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Plasmid-Mediated Aniline Assimilation by *Pseudomonas* sp. B10

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Summary An aniline-utilizing microorganism identified as a species of *Pseudomonas* was isolated from soil contaminated highly with aniline and urea-herbicide. This strain was able to utilize aniline as the sole source of carbon and energy, and was shown to harbor a single large plasmid mediating the aniline assimilation. Subsequent plasmid-curing of this bacterium resulted in the abolishment of the aniline utilizing phenotype and the loss of catechol-C2,3O-oxygenase. The reestablishment of the plasmid, denoted pB10, in cured *Pseudomonas* sp. via filter surface mating, resulted in restoration of the aniline assimilation abilities and enzyme activity.

Key words: Aniline, degradation, catechol-C2,3O-oxygenase, plasmid

Aniline and its derivatives are widely used in the chemical industry in a variety of processes such as the production of dyes, synthesis of chemical compounds, and development of aniline-based herbicides. As a result of these industrial activities, aniline-containing compounds and complexes are discharged into the environment as industrial wastes. Considerable attention has therefore been devoted to environmental pollution due to aniline [4, 12, 17]. The detoxification of aniline-containing waste is largely performed by chemical treatment. However, the chemical treatment of waste fluids containing anilines has an associated problem of generating other toxic by-products. Accordingly, biodegradation is considered as a potential alternative method for reducing the accumulation and persistence of these chemicals.

The bacterial degradation pathway of aniline has been previously reported. The metabolization of anilines proceeds via primary oxygenation and ammonium elimination by an aniline oxygenase leading to the corresponding catechol. The resulting catechol is then degraded through either the *ortho-* [3, 16, 23] or the *meta*-cleavage pathways [9, 15, 18].

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The involvement of a naturally occurring plasmid in the degradation of aromatic compounds has been extensively documented. The dissemination of biodegradative genes by bacterial plasmids has led to the rapid evolution of certain specified strains capable of using these xenobiotic compounds as substrates. Recently, several authors [1, 10, 11, 18] have also recognized plasmids involved in aniline degradation. Thus, plasmids are thought to play an important role in the evolution of aniline-containing compound-degrading capabilities. However, some are known to be the plasmid-mediated degradation of anilines [1, 10, 11, 19].

This report describes the utilization of aniline by *Pseudomonas* sp. strain B10. Furthermore, the role of a plasmid in the aniline degradation is also indicated.

Materials and Methods

Bacterial Strains and Culture Conditions

The isolated *Pseudomonas* strains, mutants, and transconjugants plus the plasmid used in this study are all listed in Table 1. The *Pseudomonas* sp. strain B10 isolated using enriched cultures from soil was selected for its ability to utilize aniline as a carbon and energy source [8].

The rifampicin-resistant (rif^r) mutant of B10M was isolated by plating a dense culture of this strain onto LB plates containing 25 μg of rifampicin per ml. The plates were incubated for 2 to 4 days at 28°C. A single-mutant colony was restreaked onto the same medium for final purification. The mutant was then used as the primary recipient in the mating assays.

Culture Conditions

The minimal salt medium (MS) used throughout this study was prepared as following (g/l): $(NH4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.05; Na_2HPO_4, 2.44; and KH_2PO_4, 1.52 g. The pH was adjusted to 6.8 and the medium was sterilized prior to the addition of organic substrates. $(NH4)_2SO_4$ was omitted from the MS if aniline was used as the sole source of nitrogen. The complete medium used

Table 1. Bacterial strains and plasmids used in this study.

