteristics of Congo Red Decolorization by *Staphylococcus* sp. EY-3

The cells were grown aerobically at 35°C for 15 h in MY medium (pH 7.0). To determine the effects of physicochemical factors on Congo Red decolorization, precultured cells were inoculated at 1% (v/v) into 500-ml flasks containing 100 ml MY medium (pH 7.0) with 0.5% glucose, 0.2% yeast extract, and 140 μM Congo Red, and aerobically incubated at various temperatures or in the same medium adjusted to different pH values. Decolorization of culture supernatant after centrifugation at 10,000 ×*g* for 20 min was measured at λ_{max} (506 nm) using a scanning spectrophotometer (Ultrospec 3000 UV/VIS, Pharmacia, Sweden). Decolorizing activity was expressed in terms of the percentage decolorization by the same method as described previously [1]. Decolorization activity was calculated as follows: Decolorization (%)=[(initial absorbance)-(observed absorbance)/(initial absorbance)]×100. All experiments were conducted in triplicate. The uninoculated sterile medium was used as a control. Cell growth was monitored by measurement of optical density of culture medium at 580 nm. Dry cell weight was determined after washing the cell pellets by centrifugation with sterile water and drying overnight at 80°C. As shown in Table 2, *Staphylococcus* sp. EY-3 almost completely decolorized 140 μM Congo Red within 48 h of incubation at 30, 35, and 40°C under aerobic conditions. Cell concentration increased also in proportion to increase of decolorization rate. Moreover, even at high dye concentration of 1.4 mM (dye 1 g/l), more than 82% of the color was removed under the same conditions, whereas less than 24% was decolorized at higher dye concentrations of 7.2 mM and 14.3 mM, because of growth inhibition effect of these high dye concentrations. Effects of pH values on the decolorization of Congo Red by *Staphylococcus* sp. EY-3 showed the results similar to those by temperatures (Table 3). Although the highest color removal was detected

Table 2. Decolorization (%) of Congo Red by *Staphylococcus* sp. EY-3 after 48 h of growth at different temperatures and dye concentrations under aerobic conditions.

Temperature (°C)	Dye concentration (mM)				
	0.14	0.7	1.4	7.2	14.3
20	75 (1.96)[a]	67 (1.52)	58 (0.97)	14 (0.12)	6 (0.04)
30	97 (2.96)	84 (2.60)	83 (2.32)	23 (0.31)	13 (0.12)
35	98 (3.02)	87 (2.82)	83 (2.34)	24 (0.32)	13 (0.12)
40	96 (2.94)	84 (2.64)	82 (2.26)	23 (0.31)	12 (0.12)
45	85 (2.63)	76 (1.85)	66 (1.48)	21 (0.28)	10 (0.07)

[a]Dry cell weight (mg/ml) after 48 h of growth; the variation in dry cell weight of triplicates was 0.01 to 0.05 mg/ml.
The variation in decolorization by triplicates was 0.01 to 0.15%.

Table 3. Decolorization (%) of Congo Red by *Staphylococcus* sp. EY-3 after 48 h of growth at different initial pH's, and dye concentrations under aerobic conditions.

Initial pH	Final pH[a]	Dye concentration (mM)				
		0.14	0.7	1.4	7.2	14.3
5.0	5.0±0.04	86 (2.50)[b]	80 (2.25)	70 (1.73)	12 (0.17)	5 (0.04)
5.5	5.1±0.02	93 (2.87)	84 (2.46)	72 (1.87)	17 (0.26)	7 (0.04)
6.0	5.1±0.05	94 (2.86)	87 (2.53)	74 (1.87)	22 (0.30)	12 (0.13)
6.5	5.1±0.04	96 (2.91)	90 (2.75)	79 (2.07)	25 (0.31)	13 (0.14)
7.0	5.1±0.07	98 (2.95)	92 (2.76)	82 (2.08)	27 (0.32)	16 (0.15)
7.5	5.2±0.03	97 (2.94)	91 (2.78)	80 (2.10)	27 (0.31)	16 (0.14)
8.0	5.3±0.02	91 (2.77)	86 (2.52)	72 (1.88)	23 (0.31)	13 (0.13)

