J. Microbiol. Biotechnol. (2002), **12**(3), 437–443

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Identification of *Stenotrophomonas maltophilia* LK-24 and its Degradability of Crystal Violet

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Received: December 1, 2001
Accepted: April 1, 2002

Abstract A number of soil and wastewater samples were collected from the vicinity of an effluent treatment plant for the chemical industry. Several microorganisms were screened for their ability to decolorize the triphenylmethane group of dyes. As a result, a novel crystal violet dye-degrading strain LK-24 was isolated. Taxonomic identification including 16S rDNA sequencing and phylogenetic analysis indicated that the isolate had a 99.5% homology in its 16S rDNA base sequence with *Stenotrophomonas maltophilia.* The triphenylmethane dye, crystal violet, was degraded extensively by growing cells of *Stenotrophomonas maltophilia* LK-24 in agitated liquid cultures, although their growth was strongly inhibited in the initial stage of incubation. This group of dyes is toxic, depending on the concentration used. The dye was significantly degraded at a relatively lower concentration, below 100 $\mu g\ ml^{-1}$, yet the growth of the cells was totally suppressed at a dye concentration of 250 $\mu g\ ml^{-1}$. The degradation products of crystal violet were identified as 4,4'-bis(dimethylamino)-benzophenone and ρ-dimethylaminophenol by Gas chromatography-Mass spectrometry. The 4,4'-bis(dimethylamino)-benzophenone was easily obtained in a reasonable yield, as it was not metabolized further by *S. maltophilia* LK-24; however, the ρ-dimethylaminophenol was not easily identifiable, as it was further metabolized

Key words: Biodegradation, crystal violet, metabolites, *Stenotrophomonas maltophilia*

The triphenylmethane group of dyes is extensively used in human and veterinary medicine, textile dyeing, dyestuff manufacturing industries, as a biological stain, and in paper printing. Yet, unfortunately, wastewater treatment facilities are often unable to completely remove commercial dyestuffs; for example, the triphenylmethane group of dyes, including crystal violet, from contaminated wastewater, thereby contributing to the pollution of aqueous habitats [9, 18]. The presence of a very low concentration of the triphenylmethane group of dyes in effluent is highly visible and undesirable [5]. Many dyes are difficult to degrade due to their complex structure and synthetic origin. Therefore, the microbial degradation of the triphenylmethane group of dyes has received considerable attention from the viewpoint of treating industrial wastewater containing such dyes. The wood-rotting fungus, *Phanerochaete chrysosporium* [1, 3, 12, 14], and *Aspergillus sojae* [6] have been shown to exhibit a microbial demethylation reaction of dyes. *Pseudomonas pseudomallei* [19], *Bacillus subtilis* [18], *Nocardia corallina* [16], and *Enterobacter cloacae* [4] have also been investigated for their ability to decolorize several triphenylmethane dyes. Roth *et al.* [11] isolated twenty-one *Mycobacterium* strains that exhibited decolorizing activity with synthetic dyes. However, very few reports are available on the biodegradation products or intermediates of triphenylmethane dyes. Accordingly, we report in the current paper the isolation and examination of microorganisms able to decolorize crystal violet, the taxonomical identification of a new isolate by phylogenetic analysis, and the microbial degradation and digestion products of crystal violet by the Gram-negative microorganism, *Stenotrophomonas maltophilia* LK-24.

Materials and Methods

Chemicals

The crystal violet used this study was purified by preparative thin-layer chromatography (TLC) and then re-

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crystallized from ethanol. The 4,4'-bis(dimethylamino)-benzophenone (Michler's ketone) was purchased from Aldrich Chem. Co. (U.S.A.) and used without further purification, however its purity was examined by TLC and confirmed to be homogenous. The p-dimethylaminophenol was synthesized by the method of Porter and Spears [10].

