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Cloning of *catBCIJFD* Genes for Catechol Degradation into Chromosomal *pobA* and Genetic Stability of the Recombinant *Acinetobacter calcoaceticus*

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Abstract

A possible obstacle in the development of hybrid strains of *Acinetobacter calcoaceticus* by the introduction of a metabolic pathway into the chromosome is genetic instability of the resulting recombinant strains. Therefore, the possibility that the *pobA* gene can be used as a chromosomal cloning site where the transposed genes can be maintained and expressed, was explored in the present study. For this purpose, two model hybrid strains of *A. calcoaceticus* were created, in which a DNA fragment carrying *catBCIJFD* genes for catabolic degradation of catechol was inserted into *pobA* in opposite directions of each other, and their genetic stabilities were experimentally examined. Our data demonstratéd that the stability of the genes neighboring the insertions depends on the orientations of the insertions. Also, the data further indicated that the functional metabolic pathways introduced into the *pobA* can be expressed successfully as far as the insertion is engineered in an appropriate way. Concurrently, it was also proposed that the *pobA* can be used as a chromosomal cloning site, and that introduction of an useful metabolic pathway into the *pobA* may offer considerable promise to the construction of a hybrid strain with improved metabolic capabilities.

Key words: *Acinetobacter calcoaceticus*; Hybrid Strain; Genetic Stability.

I. Introduction

Recently developed genetic engineering techniques offer considerable hope to the construction of bacterial hybrid strains with desired metabolic capabilities. Among these hybrid strains, an increasing number of recombinant strains constructed by the rational combination of two catabolic pathways to improve their degradative abilities have been reported[1,2]. Most current technology for construction of useful hybrid strains involves cloning and expression of exogeneous genes encoding new metabolic pathways through appropriate plasmid systems and using *E. coli* as a host[3,4]. However, despite the fact that a large number of genes have been cloned and expressed in *E. coli*, stable maintaining or adequate expression in a non-enteric host through plasmid system is still not effective. In an effort to overcome these limitations, cloning desirable genes on bacterial chromosome instead of on plasmid has been attempted. Although it has been reported that a few recombinant bacteria have been constructed by inserting transposon carrying the genes for new metabolic functions into random sites of the chromosome[4,5], it is not apparent yet that these genes have been cloned successfully into any defined specific site of bacterial chromosome. Therefore, it is still doubtful that the newly acquired or transposed genes can be maintained and expressed stably in specialized locations of the bacterial chromosome.

Acinetobacter calcoaceticus are capable of degrading a variety of aromatic compounds including benzoate, *p*-hydroxybenzoate, quinate and shikimate. The catabolic versatility of *A. calcoaceticus*[6]

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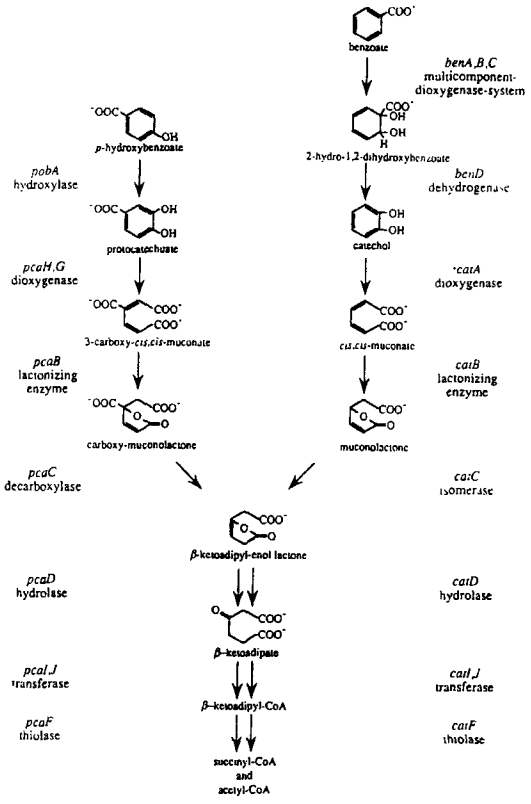

Fig. 1. The β-Ketoadipate Pathway of *A. calcoaceticus* for Dissimilation of Aromatic Compounds via Protocatechuate and Catechol.

is comparable to that of the well-studied fluorescent *Pseudomonas* species[7], and apparently plays a significant role in carbon cycling. The degradation of these aromatic compounds takes place by way of the β-ketoadipate pathway (Fig. 1)[8,9]. The β-ketoadipate pathway seems widely distributed in diverse soil bacteria such as *Psedomonas* and *Rhizobium*[8,9]. However, in the case of *A. calcoaceticus*, some enzymes involved in the β-ketoadipate pathway; β-ketoadipate enol-lactone hydrolase (*pcaIJ* or *catIJ*) and β-ketoadipyl CoA thiolase (*pcaF* or *catF*) are composed of isoenzymes. The DNA sequences of the *catIJF* and *pcaIJF* regions are nearly identical[10,11] and the similarity appears to afford opportunity for DNA sequence exchange between the two regions[12].

