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SHORT COMMUNICATIONS

Key Role of the *dca* Genes in ε-Caprolactam Catabolism in *Pseudomonas* strains

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Investigation of molecular mechanisms of the emergence and outspread of the capacity of microorganisms for oxidation of xenobiotic compounds that had not previously existed in nature is not only important for the science of evolutionary biology, but is also of practical significance for ecology, biotechnology, and metabolic engineering.

Caprolactam (ε-caprolactam, lactam of 6-aminohexanoic acid, CAP) is among the most widely used chemical compounds, the annual production of which amounts to millions of tons. Over 90% of synthesized CAP is used for production of polymer materials (polycaproamide, or capron, or nylon-6), which are further utilized in different branches of economy.

CAP is a persistent pollutant with a toxic effect on living organisms (Gross, 1984). Although bacteria capable of utilizing CAP as a sole source of carbon and energy have been described (Baxi, 2013; Sanuth et al., 2013), bacterial catabolism of CAP is a poorly studied process, the initial stages of which the currently unknown genes and enzymes participate in. As a result of study of CAP catabolism in the strain *Pseudomonas dacunhae*, the following biochemical degradation pathway has been proposed: ε-caprolactam (CAP) → 6-aminohexanoic acid (AHA) → → adipic acid (AD) → → → → Krebs cycle (Naumova, 1988). It was shown that the ability of pseudomonads to utilize CAP and its intermediates (AHA and AD) as the sole carbon and energy sources is controlled by CAP plasmids (Naumova, 1988; Esikova et al., 1990). The goal of the present study was to identify the regions of the CAP plasmid pBS270 that are responsible for the synthesis of enzymes involved in the catabolism of CAP and its intermediates, and to investigate their molecular genetic organization.

The work was performed using *Pseudomonas* strains from the collection of the Laboratory of Plasmid Biology of the Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The object of the study was the CAP biodegradation plasmid pBS270, nearly 110 kb long (Panov et al., 2013), which had been found in a CAP-degrading strain *Pseudomonas* sp. BS838 isolated from chemical industry waste (Esikova et al., 1990). Strains *P. putida* KT2442(pBS270) and *P. vignae* 1025(pBS270) were obtained by conjugative transfer of pBS270 from *Pseudomonas* sp. BS838. Bacteria were grown at 28°C in the complete Luria-Bertani medium or in the Evans mineral medium (Evans et al., 1965) containing 0.1% (wt/vol) CAP or AD as the sole carbon sources. DNA isolation, fragment cloning, PCR analysis, and DNA sequencing were performed using the conventional techniques (Sambrook et al., 1989).

An efficient approach to investigation of target genes within large plasmids is the cloning of plasmid fragments into suitable vectors and subsequent identification and analysis of the functional regions. In order to clone the fragments of pBS270, we used the pFME5mini vector, a 2.6-kb mini-replicon of the natural pFME5 plasmid, isolated from *P. fluorescens* FME5. This vector provides the replication and maintenance of different genetic constructions in pseudomonads (Volkova, 2013). The DNAs of pBS270 and pFME5mini were digested with *Bam*HI, the mixture of plasmid fragments and the vector was ligated with T4 DNA ligase, and the product was used for transformation of *P. putida* KT2442 and *P. vignae* 1025, which are normally unable to grow on CAP and its intermediates. Transformed cells were selected on the mineral medium supplemented with CAP as the substrate. The grown colonies carried the pFME5mini plasmid with an insertion of ~15 kb. The *Bam*HI fragment of pBS270 was then recloned into the *Escherichia–Pseudomonas* shuttle vector pUCP22, which made it possible to manipulate the plasmid DNA in *E. coli* cells, with the functional analysis of the obtained construction (designated pTE270-15) to be conducted in pseudomonads. The nucleotide sequencing of the cloned fragment was performed using the "primer walking" strategy.

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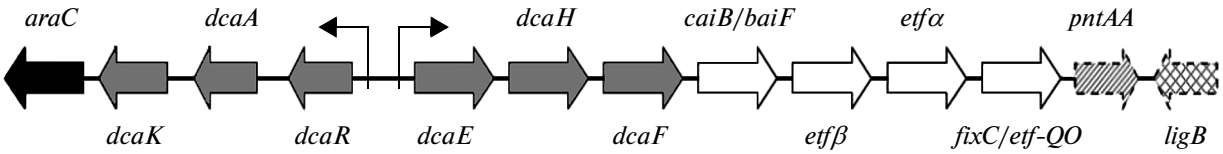

Fig. 1. Genetic organization of the *Bam*HI fragment of pBS270 (15111 bp) containing the *dca* gene cluster.