Isolation of Dye Decolorizing Organisms

Various soil and liquid samples were collected from the textile dyeing industry and screened for dye decolorization organisms. The screening of strains for dye decolorization was carried out on agar plates containing (gl^{-1}): peptone 5.0, NaCl 5.0, yeast extract 2.0, beef extract 1.0, agar 15.0, and crystal violet 100 $\mu g\ ml^{-1}$. The organisms were selected on the basis of a clear zone on the agar plate. The strains grown on the plates that decolorized the dye were then incubated in Simmons' medium (galactose 2 g; $MgSO_4$ 0.2 g; K_2HPO_4 1 g; $NH_4H_2PO_4$ 1 g, NaCl 5 g, tween 80 0.1% per 1 l) containing 100 $\mu g\ ml^{-1}$ of crystal violet at 37°C for 3–5 days on a shaker (150 rpm), and the strain with the strongest activity to degrade the triphenylmethane group of dyes was selected.

Determination of Biochemical and Physiological Characteristics of Strain LK-24

The morphological and biochemical characteristics were determined using the method of Gerhardt *et al.* [4], and carbon utilization, the optimum pH and temperature for growth, a hemolysis test, homo-hetero fermentation test, and Gram straining were further invertigated.

Identification of Strain LK-24

The chromosomal DNA was isolated by the method described elsewhere [20]. The amplification of the 16S rDNA was performed using two primers according to Stackebrandt and Liesack [15]; 5'-GAGTTTGATCCTG-GCTCAG-3' and 5'-AGAAAGGAGGTGATCCAGCC-3'. A PCR was run for 35 cycles in a DNA thermal cycler, Genetic analyzer 377 (Perkin-Elmer, Boston, U.S.A.), employing the thermal profile according to Yoon *et al.* [20]. The 16S rDNA sequence of strain LK-24 was aligned using CLUSTAL W software [8] and the evolutionary distance matrices were calculated with the DNADIST program within the PHYLIP package [3]. The sequence of representative species of the genus *Stenotrophomonas* and related taxa were cited from the GenBank database. The values of 16S rDNA similarity were calculated from the alignment, while the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method [13] based on the calculated distance matrix.

Decolorization Assays

The decolorizing activity was expressed in terms of the percentage decolorization using the modified method of Yatome *et al.* [17] and determined by monitoring decrease in absorbance at 590 nm for crystal violet. A scanning spectrophotometer (Specord 10, Carl Zeiss Technology, Jena, Germany) was used for absorbance measurements and recording the visible absorption spectra. A 1 ml sample was removed aseptically and the residual dye in the was mixture extracted with 1-butanol (2 ml). The absorbance of the residual extracted dye was measured at 590 nm after the dye was dissolved in water-saturated 1-butanol. For the control, only the broth (1 ml) was extracted with 1-butanol (2 ml). The decolorization activity was calculated as follows: decolorization (%)=[(Initial absorbance)-(Observed absorbance)/(Initial absorbance)]×100.

Identification of Degradation Products

S. maltophilia LK-24 were allowed to grow for 48 h, as described above. At this time, 100 ml of crystal violet (1.0 mg ml^{-1} in H_2O) was added to the cultures with gentle mixing to give a final concentration of 50 μM. The disappearance of crystal violet as well as metabolite formation and disappearance were monitored, and identified by TLC and gas chromatography-mass spectrometry (GC-MS). A culture collection containing precultured *S. maltophilia* LK-24 (10 ml), a LB medium (50 ml), and dye solution (50 ml) was incubated with shaking at 37°C for 48 h. The reaction mixture was centrifuged at 5,000 rpm for 15 min, and the supernatant was then loaded onto a Mono-Q ion-exchange chromatography containing 20% ethanol as a preservative in the dark. Benzene was passed through the column at room temperature for 30 min. The eluate concentrated was applied to a Kiselgel 60 F_{254} (E. Mark, U.K.) and developed with n-hexan-ethyl acetate (4:6, v/v). Finally, the identification of putative biodegradation products was performed by a GC-MS of an authentic compound. A 1 m×3 mm glass column was packed with silicone OV-17 on Chromosorb WAW DMCS. The injection port temperature was 230°C. The oven was initially maintained at 100°C for 1 min and then heated at 20°C min^{-1} to 270°C, where it remained for 30 min. A 1 m×3 mm capillary column was operated with 12 lb in^{-2} of helium as the carrier gas. The mass spectrometer was operated in the 20-V electron impact mode. The source temperature was 270°C. 4,4'-Bis(dimethylamino)-benzophenone (Michler's ketone) was identified in the benzene extract of the reaction mixture on the basis of absorbance at λ_{max} using a scanning spectrophotometer (Specord 10, Carl Zeiss Technology, Jena, Germany).

