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Increased Mutagenesis Mediated by Cloned Plasmid CAM-OCT Genes: Potential for Expanding Substrate Ranges of *Pseudomonas* spp.

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Twenty-five kilobases of *Pseudomonas* plasmid CAM-OCT DNA encoding a DNA repair gene(s) was cloned into the broad-host-range vector pVK100. The presence of the cloned genes increased the isolation frequency of *Pseudomonas putida* derivatives capable of using ethyl lactate or 3-methyl-3-buten-1-ol as their carbon source 15- and 8-fold, respectively, after UV irradiation. Ethyl lactate-utilizing strains expressed a novel intracellular hydrolase.

Pseudomonas bacteria occupy a variety of soil and water environments, due in part to their remarkable metabolic diversity. Plasmids often contribute to this diversity by encoding metabolic enzymes. One such plasmid, CAM-OCT, a >300-kb IncP-2 plasmid, encodes genes for the utilization of camphor and *n*-alkanes of 6 to 10 carbons (2, 17) and a DNA repair gene(s) that results in increases in both survival and mutagenesis after DNA damage (15). Some other *Pseudomonas* plasmids have also been reported to carry DNA repair genes (10, 11), including the IncP-2 antibiotic resistance plasmid pMG2 (14), the IncP-2 phenol-degradative plasmid pVI150 (unpublished observation), and the IncP-9 plasmid R2 (16, 22). In the current work, we sought to clone the CAM-OCT DNA repair genes and test their utility in aiding in the isolation of bacterial strains with potential industrial uses.

Isolation of DNA clones carrying a CAM-OCT UV response gene(s). Bacterial strains and plasmids used in this work are listed in Table 1. A library of cloned DNA from *Pseudomonas putida* PpS145 was prepared by partial *Hin*dIII digestion of total DNA, ligation into the *Hin*dIII site of vector pVK100 (4), in vitro lambda packaging (Stratagene), and transfection of *Escherichia coli* HB101. Cloned DNA was transferred to *Pseudomonas aeruginosa* PAC5 by triparental conjugation (8, 16) with selection for tetracycline resistance (100 μg/ml). After conjugal transfer to PAC5, the library was screened by replica plating to minimal medium with glucose and a trace of histidine. Clones carrying the error-prone repair functions were identified as those exhibiting increased UV-induced His^+ reversion as evidenced by papilla formation after incubation of replica plates irradiated with UV at a dose of 10 J/m^2. Screening was done in *P. aeruginosa* because previous experiments have suggested that UV-induced mutagenesis does not exist or occurs at extremely low levels in this species (15, 22). Seven clones with greatly increased levels of UV-induced His^+ reversion have been isolated after multiple screenings of the 25,000-member clone bank. Four clones carry *Hin*dIII fragments of 7.0, 5.9, 4.2, and 4.0 kb; the other three carry an additional 4.0-kb fragment. One of the latter was chosen for further analysis and designated pDM101.

To verify that the cloned DNA was of CAM-OCT origin, pDM101 was labeled with [^{32}P]dCTP and used to probe a Southern blot of *Hin*dIII-digested chromosomal DNAs from *E. coli* and *P. aeruginosa* and *P. putida* DNAs from strains with and without the CAM-OCT plasmid. Figure 1 shows that pDM101 hybridized only to those DNAs from strains carrying the CAM-OCT plasmid.

UV survival and UV-induced mutagenesis experiments analogous to those done earlier with *Pseudomonas* strains carrying the whole CAM-OCT plasmid (15) were performed. Survival after UV irradiation was enhanced in *P. aeruginosa* strains in a manner equivalent to that seen earlier with the whole CAM-OCT plasmid (15). Mutagenesis after UV irradiation (10 J/m^2) of either *P. aeruginosa* PAC5 derivatives or *P. putida* PpS338 derivatives, as measured by auxotroph reversion, was enhanced 20- and 7-fold, respectively, compared with strains lacking the cloned DNA. These experiments suggest that all of the genetic information required for the DNA repair functions encoded by the CAM-OCT plasmid has been cloned.

The cloned CAM-OCT genes did not exhibit homology to the well-studied *mucA* and *mucB* genes of the enterobacterial plasmid pKM101 (18, 24), as evidenced by reciprocal Southern blotting experiments at low stringency. ^{32}P-labeled pDM101 did not hybridize to pKM101 or to cloned DNA carrying *mucA* and *mucB* (pGW1700). Conversely, labeled pGW1700 did not hybridize to pDM101 but did hybridize to itself and to pKM101 (data not shown).

