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**SHORT COMMUNICATIONS**

# Selection of the *Tn5*-Induced Mutants of the Plasmid-Containing Naphthalene- and Salicylate-Degrading Strain *Pseudomonas putida* BS394(pBS216) with Growth Inhibition on Different Substrates at Low Temperatures

**V. G. Grishchenkov*, A. A. Radzion**, P. A. Medvedev*, M. I. Balina**, and A. M. Boronin***

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

***Pushchino State University, Pushchino, Moscow oblast, Russia*

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Polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, phenanthrene, biphenyl, and pyrene, belong to the class of polynuclear aromatic compounds, which are of great environmental concern because of their high toxicity, carcinogenicity, and persistence [1]. The increasing interest of researchers in the low-temperature biodegradation of these widespread pollutants is due to the fact that much of the earth's surface is exposed, either constantly or seasonally, to low temperatures.

The biodegradation of various xenobiotics, such as *n*-alkanes, mono- and polycyclic aromatic hydrocarbons, etc., can occur in a wide range of temperatures [2, 3]. On the other hand, the ability of bacterial strains to degrade PAHs (e.g., naphthalene and its intermediate salicylate) can depend on the ambient temperature [4]. The naphthalene degradation genes of *Pseudomonas* spp. are often carried by conjugative plasmids, which allows the expression of these genes to be investigated in different microorganisms, including psychrophiles. The study of the low- temperature bacterial degradation of naphthalene as a model substrate may make it possible to reveal the genetic and biochemical factors that are responsible for the low-temperature utilization of xenobiotics. One of the approaches to this goal is the obtaining and identification of transposon-induced negative mutations [5, 6].

This work deals with the derivation of *Tn*-induced thermosensitive mutants of the naphthalene- and salicylate-degrading strain *Pseudomonas putida* BS394(pBS216) with the use of the two-component system developed by Simon *et al.* [7]. The donor strain was *Escherichia coli* S17-1, whose chromosome carried the *tra* genes of the broad-host-range plasmid RP4. The *E. coli* plasmid vector pSUP2021 carried the *mob* genes of plasmid RP4 and the kanamycin-resistant ($Km^r$) transposon *Tn5*. The presence of the *tra* and *mob* genes of plasmid RP4 in *E. coli* made possible the transfer of vector pSUP2021 to bacterial cells of other species, in which, however, the vector was not stable and was eliminated. In this case, the *Tn5* marker was saved due to its insertion into the chromosome or a resident plasmid of the host.

The $Km^r$ transconjugants of strain BS394(pBS216) were produced at a frequency of $10^{-6}$/donor cells. Approximately 1% of the $Km^r$ clones had the mutant phenotype characterized by growth inhibition at low temperatures. All the mutant phenotypes could be divided into three groups (Table 1).

Group I mutants (row 1 in Table 1) were unable to grow on naphthalene, salicylate, and glucose at low temperatures. Group II mutants (row 2) could not grow even on complete LA medium. The low-temperature growth of group III mutants did not differ from their growth at 28°C but was characterized by a reversion to prototrophy.

To localize transposon insertions, the mutant clones of the first and second groups were crossed with strain BS228($Ade^-$). As follows from data in Table 2, transconjugants of different types were selected by using two selective media, one of which contained naphthalene (the marker of the biodegradation plasmid) and the other of which contained kanamycin (the *Tn5* marker).

Some transconjugants (group I, Table 2) were characterized by the inability to grow on naphthalene, salicylate, and glucose at low temperatures. Other transconjugants (group II, Table 2) were characterized by the inhibition of their growth on all the substrates tested (including the complex LA medium) by low temperatures. It should be noted that the transconjugants of both types had the $Km^r$ marker of transposon *Tn5*, whose transfer rate was comparable with that of the naphthalene plasmid marker. In other words, the above mutant phenotypes were inherited by the transconjugants that had received the naphthalene degradation plasmid and the $Km^r$ marker from the parent mutants.

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**Table 1.** The *Tn5*-induced mutants of strain BS394(pBS216) grouped according to their ability to grow on different media at 28 and 8°C

| Group | | 28°C | | | | | 8°C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | E medium | | | | L medium | E medium | | | | L medium |
| | | naphthalene | salicylate | glucose | $Km^r$ | | naphthalene | salicylate | glucose | $Km^r$ | |
| 1 | | + | + | + | + | + | – | – | – | – | + |
| 2 | | + | + | + | + | + | – | – | – | – | – |
| 3 | | + | + | + | + | + | + | + | + | + | + |
| Control strains | 394 | – | – | + | – | + | – | – | + | – | + |
| | 394 (pBS216) | + | + | + | – | + | + | + | + | – | + |

Note: Growth on agar media was evaluated by the production of a lawn of cells and separate colonies as described earlier [4]. Growth was evaluated after two and seven days of incubation at 28 and 8°C, respectively.

**Table 2.** Transconjugants of strain BS228($Ade^-$) grouped according to their ability to grow on different media at 28 and 8°C

| Group | | Derivation conditions | | Growth characteristics | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 28°C | | | | | 8°C | | | | |
| | | Selective medium | Derivation frequency | E medium | | | | L medium | E medium | | | | L medium |
| | | | | naphthalene | salicylate | glucose | $Km^r$ | | naphthalene | salicylate | glucose | $Km^r$ | |
| I | | Naphthalene | $10^{-5}$ | + | + | + | + | + | – | – | – | – | + |
| | | Km | $10^{-5}$ | | | | | | | | | | |
| II | | Naphthalene | $10^{-5}$ | + | + | + | + | + | – | – | – | – | – |
| | | Km | $10^{-5}$ | | | | | | | | | | |
| III | | Naphthalene | $10^{-5}$ | + | + | + | – | + | + | + | + | – | + |
| | | Km | $<10^{-9}$ | | | | | | | | | | |
| Control strains | 394 | – | – | – | – | + | – | + | – | – | + | – | + |
| | 394 (pBS216) | – | – | + | + | + | – | + | + | + | + | – | + |

Note: See the note to Table 1.

The transconjugants of group III (Table 2) lacked the $Km^r$ marker and were able to grow at low temperature on the substrates tested. This suggests that the mutant phenotype in the parent mutants of strain BS630 was most likely to be due to the insertion of the *Tn5* transposon into the bacterial chromosome.

To obtain further evidence that the appearance of the mutant phenotypes is due to the naphthalene degradation plasmid pBS216 with the inserted *Tn5* transposon, some of the mutant transconjugants (namely, mutants A4 and 41 from group II and mutant 56 from group I, Table 2), were treated with mitomycin C in order to eliminate the plasmid [8]. As a result, the mitomycin-treated cells lost not only the plasmid pBS216 markers but also the $Km^r$ marker of transposon *Tn5*. In this case, mutants A1 and 41 resumed the ability to grow on glucose and in the complete medium at 8°C. Moreover, the transfer of the corresponding plasmid DNA isolated from the mutant strains A4, 41, and 56 to the plasmid-cured transformants restored the mutant phenotype of growth inhibition at 8°C. The same results were obtained with the transformants derived from the wild-type strain BS228 (data not presented).

Thus, we succeeded in obtaining plasmid mutations responsible for the low-temperature inhibition of the growth of host bacteria on naphthalene, salicylate, and glucose (mutant 56), whose catabolism is known to be controlled by chromosomal determinants, as well as on the complete medium (mutants A4 and 41).

The comprehensive biochemical and molecular genetic investigation of these plasmid-containing

mutants may provide insight into the biochemical and genetic factors that are responsible for the growth of bacteria on different substrates at low temperatures.

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