Selection of *Acinetobacter calcoaceticus* Mutants Deficient in the p-Hydroxybenzoate Hydroxylase Gene (pobA), a Member of a Supraoperonic Cluster

GAIL B. HARTNETT, BEATE AVERHOFF, AND L. NICHOLAS ORNSTON*
Department of Biology, Yale University, New Haven, Connecticut 06511-8112

Received 14 May 1990/Accepted 20 July 1990

p-Hydroxybenzoate hydroxylase, the product of the *pobA* gene, gives rise to protocatechuate, which is metabolized by enzymes encoded by the *pca* operon in *Acinetobacter calcoaceticus*. Mutations in *pcaD* prevented growth of *A. calcoaceticus* with succinate in the presence of p-hydroxybenzoate. Mutants selected on this medium contained the original mutation in *pcaD* and also carried spontaneous mutations in *pobA*. These independently expressed genes were cotransformed with a frequency of 15% and thus are components of a supraoperonic cluster.

Metabolic transformations of catechol and protocatechuate form the two major branches of the β-ketoadipate pathway (12). Independently transcribed genes associated with metabolism of benzoate via catechol compose a supraoperonic cluster in *Acinetobacter calcoaceticus* (10, 11). p-Hydroxybenzoate hydroxylase (EC 1.14.13.2), encoded by the *pobA* gene (6), gives rise to protocatechuate, and enzymes encoded by the *Acinetobacter pca* operon (3) complete catabolism of this compound. Regulation of the *pobA* gene and its possible linkage to the *pca* genes in *A. calcoaceticus* had not been explored prior to this investigation. In this paper, we describe a procedure that allows direct selection of *Acinetobacter* strains carrying mutations in both *pobA* and *pcaD*, the structural gene for β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24). We show that these genes respond to different inducers yet are linked in a supraoperonic cluster.

Evidence indicating that a mutation in *pcaD* prevented growth with succinate in the presence of p-hydroxybenzoate emerged from analysis of *Acinetobacter* strain ADP230, which carries a deletion in the *pcaD* gene. The deletion was created by removing two *EcoRV* fragments, containing 1.0 kilobase pairs (kbp) of DNA, from pZR3, which contains the *pca* gene in 2.6 kbp of *Acinetobacter* DNA inserted into the HindIII locus of the multiple cloning site of pUC18 (3). The resulting plasmid, pZR301, contains *Acinetobacter* DNA solely in HindIII-*EcoRV* segments of 0.7 and 0.9 kbp, DNA insufficient in length to encode the entire *pcaD* gene product (15). Cleavage of pZR301 with HindIII produced linear DNA containing the 1.0-kbp *EcoRV*-*EcoRV* deletion, and this DNA was introduced into wild-type *A. calcoaceticus* spread upon 10 mM succinate plates. Of 200 colonies isolated from the transformed culture, 2 appear to have acquired the deletion as evidenced by inability to grow at the expense of p-hydroxybenzoate. The location of the mutation in one of these isolates, designated strain ADP230, was confirmed by demonstration that it was efficiently transformed to the wild type by the 2.6-kbp insert of *Acinetobacter* DNA carried in pZR3.

Unlike wild-type cells, strain ADP230 failed to grow with 10 mM succinate in the presence of 5 mM p-hydroxybenzoate. The same mutant phenotype was exhibited by strain ADP212, a recombinant that had acquired by transformation *Acinetobacter* DNA containing the transposon Tn5 within *pcaD* (3). Therefore, dysfunctions in *pcaD* appear to prevent growth on succinate in the presence of p-hydroxybenzoate. Mutants derived from ADP212 on p-hydroxybenzoate–succinate growth medium also grew with p-hydroxybenzoate as the sole carbon source. As judged by their sensitivity to kanamycin, these strains had regained *pcaD* function by precise excision of the transposon. The *pcaD* mutation within strain ADP230 prevented reversion, and this organism gave rise to secondary mutants that resisted the toxic effect of p-hydroxybenzoate. About 40% of these mutants remained sensitive to quinate, a compound that is metabolized to protocatechuate via a route that is independent of p-hydroxybenzoate (13; Fig. 1). Typical of members of this subset of mutants was strain ADP231, which proved to have a mutation within *pobA*.