Members of *A. calcoaceticus* are found in most natural environments[13] and seem to be able to compete well with other indigenous populations in the microbial community. In addition, one *A. calcoaceticus* isolate is a naturally competent recipient for genes transferred by transformation[14] and provides a powerful tool for genetic manipulation of the bacteria[11,15]. Recently, a number of genetic studies have been inspired by the competency of the bacteria, and genes involved in the β-ketoadipate pathway have been cloned and analyzed[10,11]. Consequently, an increasing amount of information is available on their genetic organization, regulation and evolution[8,9,16].

Such information offer an attractive possibility of using *A. calcoaceticus* in developing effective hybrid strains. For example, a hybrid strain with designed and improved metabolic capabilities can be constructed by introduction a DNA fragment carrying certain catabolic and/or anabolic functions into specialized locations within the *A. calcoaceticus* chromosome. In the developing of the hybrid strain, one of the most crucial questions to be answered is on the stability of the recombinant metabolic pathways introduced.

In the present study, the possibility that the *pobA* gene encoding *p*-hydroxybenzoate hydroxylase can be used as a chromosomal cloning site, to which a useful metabolic pathway is introduced to construct a hybrid strain of *A. calcoaceticus*, is explored. For this purpose, two recombinant strains, in which a DNA fragment carrying *catBCIJFD* genes[10] for catabolic degradation of catechol in the β-ketoadipate pathway is inserted in opposite directions of each other into the *pobA*[17], are constructed. Concurrently, the effects of these insertions on the genetic stability of the recombinants are here examined. To our knowledge this is the first substantiated report regarding the genetic stability of bacterial hybrid strains constructed in an engineered fashion by using a defined chromosomal cloning site.

II. Materials and Methods

Bacterial strains and plasmids. Descriptions of the bacterial strains of *Acinetobacter calcoace-*

ticus and plasmids used in this study are listed in Table 1. All *A. calcoaceticus* ADP strains used are derivatives of strain BD413[14], which is a remarkably competent recipient for genes transfered by natural transformation. *A. calcoaceticus* ADP230[18] is a *pcaBDK* deletion mutant of the wild-type strain ADP001. *A. calcoaceticus* ADP 900 is also a *pcaBDK* deletion mutant of strain ADP161, which carries a large deletional mutation in the *ben cat* structural gene[11]. Both ADP 903 and ADP904 were derived from ADP900 by introduction of *catBCIJFD* genes into the *pobA* structural gene.

Culture conditions. Cultures of *E. coli* were grown in LB (Luria-Bertani) broth or on LB agar containing the appropriate antibiotics for plasmid screening or maintenance (100 μg of ampicillin, 15 μg of tetracycline).

A. calcoaceticus strains were grown as described elsewhere[10]. The basal salt medium consisted of 12.5 mM Na_2HPO_4, 12.5 mM KH_2PO_4, 0.5% $(NH_4)_2SO_4$, and 10 ml of a trace element concentrated base. The latter contained 59.3 μg of $MgSO_4 \cdot 7H_2O$, 20 μg of nitrilotriaceticacid, 6.67 μg of $CaCl_2 \cdot 2H_2O$, 0.25 μg of EDTA, 1.95 μg of $ZnSO_4 \cdot 7H_2O$, 0.5 μg of $FeSO_4 \cdot 7H_2O$, 0.198 μg of $FeSO_4 \cdot 7H_2O$, 0.154 μg of $MnSO_4 \cdot H_2O$, 39.2 mg of $CuSO_4 \cdot 5H_2O$, 25 mg of Co $(NO_3)_2 \cdot 6H_2O$, 18.5 mg of Molybdic acid, and 17.7 mg of $Na_2B_4O_7 \cdot 10H_2O$ per liter. The following concentrations of carbon sources were added to the basal salt medium to compose corresponding media : succinate (10 mM), benzoate (5 mM), *p*-hydroxybenzoate (5 mM), protocatechuate (5 mM), *cis,cis*-muconate (5 mM).

For determining the genetic stability of the ADP strains, 5 ml test tube cultures of the strains in succinate broth, inoculated to OD 0.01 at 660 nm, were incubated with shaking (200rpm) for about 8 h. Inocula were from late logarithmic phase cultures which had been grown in succinate broth. When their cell density reached to about 1 of OD at 660 nm, the growth of the cells was stopped by putting the tubes into ice, and the cells diluted with the basal salt medium appropriately were then plated. Unless otherwise indicated, all broth cultures and plates were incubated at 30 °C.

