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

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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 this study. For this purpose, two model hybrid strains of *A. calcoaceticus* were created, in which a DNA fragment carrying *catBCIJFD* genes for catechol degradation of catechol was inserted into *pobA* in opposite directions of each other, and their genetic stabilities were experimentally examined. Our data demonstrated 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 *pobA* can be expressed successfully as far as the insertion is engineered in an appropriate way. Concurrently, it was proposed that the *pobA* can be used as a chromosomal cloning site, and that introduction of an useful metabolic pathway into *pobA* may offer considerable promise to the construction of a hybrid strain with improved metabolic capabilities.

**Key words:** *Acinetobacter calcoaceticus*; hybrid strain; genetic stability

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. Most current technology for construction of useful hybrid strains involves cloning and expression of exogenous genes encoding new metabolic pathways through appropriate plasmid systems and using *E. coli* as a host. However, despite the fact that a large number of genes have been cloned and expressed in *E. coli*, stable maintenance or adequate expression in a non-entropic host through a plasmid system is still not effective. In an effort to overcome these limitations, cloning desirable genes on a bacterial chromosome instead of on the plasmid has been attempted. Although it has been reported that a few recombinant bacteria have been constructed by inserting transposons carrying the genes for new metabolic functions into random sites of the chromosome, 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* is comparable to that of the well-studied fluorescent *Pseudomonas* species, and apparently is significant in carbon cycling. The degradation of these aromatic compounds takes place by way of the β-ketoacipate pathway. (Fig. 1) The β-ketoacipate pathway seems widely distributed in diverse soil bacteria such as *Pseudomonas* and *Rhizobium*. However, in the case of *A. calcoaceticus*, some enzymes involved in the β-ketoacipate pathway: β-ketoacipate enol-lactone hydrodase (*pcaJI* or *catIJ*) and β-ketoacipil CoA thiolase (*pcaF* or *catF*) are composed of isoenzymes. The DNA sequences of the *catIJF* and *pcaIJF* regions are nearly identical and the similarity appears to afford opportunity for DNA sequence exchange between the two regions.

Members of *A. calcoaceticus* are found in most natural environments 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 and provides a powerful tool for genetic manipulation of the bacteria. Recently, a number of genetic studies have been inspired by the competency of the bacteria, and genes involved in the β-ketoacipate pathway have been cloned and analyzed. Consequently, an increasing amount of information is available on their genetic organization, regulation, and evolution.

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 development of the hybrid strains, one of the most crucial questions to be answered is on the stability of the recombinant metabolic pathways introduced.

In this 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*.\(^{11}\) were 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.

**Materials and Methods**

**Bacterial strains and plasmids.** Descriptions of the bacterial strains of *Acinetobacter calcoaceticus* and plasmids used in this study are listed in Table I. All *A. calcoaceticus* ADP strains used are derivatives of strain BD413,\(^{14}\) which is a very competent recipient for genes transferred by natural transformation. *A. calcoaceticus* ADP230\(^{15}\) is a *psbBDK* deletion mutant of the wild-type strain ADP001. *A. calcoaceticus* ADP900 is also a *psbBDK* deletion mutant of strain ADP161, which carries a large deletion mutation in the *ben cat* structural gene.\(^{13}\) Both ADP903 and ADP904
Table 1. Bacterial Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. calcoaceticus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP001</td>
<td>Wild type (BD413)</td>
<td>(Juni)14</td>
</tr>
<tr>
<td>ADP161</td>
<td><em>phen cat</em></td>
<td>(Doten et al)31</td>
</tr>
<tr>
<td>ADP230</td>
<td><em>pcaBDK</em></td>
<td>(Hartnett et al)18</td>
</tr>
<tr>
<td>ADP900</td>
<td><em>pcaBDK, phen cat</em></td>
<td>This study</td>
</tr>
<tr>
<td>ADP903</td>
<td><em>pcaBDK, phen cat, poh4::catBCJIFD</em></td>
<td>This study</td>
</tr>
<tr>
<td>ADP904</td>
<td><em>pcaBDK, phen cat, poh4::catBCJIFD</em></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPAN4</td>
<td>pUC18 carrying a 5.3 kb catBCJIFD fragment</td>
<td>(Shanley et al)10</td>
</tr>
<tr>
<td>pZR32</td>
<td>pZR340A, poh4::catBCJIFD</td>
<td>This study</td>
</tr>
<tr>
<td>pZR33</td>
<td>pZR340A, poh4::catBCJIFD</td>
<td>This study</td>
</tr>
<tr>
<td>pSC003</td>
<td>Same as pZR32, but in PRK415</td>
<td>This study</td>
</tr>
<tr>
<td>pSC004</td>
<td>Same as pZR33, but in PRK415</td>
<td>This study</td>
</tr>
<tr>
<td>pZR3</td>
<td>pUC18 carrying a 2.6 kb pcaBDK fragment</td>
<td>(Hartnett et al)18</td>
</tr>
<tr>
<td>pZR301</td>
<td>Same as pZR3, but the whole pohD and part of pcaBDK sequences are deleted</td>
<td>(Hartnett et al)18</td>
</tr>
<tr>
<td>pZR340</td>
<td>pUC18 carrying 3.6 kb poh4R fragment with a multicloning site in the middle of pohA</td>
<td>(DiMarco et al)17</td>
</tr>
<tr>
<td>pZR343</td>
<td>pZR340, but in PRK415</td>
<td>(Keen et al)24</td>
</tr>
<tr>
<td></td>
<td>a broad host range vector</td>
<td></td>
</tr>
</tbody>
</table>