The wild-type *pcaD* gene contained within pZR3 was introduced into ADP231 by transformation. Recombinants, selected by demanding growth with quinate, invariably failed to grow with p-hydroxybenzoate because the donor pZR3 DNA does not contain the *pobA* gene. One recombinant, strain ADP239, was used to examine inducer specificity by measurement of p-hydroxybenzoate hydroxylase (5) and protocatechuate 3,4-dioxygenase (EC 1.13.11.3) in extracts of induced cells (4). The *pobA* mutation in ADP239 prevented metabolism of p-hydroxybenzoate; growth of the strain with 10 mM succinate in the presence of 5 mM p-hydroxybenzoate produced cells in which the specific activity of protocatechuate oxygenase was 0.006 μmol/min per mg of protein. The same activity was observed in uninduced (succinate-grown) wild-type cells. Exposure of wild-type cells to 5 mM p-hydroxybenzoate during growth increased the specific activity of protocatechuate oxygenase about 25-fold to 0.157 μmol/min per mg of protein. Thus, in accord with earlier observations, expression of the *pcaHG* genes in response to p-hydroxybenzoate requires metabolism of this compound to protocatechuate (2). *p-Hydroxybenzoate* is not formed as quinate is metabolized via protocatechuate (13), and the level of *p-hydroxybenzoate* hydroxylase in quinate-grown wild-type cells is less than 0.001 μmol/min per mg of protein. Growth of wild-type cells at the expense of p-hydroxybenzoate results in an increase

* Corresponding author.
FIG. 1. Growth inhibition exerted by p-hydroxybenzoate on strains carrying a deletion in pcaD. Cells were grown with 10 mM succinate, and the potential growth inhibitor (p-hydroxybenzoate [POB] or quinate [Q]) was added at 1 h to a final concentration of 5 mM. Strain ADP230, which carries the pcaD deletion, did not grow in the presence of p-hydroxybenzoate. This growth inhibition was overcome by the pobA mutation in ADP231 (which also carries the pcaD deletion). Quinate, metabolized to protocatechuate independently of pobA, inhibited growth of ADP231.

of at least 50-fold in pobA expression so that its gene product is formed at a level of 0.051 μmol/min per mg of protein. In sum, the studies of inducer specificity fortify earlier conclusions that the pobA gene is regulated independently of the pca genes (1). The former gene is expressed in response to p-hydroxybenzoate but not in response to protocatechuate. The latter compound elicits expression of the pca genes which are not expressed in response to p-hydroxybenzoate.

Linkage of pobA and pcaD was demonstrated by transformation of strain ADP231 with chromosomal DNA from wild-type A. calcoaceticus followed by selection with quinate. Of 784 selected transformants, 114 grew with p-hydroxybenzoate and therefore had acquired from the donor both the pobA gene and the pcaD gene. This frequency of cotransformation, 15%, corresponds to a physical distance of about 10 kilobases between ben and cat alleles in the ben-cat supraoperonic cluster (10, 11; M. E. Rae, unpublished observations). The frequency of pobA-pcaD cotransformation was independent of the concentration of donor DNA and hence cannot be attributed to multiple transformation events. In a separate experiment, ADP239 served as a donor and recombinants derived from ADP230 were selected on quinate. Of 100 selected transformants that had acquired the wild-type pcaD gene, 8 had also acquired the donor pobA mutation, as demonstrated by their inability to grow with p-hydroxybenzoate.

The results show linkage of the independently regulated pobA and pca genes in a supraoperonic cluster within the Acinetobacter chromosome. Similar observations have been reported for fluorescent Pseudomonas species (7, 9, 14) in which the pca genes are organized in a relatively fragmented manner (8). Thus, evolutionary divergence of Acinetobacter and Pseudomonas spp. allowed substantial reorganization of the pca genes but resisted their separation from pobA. The growth inhibition exhibited by the Acinetobacter pcaD mutant ADP231 in the presence of quinate (Fig. 1) suggests that it should be possible to select mutations blocking quinate catabolism (13) and to explore their possible linkage to the pobA-pca cluster.

This research was supported by the U.S. Army Research Office and by the Celgene Corporation. B. Averhoff was supported by the Deutsche Forschungsgemeinschaft.

We thank L. Gregg for providing strain ADP212 and R. Burdick for technical assistance.

LITERATURE CITED