A cloned UV response gene(s) increases the ability to isolate bacterial strains with novel metabolic properties. *P. putida* PpS338 cells with and without the cloned gene(s) as well as cells carrying the CAM-OCT plasmid were plated on minimal medium, UV irradiated (10 J/m^2), and placed under selective pressure for growth on 3-methyl-1-butene-1-ol (MBO). This branched-chain alcohol is not a substrate for the alcohol dehydrogenase encoded by CAM-OCT; it can, however, be a substrate for the enzyme encoded by the chromosomal *alcA* gene. PpS338 carries an *alcA* mutation. The cloned UV response gene(s) greatly increased the ability to isolate MBO^+ colonies above that seen with PpS338 as well as PpS338(CAM-OCT) (Fig. 2). The MBO^+ phenotype in PpS338 and PpS338 (pDM101) most likely arose by a reversion mutation of the chromosomal *alcA* gene, but MBO^+ colonies isolated from the PpS338(CAM-OCT) strain could have arisen by mutation of this gene or of the *alk* genes of the CAM-OCT plasmid. Conjugation experiments designed to test for the cotransfer of the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
Strains		
P. aeruginosa		
PAC5	*his-5*	P. Clarke
PAM150	*his-5* (CAM-OCT)	This work
PAM247	*his-5* (pDM101)	This work
P. putida		
PpS338	*alcA81 trp-338*	15
PpS145	*met-145* (CAM-OCT)	5
PpS597	*alcA437 his-597*	J. Shapiro
E. coli HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	
Plasmids		
CAM-OCT	IncP2; camphor$^+$ alkane$^+$ UV$^+$	5
pVK100	Km^r Tc^r	20
pDM101	Tc^r; CAM-OCT UV genes cloned in *Hin*dIII site of pVK100	This work
pKM101	Encodes *mucA* and *mucB*	18, 24
pGW1700	*mucA* and *mucB* cloned in pBR322	18, 24

MBO$^+$ phenotype by transfer of the CAM-OCT plasmid from PpS338(CAM-OCT) to recipient PpS597 showed that the MBO$^+$ phenotype results from mutation of the plasmid *alk* genes in 2 out of 10 cases.

Similarly, the ability of the cloned UV response gene(s) to enhance the ability to isolate bacterial strains which hydrolyze ethyl lactate (MEE) was tested. This substrate serves as a model compound for the isolation of bacterial strains capable of cleaving ester compounds not typically hydrolyzed by known lipases. The presence of pDM101 enhanced the isolation of bacterial strains capable of using MEE more than 15-fold (Fig. 3). These strains were shown to express a novel intracellular hydrolase. Hydrolase activity was measured in cell extracts prepared from cultures of PpS338 and PpS338 MEE$^+$ grown in Luria broth (LB) or in LB plus MEE (0.1%). Crude extracts from 14 g of cells in 60 ml of sodium phosphate buffer (20 mM, pH 6.0) were prepared by sonication. Reaction mixtures contained 10 to 100 μl of crude extract incubated in 970 μl of sodium phosphate buffer (0.1 M, pH 7.0) plus 20 μg of MEE. Reaction mixtures were incubated for 30 min at room temperature and then analyzed by gas chromatography for the amount of hydrolyzed ester (Hewlett-Packard methyl silicon column, 25 m, 50 to 250°C). No activity was detected in PpS338 extracts, whereas extracts from PpS338 MEE$^+$ strains showed 3.5 and 45 mU of activity per ml in extracts from cultures grown in LB or LB plus MEE, respectively.

The isolation of *P. putida* strains capable of using MBO as a carbon source (Fig. 2) demonstrated the ability of the CAM-OCT DNA repair functions to increase the isolation frequency

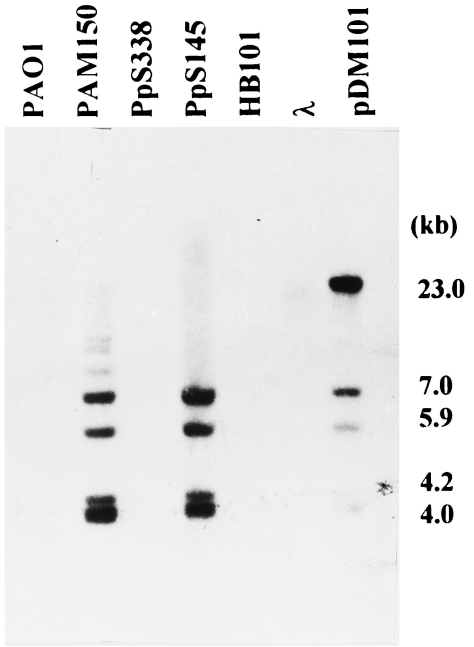

FIG. 1. Southern blot analysis of *Hin*dIII-digested DNAs isolated from *P. aeruginosa* PAO1 and PAM150 and *P. putida* PpS338 and PpS145, without and with plasmid CAM-OCT, respectively, as well as total DNA from *E. coli* HB101, with labeled pDM101 as the probe. Molecular weight markers are indicated for the *Hin*dIII fragments of isolated pDM101; the 4-kb fragment is a doublet, and the 23-kb fragment is the vector pVK100.