Strains	Plasmids and comments	Phenotype	Source
Pseudomonas sp. B10	Wild-type aniline degrader, pB10	An^+ Rif^s	[8]
Pseudomonas sp. B10 M	pB10-Cured derivatives of B10	An^- Rif^r	This study
Pseudomonas sp. B10TC	Transconjugants between B10 and B10M, pB10	An^+ Rif^r	This study

An^+, ability to degrade aniline; An^-, inability to degrade aniline; Rif^r, rifampicin resistant; Rif^s, rifampicin sensitive.

was an L-Broth containing 10 g Bacto-peptone, 5 g Bacto-yeast extract, and 5 g NaCl in 1-l distilled water (pH 7.0). The agar was added at a concentration of 1.5% for the solid media. The liquid cultures were grown in 250-ml conical flasks (working volume of 100 ml) on a rotary shaker for enzyme production and cell propagation. The rates of bacterial growth were measured spectrophotometerically at OD_{600}.

Utilization of Aniline as a Sole Source of Carbon and Nitrogen

Growth at the expense of aniline was established by demonstrating an increase in the bacterial biomass with a concomitant decrease in the concentration of aniline. The aniline concentration in the culture was determined by using a Daizo-coupling reaction [21].

Substrate Spectrum

Growth on the aniline derivative compounds was screened with a solid mineral medium. 2-Chloroaniline, 3-chloro-4-methylaniline, 3-chloroaniline, 2,3-dichloroaniline, m-toluidine, o-toluidine, 3,5-dichloroaniline, catechol, and phenol were supplied at 0.5 mM. Growth evidenced by a significant increase in the bacterial mass on the test plates was compared to that on the control plates incubated in the absence of the substrates. The plates were incubated at 30°C for 5 days. The results were confirmed by inoculating strain B10 into a 250-ml flask containing 50 ml of the mineral medium with individual substrates supplied at 0.5 mM. The culture was analyzed for evidence of increase in the turbidity of the cells using a spectrophotometer at 600 nm.

Preparation of Cell Extract and Enzyme Assay

The cells were grown to the mid-logarithmic growth phase and harvested by centrifugation (8,000 × g for 15 min at 4°C). The cell pellets were washed twice with a 50 mM phosphate buffer (pH 7.0). The cell paste was then re-suspended in the same buffer and disrupted by sonication for 20 min at 0°C. The cellular debris was removed by centrifugation (10,000 × g for 20 min at 4°C). Acetone was added at 10% (v/v) to the clear supernatant (crude extract) and the mixture was used immediately for the enzyme assay. The C230 activity was measured spectrophotometrically by the increase in A_{375} concomitant with the formation of 2-hydroxymuconic semialdehyde. The reaction mixtures contained 0.1 mM catechol and the cell extract in phosphate buffer (pH 7.0) at 24°C, and the amount of 2-hydroxymuconic semialdehyde formed was estimated as described previously by Nakazawa and Yokota [20]. Specific activity was expressed as U/mg of protein. The protein concentration was measured by the Lowry method using bovine serum albumin as a reference protein.

Plasmid Isolation

The plasmid from strain B10 and the transconjugants were isolated by the Kado and Liu method [13].

Isolation of Aniline-Assimilating Mutant

Mitomycin C was added to a final concentration of 10 mg/ml of an L-Broth culture inoculated with a loopfull of 24 h growing cells. The culture was shaken at 30°C for 18 h. A sample from the culture that showed some growth was then diluted and spread on LB plates. The colonies appearing on the plates were replicated onto aniline-minimal salt medium plates to examine the aniline-utilizing abilities. In addition, the growth of strain B10 in the LB broth medium with no aniline was used as a curing procedure to isolate the cells that lost plasmid spontaneously.

Conjugation

The mating between B10 (An^+ rif^s) as the donor and the B10M mutant (An^- rif^r) as the recipients was performed with equal cell densities of mid-log or early stationary-phase cells grown in L-Broth. To initiate the conjugation, samples containing 1 ml of both the donor and the recipient cells were filtered onto a sterile acetate cellulose filter (0.2 µm pore size). The filter was then placed on an LB plate and incubated overnight at 25°C. Next, the filter was transferred to a test tube containing 1 ml of 0.9% NaCl and the tube was vortexed to suspend the bacteria. The cell suspension was diluted and plated on a selective medium containing aniline (1 mg/ml) and rifampin (25 µg/ml). The donor and recipient cultures were also separately plated to assess the occurrence of antibiotic-resistant mutants on the transconjugant selective plates. The plates were incubated for 2 days at 30°C.