[a]pH values measured after 48 h of growth at different initial pH's and dye concentrations of 140 μM and 700 μM.
[b]Dry cell weight (mg/ml) after 48 h of growth; the variation in dry cell weight of triplicates was 0.01 to 0.05 mg/ml.
The variation in decolorization by triplicates was 0.01 to 0.13%.

at pH 7.0, there were no noticeable effects on percentage decolorization at different initial pH values within the range tested when 140 μM, 700 μM, and 1.4 mM dye concentrations were used. To determine the effects of carbon and nitrogen sources on cell growth and Congo Red decolorization, the cultures were aerobically incubated in MY medium containing various carbon or nitrogen sources at 35°C for 48 h, and the most effective decolorization rate with good cell growth was found in the presence of 0.2 to 1% of glucose and yeast extract or peptone (data not shown). Although Congo Red has been reported to be the azo dye most resistant to microbial decolorization [8], in the present study, *Staphylococcus* sp. EY-3 was able to decolorize 82% of the dye, even at a high dye concentration of 1.4 mM.

Decolorization of Azo Dyes by *Staphylococcus* sp. EY-3

To investigate whether *Staphylococcus* sp. EY-3 was able to decolorize other azo dyes, precultured cells were inoculated at 1% (v/v) into 500-ml flasks containing 100 ml MY medium (pH 7.0) with 0.5% glucose, 0.2% yeast extract, and azo dyes, and incubated for 48 h at 35°C with shaking at 250 rpm. Decolorization of culture supernatant after centrifugation at 10,000 ×*g* for 20 min was determined by monitoring the decrease in absorbance at the visible absorbance maximum of each dye. As shown in Table 4, *Staphylococcus* sp. EY-3 also showed an excellent decolorization capability against azo dyes tested.

Table 4. Decolorization (%) of azo dyes by *Staphylococcus* sp. EY-3 after 48 h of growth at 35°C under aerobic conditions.

Dyes	Dye concentration[a] (mM)	λ_{max} (nm)	Decolorization (%)
Amaranth	1.65	510	98.1
Tartrazine	1.87	410	93.8
Methyl red	3.71	514	97.6
Acid Blue 113	1.47	570	95.2

[a]Molar concentrations of each dye correspond to 1 g of dye/l.
The variation in decolorization by triplicates was 0.02 to 0.16%.

Wong and Yuen [17] reported that *Klebsiella pneumoniae* RS-13 and *Acetobacter liquefaciens* S-1 completely decolorized 371 μM methyl red at 30°C under shaking conditions within 2 days and 1 week, respectively. However, *Staphylococcus* sp. EY-3 almost (97.6%) decolorized an even higher concentration (3.71 mM) of methyl red within 48 h. These results indicate that *Staphylococcus* sp. EY-3 has a high decolorizing ability even at a higher concentration of azo dyes, compared to other azo dye-decolorizing bacteria reported previously [2]. Therefore, *Staphylococcus* sp. EY-3 isolated in this work appears to be highly promising for applications involving biodegradation of azo dyes at high concentrations.

Detection of Intermediate Metabolite

The first step in the degradation of azo compounds is usually the formation of amine derivatives, and the amine derivative benzidine is formed by degradation of Congo Red [9]. Production of benzidine from the Congo Red degradation by *Staphylococcus* sp. EY-3 was analyzed by gas chromatography with mass selective detection (GC/MSD). Thus, after incubation at 35°C for 48 h in MY medium (pH 7.0) with 0.5% glucose, 0.2% yeast extract, and 140 μM Congo Red, 2 ml of the culture were taken out and extracted three times with equal volumes of ethyl acetate. The combined extracts were evaporated at 40°C under nitrogen, and dried residues were dissolved in 100 μl of methanol and 2 μl fractions were analyzed by GC/MSD: Automated GC/MSD analysis was performed on a Hewlett-Packard Model 5890 gas chromatograph (Palo Alto, CA, U.S.A.) and 5970B MSD using HP-5 column (0.2 mm i.d.× 25 m, 0.33 μm coating thickness). The GC was operated under the following conditions: manual injection, split 1:15; injector temperature, 230°C; carrier gas, He; flow rate, 20 ml min^{-1}; oven temperature programmed from 100 to 250°C at 5°C min^{-1}; detector temperature, 280°C; and running time, 60 min. The MSD was operated at 70 eV. As shown in Fig. 1, the compound with a retention time of 12.431 min and patterns of mass spectral fragmentation was identical to

