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# Emergence of Nylon Oligomer Degradation Enzymes in *Pseudomonas aeruginosa* PAO through Experimental Evolution

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**Through selective cultivation with 6-aminohexanoate linear dimer, a by-product of nylon-6 manufacture, as the sole source of carbon and nitrogen, *Pseudomonas aeruginosa* PAO, which initially has no enzyme activity to degrade this xenobiotic compound, was successfully expanded in its metabolic ability. Two new enzyme activities, 6-aminohexanoate cyclic dimer hydrolase and 6-aminohexanoate dimer hydrolase, were detected in the adapted strains.**

Recent developments in the chemical industry have resulted in the production and distribution of various synthetic compounds. Nylon-6 is produced by ring cleavage polymerization of caprolactam, in which the monomeric unit, 6-aminohexanoate (Ahx), is combined by amide bonds with a degree of polymerization of >100, producing by-products of linear or cyclic oligomers of Ahx (called nylon oligomers). We have previously isolated two microorganisms, *Flavobacterium* sp. strain KI72 (7) and *Pseudomonas* sp. strain NK87 (6), that grow with the Ahx cyclic dimer (Acd) as the sole source of carbon and nitrogen. The degradation of xenobiotic compounds is highly dependent on specific enzymes, i.e., 6-aminohexanoate cyclic dimer hydrolase (enzyme I [EI]) (8), 6-aminohexanoate dimer hydrolase (EII) (9), and endo-type 6-aminohexanoate oligomer hydrolase (EIII) (11), and the responsible genes are encoded on plasmids (12, 13, 15). If a new metabolic ability could be directly evolved under laboratory conditions, it would be interesting from the standpoint of enzyme evolution and would also provide a good system to study the adaptation of microorganisms to xenobiotic compounds. In this study, we investigated the possibility of creating a new metabolic activity that would degrade the Ahx oligomer in a strain that is not inherently capable of such degradation.

*Pseudomonas aeruginosa* PAO was clinically isolated in New Zealand and has been well studied biochemically and genetically as a standard strain of *Pseudomonas* (5). The wild-type PAO1 did not use Acd (Fig. 1) and the Ahx linear dimer (Ald) (data not shown); therefore, this strain was used to study whether microorganisms can acquire the ability to metabolize nylon oligomers experimentally. *P. aeruginosa* PAO1 was grown on M9 minimal medium (18) containing 2 g of glucose and 1 g of $NH_4Cl$ per liter (M9-Glu medium). Various dilutions of the culture broth were spread on M9-Ahx plates (M9 minimal plates containing 2 g of Ahx per liter as the sole carbon and nitrogen source). After 9 days of incubation at 30°C, hypergrowing colonies were obtained at a frequency of $10^{-3}$. As a control experiment, the same culture broth was spread on an M9 plate containing no carbon source. However, no colonies were observed even after 9 days of incubation. One of the hypergrowing mutants (PAO5501) was purified on an M9-Ahx plate and was cultured on Ahx minimal medium (3 g of Ahx per liter containing salt mixture A [1 g of $K_2HPO_4$, 3 g of $KH_2PO_4$, 5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3 mg of $FeCl_3$ per liter, pH 6.4]) for 3 days. The culture broth ($10^9$ cells per ml) was then transferred to Ald minimal medium (3 g of Ald per liter containing salt mixture A) in a 1% inoculation. After observing slight growth ($A_{600}$ of culture broth, 0.02 to 0.05) in the third week, this culture was retransferred to the Ald minimal medium (1% inoculation). It was only in the third month that the turbidity of the culture broth reached 1 at $A_{600}$. Strain PAO5502 selected from this culture on the Ald minimal plate (Ald minimal medium containing 1.3% agar) was able to grow to approximately $10^9$ cells per ml in either Ald or Acd minimal medium (3 g of Acd per liter containing salt mixture A) within only 4 days. The newly obtained abilities of PAO5502 were retained even after five successive cultivations on Glu minimal medium (3 g of glucose plus 1 g of $NH_4Cl$ per liter containing salt mixture A).