Screening of Plasmid for Antibiotic and Heavy Metal Resistances

The presence of plasmid-encoded resistant genes was assessed by streaking the plasmid containing strain B10

onto LB plates containing different concentrations of antibiotics or heavy metals (Table 3). The plasmid-less strain B10M was used as the control. The growth of the cells in the repeated transfer of the plasmid-loaded strain into single colonies and the absence of such growth in the plasmid-less strain was taken as evidence for the presence of the respective resistance genes.

RESULTS AND DISCUSSION

The enrichment procedure yielded a pure culture, designated B10, that was able to grow on aniline as the sole source of carbon and energy. This strain was identified as a *Pseudomonas* sp. [8].

Growth Kinetics

Examination of the growth curves of strain B10 indicated that after a short lag of approximately 8 h, this isolate was able to grow in a minimal medium containing aniline as the sole carbon and energy sources (Fig. 1). Similar growth studies using a different concentration (0.2 to 1 mg/ml) of aniline showed a corresponding increase in turbidity, thereby suggesting that the growth was directly proportional to the aniline concentration (Fig. 2). As the concentration of aniline increased above 1 mg/ml, the B10 cells exhibited a slower rate of growth (Fig. 2). This may have been due in part to saturation of the enzymes responsible for the aniline degradation and in part to the toxicity of aniline itself. No growth was observed in the minimal medium inoculated with the B10 strain without added aniline (Fig. 1). This was further proof for the growth of B10 at the expense of aniline.

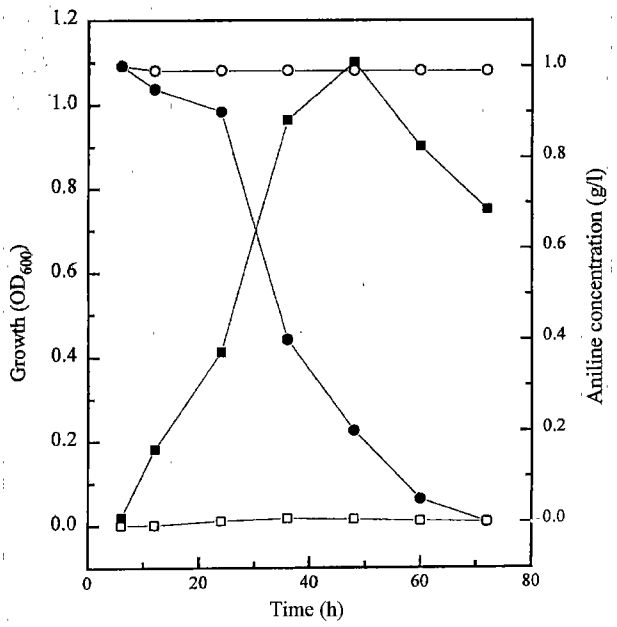

Fig. 1. Utilization of aniline by growing cells of *Pseudomonas* sp. strain B10.
Aniline concentration was determined as described in Materials and Methods. Symbols: (○), concentration of aniline in uninoculated flask; (●); concentration of aniline in inoculated flask; (□), OD_{600} of uninoculated flask; (■), OD_{600} of inoculated flask.