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Relevent genotype or description	Source or reference
A. calcoaceticus		
ADP001	Wild type (BD413)	(Juni)[14]
ADP161	*Δ ben cat*	(Doten *et al.*)[11]
ADP230	*ΔpcaBDK*	(Hartnett *et al.*)[18]
ADP900	*ΔpcaBDK, Δben cat*	This study
ADP903	*ΔpcaBDK, Δben cat, pobA::catBCIJFD*[a]	This study
ADP904	*ΔpcaBDK, Δben cat, pobA::catBCIJFD*[a]	This study
Plasmids		
pPAN4	pUC18 carrying a 5.3 kb *catBCIJFD* fragment	(Shanley *et al.*)[10]
pZR32	pZR430A, *pobA::catBCIJFD*[a]	This study
pZR33	pZR430A, *pobA::catBCIJFD*[a]	This study
pSC003	Same as pZR32, but in pRK415	This study
pSC004	Same as pZR33, but in pRK415	This study
pZR3	pUC18 carrying a 2.6 kb *pcaBDK* fragment	(Hartnett *et al.*)[18]
pZR301	Same as pZR3, but the whole *pcaD* and part of *pcaBK*	(Hartnett *et al.*)[18]
pZR430	pUC18 carrying 3.6 kb *pobAR* fragment with a multicloning site in the middle of *pobA*	(DiMarco *et al.*)[17]
pZR433	Same as pZR430, but in pRK415 a broad host range vector (incP, Tet^R)	(Keen *et al.*)[26]

[a] For strains ADP903, ADP904 and plasmids pZR32 and pZR33 plasmids, the arrows indicate the orientation of the insertion to the direction of *pobA* gene transcription.

For measurement of maximum specific growth rates (μ_{max}) on *cis,cis*-muconate of the ADP strains, a set of cultures containing fifty-mililiter (ml) of minimal broth with *cis,cis*-muconate as a sole carbon source in 250-ml Erlenmeyer flasks were employed. Cultures were inoculated and incubated as mentioned above. Cell growth was monitored by measuring the optical density of each culture at 660nm. Based on these values of cell densities, maximum specific growth rates, defined as a specific growth rate at an exponential phase, were calculated by the methods described elsewhere[19].

Enzymes and chemicals. The *cis,cis*-muconate

was a gift from the Celanese Research Co., New Jersey, USA. All other chemicals were obtained commercially at the highest purity available. The enzymes for the DNA manipulations were used as suggested by the supplier.

DNA *manipulation and bacterial transformation*. The plasmids were purified by the procedure described by Kraft *et al*[20]. The manipulations of the plasmids were performed according to the methods of Sambrook *et al*[21]. The procedure used for the transformation of the *E. coli* was described by Hanahan[22].

The *A. calcoaceticus* ADP strains were transformed with appropriate DNA fragments by the procedure of Neidle and Ornston[23]. The recipient ADP strain was grown overnight in 5 ml of succinate medium. To the culture, 10 μl of sterile 1M succinate was added, and the cells were incubated at 30 °C for 30 min, at which point they were ready for transformation. Then, 100 μl of the recipient was spotted on a succinate medium plate, to which donor DNA (ranging from 100 ng to 1 μg in less than 20 μl distilled water) was added directly. After incubation at 30 °C for more than 8 h, the cells were collected and then spread on a selective medium. As a control, a recipient, to which no DNA was added was plated on a selective medium.

Construction of vectors carrying catBCIJFD genes. The vectors carrying *catBCIJFD* genes were constructed as depicted in Fig. 2. Plasmid pZR430[24] containing a 3.6 kb fragment of *pobAR* and 0.3 kb fragment of multicloning site in the middle of *pobA* structural gene was used for this purpose. The 5.3 kb EcoRI fragment carrying the *catBCIJFD* genes from pPAN4[10] was subcloned into the *Eco*RV site of pZR430 in both orientations, forming pZR32 or pZR33 respectively. Plasmid pZR32 and pZR33 are identical except that the fragment carrying the *catBCIJFD* genes is inserted in opposite orientations with regard to the direction of the *pobA* gene transcription. The restriction enzyme used to demonstrate the orientation of the *catBCIJFD*

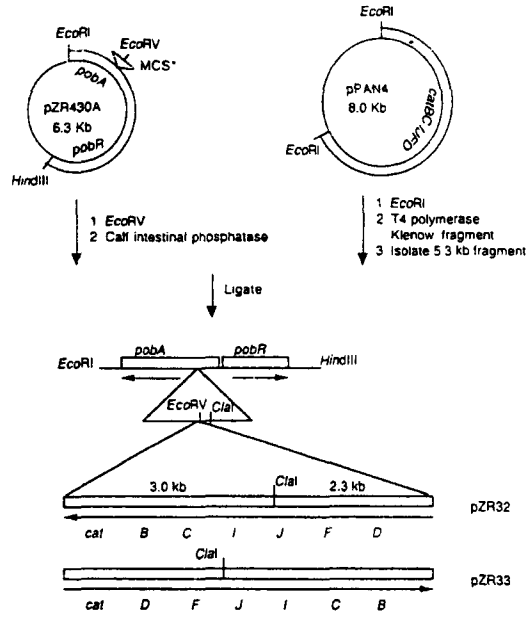

Fig. 2. Construction of the Suicide Cloning Vectors Used for Delivery of the *catBCIJFD* Genes to the *A. calcoaceticus* Chromosome.
Details of the construction are described in Materials and Methods. Low melting temperature-agarose gel electrophoresis was carried out by the standard procedure as described elsewhere (Sambrook *et al.*).[21] MCS* represents multicloning site (DiMarco *et al.*).[17]

insertions is *Cla*I.

