

Application of *p*-Toluidine in Chromogenic Detection of Catechol and Protocatechuate, Diphenolic Intermediates in Catabolism of Aromatic Compounds

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In the presence of *p*-toluidine and iron, protocatechuate and catechols yield color. Inclusion of *p*-toluidine in media facilitates the screening of microbial strains for alterations affecting aromatic catabolism. Such strains include mutants affected in the expression of oxygenases and *Escherichia coli* colonies carrying cloned or subcloned aromatic catabolic genes which encode enzymes giving rise to protocatechuate or catechol. The diphenolic detection system can also be applied to the creation of vectors relying on insertion of cloned DNA into one of the latter marker genes.

Research into the genetics of aromatic catabolism in microbes has blossomed over the past decade. Recognition of the roles that microbes can play in industrial chemical conversions, for example, in producing specific isomers (8, 21) and in degrading environmental contaminants (1, 11, 24), has given impetus to this focus. A major source of simple and complex aromatic compounds in soil and water is the plant polymer lignin. This compound is estimated to be the second most abundant organic material on earth and thus represents a tremendous source of biomass and energy production (6). Living plants produce a diverse array of aromatic compounds, some of which are known to be associated with plant cell walls or released into the environment (7, 14, 22, 33, 38-40). Members of the family *Rhizobiaceae*, which interact with plants as pathogens or symbionts, utilize aromatics as sources of carbon and energy (4, 5, 15, 27) and recognize many of these growth substrates as signal molecules (29, 30), undergoing chemotaxis in response to them. Specific aromatics produced by plants induce the nodulation and virulence genes in this group of bacteria (32). In members of the family *Rhizobiaceae* and many other microbial groups, numerous aromatic compounds are ultimately degraded to one of the *ortho*-diphenolics catechol or protocatechuate. Subsequently, the two phenolics are cleaved by *ortho*-3,4-dioxygenases and processed through the β -ketoadipate pathway to tricarboxylic acid cycle intermediates (27, 28). Some microbes possess a *meta*-cleavage route for the catabolism of protocatechuate or catechol; one route for processing such toxic environmental pollutants as toluene and xylenes is via catechol with subsequent cleavage by a *meta*-2,3-dioxygenase (12, 34, 41).

Chromogenic substrates are used in bacterial diagnostics (3, 20) and are tools in molecular biology, immunoblotting, and immunocytochemistry (35). Two well-known examples include the substrate analogs 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium (NBT), acted upon by β -galactosidase and alkaline phosphatase, respectively, which yield colored products. Catechol is used as a histochemical indicator in conjunction with the *meta*-dioxygenase gene: the *Pseudomonas putida xylE* gene product, catechol 2,3-dioxygenase, converts catechol into a yellow hydroxymuconic semialdehyde (16, 19, 42). In this report, I describe the use of *p*-toluidine to detect protocatechuate or catechol accumulation in the medium around cells.

Although, unlike BCIP or X-Gal, the detection system is not localized to cells, it is sufficiently sensitive to be used in similar ways. Conditions for the use of *p*-toluidine as an indicator and some applications of the method are described.

Conditions for chromogenic detection of protocatechuate and catechol. Bacterial strains used in the study are listed in Table 1. The minimal medium employed has been previously described (27). It was supplemented with thiamine-HCl at 1 $\mu\text{g ml}^{-1}$, calcium pantothenate at 2 $\mu\text{g ml}^{-1}$, and biotin at 0.5 $\mu\text{g ml}^{-1}$ for growth of *Rhizobium leguminosarum* biovar trifolii. Minimal medium for growth of *Escherichia coli* S17-1 was supplemented with proline at 2.5 mM and thiamine at 1 $\mu\text{g ml}^{-1}$; antibiotics were ampicillin at 100 $\mu\text{g ml}^{-1}$ or tetracycline at 12.5 $\mu\text{g ml}^{-1}$. Ferric chloride was prepared as a 0.5 M solution and filter sterilized. Carbon sources were prepared as 0.05 to 0.5 M solutions, neutralized, and filter sterilized. The source of *p*-toluidine was the Sigma Chemical Co., St. Louis, Mo.

