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Molecular classification of IncP-9 naphthalene degradation plasmids

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Abstract

A large collection of naphthalene-degrading fluorescent *Pseudomonas* strains isolated from sites contaminated with coal tar and crude oil was screened for the presence of IncP-9 plasmids. Seventeen strains were found to carry naphthalene catabolic plasmids ranging in size from 83 to 120 kb and were selected for further study. Results of molecular genotyping revealed that 15 strains were closely related to *P. putida*, one to *P. fluorescens*, and one to *P. aeruginosa*. All catabolic plasmids found in these strains, with the exception of pBS216, pSN11, and p8909N-1, turned out to belong to IncP-9 β-subgroup. Plasmids pBS216, pSN11, and p8909N-1 were identified as members of IncP-9 δ-subgroup. One plasmid, pBS2, contains fused replicons of IncP-9β and IncP-7 groups. RFLP analyses of the naphthalene catabolic plasmids revealed that organisation of the replicon correlates well with the overall plasmid structure. Comparative PCR studies with conserved oligonucleotide primers indicated that genes for key enzymes of naphthalene catabolism are highly conserved among all studied plasmids. Three bacterial strains, *P. putida* BS202, *P. putida* BS3701, and *P. putida* BS3790, were found to have two different salicylate hydroxylase genes one of which has no similarity to the "classic" enzyme encoded by *nahG* gene. Discovery of a large group of plasmid with unique nahR suggested that the regulatory loop may also represent a variable part of the pathway for catabolism of naphthalene in fluorescent *Pseudomonas* spp.

Keywords: IncP-9 plasmids; Fluorescent *Pseudomonas*; Biodegradation; Naphthalene; Catabolic genes

1. Introduction

Plasmids are extrachromosomal DNA replicons that represent an important part of the bacterial gene pool and play a role in the adaptation of

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microorganisms to constantly changing environmental conditions. They control a wide array of genetic traits including resistance to antibiotics and the ability to metabolise complex carbon sources (Thomas, 2000). *Pseudomonas* species are long known to harbour a variety of plasmids that belong to 14 distinct incompatibility groups (Boronin, 1992). Catabolic plasmids that are frequently found in *Pseudomonas* spp. control utilisation of different polycyclic aromatic hydrocarbons including naphthalene (Yen and Serdar, 1988). The plasmid genes for catabolism of naphthalene are organised into two operons that exist on a plasmid as parts of composite transposons (Tsuda and Iino, 1990). Based on this fact, one would expect naphthalene operons to be equally distributed in *Pseudomonas* spp. among plasmids of all incompatibility groups. However, *Pseudomonas* plasmids that control catabolism of naphthalene and other aromatic hydrocarbons fall almost exclusively into the incompatibility groups IncP-9, IncP-7, and IncP-2 (Kochetkov and Boronin, 1984; Thomas, 2000). Plasmid incompatibility is tightly linked to the structure of the basic replicon. It affects the fate of a plasmid in the environment, the range of bacterial hosts and the probability of co-existence of different plasmid-controlled traits in a single bacterial strain.

Despite the fact that genes for initial steps of naphthalene catabolism have have been cloned from many *Pseudomonas* strains, the extent of diversity among naphthalene plasmids, the underlying mechanisms of evolution, and the taxonomic affiliation of bacterial hosts are still poorly understood.

We report here the molecular classification of IncP-9 naphthalene degradation plasmids based on characterisation of the key genes for catabolism of aromatic hydrocarbons, individual plasmid replicons, and their bacterial hosts.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Bacteria were routinely cultured at 28 °C in Luria–Bertani (Sambrook et al., 1989) or Evans mineral salt medium (Evans et al., 1970) supplemented with an appropriate carbon source. Salicylic acid and 1-hydroxy-2-naphthoate were used as growth substrates at a final concentration of 1 g/L while phenanthrene was used at a concentration of 0.2 g/L. Naphthalene was provided as a carbon source by placing a small amount of naphthalene crystals on the lids of inverted Petri dishes.

2.2. DNA manipulations

Standard methods were used for plasmid DNA isolation (Birnboim and Doly, 1979), restriction enzyme digestion, and agarose gel electrophoresis (Ausubel et al., 1999). Total DNA from *Pseudomonas* cultures was isolated and purified by using a cetyltrimethylammonium bromide miniprep procedure (Ausubel et al., 1999). Hybridisation analyses were carried out as described previously (Kosheleva et al., 2003). Conjugal mating was carried out on solid medium, using *Pseudomonas putida* KT2440 (Nah^-Sal^- *gfp*Km^R) as a recipient strain (Dunn and Gunsalus, 1973).

2.3. PCR analyses

Amplifications were performed with a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA). The PCR mixture contained 1× Reaction Buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 5.0% dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO), 200 μM each of dGTP, dATP, dTTP, and dCTP, 20 pmol of each primer, 2.0 U of *Taq* DNA polymerase (Promega), and 20–100 ng of purified genomic or plasmid DNA.

IncP-9 replicons were detected and characterised by using oligonucleotide primers and PCR conditions described earlier by Krasowiak et al. (2002). Genes encoding for the large subunit of naphthalene-1,2-dioxygenase, *nahAc*, were detected by using PCR with oligonucleotide primers Ac149f and Ac1014r as described by Ferrero et al. (2002). The gene for catechol 2,3-dioxygenase, *nahH*, was detected by PCR with conserved oligonucleotide primers 23OF and 23OR according to Wilkstrom et al. (1996). The genes for salicylate hydroxylase were detected by PCR with primers shc1_up (5′-CGG CKT THG GTG ARG TCG GTG C-3′) and shc1_lo (5′-GGC GAG GAA RTA GGC GTC CTC AAG-3′), which amplified a 892-bp fragment of the *nahG* gene. Finally, the plasmid-borne regulatory gene *nahR* was detected by PCR with primers nahR_1f (5′-ATG GAA CTG CGT GAC CTG G-3′) and nahR_585r (5′-GCC GTA GGA ACA GAA GCG-3′), which were designed to amplify a 585-bp fragment of *nahR*.

Genotypes of individual bacterial strains were characterised by using amplified ribosomal DNA restriction analysis (ARDRA) and rep-PCR fingerprinting. Amplification of a nearly full-length 16S rDNA was performed by PCR with conserved eubacterial primers 8f and 1492r (Weisburg et al., 1991). Amplified rDNA was digested with restriction endonucleases (*Rsa*I, *Msp*II, and *Hae*III from MBI Fermentas, Vilnius, Lithuania), separated on 2% agarose gel and analyzed. Genomic fingerprinting was carried out with BOXA1R primer according to Dombek et al. (2000). Data were analyzed with GelCompar version 4.0 software (Applied Maths, Kortrijk, Belgium) by correlation-based clustering.