The fragment was 15111 bp long and contained 13 open reading frames (ORFs); two of them were incomplete (Fig. 1). Putative functions of the protein products encoded by these ORFs were identified based on their homology to known nucleotide and amino acid sequences using BLAST search

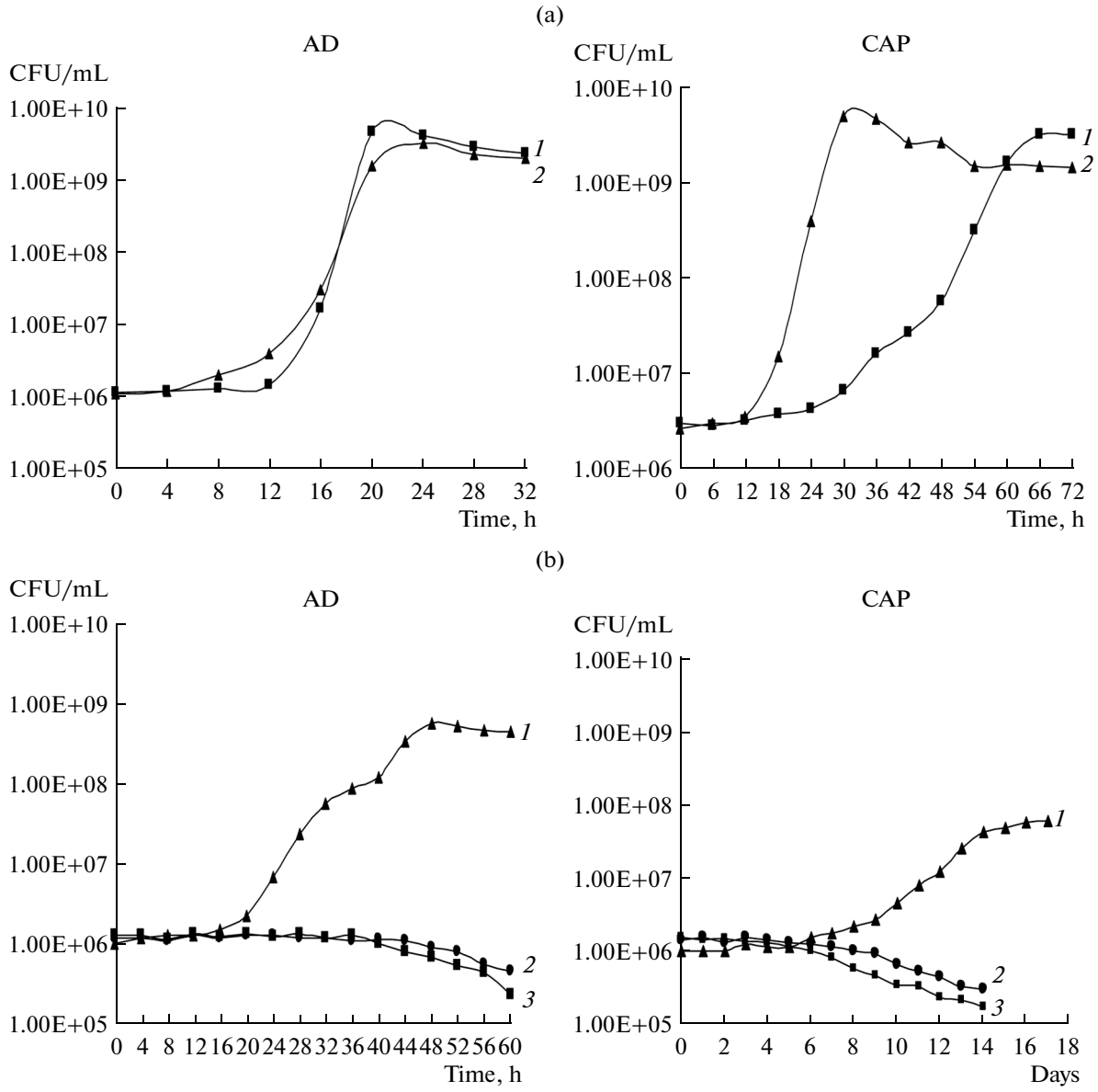

Fig. 2. Growth of *Pseudomonas* strains in a mineral medium containing adipic acid (AD) and ε-caprolactam (CAP) as the sole sources of carbon and energy. Plasmid-carrying derivatives of *P. putida* KT2442 (a): *P. putida* KT2442(pTE270-15) (*1*) and *P. putida* KT2442(pBS270) (*2*); plasmid-free strains (b): *P. aeruginosa* PAO1 (*1*); *P. putida* KT2442 (*2*); and *P. vignae* 1025 (*3*).