For strains ADP903 and ADP904 and plasmids pZR32 and pZR33 plasmids, the arrows indicate the orientation of the insertion to the direction of pohA gene transcription.

were derived from ADP900 by introduction of catBCJIFD genes into the pohA 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 Na2HPO4, 12.5 mM KH2PO4, 0.5% (NH4)2SO4, and 10 ml of a trace element concentrate base. The latter contained 59.3 g of MgSO4 · 7H2O, 20 g of nitrolotriacetic acid, 6.67 g of CaCl2 · 2H2O, 0.25 g of EDTA, 1.95 g of ZnSO4 · 7H2O, 0.5 g of FeSO4 · 7H2O, 0.198 g of FeCl3 · 6H2O, 0.154 g of MnSO4 · H2O, 3.2 mg of CuSO4 · 5H2O, 25 mg of Na2S2O3 · 5H2O, 18.5 mg of molybdate acid, and 17.2 mg of Na2B4O7 · 10H2O per liter. The following concentrations of carbon sources were added to the basal salt medium to compose the corresponding media: succinate (10 mM), benzene (5 mM), p-hydroxybenzate (5 mM), protocatechuate (5 mM), cis,cis-muconate (5 mM).

For measuring 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 (200 rpm) for about 8h. Inocula were from late logarithmic phase cultures that 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 were appropriately diluted with the basal salt medium were then plated. Unless otherwise indicated, all broth cultures and plates were incubated at 30°C.

For measurement of maximum specific growth rates (µmax) on *cis,cis*-muconate of the ADP strains, a set of cultures containing 50 ml of minimal broth with *cis,cis*-muconate as a sole carbon source in 250-ml Erlenmeyer flasks were used. Cultures were inoculated and incubated as mentioned above. Cell growth was monitored by measuring the optical density of each culture at 660 nm. Based on these values of cell densities, maximum specific growth rates, defined as a specific growth rate at exponential phase, were calculated by the methods described elsewhere.18

**Enzymes and chemicals.** The *cis,cis*-muconate was a gift from the Celanese Research Co., New Jersey, U.S.A. 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 method of Kunitz et al.20 The manipulations of the plasmids were done by 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 Orston.23 The recipient ADP strain was grown overnight in 5 ml of succinate medium. To the culture, 10 µl of sterile 1 M 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 (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 catBCJIFD genes.** The vectors carrying catBCJIFD genes were constructed as depicted in Fig. 2. Plasmid pZR34024 containing a 3.6-kb fragment of poh4R and a 0.3-kb fragment of multicloning site in the middle of pohA structural gene was used for this purpose. The 5.3-kb EcoRI fragment carrying the catBCJIFD genes from pPAN410 was subcloned into the EcoRV site of pZR430 in both orientations, forming pZR32 or pZR33 respectively. Plasmid pZR32 and pZR33 are identical except that the fragment carrying the catBCJIFD genes is inserted in opposite orientations with regard to the direction of the pohA gene transcription. The restriction enzyme used to demonstrate the orientation of the catBCJIFD insertions is Clal.

