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

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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 *Eco*RV fragments, containing 1.0 kilobase pairs (kbp) of DNA, from pZR3, which contains the *pcaD* gene in 2.6 kbp of *Acinetobacter* DNA inserted into the *Hin*dIII locus of the multiple cloning site of pUC18 (3). The resulting plasmid, pZR301, contains *Acinetobacter* DNA solely in *Hin*dIII-*Eco*RV segments of 0.7 and 0.9 kbp, DNA insufficient in length to encode the entire *pcaD* gene product (15). Cleavage of pZR301 with *Hin*dIII produced linear DNA containing the 1.0-kbp *Eco*RV-*Eco*RV 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 Tn*5* 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

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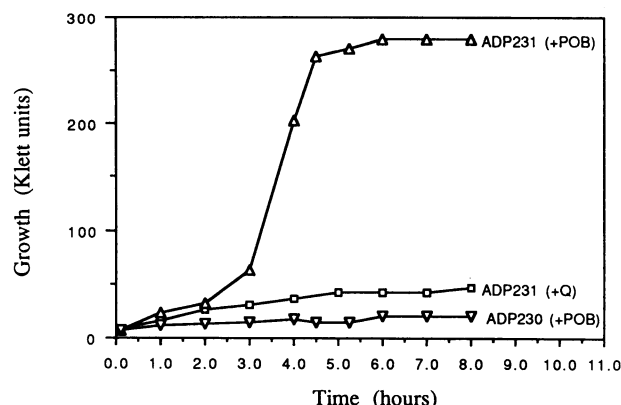

FIG. 1. Growth inhibition exerted by *p*-hydroxybenzoate on strains carrying a deletion in *pcaD*. Cells were grown with 10 mM succinate, and the potential growth inhibitor (*p*-hydroxybenzoate [POB] or quinate [Q]) was added at 1 h to a final concentration of 5 mM. Strain ADP230, which carries the *pcaD* 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.

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