[http://blast.ncbi.nlm.nih.gov/Blast.cgi]. As a result, pTE270-15 was found to contain a cluster of six genes whose order was similar to the previously characterized *dca* operon (for DiCarboxylic Acid) in the genome of *Acinetobacter* sp. ADP1 (Parke et al., 2001). Thus, the CAP plasmid pBS270 was shown to carry *dcaKAREHF* genes, which encode the proteins participating in oxidation and transportation of higher dicarboxylic acids, including AD (see the scheme of CAP catabolism). The genes lying downstream from *dcaEHF* encode the products showing high level of identity to *P. putida* SJ3 proteins CaiB/BaiF, Etfα, and Etfβ (94, 87, and 91% identical amino acids, respectively), and to FixC from *P. taiwanensis* SJ9 (94% identity). The available data suggest that these enzymes and protein acceptors may be involved in electron transfer in redox reactions associated with metabolism of dicarboxylic acids or specific substrates (Watmough et al., 2010). The protein encoded by the ORF lying downstream from *dcaK* exhibited most identity to the AraC transcriptional regulator from *Ralstonia* sp. 5747 (53% identical amino acids).

Analysis of growth dynamics of *P. putida* KT2442(pBS270) and *P. putida* KT2442(pTE270-15) showed that both plasmids supported the similar parameters of cell growth on the mineral medium containing AD as a sole carbon source. At the same time, in the CAP-containing medium, the *P. putida* KT2442(pTE270-15) culture had a longer lag phase than *P. putida* KT2442 (pBS270) (Fig. 2a). The same results were obtained for the analogous derivatives of *P. vignae* 1025 (data not shown). This difference in the growth dynamics is related to the fact that the native pBS270 plasmid, as it was shown previously, contains all the genetic information required for complete utilization of the xenobiotic (Esikova et al., 1990). Since pBS270-15 contains only the *dca* operon, CAP degradation by corresponding strains depends on the contribution of two genetic systems being in a *trans* position: *dca* genes are located on the plasmid, while the genes responsible for synthesis of enzymes involved in the primary stages of CAP catabolism are encoded by the bacterial chromosome.

It is known that the ability to oxidize higher dicarboxylic acids, including AD, is not intrinsic for the *Pseudomonas* strains. Only a few strains, such as *P. aeruginosa* PAO1, have been shown to possess this property (Parke et al., 2001; Stanier et al., 1966). It should be noted that the genome of *P. aeruginosa* ÐÀÎ1 contains the genes homologous to the *dca* genes of *Acinetobacter* sp. ADP1 (Stover et al., 2000). The obtained results made us suppose that the presence of *dca* genes located either on a plasmid or on the chromosome is a key factor enabling pseudomonads to acquire the ability to catabolize caprolactam. Analysis of *P. aeruginosa* PAO1 growth on a CAP-containing mineral medium confirmed this hypothesis. As shown in Fig. 2b, after long-term cultivation, the AD-utilizing strain *P. aeruginosa* PAO1 exhibited growth on the mineral medium containing CAP as a sole carbon source, whereas *P. putida* KT2442 and *P. vignae* 1025, which were initially unable to utilize AD, remained unable to degrade this xenobiotic.

Thus, the caprolactam biodegradation plasmid was found to contain a cluster of *dca* genes encoding the enzymes responsible for oxidation of higher dicarboxylic acids. These results provide the first data on the organization of genes involved in catabolism of CAP and located on a CAP plasmid. It was shown that the presence of the *dca* genes located either on plasmids or on the chromosome is necessary for catabolism of caprolactam in *Pseudomonas* strains. Interestingly, in *Acinetobacter* sp. ADP1, the *dca* operon is a part of a chromosomal genomic "island of catabolic diversity" encoding synthesis of the enzymes participating in biodegradation of different aromatic compounds (Parke et al., 2001). Genomic islands, along with plasmids and transposons, are thought to play an important role in the horizontal transfer of catabolic genes in bacterial populations (Juhas et al., 2009). The presence of *dca* genes within pBS270 is one more example confirming the involvement of plasmids in the emergence and outspread of novel catabolic pathways.

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