Strains PAO1, -5501, and -5502 exhibited similar growth rates on Glu minimal medium: 0.29, 0.28, and 0.27 $h^{-1}$, respectively, at the logarithmic phase (Fig. 1). However, only PAO5502 grew to up to $10^9$ cells per ml within 3 to 4 days of cultivation in the Acd minimal medium, while PAO1 and PAO5501 showed no detectable growth under the same conditions. These results suggest that PAO5502 acquires some genetic alterations in the course of acquiring the new metabolic activity and that the extraordinary lag is not due to very slow growth of the parental strain. The growth rates of PAO5502 on Ald and Acd minimal media were 0.1 and 0.03 $h^{-1}$, respectively.

Strains PAO1, PAO5501, and PAO5502 exhibited similar colony morphologies on Glu minimal plates and similar levels of resistance toward phenanthroline (150 μg/ml) and various antibiotics (ampicillin [400 μg/ml], kanamycin [400 μg/ml], carbenicillin [100 μg/ml], tetracycline [15 μg/ml], streptomycin [50 μg/ml], and chloramphenicol [25 μg/ml]). The cytochrome *c* oxidase activity and production of fluorescent pigment by these three strains were both in agreement with the standard characteristics of *P. aeruginosa*. Moreover, two-dimensional gel electrophoresis (14) of the total proteins in cell lysates of PAO1, PAO5501, and PAO5502 obtained from cells grown in Glu minimal medium for 16 h showed similar patterns (data not shown). These results indicate that *P. aeruginosa* PAO5501 and PAO5502 are true derivatives of PAO1.

To check whether strain PAO5502 used the pathway of Acd utilization found in *Flavobacterium* sp. strain KI72 (8, 9), the hydrolytic activities of Acd (EI) and Ald (EII) were analyzed by thin-layer chromatography. Cells were grown on Glu or Acd minimal medium, harvested by centrifugation, and washed with buffer A (20 mM potassium phosphate buffer containing 10% glycerol, pH 7.2). Approximately $10^9$ cells were sus-

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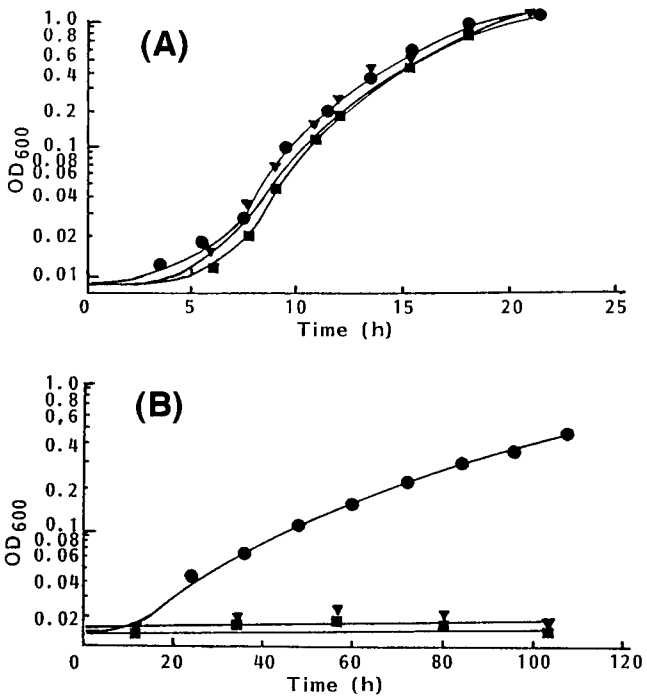


FIG. 1. Growth of *P. aeruginosa* in minimal liquid medium. After strains PAO1, PAO5501, and PAO5502 were precultured at 30°C for 2 days on Glu minimal medium (see text), using a reciprocal shaker (100 strokes $min^{-1}$), the cells were inoculated into fresh Glu (A) or Acd (B) minimal medium (inoculation size, 1%), and cultivation was continued under the same conditions. Cell densities were measured by $A_{600}$. ▼, PAO1; ■, PAO5501; ●, PAO5502. $OD_{600}$, optical density at 600 nm.