Since pZR32 and pZR33 are derived from pZR430 carrying *col*EI replication origin, the plasmids can not be maintained stable in *Acinetobacter*. Consequently, these suicide plasmids carrying *pobA* and *pobR* sequences flanking the *catBCIJFD* insertion can be used for easy delivery of the inserted genes to the *A. calcoaceticus* chromosome.

III. Results and Discussion

1. Construction of ADP900 strain deleted for pcaBDK genes.

The mutant in which a *catBCIJFD* fragment is inserted in the middle of *pobA* on the chromosome of *A. calcoaceticus* has no selection advantage over its parent type. Therefore, it could take a long time to isolate the insertion mutants by incubating each cell onto a *p*-hydroxybenzo-

ate plate and a *cis,cis*-muconate plate after it is transformed with pZR32 and pZR33 DNA, and then identify colonies that can grow with *cis,cis*-muconate as a sole carbon source but can not grow with *p*-hydroxybenzoate.

To bypass this time-consuming step, the *pcaBDK*-deletion mutant of the ADP161 strain[11] was constructed as an initial step (Fig. 1 and Fig. 3). The recipient cells of the ADP161 strain were transformed with pZR301 (Table 1) linearized with *Hind*III. The pZR301 was created by removing two *Eco*RV fragments, containing 1.0 kb of DNA from pZR3[18]. However, pZR301 still contains the part of the *pcaBK* genes in the 1.6 kb of *Acinetobacter* DNA inserted into the *Hind*III locus of pUC18[18]. Therefore, it was expected that the *pcaBDK* deletion can be introduced to the chromosome by homologous recombination (marker exchange) in transformed cells. The transformed cells were plated onto succinate plates and the colonies which appeared were transfered onto a *p*-hydroxybenzoate plate. An isolated mutant that can not grow with *p*-hydroxybenzoate was expected to be a *pcaBDK*-deletion mutant, and was designated as an ADP 900 strain (Fig. 3). The creation of the *pcaBDK*-deletion in the ADP900 strain was confirmed by isolating the revertant that can grow at the expense of *p*-hydroxybenzoate after transformation of ADP900 with pZR3 DNA (data not shown).

2. Cloning of catBCIJFD genes into the chromosomal pobA of A. calcoaceticus.

The *pcaBDK*-deletion of the ADP900 strain would block catabolic degradation of *p*-hydroxybenzoate (or protocatechuate) and accumulate the toxic intermediate, 3-carboxy-*cis,cis*-muconate, in the cells (Fig. 1). Therefore, the ADP900 strain was not able to grow with succinate in the presence of *p*-hydroxybenzoate. However, the mutant cells in which the *pobA* gene was disrupted by the insertion could grow in the presence of *p*-hydroxybenzoate since *p*-hydroxybenzoate could not be further metabolized (Fig. 1 and Fig. 3). Based on this information, two recombinant strains from the ADP900, in which two *ca-*

