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# Physiological Changes and *alk* Gene Instability in *Pseudomonas oleovorans* during Induction and Expression of *alk* Genes

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**The *alk* genes of *Pseudomonas oleovorans*, which is able to metabolize alkanes and alkenes, are organized in *alkST* and *alkBFGHJKL* clusters, in which the expression of *alkBFGHJKL* is positively regulated by AlkS. Growth of the wild-type strain GPo1 and *P. oleovorans* GPo12 *alk* recombinants on octane resulted in changes of cellular physiology and morphology. These changes, which included lower growth rates and a reduction of the number of CFU due to filamentation, were also seen when the cells were grown on aqueous medium, and the *alk* genes were induced with dicyclopropylketone, a gratuitous inducer of the *alk* genes. These effects were seen only for recombinants carrying both *alkST* and *alkBFGHJKL* operons. Deletion of parts of either *alkB* or *alkJ*, which encode two major Alk proteins located in the cytoplasmic membrane, modified but did not eliminate the effects described above, suggesting that they were due to induction and expression of several *alk* genes. Continuous growth of the cells in the presence of dicyclopropylketone for about 10 generations led to inactivation, but not elimination, of the *alk* genes. This resulted in a return of the recombinants to normal physiology and growth.**

*Pseudomonas oleovorans* is able to grow on alkanes and alkenes (1). Its catabolic OCT plasmid contains two operons that encode the genes necessary for alkane oxidation (Fig. 1). Both operons have been sequenced (11, 17, 18, 27, 29), and it has been shown that the expression of operon *alkB-L* is positively regulated by AlkS (10, 29). The alkane hydroxylase system, which catalyzes the first step of alkane oxidation (23), consists of three components: alkane hydroxylase (AlkB), rubredoxin (AlkG), and rubredoxin reductase (AlkT) (11, 17, 18, 26). The enzymes which catalyze the subsequent oxidation and catabolic steps are encoded by *alkJ*, *alkH*, and *alkK* (17, 27). *alkF* encodes a nonfunctional rubredoxin (17), while *alkL* encodes an outer membrane protein which is not essential for growth on octane (27). When the *alk* system is induced with octane or the gratuitous inducer dicyclopropylketone (DCPK), about 35,000 copies of AlkB (12) and 10,000 copies of AlkJ (27) per cell are found in the cytoplasmic membrane. The former represents 25 to 30%, while the latter represents about 10% of total cytoplasmic membrane proteins.

When *P. oleovorans* is grown on alkanes or alkenes, the cells change their physiology, membrane morphology (8, 24), and membrane properties (7). Changes of membrane lipid fatty acid composition resulted both from exposure of the cells to organic solvents and from the induction and expression of *alkB* (6). Induction of *alkB* and insertion of alkane hydroxylase into the cytoplasmic membrane have also been found to alter the physiology of *Escherichia coli alk*$^+$ recombinants, by inducing, for example, reductions in growth rate and appearance of membrane vesicles in the cytoplasm (15, 22).

To explore the effects of induction and expression of the *alk* genes, and especially *alkB*, we have compared the physiological and morphological changes of *Pseudomonas* hosts during batch and continuous growth in the presence of DCPK, a gratuitous inducer of the *alk* genes, which permits separating *alk* induction effects from solvent effects of octane or its oxidation products, such as 1-octanol, on the cells.

In this paper we show that, following induction and expression of *alk* genes, there was a reduction of growth rate and formation of filaments, effects which were reversed after continuous induction of the *alk* genes for 10 to 15 generations, resulting in the disappearance of filaments and a return to normal physiology.

## MATERIALS AND METHODS

**Strains and plasmids.** *P. oleovorans* wild-type strain GPo1 and its OCT plasmid-cured variant GPo12 (6, 16) were used throughout the experiments. The plasmids depicted in Fig. 1 were described previously (6, 27).

