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Genetic Analysis of Supraoperonic Clustering by Use of Natural Transformation in *Acinetobacter calcoaceticus*

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DNA within *Escherichia coli* colonies carrying cloned *Acinetobacter calcoaceticus* genes transforms mutant *A. calcocaceticus* cells with high efficiency. Therefore, *E. coli* colonies containing such cloned genes can be identified by replica plating onto a lawn of *A. calcoaceticus* mutant cells. Transformation of *A. calcoaceticus* also facilitates gap repair and thus allows recovery of specified chromosomal segments in recombinant plasmids. These procedures were used to demonstrate the clustering of *A. calcoaceticus* genes required for utilization of *p*-hydroxybenzoate. Chromosomal linkage of the bacterial genes, contained in different operons separated by about 10 kbp of DNA, may have been selected on the basis of their physiological interdependence.

Members of the nutritionally versatile bacterial species *Acinetobacter calcoaceticus* (2, 12) are widely distributed in the natural environment (1). Examination of the genetic basis for the metabolic diversity of these bacteria has been facilitated by the properties of one isolate that exhibits extraordinarily high competence for natural transformation (8–12, 16–18). This trait obviates the need for selection in the isolation of recombinants that have acquired engineered chromosomal DNA (3) and allows mutant alleles to be located within 200 bp of known DNA sequence (8).

The *pobA* gene encodes *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2), an enzyme that converts its substrate to protocatechuate (6, 22). This metabolite is converted to citric acid cycle intermediates by six enzymes encoded by the *pca* operon in *A. calcoaceticus* (4). The *pobA* gene and the *pca* operon are expressed independently, as indicated by their response to different metabolites as inducers (9). Despite the difference in transcriptional controls, the *pobA* gene and the *pcaD* gene are cotransformed with a frequency of 15% (9). This indirect evidence suggested that the separately regulated genes are components of a supraoperonic cluster in which the different transcriptional units are separated by about 10 kbp within the *A. calcoaceticus* chromosome (9). As described here, the validity of this inference was established by cloning the *pobA* gene and then recovering a chromosomal fragment in which the gene is carried along with a portion of the *pca* operon.

We here describe use of natural transformation to screen for *Escherichia coli* colonies carrying the cloned *A. calcoaceticus pobA* gene. We also demonstrate use of gap repair, a technique that requires transformation with linearized plasmid DNA (7, 14, 15, 19), to isolate from the *A. calcoaceticus* chromosome a 12.3-kbp segment of DNA that bridges the *pobA* gene and the *pca* operon. These independently transcribed genetic regions have been coselected in *A. calcoaceticus* because their combined functions are required for utilization of *p*-hydroxybenzoate as a growth substrate. Physical linkage of the genes demonstrates that, as with some other prokaryotic genes with coevolved physiological functions, the *pobA* gene and the *pca* operon form a supraoperonic cluster (17, 18, 20, 23–25).

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MATERIALS AND METHODS

Screening by replica plating for *E. coli* clones containing plasmid-borne *A. calcoaceticus pob* DNA. Chromosomal DNA from wild-type *A. calcoaceticus* ADP1 was digested with *Pst*I, and the resulting fragments were ligated to the multiple cloning site of pUC18. The ligated material was introduced into *E. coli* DH5α, and the plasmids were selected by demanding ampicillin resistance (21). The resulting *E. coli* colonies were screened for the presence of *pob* DNA by assessment of the ability of replicas of the *E. coli* colonies to transform the *pob-1* mutant *A. calcoaceticus* ADP239 (9) so that recombinants grew with *p*-hydroxybenzoate (Fig. 1). The activity of the hydroxylase encoded by *pobA* cloned in *E. coli* was determined by published procedures (6).