Since pZR32 and pZR33 are derived from pZR430 carrying colEl replication origin, the plasmids cannot be maintained stably in *Acinetobacter*. Consequently, these suicide plasmids carrying poh4R and pohR sequences flanking the catBCJIFD insertion can be used for easy delivery of the inserted genes to the *A. calcoaceticus* chromosome.

**Results and Discussion**

**Construction of ADP900 strain deleted for pcaBDK genes.**

The mutant in which a catBCJIFD fragment is inserted...
in the middle of pohA on the chromosome of \textit{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 on a \(p\)-hydroxybenzoate 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 cannot
grow with \(p\)-hydroxybenzoate.

To bypass this time-consuming step, the \textit{pcaBDK}-deletion
mutant of the ADP161 strain\(^{18}\) was constructed as an initial
step (Figs. 1 and 3). The recipient cells of the ADP161 strain
were transformed with \(pZR301\) (Table 1) linearized with
\textit{HindIII}. The \(pZR301\) was created by removing two \textit{EcoRV}
fragments, containing 1.0 kb of DNA from \(pZR3\).\(^{18}\) However,
\(pZR301\) still contains the part of the \textit{pcaBK} genes
in the 1.6 kb of \textit{Acinetobacter} DNA inserted into the \textit{HindIII}
locus of \(pUC18\).\(^{18}\) Therefore, it was expected that the
\textit{pcaBDK} deletion could be introduced to the chromosome
by homologous recombination (marker exchange) in
transformed cells. The transformed cells were plated onto
succinate plates and the colonies that appeared were
transferred onto a \(p\)-hydroxybenzoate plate. An isolated
mutant that cannot grow with \(p\)-hydroxybenzoate was
expected to be a \textit{pcaBDK}-deletion mutant, and was
designated as an ADP900 strain (Fig. 3). The creation of
the \textit{pcaBDK}-deletion in the ADP900 strain was confirmed
by isolating the revertants that could grow at the expense
of \(p\)-hydroxybenzoate after transformation of ADP900 with
\(pZR3\) DNA (data not shown).

Cloning of \textit{catBC1JFD} genes into the chromosomal \(pohA\) of
\textit{A. calcoaceticus}

The \textit{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 \(pohA\) gene was disrupted by the insertion could grow in
the presence of \(p\)-hydroxybenzoate since \(p\)-hydroxy-
benzoate could not be further metabolized (Figs. 1 and 3).
Based on this information, two recombinant strains from
the ADP900, in which two \textit{catBC1JFD} fragments were
inserted in opposite directions of each other into the \(pohA\)
were constructed. The ADP900 strain was transformed with
\(pZR32\) or \(pZR33\) DNA linearized using \textit{EcoRI}, and the
transformants were plated on a selective media containing
both succinate and \(p\)-hydroxybenzoate. The colonies that
appeared on this succinate/\(p\)-hydroxybenzoate plate were
transferred onto \(cis,cis\)-muconate plates. The transformants
that could grow at the expense of \(cis,cis\)-muconate as a sole
carbon source were isolated and designated as ADP903
and ADP904, according to the plasmid \(pZR32\) or \(pZR33\) used,
respectively (Fig. 3).

The ADP903 and ADP904 strains showed characteristics of
\(pohA^+\) and \textit{catBC1JFD} \(^*\) genotypes. Because \textit{catBC1JFD}
genomes were carried by plasmids which are unstable in
\textit{Acinetobacter}, it was expected that their ability to grow on
\(cis,cis\)-muconate was due to the insertion of \textit{catBC1JFD}
into the \(pohA\), which can occur by homologous recombina-
tion between \(pohA\) sequences of plasmid and chromo-
some. However, other possibilities including a nonsense
or missense mutation(s) in the \(pohA\) and simultaneous inser-
tion of \textit{catBC1JFD} genes into other (non-\(pohA\)) sites could
not be ruled out.