**Media and growth conditions. (i) Batch cultures.** Overnight precultures grown on $E_2$ medium (20) supplemented with different carbon sources or 0.4% (wt/vol) yeast extract were transferred to 50 ml of fresh medium, giving an initial cell density of about 0.06 mg/ml. The cells were grown at 30°C in a 200-rpm shaker. To induce *alk* genes, 0.05% (vol/vol) DCPK was added 1 h after inoculation at a cell density of about 0.1 mg/ml. The cell density was determined as described before (28). Tetracycline was added to a final concentration of 12.5 μg/ml when appropriate.

**(ii) Continuous cultures.** The cells were grown in minimal medium (6) containing 1.1% (wt/vol) trisodium citrate dihydrate with or without 0.4% (wt/vol) yeast extract, and the continuous cultures were run as described in reference 6. Continuous operation and DCPK induction were started towards the end of the exponential growth phase, at cell densities of about 0.4 to 0.5 mg/ml (no yeast extract) or 0.8 to 1.0 mg/ml (yeast extract added). For growth of recombinant strains, 12.5 μg of tetracycline per ml was added to the medium.

**Viable cells and growth phenotype.** The number of viable cells was estimated by plating serial dilutions on Luria-Bertani (LB) plates with or without 12.5 μg of tetracycline per ml. For all the recombinant strains, the number of viable cells counted on LB plates was identical to that on LB plates containing tetracycline, indicating that no tetracycline-sensitive mutants appeared during continuous growth.

To determine the percentage of cells that could still grow on octane, at least 100 clones randomly selected from LB plates containing tetracycline were replica plated to $E_2$ medium plates and incubated in a closed tin saturated with octane vapor for 2 days.

**Plasmid detection.** Plasmid DNA was extracted by the method of Birnboim and Doly (4) and detected by agarose gel electrophoresis.

**Detection of expression of *alkB*.** To detect the expression of AlkB, total cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide) as described by Laemmli (19) and stained with Coomassie blue. The relative amount of AlkB was estimated by densitometry (Molecular Dynamics).

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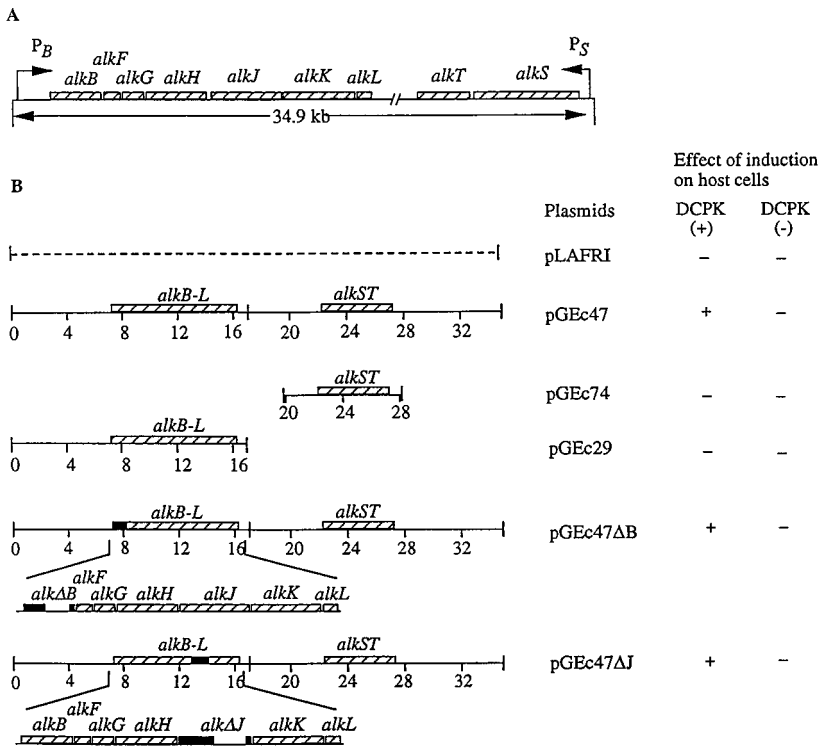