Assessment of Cell Growth

The growth of *S. maltophilia* LK-24 with the dye solution was monitored by the formation of visible colonies on agar plates. Cell-viability counts were performed by serially diluting the culture mixture and plating the dilution on a minimal medium agar plate containing KH_2PO_4 4.3 g, K_2HPO_4 3.4 g, NH_4NO_3 2.0 g, $MgCl_2$ 0.16 g, $CaCl_2{\cdot}2H_2O$

Table 1. Biochemical and physiological characteristics of strain LK-24.

Cell type	Rod
Gram staining	Negative
Biological characteristics	
Catalase	Positive
Hemolysis	Negative
Motility	Negative
Homo-hetero fermentation	Hetero
Carbon source utilization	Amygdalin, arabinose, cellulose, fructose, galatose, gluconate, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose, sorbitol, sucrose, trehalose
Optimum pH for growth	6.0
Optimum temperature for growth	37–40°C

0.02 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and deionized water 1 l. After incubation at 37°C and pH 7.0 for 5 days, the colony numbers were counted. The cell numbers were estimated using a bacterial counting chamber.

RESULTS AND DISCUSSION

Isolation and Characterization of Strain LK-24

A number of strains were isolated from the wastewater, returned sludge, and soil collected from the textile dyeing industrial complexes. Twelve isolates exhibited ability to degrade crystal violet on the agar plates, and strain LK-24 was selected as the strongest in decolorizing triphenylmethane dyes. The characteristics of strain LK-24 are shown in Table 1. Strain LK-24 was found to be very similar to *Xanthomonas* sp. or *Stenotrophomonas* sp. in its biochemical and physiological characteristics. There have been a few reports on the biodegradation of triphenylmethane dyes by bacteria, viz, *Pseudomonas pseudomalli*, *Bacillus subtilis*, *Nocardia corallina* [17, 18, 19], and *Enterobacter cloacae* [5], however, the present study seems to be the first to report that *Xanthomonas* sp. or *Stenotrophomonas* sp. also have the ability to degrade crystal violet.

Identification of Strain LK-24

The 16S rDNA sequence was analyzed to determine which of the *Stenotrophomonas* species cited in the GenBank