To prove the insertion of \textit{catBC1JFD} genes into the \(pohA\),
the gap repair method\(^{11,25}\) using the characteristic natural
transformation capability of \textit{A. calcoaceticus} was used (Fig.
4). \(pZR33\) carries a 3.6-kb \(pohAR\) fragment, the same as
\(pZR30\), but in \(pRK415\) (Table I). Since \(pRK415\) is a broad
host range vector and has an \textit{incP} replication origin,\(^{27}\)
\(pZR33\) can be maintained stably in \textit{A. calcoaceticus}.
Plasmid \(pZR33\) DNA was linearized by \textit{BamHI} and \textit{XhoI}
digestion. The linearized DNA was used to transform \textit{A. calcoaceticus} ADP903 and ADP904. The transformed \textit{A. calcoaceticus} strains were spread on LB plates containing
tetracycline. It was expected that the transformants in which
the gap in the linearized \(pZR33\) was repaired to circular
plasmids 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 \(pZR33\), and the size and restriction
sites of the plasmids were analyzed. When the plasmids
were digested with \textit{BamHI} and \textit{XhoI}, a 5.3-kb insertion
fragment could be observed on the agarose gel electrophoresis
(Fig. 4). This result strongly supports the idea that the \textit{A. calcoaceticus} ADP903 and ADP904 strains each contains
a \textit{catBC1JFD} DNA fragment in the middle of the \(pohA\).

Genetic stability of the recombinant \textit{A. calcoaceticus}

To examine the genetic stability of the ADP903 and
ADP904 strains, the effects of \textit{catBC1JFD} insertions on the
stability of the \textit{pcaHG} genes encoding for protocatechuate
dioxygenase were explored. As mentioned previously,
\textit{pcaBDK} deletion inhibits the growth of \textit{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 de-
gradation of protocatechuate to 3-carboxy-cis,cis-muconate
allow the strains carrying the \textit{pcaBDK} deletion to grow in
the presence of protocatechuate (Figs. 1 and 3). Thus,
to measure the genetic stability of \textit{pcaHG} genes, ADP strains
carrying the \textit{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 that
appeared on the protocatechuate succinate plate were
counted after 36 h of incubation and were used to determine
the reversion rates. The reversion rates were then used as
an index for the stability of \textit{pcaHG} genes in ADP strains
deleted for \textit{pcaBDK} genes as shown in Table II.

From the results shown in Table II, it seemed likely that
the effects of \textit{catBC1JFD} insertions on the stability of \textit{pcaHG}
genomes depend on the orientations of the insertions.
Apparently, an insertion in the opposite orientation to the
direction of the \textit{pcaIJF} transcription has no significant effect
on the stability of \textit{pcaHG}. However, the stability of \textit{pcaHG}
genomes in ADP903, in which \textit{catBC1JFD} genes are inserted
in the same direction as the \textit{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 orientations to each other in the wild.
Fig. 3. Schematic Representation of the pca and poh Supraoperons Described in This Report.  
The wild-type pca and poh supraoperon is shown on top with the designated individual genes. The mutant pca and poh supraoperons constructed by deletion of pcaBDK and insertion of catBCIJFD into poh-A are shown below.
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 crossover. Abbreviations: B, BamHI; X, XhoI. B. From the comparison of ethidium bromide profile of the pSC003, pSC004, and pZK43 DNA digested with BamHI and XhoI, the presence of the 5.3 kb catBCIJFD fragment in the pSC003 and pSC004 is observed. The migrations of a DNA digested with HindIII are indicated to the left in kb as molecular size standards.

<table>
<thead>
<tr>
<th>Location of catBCIJFD</th>
<th>Instability *</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP230</td>
<td>1.8 × 10^-7</td>
</tr>
<tr>
<td>ADP900</td>
<td>3.1 × 10^-7</td>
</tr>
<tr>
<td>ADP903</td>
<td>6.3 × 10^-7</td>
</tr>
<tr>
<td>ADP904</td>
<td>9.6 × 10^-7</td>
</tr>
</tbody>
</table>

* 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.

Table II. Effects of Insertion of catBCIJFD in pobA on the Stability of pcaHG

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

<table>
<thead>
<tr>
<th>Location of catBCIJFD</th>
<th>catBCIJFD mutants (%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP230</td>
<td>10.0 *</td>
</tr>
<tr>
<td>ADP900</td>
<td>N.D. *</td>
</tr>
<tr>
<td>ADP903</td>
<td>95.0</td>
</tr>
<tr>
<td>ADP904</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* 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.

* Not determined since the het cat region of ADP900 strain had been deleted.