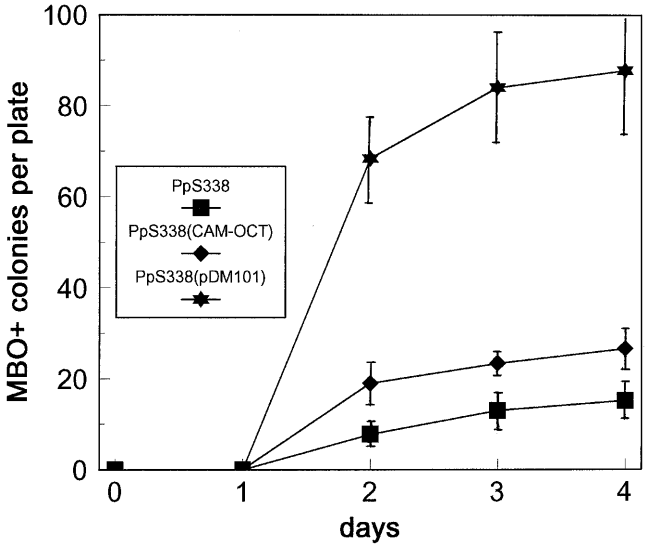

FIG. 2. UV-induced adaptation for growth of *P. putida* PpS338 and derivatives on MBO. Growth of bacterial variants on novel substrates was achieved as previously described (6). Cells (2×10^7) were spread on minimal medium with no carbon source. Plates were UV irradiated (10 J/m^2), placed in closed cans containing vials of MBO, and then incubated at 32°C. Individual colonies arising over time were counted. Results are the averages of six separate determinations and are expressed as MBO$^+$ colonies per plate ± the standard error of the mean.

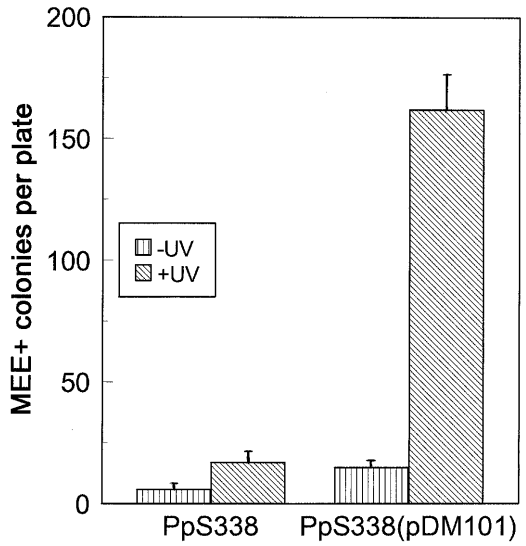

FIG. 3. UV-induced adaptation for growth of *P. putida* PpS338 and derivatives carrying pDM101 on MEE. Cells (2×10^7) were spread on minimal medium with MEE (0.1%). One set of plates was not treated with UV light, and another set was UV irradiated at a dose of 10 J/m^2. Results are the averages of three separate determinations and are expressed as MEE$^+$ colonies per plate (± the standard error of the mean) appearing after 7 days of incubation at 32°C.

of strains with differing enzyme specificities. Although the majority of the MBO$^+$ strains represented reversion of the *alcA* chromosomal mutation, 20% of those isolated in strains carrying CAM-OCT were plasmid encoded and presumably arose by mutation of the plasmid genes involved in alcohol oxidation. Current work is aimed at further study of these strains. Besides the potential degradative role, the CAM-OCT *alk* genes can serve a useful role in certain biotransformations when other substrates are provided (23). For example, alkenes can be oxygenized to epoxides, which can serve as the building blocks for the synthesis of pharmaceutical compounds. Strains with extended substrate ranges could conceivably expand the utility of such biotransformations to provide novel compounds.

The isolation of MEE$^+$ *P. putida* strains (Fig. 3) demonstrated the ability of the cloned CAM-OCT DNA repair gene(s) to enhance isolation of strains with previously unidentified enzyme specificities. The genetic and biochemical nature of the novel intracellular hydrolase expressed by these strains is currently under investigation. The isolation of bacterial strains expressing unique hydrolases can have a variety of roles in industrial applications, from their use as lipases to their use in the synthesis of unique esters (1, 12, 19).

Bacterial strains are often identified and used in industry for the production of enzymes for a variety of applications, such as chemical degradation and biocatalysis (3, 9). Bacterial strains which encode functions for the breakdown of novel substrates as well as for the catalysis of novel reactions pose a challenge for biotechnology. Typically this results in the need to screen for novel strains or to improve strains which are already in hand, both potentially time-consuming processes (6, 21). The cloned CAM-OCT DNA repair functions served to speed up the adaptation process for the isolation of laboratory strains with novel properties. Presumably, with the appropriate selection procedures, similar methods could be applied in additional strain improvements in pseudomonads and related bacteria. Given the rather remarkable metabolic diversity of pseudomonads and the role that plasmids often play in that diversity (7), it is tempting to speculate that in a similar manner in nature, plasmid-encoded error-prone DNA repair pathways may contribute to metabolic diversity as a result of their effects on mutation frequencies (13). The full exploitation of this system for accelerated evolution of bacterial strains to increase the ease of isolation of novel bacterial strains will require a more complete understanding of the nature of the DNA repair genes expressed by the CAM-OCT plasmid.

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