In working with BCIP, it became apparent that the salt, *p*-toluidine, in one source of BCIP was acting as an indicator for the presence of protocatechuate or catechol. Investigation of conditions for taking advantage of this revealed that color development with *p*-toluidine in agar occurred in minimal medium, but in Luria broth (LB) medium (36) color was greatly reduced. The sole component of minimal medium required for color development was iron. The use of iron to detect protocatechuate in a spot test in liquid has been described (13). In testing the limits of detection of diphenolics on solidified minimal medium, described below, neither iron nor *p*-toluidine alone supported the color development characteristic of diphenolics in the presence of iron and *p*-toluidine. Addition of iron as ferric chloride at a final concentration of 1.5 mM permitted color development with *p*-toluidine on LB medium and doubled the color development on minimal medium without having a toxic effect on cells. For the work described in this note, minimal medium was employed because it gave a clear background. Unless specifically mentioned, iron supplementation of minimal medium was not used.

A 1 M solution of *p*-toluidine in *N,N*-dimethylformamide was found to be stable over a prolonged period at 5°C in the dark. Usually, an aliquot of the stock solution was spread on plates prior to inoculating cells. Full color development was

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>A. calcoaceticus</i>		
ADP1	Wild type (BD413)	17
ADP6	<i>pcaA3006</i> ; derived from ADP1	9
ADP139	<i>catA3139</i> ; derived from ADP125, <i>pcaE3125</i>	23
<i>A. tumefaciens</i>		
A348	Wild type	18
ADO2032	Km ^r ; Tn5 mutant of A348; <i>pcaA</i>	26
<i>E. coli</i> S17-1	<i>recA pro hsdR RP4-Tc::Mu-Km::Tn7</i> integrated into the chromosome	37
<i>P. putida</i>		
PRS2000	Wild type	25
PRS3000	<i>pcaA</i> mutant of PRS2000	L. N. Ornston
<i>R. leguminosarum</i> biovar trifolii		
MNF9000	Wild type	31
MNF9010	Km ^r ; Tn5 mutant of MNF9000; <i>pcaA</i>	31
Plasmids		
pZR405	Ap ^r ; 3.35-kb <i>SstI-HindIII</i> insert encoding <i>pobA</i> from <i>A. calcoaceticus</i> in pUC18	2
pZR503	Tet ^r ; 17.8-kb insert encoding <i>pcaA</i> (<i>pcaHG</i>) as well as <i>pobA</i> from <i>A. calcoaceticus</i> in pRK415-1	2

a relatively slow process, taking hours. *p*-Toluidine is a solid at room temperature. However, owing to its toxicity and volatility (mp, 44°C), petri dishes containing the compound were incubated within a crisper at 30°C, even when *E. coli* strains were being screened. A *p*-toluidine concentration of 50 µg ml⁻¹ was sufficient to yield an intense dark brown color with protocatechuate on minimal medium. At this level, an adverse effect on growth of any of the bacterial strains listed in Table 1 was not obvious.

Specificity and characteristics of *p*-toluidine color complex. In liquid minimal medium, *p*-toluidine was observed to form a dark brown precipitate with protocatechuate or catechol. After isolation by centrifugation and solution in methanol, 2-propanol, or *N,N*-dimethylformamide, the complexes became a pink to tan color. On solidified minimal medium containing *p*-toluidine, 4-chlorocatechol, catechol, and protocatechuate yielded a dark brown color and 4-methylcatechol turned red. Protocatechuate produced by cells growing on LB medium containing *p*-toluidine and iron turned deep purple.

When diphenolics were applied in a volume of 1 µl to solidified *p*-toluidine minimal medium, as little as 2.5 nmol of catechol, 4-chlorocatechol, and protocatechuate could be detected. Other compounds were tested by spotting approximately 25 µl of stock solutions onto solidified minimal medium containing *p*-toluidine. The following compounds, tested with and without added ferric chloride, failed to give the characteristic intense color development: intermediates of the β-ketoadipate pathway [β-carboxy-*cis,cis*-muconate, β-ketoadipate, *cis,cis*-muconate, and (+)-muconolactone], the hydroaromatics shikimate and quinate, and the aromatic compounds anthranilate, benzoate, benzylamine, 4-chlorobenzoate, *p*-coumarate, ferulate, *p*-hydroxybenzoate, D-(-)-mandelate, L-(+)-mandelate, phenol, β-resorcyate, salicylate, syringate, terephthalate, *m*-toluate, *p*-toluate, and vanillate.