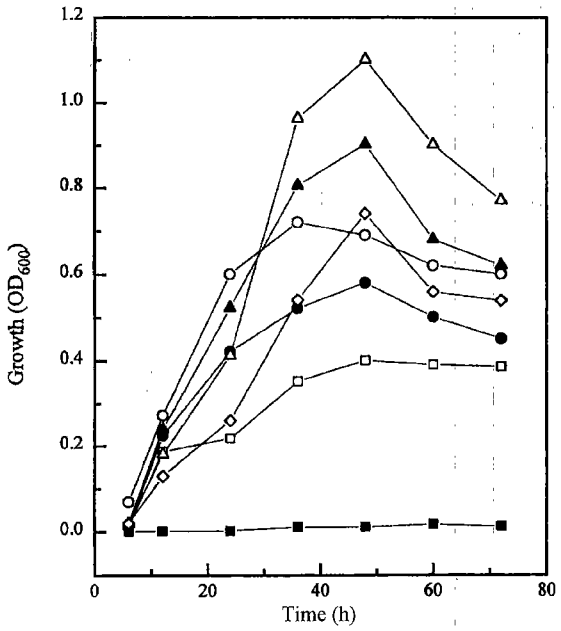

Fig. 2. Growth of *Pseudomonas* sp. strain B10 in minimal medium with aniline as the sole source of carbon and nitrogen.
Concentrations of aniline are: (■), no aniline; (□), 0.2 mg/ml; (●), 0.4 mg/ml; (○), 0.6 mg/ml; (▲), 0.8 mg/ml; (△), 1 mg/ml; (◇), 1.2 mg/ml.

Aniline Utilization

The assimilation of aniline by *Pseudomonas* sp. strain B10 was confirmed by its removal from the aniline-mineral salts medium, with a corresponding increase in the bacterial biomass (Fig. 1). After an 8 h lag phase, the concentration of aniline decreased slightly during the ensueing 24 h and then dramatically over another 48 h. During the stationary phase, the amount of aniline decreased slowly to a level no longer detectable by an HPLC (data not shown).

Enzyme Activities

During growth, the color of the culture gradually turned yellowish, which probably indicated the formation of an intermediate, 2-hydroxymuconic semialdehyde, from aniline [2, 10]. Therefore, it was presumed that the aniline was metabolized *via* catechol, thereby involving the *meta*-cleavage of the benzene ring [10]. To determine if B10 degraded aniline in an analogous manner and possessed *meta*-cleavage activity, the enzyme activity of catechol 2,3-dioxygenase (C2,3O) in the crude cell extract was determined. The data presented in Table 2 indicate that this was indeed the case. A comparison of the enyzme activity in the cells grown in

Table 2. Specific activities of *meta*-cleaving enzyme C2,3O dioxygenase in crude extracts of strain B10 and its derivatives grown on different media. Enzyme is assayed as described in Materials and Methods.

Growth medium	Strain B10	Strain B10M	Strain B10TC
Aniline	18.8	0.001	18.8
Succinate	0.001	0.001	0.001
L-Broth	ND	ND	ND

Enzyme activity is given in U/mg of protein.
ND=not detected.

the L-Broth or the minimal medium containing succinate as the sole carbon source, with that in the cells grown in the presence of aniline, clearly demonstrated that C2,3O dioxygenase was induced above basal levels in the presence of aniline. In contrast, no catechol 1,2 dioxygenase was found (data not shown).

Growth on Other Aromatic Compounds

In addition to utilizing aniline, strain B10 was shown to grow with both phenol and catechol. In contrast, no growth was observed with aminophenols, 2-chloroaniline, 3-chloro-4-methylaniline, 3-chloroaniline, 2,3-dichloroaniline, m-toluidine, o-toluidine, and 3,5-dichloroaniline. Similar restrictions on the degradation of differentially substituted aromatic amines have been reported for other aniline-degrading bacteria [10].

Plasmid-Mediated Aniline Assimilation in Strain B10

Pseudomonas sp. strain B10 was investigated for its plasmid DNA using the procedure as in Materials and Methods. The agarose gel electrophoresis of the cell lysate revealed that strain B10 carried one large plasmid (Fig. 3, lane 2). This plasmid was designated as pB10.