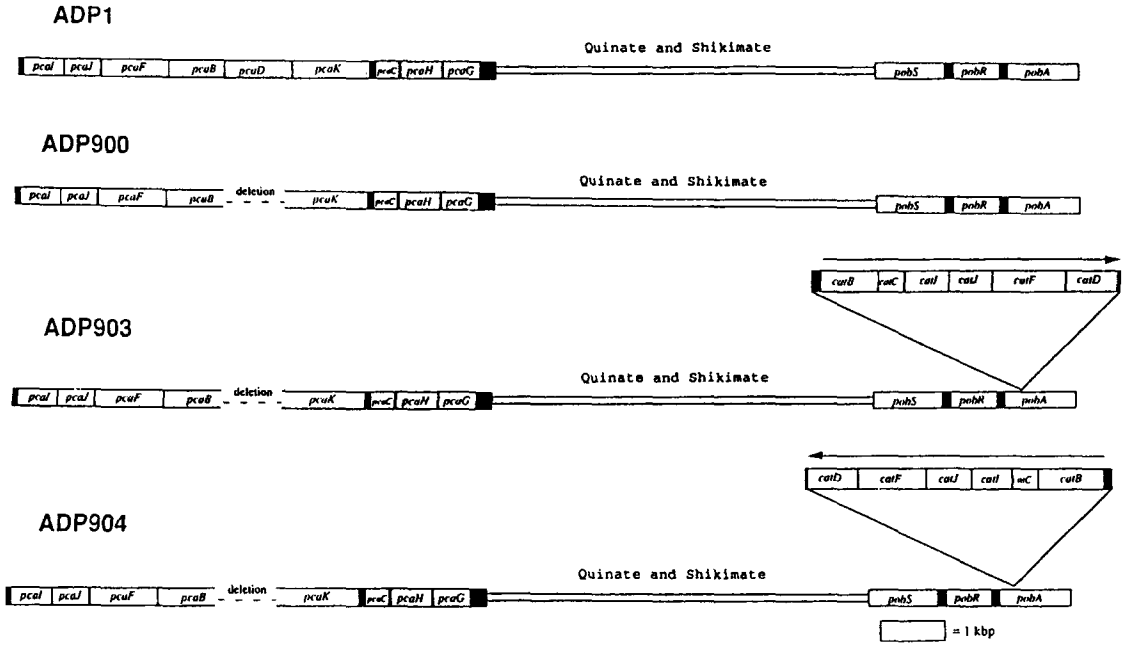

Fig. 3. Schematic Representation of the *pca* and *pob* Supraoperons Described in This Report.
The wild-type *pca* and *pob* supraoperon is shown on top with the designated individual genes. The mutant *pca* and *pob* supraoperons constructed by the deletion of *pcaBDK* and insertion of *catBCIJFD* into *pobA* are shown below.

tBCIJFD fragments were inserted in opposite directions of each other into the *pobA* were constructed successfully. The ADP900 strain was transformed with pZR32 or pZR33 DNA linearized using EcoRI, and the transformants were plated on a selective media containing both succinate and *p*-hydroxybenzoate. The colonies which appeared on this succinate/*p*-hydroxybenzoate plate were transfered onto *cis,cis*-muconate plates. The transformants that can grow at the expense of *cis,cis*-muconate as a sole carbon source were isolated and designated as ADP903 or ADP 904, according to the plasmid pZR32 or pZR33 used, respectively (Fig. 3).

The ADP903 and ADP904 strains showed characteristics of *pobA*$^-$ and *catBCIJFD*$^+$ genotypes. Because *catBCIJFD* genes were carried by plasmids which are unstable in *Acinetobacter*, it was expected that their ability to grow on *cis,cis*-muconate was due to the insertion of *catBCIJFD* into the *pobA*, which can occur by homologous recombination between *pobAR* sequences of plasmid and chromosome. However, other possibilities including a nonsense or missense mutation (s) in the *pobA* and simultaneous insertion of *catBCIJFD* genes into other (non-*pobA*) sites could not be ruled out.

In order to prove the insertion of *catBCIJFD* genes into the *pobA*, the gap repair method11[25], using the characteristic natural transformation capability of *A. calcoaceticus* was employed (Fig. 4). pZR433 carries a 3.6 kb *pobA*R fragment, the same as pZR430, but in pRK415 (Table 1). Since pRK415 is a broad host range vector and has an incP replication origin[26], pZR433 can be maintained stably in *A. calcoaceticus*. Plasmid pZR433 DNA was linearized by *Bam*HI and *Xho*I digestion. The linearized DNA was used to transform *A. calcoaceticus* ADP903 and ADP904. The transformed *A. calcoaceticus* strains were spread on LB plates containing tetracycline. It was expected that the transformants in which the gap in the

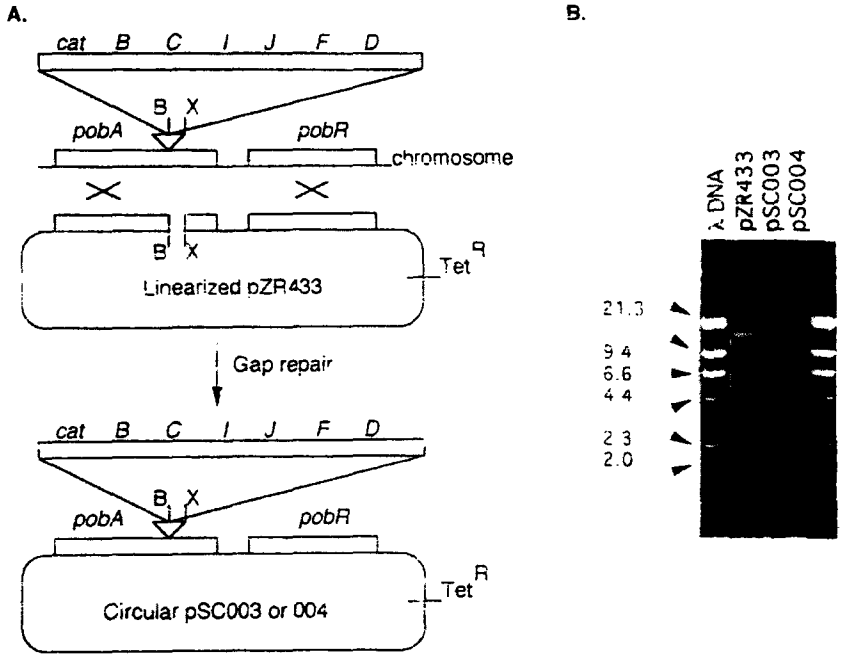

Fig. 4. Recovery of a 5.3 kb *catBCIJFD* DNA Fragment Lying in the *A. calcoaceticus pobA*.
A. The possible mechanism of gap repair by homologous double cross over. *Abbreviations*: B, *Bam*HI: X, *Xho*I. B. From the comparison of ethidium bromide profile of the pSC003, pSC004 and pZR433 DNA digested with *Bam*HI and *Xho*I, the presence of the 5.3 kb *catBCIJFD* fragment in the pSC003 and pSC004 is observed. The migrations of λ DNA digested with *Hind*III are indicated to the left in kb as molecular size standards.