Application of *p*-toluidine to detect mutant strains. One use of *p*-toluidine is to identify mutant strains of bacteria. Figure 1 illustrates the amplification of catechol and protocatechu-

ate accumulation around cells in the presence of *p*-toluidine plus benzoate or *p*-hydroxybenzoate and the differentiation between mutant and wild-type strains. No color was observed around cells incubated with *p*-toluidine alone. An example of the amplification and acceleration of protocatechuate or catechol detection is the fact that a *catA* mutant strain of *Acinetobacter calcoaceticus* ADP139, patched onto medium containing 2.5 mM benzoate and *p*-toluidine, yielded a dark color in the surrounding medium within 5 h. After 2 days at 30°C, the medium was intensely dark brown in the area of the patch, whereas the same sized patch on medium containing benzoate but no *p*-toluidine yielded a uniformly pale grey color over an area that was six times larger. Generally, within less than 24 h on *p*-toluidine medium containing *p*-hydroxybenzoate or quinate as the sole

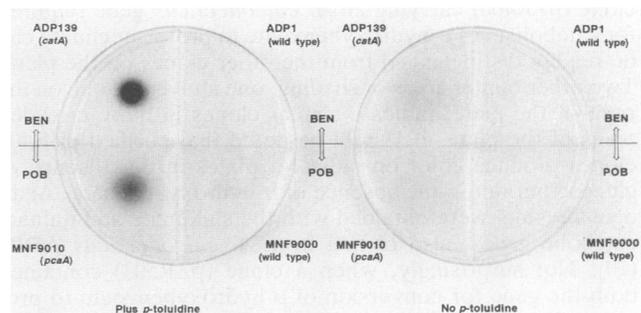


FIG. 1. Amplification of protocatechuate and catechol detection by incorporation of *p*-toluidine in medium. Each petri dish contains four sectors: the top two sections of each plate contain benzoate at 0.5 mM; the bottom two sections contain *p*-hydroxybenzoate at 2 mM. The aromatics provide the sole source of carbon in the media. All sectors of the left-hand petri dish contain *p*-toluidine at 50 µg ml⁻¹; the petri dish on the right is free of the compound. Cells were inoculated on each sector as a 2-mm patch, with the cells on benzoate incubated overnight at 30°C and cells on *p*-hydroxybenzoate incubated for 2 days.

carbon source, a brown color surrounded patches of *pcaA* mutant strains of *Agrobacterium tumefaciens* ADO2032, *R. leguminosarum* biovar trifolii MNF9010, *A. calcoaceticus* ADP6, *P. putida* PRS3000, and even a *pcaA* mutant strain of the slowly growing species *Bradyrhizobium japonicum* (26). Clearly, in these strains, degradation of *p*-toluidine does not occur to any extent that interferes with its use as an indicator.

To differentiate between mutant and wild-type colonies with certain bacterial strains and growth substrates, it may be necessary to adjust the aromatic substrate concentrations. For example, wild-type *A. calcoaceticus* ADP1 excretes some protocatechuate when the growth substrate is 5 mM shikimate; thus it is helpful to lower the hydroaromatic concentration to 0.5 mM in order to differentiate between mutant and wild-type strains.

In testing the limits of detection with cells, it was found that as little as 20 μ M *p*-hydroxybenzoate in minimal medium resulted in some color development around a patch of *A. calcoaceticus* ADP6 blocked in the catabolism of protocatechuate. As low as 1 μ M benzoate yielded detectable color around a patch of *A. calcoaceticus* ADP139 blocked in catechol oxygenase within 24 h, with color intensifying over time. These tests did not require addition of a source of carbon and energy. In fact, cells produced less protocatechuate when succinate at 10 mM was added than when the media contained only the low concentration of *p*-hydroxybenzoate with or without glucose at 5 mM.