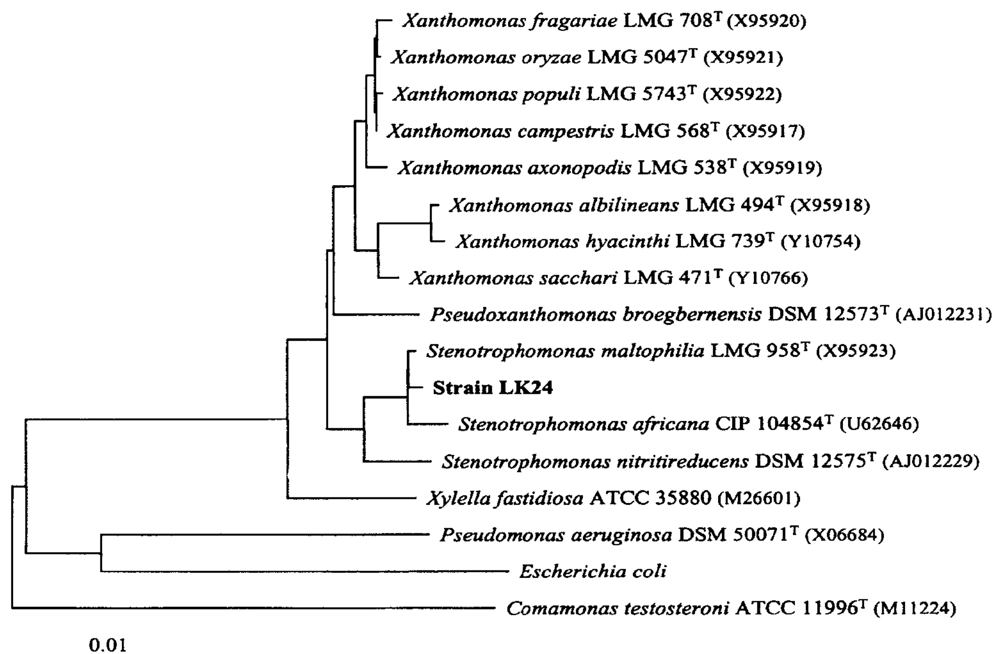

Fig. 1. Phylogenetic tree based on 16S rDNA sequences, showing position of strain LK-24 and reference Protobacterial organisms. The diagram shows the position of strain LK-24, the type of strains of certain *Stenotrophomonas* species, and representatives from various other related taxa. Scale bar represents 0.01 substitutions per nucleotide position.

Table 2. Levels of 16S rDNA similarity between strain LK-24, type strains of certain *Stenotrophomonas* species, and representatives from other related taxa.

Strain	% of Similarity															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 Strain LK-24																
2 *Stenotrophomonas maltophilia* LMG 958^T	99.5															
3 *Stenotrophomonas africana* CIP 104854^T	98.8	99.0														
4 *Stenotrophomonas nitritireducens* DMS 12575^T	97.4	97.5	96.6													
5 *Xanthomonas campestris* LMG 568^T	97.1	97.1	96.5	96.9												
6 *Xanthomonas albilineans* LMG 494^T	95.1	95.3	94.5	95.2	97.8											
7 *Xanthomonas axonopodis* LMG 538^T	96.4	96.5	96.0	96.3	99.3	07.9										
8 *Xanthomonas fragariae* LMG 708^T	96.7	96.8	96.3	96.5	99.6	97.4	99.1									
9 *Xanthomonas oryzae* LMG 5047^T	96.9	97.0	96.4	96.8	99.9	97.7	99.2	99.6								
10 *Xanthomonas populi* LMG 5743^T	96.9	96.9	96.4	96.7	99.9	97.6	99.2	99.5	99.7							
11 *Xanthomonas sacchari* LMG 471^T	96.6	96.8	95.8	96.5	98.7	98.3	98.3	98.3	98.6	98.5						
12 *Xanthomonas hyacinthi* LMG 739^T	95.0	95.2	94.5	95.0	97.7	99.5	97.9	97.3	97.6	97.5	98.1					
13 *Xylella fastidiosa* ATCC 35880	94.0	94.2	94.0	93.8	05.4	95.4	95.2	95.1	95.2	95.2	94.8	95.0				
14 *Pseudoxanthomonas broegbernensis* DSM12573^T	96.5	96.5	95.7	95.5	97.4	95.6	97.1	97.1	97.3	97.2	96.8	95.7	94.4			
15 *Pseudomonas aeruginosa* DSM 50071^T	84.8	85.2	84.7	85.0	85.5	85.9	85.3	85.4	85.5	85.4	85.7	85.8	94.6	85.8		
16 *Escherichia coli*	83.5	82.7	82.3	82.9	84.8	85.0	84.8	84.9	84.9	84.8	84.5	84.5	83.1	83.8	85.8	
17 *Comamonas testosteroni* ATCC 11996^T	83.7	83.7	82.9	83.8	83.4	83.3	83.2	83.2	83.4	83.1	83.3	83.2	83.1	83.8	83.0	81.9

^{T}Type strain.