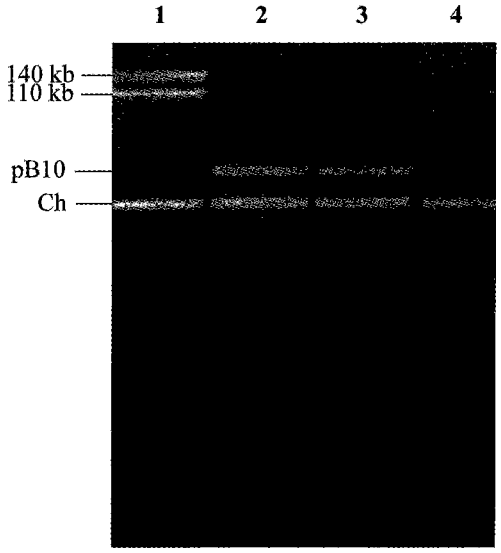

Fig. 3. Plasmid profiles of strain B10 and its derivatives.
Lanes: 1, *Shigella flexneri* 49 used as size standard; 2, wild-type strain B10; 3, B10TC transconjugant obtained by mating B10 with B10M; 4, B10M mutant from mitomycin C-treated culture. Ch, chromosomal DNA.

Existing genetic evidence suggests that a significant proportion of the pathways specifying the catabolism of anilines by bacteria are encoded by plasmids [1, 10, 11, 19]. Therefore, this may also be the case for the aniline degradation encoded by B10.

Two approaches were used to prove that the aniline degradation in strain B10 was determined by plasmid genes. The first approach was based on the idea that elimination of the plasmid containing the aniline-degrading genes should eliminate the capacity for growth on aniline-containing media. However, since the aniline-degrading genes can be partially located on the chromosome, the plasmid location of the aniline-degrading genes was distinctly proven by the simultaneous transfer of the plasmid and the trait of aniline degradation (An^+) into an aniline degradation deficient mutant (An^-).

To eliminate the plasmid, B10 was grown in a batch culture in a LB medium under nonselective conditions, i.e. in the presence of mitomycin C or without aniline. Mutants unable to utilize aniline as the sole source of carbon and nitrogen appeared at a high frequency from the wild-type strain B10 by curing with mitomycin C. The frequency of the loss of the An^+ phenotype in strain B10 during successive growth on LB was less than 0.2%, and this increased up to 5% when mitomycin C was present during growth. Additionally, there was no reversion to the An^+ phenotype from An^-. If a revertant did occur, it did so at a frequency well below 10^{-8}. The plasmid was found to be absent in those strains that could no longer grown on aniline (Fig. 3, lane 4). Moreover, the aniline grown B10M cells showed no C2,3O dioxygenase activity (Table 2). These results indicate that the loss of the plasmid caused the concomitant loss of aniline assimilation (Fig. 4) and the loss of C2,3O dioxygenase activity. One of these mutant strains, designated B10M, was subjected to biochemical tests (data not shown) and appeared identical to the wild-type in all respects, except for the loss of the aniline assimilation ability.

Conjugational Transfer of pB10 Plasmid

The ability of an An^+ marker to be transferred in bacterial crosses was investigated. The nonrevertible An^- derivative of B10 (B10M) was chosen as the recipient and strain B10 as the donor. A rifampicin-resistant mutant of recipient (B10M) was obtained as described in Materials and Methods. An^+ transconjugants with a low frequency were selected from a number of bacterial crosses based on their growth on a minimal medium supplemented with aniline as the growth substrate. The transconjugates obtained were all rifampicin-resistant, showing that they represented true transconjugants and not the donor. The ability to utilize aniline was transferred from B10 (pB10, An^+) to B10M (plasmid free, An^-). The