Application of *p*-toluidine to screen *E. coli* recombinants. The protocatechuate-catechol amplifier has also been used successfully to distinguish *E. coli* S17-1 colonies carrying a cloned gene(s) encoding an enzyme(s) that gives rise to protocatechuate. Strain S17-1, like other *E. coli* strains, has no background of endogenous aromatic catabolism. Since it is possible that *p*-toluidine could mutagenize cloned DNA, it is advisable to work off a master plate. Colonies were replica plated from LB medium onto minimal medium containing appropriate antibiotics and nutritional supplements. The medium also contained 5 mM glucose as the carbon and energy source, *p*-toluidine, and an aromatic or hydroaromatic at a concentration of 2 or 3 mM. Figure 2 shows the results of overnight growth of *E. coli* colonies on minimal medium containing *p*-toluidine at 100 μ g ml⁻¹ and 3 mM *p*-hydroxybenzoate. Towards the top of the plate, a single clone (pZR405) carrying an *A. calcoaceticus* gene required for catabolism of *p*-hydroxybenzoate to protocatechuate can be readily distinguished from the other clones on the plate. Two other fainter areas of shading, one above and one on the right of the plate, indicate similar clones in more crowded areas of the plate. It should be noted that purified pZR405 clones produce color on indicator plates in the absence of glucose but not in the absence of *p*-hydroxybenzoate. Analogous results were obtained with the shikimate and quinate catabolic genes, also cloned from *A. calcoaceticus* ADP1 (10). Not surprisingly, when a clone (pZR503) contained both the gene for conversion of *p*-hydroxybenzoate to protocatechuate and the oxygenase gene that acts on protocatechuate, color development on indicator plates was greatly reduced.

The discovery of a protocatechuate-catechol detection system opens the door to a whole new group of genes as tools in molecular biology. In *E. coli*, the chromogenic system can be used to screen for inserts in vectors created with marker genes, such as *pobA* (2), encoding enzymes which give rise to one of the diphenolic intermediates. The marker genes may also serve as effective reporter genes in

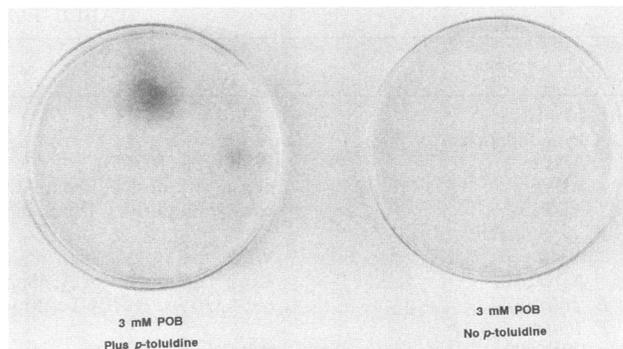


FIG. 2. Identification of *E. coli* clones producing protocatechuate on medium containing *p*-toluidine. Both petri dishes contained supplemented minimal medium, as described in the text, with glucose as the carbon and energy source; they also contained *p*-hydroxybenzoate (POB) at 3 mM. The plate on the left contained *p*-toluidine as well, at 100 μ g ml⁻¹. *E. coli* S17-1 cells containing plasmids were replica plated from LB medium containing ampicillin onto the minimal medium. The darkened areas around colonies in the upper center and at the right on the left-hand plate were produced by the *p*-toluidine-protocatechuate chromophore and indicate clones containing the *pobA* gene required for conversion of *p*-hydroxybenzoate to protocatechuate. Variability in the intensity of color is related to the size and degree of crowding of the colonies.

appropriate genetic backgrounds lacking *pcaA*, *catA*, or *xylE*.

In conclusion, *p*-toluidine has the advantages of being inexpensive, simple to use, sensitive, and stable but the disadvantage of being highly toxic to people. The protocatechuate-catechol amplifier is applicable to many aspects of aromatic catabolism in diverse microbes. Its efficacy in cloning and subcloning genes giving rise to protocatechuate, catechol, or substituted catechols in *E. coli* has been demonstrated, and it can be exploited for investigations on the regulation of such genes. Its sensitivity in detecting very low levels of catechols and protocatechuate points to a potential role in ecological studies. The *p*-toluidine chromogenic system should give further impetus to the use of aromatic catabolic genes and regulatory elements as productive and tractable models for studying the evolution and regulation of genes.

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