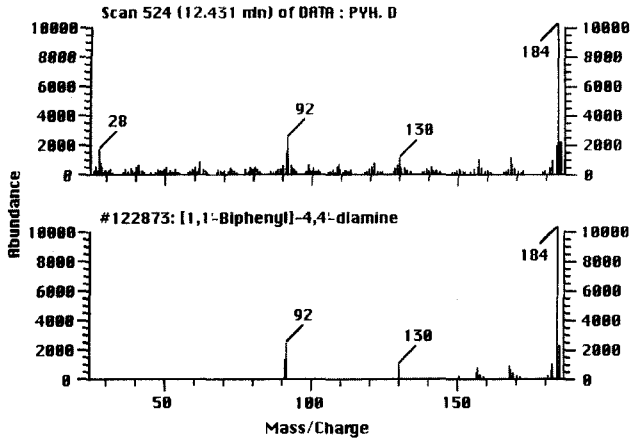

Fig. 1. GC/MSD total and specific ion trace of ethyl acetate-extractable metabolites formed from Congo Red by *Staphylococcus* sp. EY-3.

authentic benzidine, [1,1'-biphenyl]-4'4-diamine. Because benzidine is soluble in the organic solvent dichloromethane, it is not contained in the culture medium. Therefore, this result indicates that benzidine was formed as a metabolite of Congo Red by *Staphylococcus* sp. EY-3. Decolorization of the dye may take place in two ways; either adsorption on the microbial biomass or biodegradation of the dyes by the cells [21]. Dye adsorption may be evident from the inspection of the bacterial growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. *Staphylococcus* sp. EY-3 cells cultured for 48 h with azo dyes tested were found to be colorless. Decolorization assay of butanol extract of the cell pellets after incubation for 48 h showed that none of the azo dyes tested were significantly detected in cell pellets (data not shown). These results, therefore, indicate that the color removal by this strain is due to biodegradation.

It is generally recognized that the mechanism of bacterial decolorization of azo dyes is triggered by azoreductase, and that the aerobic reductive metabolism of azo dyes requires specific enzymes (aerobic azoreductases), which catalyze the NAD(P)H-dependent reduction of azo compounds to the corresponding amines [2, 19, 20]. Therefore, purification and biochemical characterization of azoreductase from *Staphylococcus* sp. EY-3 should help clarify the exact mechanism of Congo Red degradation by azoreductase.

Acknowledgment

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program (Grant MG02-0301-005-2-2-0) from THE Ministry of Science & Technology, Republic of Korea.