linearized pZR433 was repaired to circular plasmid could form colonies on the LB-tetracycline plate. The plasmids pSC003 and pSC004 were isolated from the colonies derived from ADP903 and ADP904, respectively by transformation with pZR 433, and the size and restriction sites of the plasmids were analyzed. When the plasmids were digested with *Bam*HI and *Xho*I, a 5.3 kb insertion fragment could be observed on the agarose gel electrophoresis (Fig. 4). This result strongly supports that the *A. calcoaceticus* ADP903 and ADP 904 strains each contains a *catBCIJFD* DNA fragment in the middle of the *pobA*.

3. Genetic stability of the recombinant A. calcoaceticus.

In order to examine the genetic stability of the ADP903 and ADP904 strains, the effect of *catBCIJFD* insertions on the stability of the *pcaHG* genes encoding for protocatechuate dioxygenase was explored. As mentioned previously, *pcaBDK* deletion inhibits the growth of *A. calcoaceticus* in the presence of protocatechuate by accumulating 3-carboxy-*cis,cis*-muconate. However, any mutations that inhibit functional expression of enzymes involved in the catabolic degradation of protocatechuate to 3-carboxy-*cis,cis*-muconate can allow the strains carrying *pcaBDK* deletion to grow in the presence of protocatechuate (Fig. 1 and Fig. 3). Thus, to determine the genetic stability of *pcaHG* genes, ADP strains carrying *pcaBDK* deletion were cultivated in succinate and diluted to spread on the plate containing both succinate and protocatechuate as described in Materials and Methods. The number of the revertants of each ADP strain which appeared on the protocatechuate/succinate plate were counted after 36 h incubation and were used to determine the reversion rates. The reversion rates were then used as an index for the stability of *pcaHG* genes in ADP strains deleted for *pcaBDK* genes as shown in Table 2.

From the results shown in Table 2, it seemed likely that the effect of *catBCIJFD* insertions on the stability of *pcaHG* genes depends on the orientations of the insertions. Apparently, an insertion in the opposite orientation to the direction of the *pcaIJF* transcription has no significant effect on the stability of *pcaHG*. However, the stability of *pcaHG* genes in ADP903, in which *catBCIJFD* genes are inserted in the same direction as the *pcaIJF* transcription, was lower by 30 fold than that of the strain ADP900. From the data showing the lowest frequency of reversion rate of the strain ADP230 on the succinate/protocatechuate plate, it is indicated that the two homologous gene clusters may be located in opposite orientation to each other in the wild type chromosome of *A. calcoaceticus*. It is also possible that the DNA fragment which resides between the two gene clusters encodes essential functions, such as enzymes involved in ATP synthesis for the growth of the bacteria, and thus can be kept stable by some mechanisms. Those mechanisms may involve ways that organize secondary and/or tertiary structure of the bacterial chromosomes. Experiments which show that the *pcaHG* genes in the strain ADP900 carrying a large deletion, including *catIJF* and thus having no sequences homologous to *pcaIJF*, are not significantly more stable than in the strain ADP230 indicate again that the presense of *catIJF* genes does not hamper the stability of *pcaGH* as far as they are located in the wild type position.

In order to determine the stability of *catBCIJFD* genes in *pobA*, each ADP strain was spread on succinate plate and *cis,cis*-muconate plate, and the numbers of colonies appeared on both plats were compared. However, it was impossible to observe any differences between the numbers of colonies appeared on the respective plates. This

Table 2. Effect of Insertion of *catBCIJFD* in *pobA* on the Stability of *pcaHG*.

Location of *catBCIJFD*	Instability[a]
ADP230	1.8×10^{-7}
ADP900	3.1×10^{-7}
ADP903	6.3×10^{-6}
ADP904	9.6×10^{-7}

[a]Number of revertants on plate containing both 10 mM succinate and 5 mM protocatechuate divided by number of total cells appearing on 10 mM succinate plate. Details are discussed in Results and Discussion.