Construction of plasmids used for gap repair. The following procedures were used to construct pZR502, which was used to recover DNA lying between the *pca* and *pob* regions from the *A. calcoaceticus* chromosome (see Fig. 3A). Cleavage of pZR408 (see Fig. 2) with *Eco*RI and *Hin*dIII yielded the *pobA* gene within the 2.7-kbp *Acc*I-*Sst*I fragment flanked by portions of the multiple cloning site acquired from pUC18. The multiple cloning site contributed a unique *Pst*I site proximal to the position of *Hin*dIII cleavage in pZR408. This *Pst*I site, conserved through the following constructions, was used to separate *pobA* from *pcaD* during linearization of pZR502. The 2.7-kbp *Eco*RI-*Hin*dIII DNA fragment from pZR408 was ligated into the multiple cloning site of the broad-host-range plasmid pRK415 (13). The resulting plasmid, pZR501, was introduced into *E. coli* by transformation and selected by demanding expression of the tetracycline gene derived from pRK415. Presence of the *pobA* gene in pZR501 was detected by replica plating onto the *A. calcoaceticus* mutant strain ADP239 followed by selection for growth with *p*-hydroxybenzoate (Fig. 1). The 2.8-kbp *Hin*dIII fragment containing the *pcaD* gene was introduced from pZR3 (4) into the *Hin*dIII site of pZR501, giving rise to pZR502, and this plasmid was selected in *E. coli* DH5α on the basis of tetracycline resistance. The presence of both *pobA* and *pcaD* within the selected *E. coli* strain DH5α(pZR502) was confirmed by replica plating onto mu-

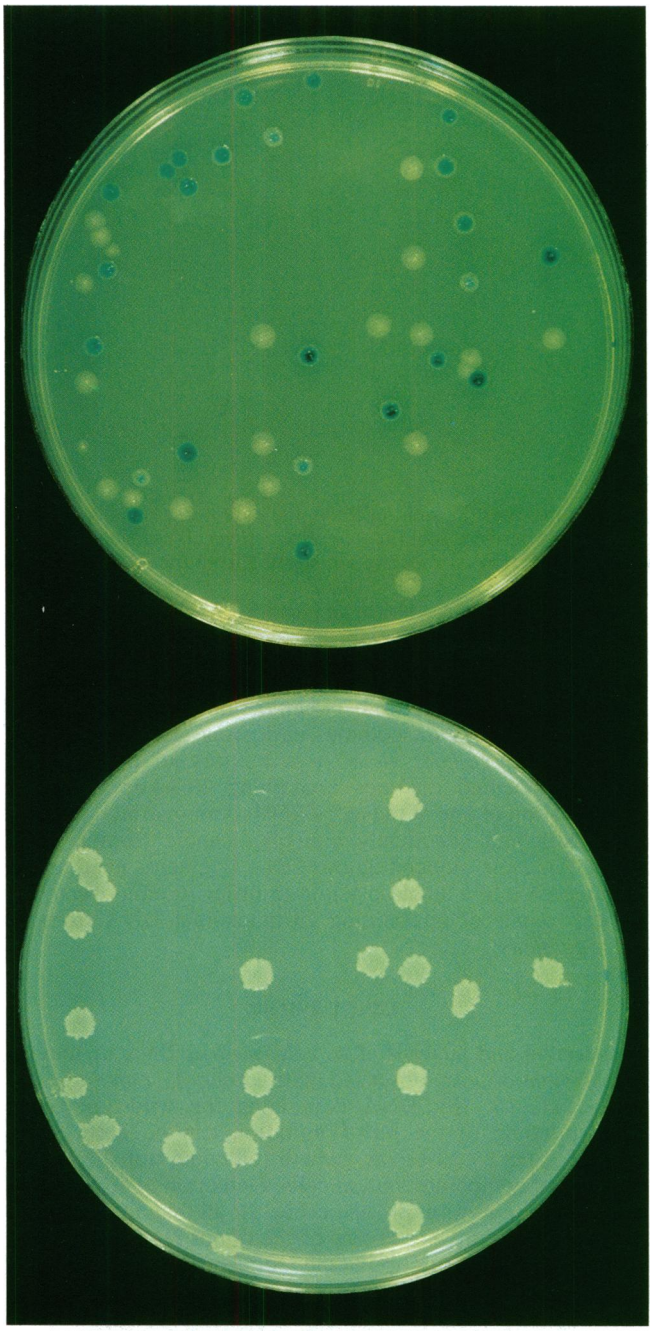

FIG. 1. Screening by replica plating for *E. coli* clones containing the plasmid-borne *A. calcoaceticus pobA* gene. (Top) *E. coli* DH5α colonies carrying pUC18 are blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyramoside) plates, a consequence of expression of the plasmid-borne *lacZ* gene (20). Disruption of *lacZ* by the *A. calcoaceticus pobA*-containing DNA insert in pZR400 results in white *E. coli* colonies. (Bottom) The presence of wild-type *pobA* DNA in pZR400 is revealed by the capacity of the white *E. coli* colonies to transform the *pobA*-deficient mutant *A. calcoaceticus* ADP239 to the wild type. The *E. coli* colonies shown on the top were transferred by replica plating to the recipient *A. calcoaceticus* mutant strain, which had been spread upon solidified mineral medium in which 5 mM *p*-hydroxybenzoate was the sole carbon source.

tant *A. calcoaceticus* strains in which the respective genes were dysfunctional.