pended in 200 μl of buffer A and lysed by repeated freezing and thawing (three times). Cell lysis was ensured by the addition of 20 μl of 10% Triton X-100. The lysate obtained was used as the crude enzyme solution. The crude enzymes (220 μl) were mixed with 220 μl of 20 mM Acd (EI assay) or 20 mM Ald (EII assay) in buffer A, and the mixtures were incubated at 30°C. After 20 h of incubation, 1 μl of the reaction mixture was spotted on a thin-layer chromatographic plate ($60F_{254}$; Merck Co. Ltd). The plates were developed with *n*-propanol–water–ethyl acetate–aqueous ammonia (24:12:4:1), and the reaction products were detected with ninhydrin (6, 7). A major spot corresponding to authentic Ahx was found in the reaction mixture containing Ald and the lysate of PAO5502 but not in that containing the lysate of PAO1 (Fig. 2). In the reaction

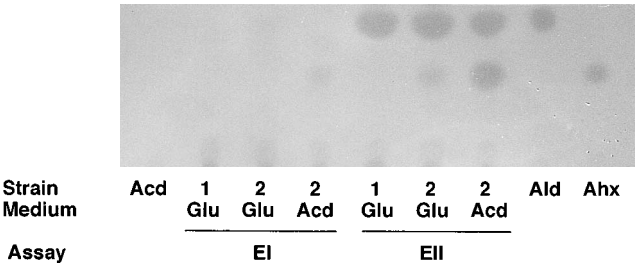


FIG. 2. Thin-layer chromatography for detection of degradation products from Acd and Ald. Cells (*P. aeruginosa* PAO1 and PAO5502) grown in Glu minimal medium (Glu) or Acd minimal medium (Acd) were lysed, and the lysate was incubated with 10 mM Acd (EI assay) or 10 mM Ald (EII assay): 1, PAO1; 2, PAO5502.

mixture containing Acd and the lysate from PAO5502, a spot for Ahx was found after 20 h of reaction. On the other hand, spots for Ald and Ahx were not found in the mixture containing the lysate from PAO1. These results demonstrate that the ability of *P. aeruginosa* PAO5502 to utilize Acd was due to the existence of a newly evolved enzymatic system responsible for hydrolyzing Acd into Ald and, furthermore, into Ahx. The enzymes seem to be partially inducible, since enzyme activities in the cells grown on Acd minimal medium were higher than those in cells grown on Glu minimal medium (Fig. 2).

The adaptation of microorganisms to nonphysiological substrates has been extensively studied, and several molecular bases have been proposed: (i) alteration of substrate specificity of an enzyme (amidase/*P. aeruginosa*) (1), (ii) activation of a cryptic gene by mutation in the promoter region (evolved β-galactosidase/*Escherichia coli*) (3), and (iii) alteration of regulator specificity (*xylS*/*Pseudomonas* sp.) (16). Though a molecular basis for the emergence of nylon oligomer metabolism in PAO5502 is still unknown, it is probable that the basic mechanisms acting during environmental stress are involved in this adaptation. Recently, it was reported that simple polymerase errors increase in the adaptive mutation from $Lac^-$ to $Lac^+$ in *E. coli* (2, 17) and that molecular mechanisms by which adaptive mutation occurs include recombination (4). Lenski and Mittler have observed a 10,000-fold increase in Mu element excision due to starvation (10). Since the nylon oligomer has no detectable toxicity toward microorganisms, the wild-type cells could be maintained in a starved condition for a long period. After the cells accumulated the required genetic alteration to make a cryptic region active, cells grew in the nylon oligomer medium. The high frequency ($10^{-3}$) of the hypergrowing mutants of parental strain PAO1 on medium containing Ahx might be a result of a high mutation rate under the condition of starvation.