indicated that the *catBCIJFD* genes in *pobA* of each ADP strain were very stable and the rate of appearance of colonies that cannot grow on *cis, cis*-muconate due to the instability of the genes would be too low to be observed. However, the relationship between instabilities of *pcaHG* and *catBCIJFD* genes was examined and the results gave us an insight on the stability of *catBCIJFD* genes of each ADP strain. The forty revertants that can grow in the presence of protocatechuate and thus apparently carry mutations in *pcaHG*, were transfered onto *cis,cis*-muconate plate to test their ability to use the compound. As shown in Table 3, 95% of revertants of ADP903 could not grow on *cis,cis*-muconate. Apparently it is likely that the instability of *catBCIJFD* genes was caused by the same mechanisms which lead to the inactivation of *pcaHG* genes. However, it appeared that most of revertants of ADP230 and ADP904 could utilize *cis,cis*-muconate to grow and that the instability of *catBCIJFD* genes of these revertants doess not have a close relationship to that of *pcaHG*. From these results, it was suggested that inactivation of *catBCIJFD* genes of ADP904 was not relevant to that of *pcaHG* and occured independently by chance. Consequently, it was proposed that the instability of *catBCIJFD* genes in *pobA* of ADP904 is stable and its instability would be similar with that of ADP230 in which *catBCIJFD* genes are located in wild-type position.

It has been reported that the sequence of the *pcaIJF* is very homologous to that of *catIJF* and more than 90% of the sequences are overlapped[10, 11]. From this information, although the specific nature of the revertant derived from the strain ADP903 has not yet been demonstrated, it seems likely that the homologous recombinational excision out of *pcaHG* is mainly responsible for the appearance of the revertants. It also appears that these two homologous gene clusters are unlinked by transformation and therefore appear to be separated more than 20 kb (L. N. Ornston, unpublished observation). As shown in Fig. 3, the distance between the *pcaIJFe* and *catIJF* sequences does not exceed 15 kb in the strain ADP 904. Experiments comparing the reversion rates of strain ADP230 and ADP904 on the succinate/protocatechuate plate did not show significant differences in the stability of the *pcaHG* genes of the two ADP strains. However, to understand clearly the influence of the *catIJF* location on the stability of *pcaGH*, construction of more mutant strains in which *catBCIJFD* genes are inserted at measured distances from the *pcaIJF* genes, such as at the quinate or shikimate catabolic genes (Fig. 3), are required.

Table 3. Instability of *catBCIJFD* Genes in *pcaHG* Mutants of Each ADP Strains.

Location of *catBCIJFD*	*catBCIJFD* mutants(%)[a]
ADP230	10.0
ADP900	N.D[b]
ADP903	95.0
ADP904	15.0

[a]Obtained from the number of *pcaHG* mutants that cannot grow on plates containing 5 mM cis,cis-muconate among total *pcaHG* mutants tested. Details are described in Results and Discussion.
[b]Not determined since the *ben cat* region of ADP900 strain had been deleted.

Table 4. Comparion of Maximum Specific Growth Rate (μ_{max}) of the ADP Strains with *cis,cis*-muconate as a Sole Carbon Source.

Location of *catBCIJFD*	$\mu_{max}(h^{-1})$[a]
ADP230	0.844
ADP900	ND[b]
ADP903	0.862
ADP904	0.842

[a]Specific growth rate at an exponential phase. Details are described in Materials and Methods.
[b]As the same as in Table 3.

4. Intracellular levels of the activities of catBCIJFD gene products.

Since ADP903 and ADP904 are of *Δben cat* background, it is reasonable to expect that the *catBCIJFD* genes in chromosomal *pobA* are stable and are expressed to produce functional proteins for dissimilation of *cis,cis*-muconate to form colonies on the *cis,cis*-muconate plate. Based on the result that both the ADP903 and ADP904 in which *catBCIJFD* genes are oriented opposite to each other can grow on a *cis,cis*-muconate plate,

it is likely that the *catBCIJFD* genes are able to be transcribed under their own promoter instead of a *pobA* promoter. Furthermore, the *catBCIJFD* genes in *pobA* seemed to be expressed without a CatM protein. This is interesting because CatM is presently suspected as a repressor protein that participates in cat gene regulation[27].

To compare the level of *catBCIJFD* gene expression in cells, the maximum specific growth rate(μ_{max}) of ADP strains were determined. When grown with *cis,cis*-muconate as a sole carbon source, the ADP903 and ADP904 showed the μ_{max} at roughly the same level as the wild-type ADP230 (Table 4). Since the ADP strains compared are genotypically the same except for different locations of *catBCIJFD* genes, and the active products of *catBCIJFD* are essential to grow using *cis,cis*-muconate, it is a reasonable assumption that the level of μ_{max} can reflect the expression level of *catBCIJFD* genes in cells. Thus it appeared that the levels of *catBCIJFD* gene expression in ADP903 and ADP904 are also roughly same as that of ADP230. The level of μ_{max} even in ADP903 in which the *catBCIJFD* genes are inserted into *pobA* in parallel orientation to the transcription of *pobA* is the same as that in ADP904 containing the gene segment in the opposite direction to *pobA* transcription. Perhaps they showed the same level of μ_{max} because the *pobA* promoter is not induced in the absence of *p*-hydroxybenzoate[24] and the expression of *catBCIJFD* genes can only be induced by their own promoter involved in the gene segment. Although the conclusions that can be drawn from the μ_{max} comparison analysis are limited, it appears that the levels of *catBCIJFD* gene expression in ADP strains containing the gene segment transposed in different locations and in different orientations are sufficient and are roughly the same.