Demonstration of gap repair with donor DNA carried on two different linearized derivatives of pRK415 (see Fig. 3B) required construction of pZR501, described in the preceding paragraph, and pZR1030. The latter plasmid was formed by introduction of the 2.8-kbp *Hin*dIII fragment from pZR3 into pRK415. After ligation, the recombinant plasmid was introduced into *E. coli* with subsequent selection for tetracycline resistance.

Gap repair with a single donor plasmid. Cleavage of pZR502 with *Pst*I produced a 16-kbp linear DNA fragment in which the vector pRK415 sequence was flanked by DNA containing the *pobA* and *pcaD* genes; about 0.5 μg of this DNA was spread with roughly 5×10^7 wild-type *A. calcoaceticus* cells upon plates containing mineral medium supplemented with 10 mM succinate. After 6 h of incubation at 37°C, the culture was suspended in 0.5 ml of sterile medium and spread upon plates containing Luria broth supplemented with 25 μg of tetracycline per ml.

Gap repair with a combination of two different donor plasmids. Prior to gap repair, pZR501 was linearized with *Hin*dIII, and pZR1030 was linearized with *Eco*RI. The two linearized plasmids (about 0.5 μg each) were spotted with about 5×10^3 viable cells on a plate containing 10 mM succinate. The relatively low cell concentration was chosen so as to optimize the likelihood of transformation of single cell recipients by multiple donor DNA fragments. After overnight incubation, the cells were resuspended and spread upon plates containing 25 μg of tetracycline per ml and 1.8% Bacto Agar.

RESULTS

Properties of *A. calcoaceticus* chromosomal fragments containing portions of the *pobA* gene. In three different experiments, about 1,000 *E. coli* colonies containing plasmids with *A. calcoaceticus* DNA inserts were screened by replica plating, and in each case one colony possessed DNA that transformed the *pob-1* mutant strain ADP239 to the wild type. Plasmid DNA from the three colonies exhibited overlapping restriction patterns. A 12.8-kbp *Pst*I insert within the multiple cloning site of pUC18 was carried in one of the plasmids, pZR400. This plasmid does not appear to carry *pca* genes because it fails to transform *A. calcoaceticus* strains carrying mutations in these genes (4) to the wild type. The properties of subclones derived from pZR400 are summarized in Fig. 2. Three subclones, pZR404, pZR405 and pZR408, resemble the parental plasmid in that they are expressed in *E. coli pobA* at levels of about 10% of those found in fully induced cultures of *A. calcoaceticus*. The orientation of subcloned DNA in these plasmids is opposite to that in the parental plasmid, and none of the plasmids exhibits normal regulation of the inserted gene in response to isopropylthiogalactoside, which elicits transcription from the *lac* promoter in the vector plasmid. This indicates that another promoter, probably in the inserted *A. calcoaceticus* DNA, is responsible for *pobA* expression from the plasmids.

Replicas of *E. coli* colonies carrying the aforementioned plasmids transform the *A. calcoaceticus pob-1* mutant strain ADP239 to the wild type (Fig. 1). This trait is shared by *E. coli* colonies carrying pZR411 or pZR412 but not by those carrying pZR406, pZR407, pZR409, or pZR410. It therefore is likely that only the former set of plasmids contain DNA significantly overlapping the mutant *pob-1* allele.