In the present study, it was shown that microorganisms can acquire an entirely new ability to metabolize xenobiotic compounds such as a by-product of nylon manufacture through the process of adaptation. The artificial expansion of the metabolic diversity of microorganisms toward various unnatural compounds would be important in terms of biodegradation of environmental pollutants.

## REFERENCES

1. **Clarke, P. H., and J. H. Slater.** 1986. Evolution of enzyme structure and function in *Pseudomonas*, p. 71–144. *In* J. R. Sokatch (ed.), The bacteria, vol. 10. The biology of *Pseudomonas*. Academic Press, Inc., Orlando, Fla.
2. **Foster, P. L., and J. M. Trimarchi.** 1994. Adaptive reversion of a frameshift mutation in *Escherichia coli* by simple base deletions in homopolymeric runs. Science **265:**407–409.
3. **Hall, B. G., and N. D. Clarke.** 1977. Regulation of newly evolved enzymes. III. Evolution of *ebg* repressor during selection for enhanced lactose activity. Genetics **85:**193–201.
4. **Harris, R. S., S. Longerich, and S. M. Rosenberg.** 1994. Recombination in adaptive mutation. Science **264:**258–260.
5. **Holloway, B. W., and A. F. Morgan.** 1986. Genome organization in *Pseudomonas*. Annu. Rev. Microbiol. **40:**79–105.
6. **Kanagawa, K., M. Oishi, S. Negoro, I. Urabe, and H. Okada.** 1993. Characterization of the 6-aminohexanoate-dimer hydrolase from *Pseudomonas* sp. NK87. J. Gen. Microbiol. **139:**787–795.
7. **Kinoshita, S., S. Kageyama, K. Iba, Y. Yamada, and H. Okada.** 1975. Utilization of a cyclic dimer and linear oligomers of ε-aminocaproic acid by *Achromobacter guttatus* KI72. Agric. Biol. Chem. **39:**1219–1223.
8. **Kinoshita, S., S. Negoro, M. Muramatsu, V. S. Bisaria, S. Sawada, and H. Okada.** 1977. 6-Aminohexanoic acid cyclic dimer hydrolase: a new cyclic amide hydrolase produced by *Achromobacter guttatus* KI72. Eur. J. Biochem. **80:**489–495.
9. **Kinoshita, S., T. Terada, T. Taniguchi, Y. Takene, S. Masuda, N. Matsunaga, and H. Okada.** 1981. Purification and characterization of 6-aminohexanoic acid oligomer hydrolase of *Flavobacterium* sp. KI72. Eur. J. Biochem. **116:**547–551.
10. **Lenski, R. E., and J. E. Mittler.** 1993. The directed mutation controversy and neo-Darwinism. Science **259:**188–194.

11. **Negoro, S., S. Kakudo, I. Urabe, and H. Okada.** 1992. A new nylon oligomer degradation gene (*nylC*) on plasmid pOAD2 of *Flavobacterium* sp. strain KI725. J. Bacteriol. **174:**7948–7953.
12. **Negoro, S., H. Shinagawa, A. Nakata, S. Kinoshita, T. Hatozaki, and H. Okada.** 1980. Plasmid control of 6-aminohexanoic acid cyclic dimer degradation enzymes of *Flavobacterium* sp. KI72. J. Bacteriol. **143:**238–245.
13. **Negoro, S., T. Taniguchi, M. Kanaoka, H. Kimura, and H. Okada.** 1983. Plasmid-determined enzymatic degradation of nylon oligomers. J. Bacteriol. **155:**22–31.
14. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
15. **Okada, H., S. Negoro, H. Kimura, and S. Nakamura.** 1983. Evolutionary adaptation of plasmid-encoded enzymes for degrading nylon oligomers. Nature (London) **306:**203–206.
16. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA **83:**8467–8471.
17. **Rosenberg, S. M., S. Longerich, P. Gee, and R. S. Harris.** 1994. Adaptive mutation by deletions in small mononucleotide repeats. Science **265:**405–407.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.).** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.