Based on the results described above, it is apparent that the inserted genes in *pobA* would not effect negatively on the stability of the neighboring genes as far as the orientation of the insertion was appropriate. It also appears that inserted genes carrying their own promotor sequence can be expressed successfully. Consequently, it is proposed that the *pobA* site can be used successfully as a chromosomal cloning site in the construction of hybrid strains with improved metabolic capabilities by cloning useful pathways into the chromosome. Even if the sequences of the genes planed to be inserted are suspected of being homologous to any members of the host chromosomal genes, the *pobA* site still seems to be useful as a chromosomal cloning site through the manipulation of the orientation of the insertion.

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References

1. L. P. Wackett, M. J. Sadowsky, L. M. Newman, H. G. Hur and S. Li, *Nature.* **368**, 627-629, (1994).
2. J. Y. Lee, K. W. Jung, S. H. Choi and H. S. Kim, *Appl. Envrion. Micrbiol.*, **61**, 2211-2217,(1995).
3. R. B. Winter, K. M. Yen and B. D. Ensley, *Bio/Technology*, **7**, 282-285, (1989).
4. V. Lorenzo, *Current Opinon in Biotechnol.*, **3**, 227-231, (1992).
5. F. Rojo, D. H. Dieper, K. H. Engesser, H. J. Knackmuss and K. N. Timmis, *Science*, **238**, 1395-1398, (1987).
6. P. Baumann, M. Doudoroff, and R.Y.Stanier, *J. Bacteriol.*, **95**, 1520-1541, (1968).
7. R. Y. Stanier, N. J. Palleroni, and M. Doudoroff, *J. Gen. Microbiol.*, **43**, 159-271,(1966).
8. L. N. Ornston, J. Houghton, E. L. Neidle, and L. A. Gregg, in "Pseudomonas: Biotransformation, Pathogens, and Evolving Biotechnology", eds. by S. Silver *et al.*, American Society for Microbiology, Washington, D.C., 1990, pp.207-225.
9. L. N. Ornston and E. L. Neidle, in "The Biology of *Acinetobacter*", eds. by K. Towner, E. Begogne-Berezin, and C. A. Fewson, FEMS Symposia Series, Plenum press, New York, 1991, pp. 201-238.

10. M. S. Shanley, E. L. Neidle, R. parales, and L. N. Ornston, J. *Bacteriol.*, 165, 557-563,(1986).
11. R. C. Doten, K. L. Ngai, D. J. Mitchell, and L. N. Ornston, *J. Bacteriol.*, **169**, 3168-3174,(1987).
12. R. C. Doten, J. A. Gregg-Jolly, and L. N. Ornston, *J. Bacteriol.*, **169**, 3175-3180,(1987).
13. P. Baumann, *J. Bacteriol.*, **96**, 39-42,(1968).
14. E. Juni, *J. Bacteriol.*, **112**, 917-931,(1972).
15. L. Gregg-Jolly and L. N. Ornston, *J. Bacteriol.*, **172**, 6192-6172,(1990).
16. L. N. Ornston, E. L. Neidle, and J. E. Houghton, in "The Bacterial Chromosome", eds. by M. Riley and K. Drlica, American Society for Microbiology, Washington, D.C., 1990, pp.325-334.
17. A. DiMarco, B. Averhoff, E. Kim, and L.N.Ornston, *Gene*, **125**, 25-33, (1993).
18. G. B. Hartnett, B. Averhoff, and L. N. Ornston, *J. Bacteriol.*, **171**, 6160-6162,(1990).
19. R.Y. Stanier, J.L. Ingraham, M.L. Wheelis, and P. R. Painter, in "The microbial world", 5th Ed, Prentice Hall, New Jersey, 1986, pp.183-195.
20. R. Kraft, J. Tardiff, K. S. Krauter, and L. A. Leinwand, *BioTechniques*, **6, 544-547,(1988)**.
21. J. Sambrook, E. F. Fritsch, and T. Maniatis, in "Molecular cloning: A laboratory manual", 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989.
22. D. Hanahan, *J. Mole. Biol.*, **166**, 557-580,(1983).
23. E. L. Neidle and L. N. Ornston, *J. Bacteriol.*, **168**, 815-820,(1986).
24. A. DiMarco, B. Averhoff, and L. N. Ornston, *J. Bacteriol.*, **175**, 4499-4506, (1993).
25. B. Averhoff, L. Gregg-Jolly, D. Elsemore, and L. N. Ornston, *J. Bacteriol.*, **174**, 200-204,(1992).
26. N, T. Keen, S. Tamaki, D. Kobayashi, and D. Trollinger, *Gene*, **70**, 191-197,(1988).
27. E. L. Neidle, C. Hartnett, and L. N. Ornston, *J. Bacteriol.*, **171**, 5410-5421,(1989).