Recovery of chromosomal DNA lying between the *pca* and

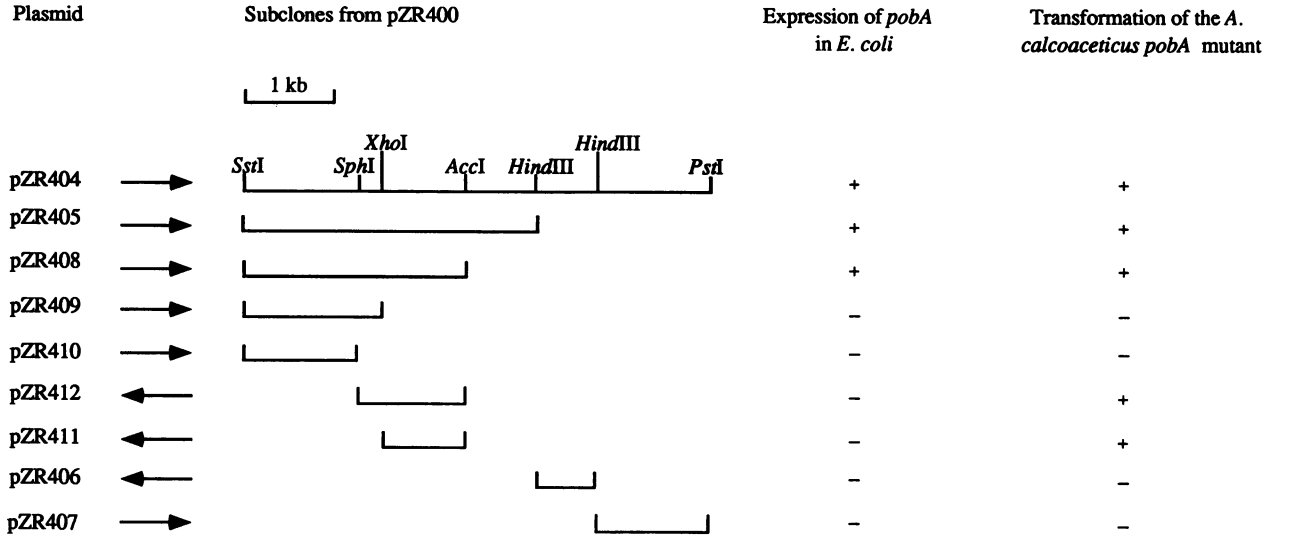

FIG. 2. Presence of *pobA*-associated DNA and enzyme activities in *E. coli* subclones. Plasmid pZR400, the parent of plasmids carrying the inserts depicted, contains the *pobA* gene in a 15.5-kbp *Pst*I restriction fragment derived from the *A. calcoaceticus* chromosome. One of the *Pst*I sites in pZR400 is shared by pZR404 and pZR407. Arrows indicate the direction of transcription starting from the *lac* promoter of pUC18.

***pob* regions by gap repair.** Noncontiguous chromosomal fragments, containing *pcaD* and *pobA*, were ligated into the broad-host-range plasmid pRK415 to form pZR502; *Pst*I linearized the plasmid by cleaving it at a unique site between the two fragments (Fig. 3A). Transformation of *A. calcoaceticus* with the linear DNA followed by selection for pRK415-encoded tetracycline resistance demanded replication of the plasmid in circular form. The selection produced about 50 colonies. Examination of 12 of the colonies revealed the presence of 28.3-kbp plasmids. One of these, pZR503, was selected for analysis of the 12.3-kbp DNA segment that appeared to have been acquired from the chromosome by gap repair. This DNA segment contained 6 kbp corresponding to one end of a previously isolated 11-kbp *Eco*RI fragment carried as an insert within pZR1 (4). The latter fragment carries all of the structural genes in the *pcaIJFB DCHG* operon; the only *pca* genes in the 6 kbp of DNA shared by pZR1 and pZR503 are those expressed downstream from *pcaD*. Among these is *pcaG*, and the presence of DNA encoding this gene was demonstrated by the capacity of *E. coli* colonies carrying pZR503 to transform ADP6, a *pcaG*-deficient *A. calcoaceticus* mutant strain (7, 8). The gene *pcaJ* lies upstream from *pcaD*. Transformation of the *pcaJ*-deficient strain ADP141 provided evidence for the presence of this gene in pZR1 (4), and the absence of such transformation indicated that the gene is not contained in pZR503.