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 insight into the stability of catBCIJFD genes of each ADP strain. The forty revertants that grew in the presence of protocatechuate, and thus apparently carry mutations in pcaHG, were transferred onto cis,cis-muconate plates to test their ability to use the compound. As shown in Table III, 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 the revertants of ADP230 and ADP904 could use cis,cis-muconate to grow and that the instability of catBCIJFD genes of these revertants does not have a close relationship.
to that of pcaHG. From these results, it was suggested that inactivation of catBC1JFD genes of ADP904 was not relevant to that of pcaHG and occurred independently by chance. Consequently, it was proposed that the instability of catBC1JFD genes in pohA of ADP904 is stable and its instability would be similar to that of ADP230, in which catBC1JFD genes are in the wild-type position.

It has been reported that the sequence of the pcaIIF is very similar to that of catIJF and more than 90% of the sequences overlapped.\textsuperscript{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 similar gene clusters are unlikely by transformation and therefore appear to be separated by more than 20 kb (L. N. Ornston, unpublished observation). As shown in Figure 3, the distance between the pcaIIF and catIJF sequences does not exceed 15 kb in the strain ADP904. 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 pcaHG, construction of more mutant strains in which catBC1JFD genes are inserted at measured distances from the pcaIIF genes, such as at the quinate or shikimate catabolic genes (Fig. 3), are required.

**Intracellular levels of the activities of catBC1JFD gene products**

Since ADP903 and ADP904 are of \textit{Ahen} cat background, it is reasonable to expect that the catBC1JFD genes in chromosomal pohA are stable and are expressed to produce functional proteins for dissimilation of cis,cis-muconate, and so form colonies on the cis,cis-muconate plate. Since both the ADP903 and ADP904 in which catBC1JFD genes are oriented opposite to each other can grow on a cis,cis-muconate plate, it is likely that the catBC1JFD genes can be transcribed under their own promoter instead of a pohA promoter. Furthermore, the catBC1JFD genes in pohA seemed to be expressed without a CatM protein. This is interesting because CatM is presently suspected to be a repressor protein that participates in cat gene regulation.\textsuperscript{27}

To compare the levels of catBC1JFD gene expression in cells, the maximum specific growth rates ($\mu_{max}$) of ADP strains were measured. When grown with cis,cis-muconate as a sole carbon source, the ADP903 and ADP904 showed the $\mu_{max}$ at roughly the same level as the wild-type ADP230 (Table IV). Since the ADP strains compared are genotypically the same except for different locations of catBC1JFD genes, and the active products of catBC1JFD are essential to growth using cis,cis-muconate, it is a reasonable assumption that the level of $\mu_{max}$ reflects the expression level of catBC1JFD genes in cells. Thus it appeared that the levels of catBC1JFD gene expression in ADP903 and ADP904 are also roughly same as that of ADP230. The level of $\mu_{max}$ even in ADP903, in which the catBC1JFD genes are inserted into pohA in parallel orientation to the transcription of pohA, is the same as that in ADP904 containing the gene segment in the opposite direction to pohA transcription.

**Table IV. Comparison of Maximum Specific Growth Rate ($\mu_{max}$) of the ADP Strains with cis,cis-Muconate as a Sole Carbon Source**

<table>
<thead>
<tr>
<th>Location of catBC1JFD</th>
<th>$\mu_{max}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP230</td>
<td>0.844</td>
</tr>
<tr>
<td>ADP900</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td>ADP903</td>
<td>0.862</td>
</tr>
<tr>
<td>ADP904</td>
<td>0.842</td>
</tr>
</tbody>
</table>

$^a$ Specific growth rate at an exponential phase. Details are described in Materials and Methods.

The same as in Table III.

Perhaps they showed the same level of $\mu_{max}$ because the pohA promoter is not induced in the absence of p-hydroxybenzoate\textsuperscript{24} and the expression of catBC1JFD genes can only be induced by their own promoter involved in the gene segment. Although the conclusions that can be drawn from the $\mu_{max}$ comparison analysis are limited, it appears that the levels of catBC1JFD 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 pohA would not negatively affect 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 promoter sequence can be expressed. Consequently, it is proposed that the pohA site can be used 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 planned to be inserted are suspected of being similar to any members of the host chromosomal genes, the pohA 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**