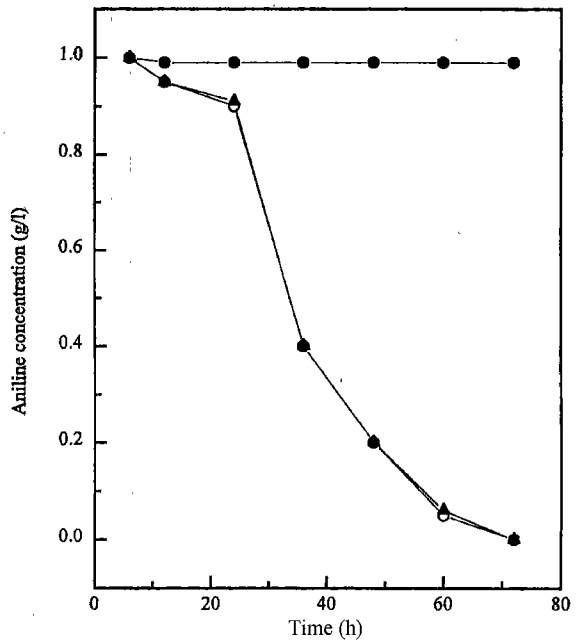

Fig. 4. Degradation of aniline by wild-type, cured, and transconjugant strains of strain B10.
Ms medium containing aniline (1 mg/ml) was inoculated with wild-type strain B10 (○); a cured derivative, B10M (●); or a transconjugant strain, B10TC, (▲) obtained by mating B10 with B10M.

frequency of the plasmid transfer ranged from 10^{-5} to 10^{-4} per donor cell. The transconjugants which had acquired the ability to utilize aniline, contained a plasmid which was shown to be identical to pB10 by agarose electrophoresis (Fig. 3, lane 3). Moreover, the resulting transconjugants were found to have a similar catabolic enzyme induction (Table 2) and to be able to grow as efficiently on aniline as the original strain (Fig. 4). This provided additional evidence that pB10 functionally mediated aniline utilization. One of these transconjugants was designated as B10TC.

Screening for Markers on Plasmid pB10

In many cases, antibiotic resistancy, heavy metal resistancy, and the capacity for xenobiotic degradation are known to be determined by plasmid genes [5-7]. Table 3 showed that plasmid pB10 did not exhibit resistance to any of the antibiotics or heavy metals.

In summary, *Pseudomonas* sp. strain B10 can grow with aniline as the sole source of carbon and nitrogen. The observed growth suggested that this process is a highly efficient assimilation of aniline as compared with other isolated strains [16]. This study revealed that the ability of *Pseudomonas* sp. strain B10 to utilize aniline is encoded by plasmid genes. The data presented in this paper also demonstrated that aniline is degraded *via* catechol, which appears to be the case in all published papers on aniline-utilizing bacteria. Kaminski *et al.* [14] reported on the aniline-utilizing pseudomonad, SB3, in which catechol is degraded by the *meta*-cleavage pathway, and the C2,3O enzyme is constitutive, which is uncommon for catabolic enzymes. B10 also appears to convert catechol *via* the *meta*-pathway; however, in this case, the enzyme appears to be inducible. This is the more usual situation in *Pseudomonas*, which contains plasmids that degrade aromatic compounds [1, 10, 22].

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Table 3. Antibiotic and heavy metal resistance of *Pseudomonas* sp. strain B10 and its mutant B10M.

Antibiotics	Wild-type B10	Mutant B10M	Heavy metals 100 μg/ml	Wild-type B10	Mutant B10M
Ampicillin 10 μg	-	-	Cadmium (Cd^{+2})	-	-
Erythromycin 15 μg	+	+	Zinc (Zin^{+2})	+	+
Gentamycin 10 μg	+	+	Cobalt (Co^{+2})	+	+
Kanamycin 10 μg	-	-	Nickel (Ni^{+2})	-	-
Novobiocin 30 μg	-	-	Mercury (Hg^{+2})	-	-
Penicillin 30 μg	-	-			
Streptomycin 10 μg	+	+			

for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134 (pJP4). *J. Bacteriol.* **161:** 85-90.

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