The ease with which the *A. calcoaceticus* strain undergoes natural transformation demonstrates its vigorous capacity to recombine DNA. This capability raised the possibility that natural recombination between separate linear pRK415 derivatives, one (pZR1030) carrying the *pcaD* gene and the other (pZR501) carrying the *pobA* gene, might give rise to recombinants carrying both genes with a frequency sufficient to allow recovery of chromosomal genes by gap repair. As illustrated in Fig. 3B, the recombination system of *A. calcoaceticus* achieved this genetic feat and produced from two different donor plasmids, each carrying a copy of a different chromosomal fragment, recombinant plasmids carrying both donor chromosomal fragments connected by a copy of the DNA that lies between them in the chromosome. Tetracycline-resistant colonies appearing after exposure of wild-type *A. calcoaceticus* to a mixture of pZR501 and pZR1030 yielded colonies containing 28.3-kbp plasmids. Examination of one of these, pZR504, demonstrated that it is physically and genetically indistinguishable from pZR503 (Fig. 3A). Thus, noncontiguous DNA fragments need not be engineered into a single plasmid in order to effect recovery by gap repair of intervening chromosomal DNA from *A. calcoaceticus*.

DISCUSSION

Detection and analysis of *A. calcoaceticus* DNA by natural transformation. As observed with other *A. calcoaceticus* genes (11, 16, 18), DNA containing the wild-type allele corresponding to the *pob-1* mutation can be detected in crude lysates by assessing growth following transformation in lawns of competent mutant cells. Detection of donor DNA does not require its expression (Fig. 2); all that appears to be needed for effective recovery of the donor DNA is a region that overlaps the mutation in the recipient strain. Subcloning of donor DNA permits fairly precise localization of the donor gene. Present evidence allows the wild-type allele corresponding to the *pob-1* mutation to be placed within the 1.1-kbp *Xho*I-*Acc*I insert carried within pZR411 (Fig. 2). Yet to be established is whether the mutation lies within the *pobA* structural gene or in a nearby gene required for *pobA* expression. The presence of the *pobA* gene in the 2.7-kbp *Acc*I-*Sst*I insert of pZR408 is clearly established by expression of the gene's product in *E. coli* (Fig. 2). Gap repair yielded 12.3 kbp of chromosomal DNA lying between this fragment and the *Hin*dIII segment containing the *pcaD* gene (Fig. 3). Thus, as predicted from the cotransformation frequency of 15% (9), about 10 kbp of DNA lies between *pobA* and *pcaD*.