References

1. An, S.-Y., S.-K. Min, I.-H. Cha, Y.-L. Choi, Y.-S. Cho, C.-H. Kim, and Y.-C. Lee. 2002. Decolorization of triphenylmethane and azo dyes by *Citrobacter* sp. *Biotechnol. Lett.* **24:** 1037-1040.
2. Banat, I. M., P. Nigam, D. Singh, and R. Marchant. 1996. Microbial decolorization of textile-dye-containing effluents: a review. *Biores. Technol.* **58:** 217-227.
3. Blümel, S., H. J. Busse, A. Stolz, and P. Kampfer. 2001. *Xenophilus azovorans* gen. nov., sp. nov., a soil bacterium that is able to degrade azo dyes of the Orange II type. *Int. J. Syst. Evol. Microbiol.* **51:** 1831-1837.
4. Blümel, S., B. Mark, H. J. Busse, P. Kampfer, and A. Stolz. 2001. *Pigmentiphaga kullae* gen. nov., sp. nov., a novel member of the family *Alcaligenaceae* with the ability to decolorize azo dyes aerobically. *Int. J. Syst. Evol. Microbiol.* **51:** 1867-1871.
5. Brown, D. and B. Hamburger. 1987. The degradation of dyestuffs. III. Investigations of their ultimate degradability. *Chemosphere* **16:** 1539-1553.
6. Chung, K. T. and C. E. Cerniglia. 1992. Mutagenicity of azo dyes: Structure-activity relationships. *Mutat. Res.* **277:** 201-220.
7. Coughlin, M. F., B. K. Kinkle, and P. L. Bishop. 1999. Degradation of azo dyes containing aminonaphthol by *Sphingomonas* sp. strain 1CX. *J. Ind. Microbiol. Biotechnol.* **23:** 341-346.
8. Cripps. C., J. A. Bumpus, and S. D. Aust. 1990. Biodegradation of azo dyes by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56:** 1114-1118.
9. Diniz, P. E., A. T. Lopes, A. R. Lino, and M. L. Serralheiro. 2002. Anaerobic reduction of a sulfonated azo dye, congo red, by sulfate-reducing bacteria. *Appl. Biochem. Biotechnol.* **97:** 147-163.
10. Gregory, P. 1993. Dyes and dyes intermediates, pp. 544-545. *In* J. I. Kroschwitz (ed.), *Encyclopedia of Chemical Technology*, vol. **8**. John Wiley & Sons, New York, U.S.A.
11. Kim, J.-D., H.-Y. An, J.-H. Yoon, Y.-H. Park, F. Kawai, C.-M. Jung, and K.-H. Kang. 2002. Identification of *Clostridium perfringens* AB&J and its uptake of bromophenol blue. *J. Microbiol. Biotechnol.* **12:** 544-552.
12. Meehan, C., A. J. Bjourson, and G. McMullan. 2001. *Paenibacillus azoreducens* sp. nov., a synthetic azo dye decolorizing bacterium from industrial wastewater. *Int. J. Syst. Evol. Microbiol.* **51:** 1681-1685.
13. Reife, A. 1993. Dyes; environmental chemistry, p. 754. *In* J. I. Kroschwitz (ed.), *Encyclopedia of Chemical Technology*, vol. **8**. John Wiley & Sons, New York.
14. Schleifer, K. H. 1986. Gram-positive cocci; Genus IV. *Staphylococcus*, pp. 1013-1035. *In* Sneath P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. **2**. Williams & Wilkins, Baltimore.
15. Shaul, G. M., T. J. Holdsworth, C. R. Dempsey, and K. A. Dostal. 1991. Fate of water soluble azo dyes in the activated sludge process. *Chemosphere* **22:** 107-119.

16. Suzuki, Y., T. Yoda, A. Ruhul, and W. Sugiura. 2001. Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil. *J. Biol. Chem.* **276:** 9059-9065.
17. Wong, P. K. and P. Y. Yuen. 1998. Decolorization and biodegradation of N,N'-dimethyl-p-phenylenediamine by *Klebsiella pneumoniae* RS-13 and *Acetobacter liquefacines* S-1. *J. Appl. Microbiol.* **85:** 79-87.
18. Woo, S.-W., J.-S. Cho, B.-K. Hur, D.-H. Shin, K.-G. Ryu, and E.-K. Kim. 2003. Hydrogen peroxide, its measurement and effect during enzymatic decoloring of congo red. *J. Microbiol. Biotechnol.* **13:** 773-777.
19. Zimmermann, T., H. G. Kulla, and T. Leisinger. 1982. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.* **129:** 197-203.
20. Zimmermann, T., F. Gasser, H. G. Kulla, and T. Leisinger. 1984. Comparison of two azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.* **138:** 37-43.
21. Zhou, W. and W. Zimmermann. 1993. Decolorization of industrial effluents containing reactive dyes by actinomycetes. *FEMS Microbiol. Lett.* **107:** 157-162.