matched strain LK-24 with the highest homology. The sequence data were aligned to construct a phylogenetic tree, and the tree constructed using the neighbor-joining method is shown in Fig. 1. The phylogenetic position of strain LK-24 was then compared with certain *Stenotrophomonas* species and related taxa in a dendrogram. In the phylogenetic tree, strain LK-24 was the closest to *S. maltophilia* LMG 958 and part of a robust monophyletic cluster with *Stenotrophomonas africana* CIP 104854 and *Stenotrophomonas nitritireducens* DMS 12575. The extent of sequence similarity of strain LK-24 within the monophyletic cluster was greater than 97% (Table 2). The sequence of strain LK-24 was almost identical to that of *S. maltophilia* LMG 958 with 99.5% similarity. Hence, this appears to be the first report that *S. maltophilia* LK-24 has the ability to degrade the triphenylmethane group of dyes.

Decolorization of Crystal Violet

Crystal violet was strongly decolorized by *S. maltophilia* LK-24 after 25–30 h of incubation. The compound was totally decolorized after 30 h of incubation at which point the cell growth was the highest (Fig. 2), although the cell growth was initially strongly inhibited. Moreover, the number of cells significantly increased after 15 h of inoculation. On continued incubation for 24 h, the level of crystal violet was reduced by over 50% of the initial value and the cells exhibited good growth in the culture broth which contained lower concentration of the dye solution (25 and 50 µg ml^{-1}). Therefore, the decolorization rate was dependent upon the initial concentration of dye solution in the culture medium. The decolorization decreased by less than 30% when the initial concentration of crystal violet was increased (100 µg ml^{-1}), and the cell growth was completely inhibited at 200 µg ml^{-1}. Adams [1] reported that crystal violet exhibits an antibacterial action against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Bacillus subtilis*, when the dye is used at a concentration of 1–6 µM. In 1991, Yatome *et al.* [18] stated that the decolorization of crystal violet occurs at a very low concentration of dye (below 10 µg ml^{-1}) when *Bacillus subtilis* was treated. The cell growth and avidity of decolorization of *Nocardia corallina* were completely inhibited at 10 µg ml^{-1} of crystal violet [17]. In the current work, the decolorization rate of

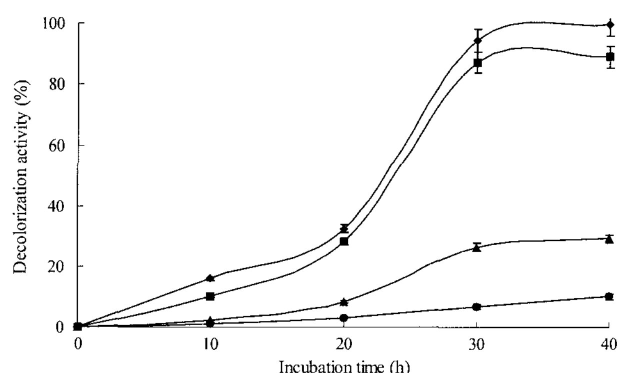

Fig. 2. Time course of decolorization by *Stenotrophomonas maltophilia* LK-24 incubated in LB (pH 7.0) containing various concentrations (25, 50, 100, and 200 µg ml^{-1}) of crystal violet at 37°C.

The initial cell concentration of 100 µl was adjusted to approximately 1.3 at 660 nm. Symbols are: (◆) 25 µg ml^{-1}; (■) 50 µg ml^{-1}; (▲) 100 µg ml^{-1}; (●) 200 µg ml^{-1}.

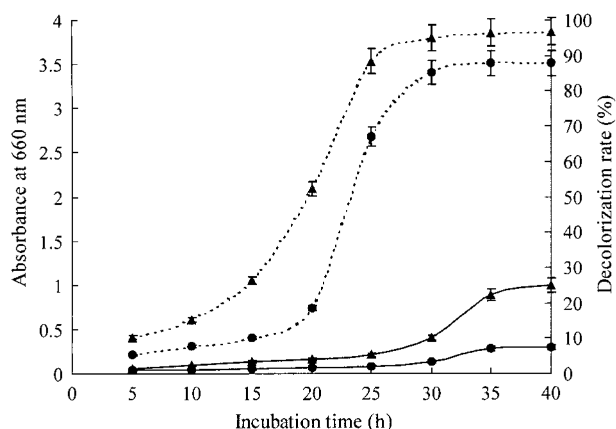

Fig. 3. Time course of decolorization of crystal violet and cell concentration in *S. maltophilia* LK-24 culture.