E. coli colonies carrying cloned *A. calcoaceticus* DNA can serve directly as donors in the natural transformation of

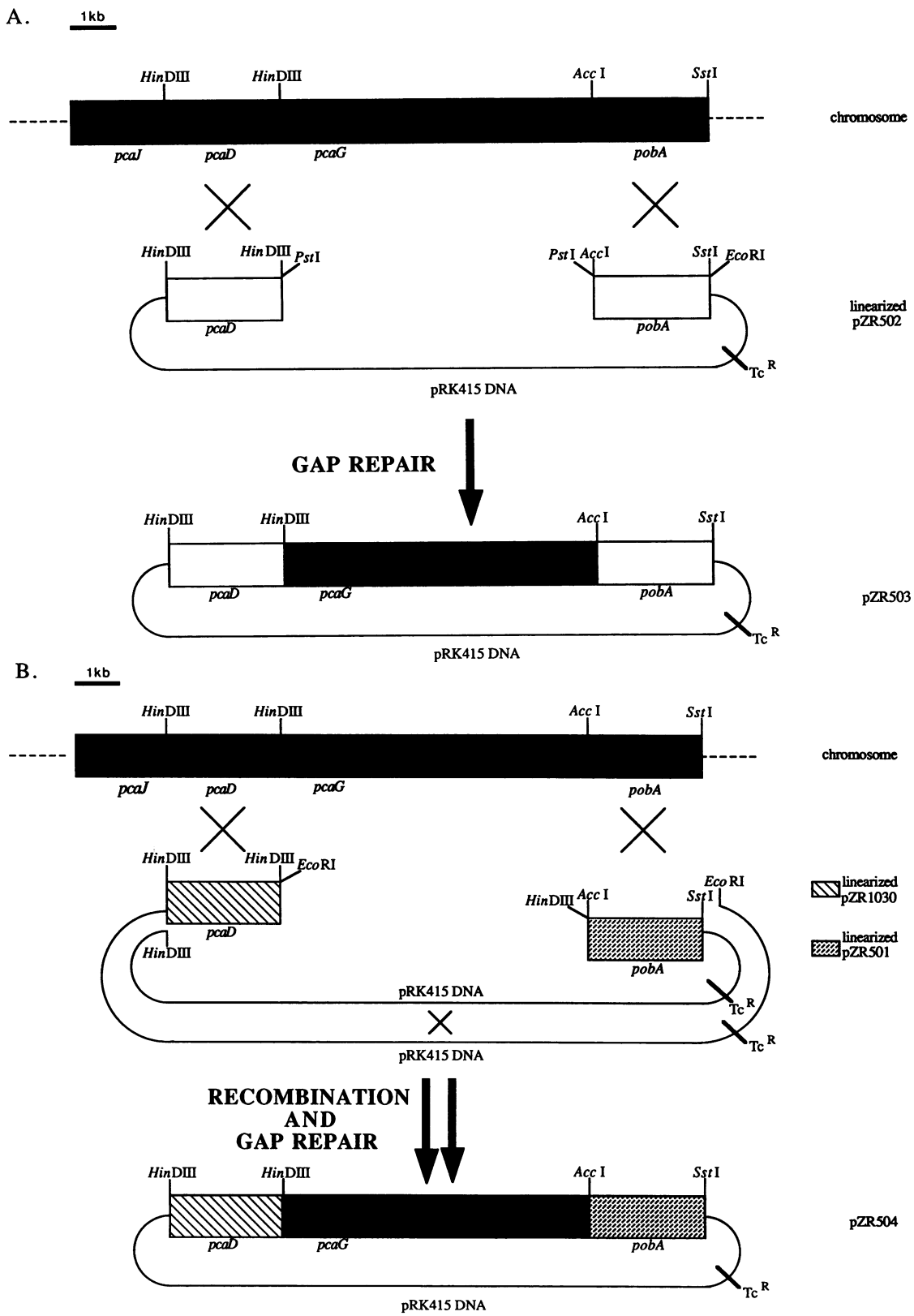

FIG. 3. Use of gap repair to isolate DNA lying between the *pca* genes and the *pobA* gene from the *A. calcoaceticus* chromosome. (A) A single donor DNA fragment, linearized pZR502, was used to recover DNA lying between *pcaD* and *pobA* from the chromosome. (B) Gap repair using as a donor a combination of two different linearized pRK415 derivatives, one carrying *pcaD* (pZR1030) and the other carrying *pobA* (pZR501), allowed recovery of intervening DNA from the *A. calcoaceticus* chromosome.

A. calcoaceticus (Fig. 1). Apparently *E. coli* colonies grown on plates contain free DNA in quantities sufficient to achieve frequent transformation of the naturally competent *A. calcoaceticus* cells. We anticipate that the procedure will prove useful in the identification of *E. coli* colonies carrying other *A. calcoaceticus* genes. Over a period of 48 h, the efficiency of transformation increases with the age of the donor *E. coli* colonies. This phenomenon has not been explored quantitatively, but it raises the possibility that the release of DNA within *E. coli* colonies may give an indication of the relative health of the resident cell population. Since DNA from all members of the species *A. calcoaceticus* may serve as a donor for the naturally competent strain, it also is possible that transformation may be used to detect the presence of *A. calcoaceticus* DNA in natural environments.

Potential use of natural transformation to study the basis for selection of supraoperonic clusters. Bacterial chromosomes are structured, and the biological forces that organized the underlying components of structure are beginning to be elucidated (5). Of particular interest are the selective pressures that led to clustering of genes with coselected physiological functions (17, 18, 20, 23–25). It is possible that clustering allows global control of expression of the linked genes. For example, clustering allows unified control of several operons associated with the photosynthetic apparatus by transcriptional readthrough (23, 25). On the other hand, supraoperonic clustering may permit physical or genetic interaction between linked regions of DNA. Natural transformation makes the supraoperonic clusters of *A. calcoaceticus* particularly amenable to genetic manipulation. A promising analytical approach will be to transpose genes that evolved as components of one supraoperonic cluster to a locus within another supraoperonic cluster. Examination of the physiological and genetic properties of strains that have undergone such transpositions may yield insight into selective forces that favored clustering of genes with physiologically interdependent functions.

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