The initial cell concentrations adjusted to approximately 1.3 at 660 nm were 100 µl (---▲---) and 10 µl (—▲—) at pH 6.0 and 37°C in LB containing 50 µg ml^{-1} of crystal violet. Symbols are (▲), Rate of decolorization and (●), Cell growth of strain LK-24.

crystal violet by *S. maltophilia* LK-24 was found to be over 80% at 120 µM (50 µg ml^{-1}), which was 10–50 times more than that by *Bacillus subtilis* [18] and *Nocardia corallina* [17]. Crystal violet usually forms a unionized complex of bacteria. Since Gram-negative bacteria have high isoelectric points and less acidic components than Gram-positive organisms which usually have lower isoelectric points, *Stenotrophomonas maltophilia* LK-24 was more resistant to crystal violet. As shown in Fig. 3, the decolorization was dependent on the initial concentration of *S. maltophilia* LK-24 in the medium. Also, the decolorization rate was related to the growth phase of the precultured cells. The rates of crystal violet (50 µg ml^{-1}) decolorization in the initial log phase (10–20 h incubation), mid-log phase (20–30 h incubation), and late log phase (30–40 h incubation) was 50 ng^{-1} min^{-1}, 16 ng^{-1} min^{-1}, and 7 ng^{-1} min^{-1}, respectively. Since no increase in the cell concentration was observed within the first 10 h of incubation, 100 µl of a cell suspension adjusted to approximately 1.3 at 660 nm was used for the initial cell concentration to calculate the decolorization rate. In addition, the decolorizing activity in the cell homogenates of *S. maltophilia* LK-24 was observed when incubated in LB.

Identification of Metabolites

The biodegradation products of crystal violet were identified by TLC and GC-MS, based on a comparison of the absorption maximum of each metabolite with that of the appropriate analytical standard. The maximum absorption of the extract from the culture mixture was observed at 348.2 nm, as clearly agreeing with that of the compared authentic sample of 4,4'-bis(dimethylamino)-benzophenone. Based on a library search of peaks, this wavelength coincided with that of 4,4'-bis(dimethylamino)-benzophenone, whereas crystal violet does not have maximum absorption

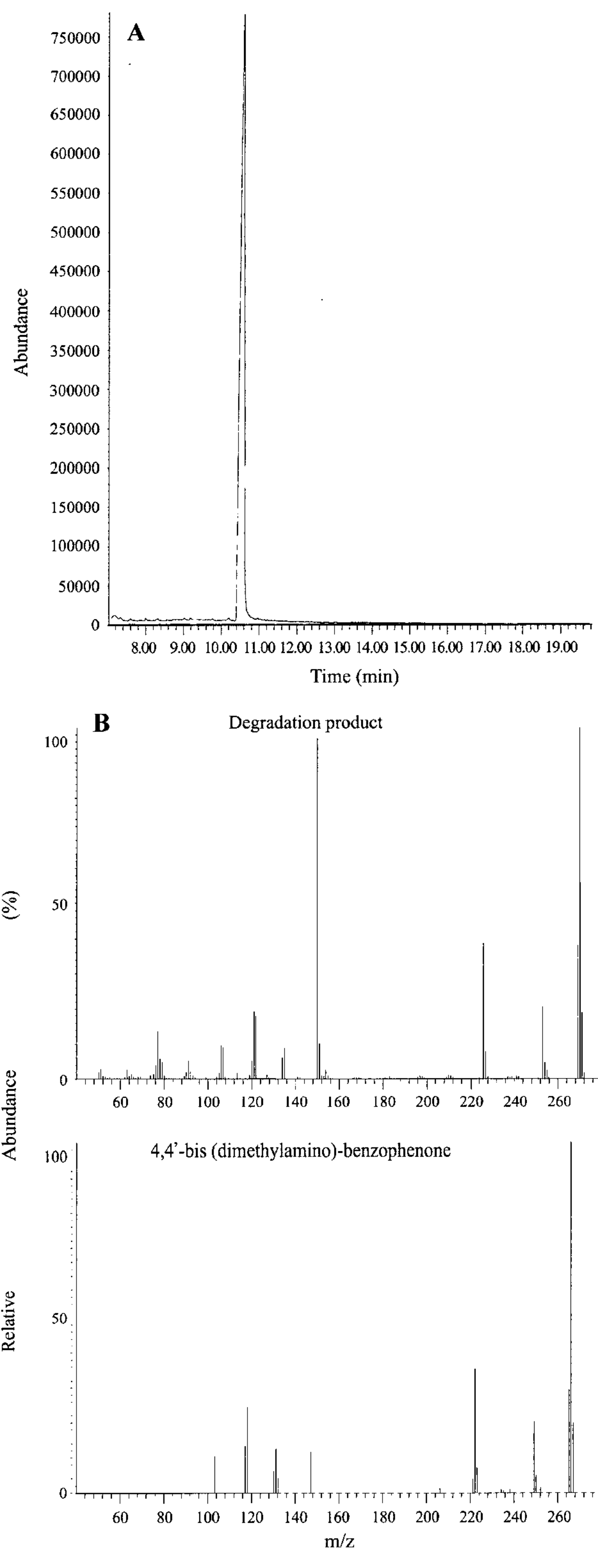

Fig. 4. GC-MS total and specific ion trace of crystal violet metabolites formed by *S. maltophilia* LK-24.

A. Total ion trace of benzene extractable metabolite formed from crystal violet by *S. maltophilia* LK-24. **B.** Mass spectra of benzene extractable metabolite formed from crystal violet by *S. maltophilia* LK-24 and authentic 4,4'-bis (dimethylamino)-benzophenone [Michler's ketone, MK].

at the same wavelength. Approximately 40 µl of benzene extract was applied in a line 2 cm from the bottom of the plate, and the TLC plate was then allowed to develop for 2 h to separate two brightly colored bands. Band I with R_f=0.70 and band II with R_f=0.35 were designated as 4,4'-bis(dimethylamino)-benzophenone and ρ-dimethylaminophenol, respectively. The R_f value of Band I was coincident with the R_f value of the authentic sample, 4,4'-bis(dimethylamino)-benzophenone, and also in close agreement with previous reports [1]. Therefore, the extract was applied onto GC-MS (chamber voltage of 20 eV and retention time of 10.6 min on GC) to confirm the structure. The GC-MS profile and specific degradation products of benzene extract after 48 h of incubation of crystal violet with *S. maltophilia* LK-24 are shown in Figs. 4A and 4B. The compound with a retention time of 10.6 min on GC and the mass spectral fragmentation patterns of the elute were identical to the authentic 4,4'-bis(dimethylamino)-benzophenone. Prominent characteristic peaks appeared at m/z (relative intensity) 269 (M+1, 27), 268 (M^+, 100), 267 (M−1, 38), 251 (25), 240 (2), 225 (10), 224 (37), 222 (12), 208 (2), 149 (15), 134 (4), 133 (14), 120 (22), and 105 (10), which were also similar to those of the authentic 4,4'-bis(dimethylamino)-benzophenone compound. A putative product having melting point of 75±1°C was revealed at m/z (relative intensity) 148 (100), 119 (M^+, 22), 118 (18), 104 (12), 91 (8), 77 (15), 63 (5), and 51(5). This appeared to be ρ-dimethylaminophenol, as reported previously [4, 8]. Therefore, it was obvious that crystal violet incubated with *S. maltophilia* LK-24 at 37°C for 48 h was converted to 4,4'-bis(dimethylamino)-benzophenone and ρ-dimethylaminophenol. The microbial degraded product 4,4'-bis(dimethylamino)-benzophenone was found to be very stable, since it was not entirely cleaved within 48 h by *S. maltophilia* LK-24 (shown in Table 3) and further metabolism of 4,4'-bis(dimethylamino)-benzophenone was not observed. Same interpretation as given previously by Yatome *et al.* [17] can also be applied here. Bumpus and Brock [2] studied biodegradation and mineralization of crystal violet by white-rot fungi, using High-Performance Liquid Chromatography to monitor the disappearance and metabolite formation from crystal violet, and they identified 3 degradation products: N,N,N',N',N"-penta, N,N,N',N"-tetra, and N,N',N"-trimethylpararosaniline. In all, crystal violet degraded into colorless products.

Table 3. Growth monitored by formation of visible colonies on agar plates after 5 days at 37°C on minimal medium containing 0.1 % NH_4NO_3 with various substrates as carbon source.

Substrates (µmol l^{-1})	Growth
Crystal violet (2.5)	+
Crystal violet (25.0)	−
MK (2.5)	−
MK (25.0)	−
DAP (2.5)	+
DAP (25.0)	+

+, slight growth; −, no visible growth. MK, 4,4'-bis(dimethylamino)-benzophenone; DAP, ρ-dimethylaminophenol.

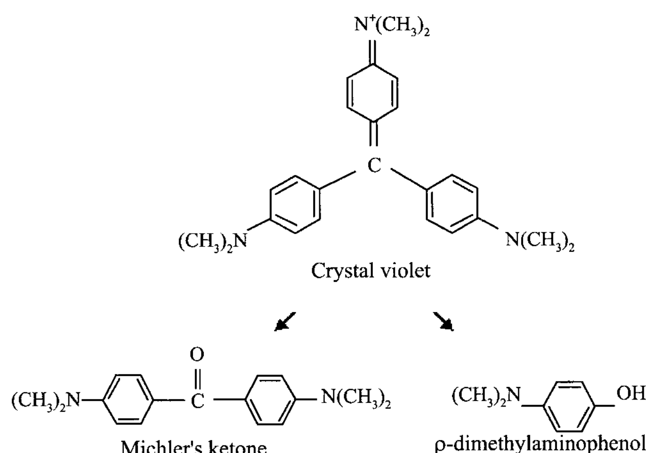

Fig. 5. Proposed pathway of formation of crystal violet metabolites by *S. maltophilia* LK-24.

Growth Kinetics of *S. maltophilia* LK-24

The further results on the metabolites of the biodegraded crystal violet are shown in Table 3. The crystal violet (N,N,N',N',N",N"-hexamethylpararosaniline) and DAP (ρ-dimethylaminophenol) were found to be slightly metabolized, whereas the MK [4,4'-bis(dimethylamino)-benzophenone] did not undergo any further cleavage by *S. maltophilia* LK-24, when treated in the minimal medium including 0.1% NH_4NO_3 as the nitrogen source. These experimental results were due to the toxicity of MK. In previous study, Yatome *et al.* [17] stated that *Nocardia corallina* was unable to use crystal violet and 4,4'-bis(dimethylamino)-benzophenone, yet could utilize ρ-dimethylaminophenol. However, in this case, the minimal medium contained 5 mM NaCl and 0.5 mM $MgSO_4 \cdot 7H_2O$, which resulted in deficiency of a nitrogen source. Hence, the bacterium, *Nocardia corallina*, could not grow in the presence of crystal violet and MK. Based on the present study, the biodegradation pathway of crystal violet by *S. maltophilia* LK-24 is proposed in Fig. 5. Based on the identification of the metabolites of crystal violet, a triphenylmethane group dye, with *S. maltophilia* LK-24, it is therefore suggested that this Gram-negative bacterium be used for the treatment of polluted industrial wastewater originating from textile dyeing and dyestuff manufacturing industries.

Acknowledgment

Korea Research Foundation Grant (KRF-99-005-G00004) supported this work.

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