FIG. 1. Organization of the *alk* genes and plasmids carrying various *alk* constructs. (A) The *alk* genes are organized in the *alkST* and the *alkBFGHJKL* clusters. The expression of the *alkB-L* operon is positively regulated by AlkS. (B) pLAFRI-derived plasmids carrying different *alk* genes. The solid bars border deletions of specific *alk* genes. The effects of growth with (+) or without (−) DCPK on the growth rate and physiology of GPo12 recombinants carrying these plasmids are indicated as follows: −, no detectable effects; +, clear effects.

**Microscopy.** A drop of culture was spread on a microscope slide, examined with phase-contrast light microscopy (Olympus BH-2 microscope) at a 1,000-fold magnification, and photographed.

## RESULTS

**Effects of induction of *alk* genes on growth rate in batch cultures.** Table 1 shows that induction of the *alk* genes with DCPK decreased the exponential growth rate ($\mu_{max}$) of *P. oleovorans* GPo1 when the cells were grown on various carbon sources such as glucose, citrate, pyruvate, and lactic acid. Figure 2 shows that DCPK as such did not influence the $\mu_{max}$ of GPo12, which contains no *alk* genes, when the cells were grown on 1% glucose, but it did when pGEc47, which contains all known *alk* genes, was transferred into GPo12, as we reported previously (6). Both *alkST* and *alkB-L* were required for this effect. Neither the vector pLAFRI nor pLAFRI-based plasmids carrying only *alkST* (pGEc74) or *alkB-L* (pGEc29) reduced the growth rate of the host significantly. To test whether this effect was related to the expression of membrane protein AlkB or AlkJ, deletion derivatives of pGEc47, pGEc47Δ*B*, and pGEc47Δ*J* were introduced into GPo12. The growth rates of these recombinants were lowered by DCPK induction but not as much as for GPo12(pGEc47). Thus, growth inhibition due to induction of the *alk* genes can be caused by several *alk* gene products.

**Effect of DCPK induction on growth and cell morphology in continuous culture.** In continuous cultures the cell density of

TABLE 1. Effects of induction of the *alk* genes with DCPK on the growth rate of *Pseudomonas* GPo1 in different media[a]

| Carbon source (g/100 ml) | $\mu_{max}$ ($h^{-1}$) | | Relative growth rate[b] |
|---|---|---|---|
| | No DCPK | With DCPK[c] | |
| Glucose (1.0) | 0.39 | 0.19 | 49 |
| Glucose (1.0) + YE (0.4) | 0.73 | 0.31 | 42 |
| Citrate[d] (2.1) | 0.39 | 0.16 | 40 |
| Pyruvate[e] (2.0) | 0.45 | 0.22 | 50 |
| Lactic acid (1.5) | 0.68 | 0.53 | 78 |

[a] The cells were grown on 50 ml of minimal $E_2$ medium in a 250-ml Erlenmeyer flask at 30°C. YE, yeast extract.
[b] Percentage of the $\mu_{max}$ of the uninduced cells.
[c] 0.05% (vol/vol).
[d] Trisodium citrate dihydrate.
[e] Sodium pyruvate.

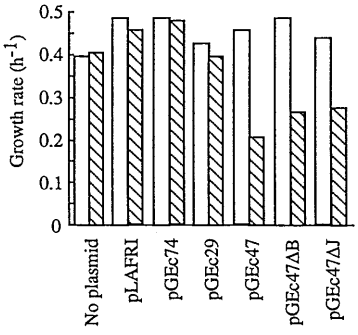


FIG. 2. Growth rate of *P. oleovorans* GPo12 carrying various recombinant plasmids in the absence (open bars) or presence (hatched bars) of DCPK (0.05% [vol/vol]). The cells were grown on 50 ml of minimal $E_2$ medium in 250-ml Erlenmeyer flasks at 30°C, with 1% (wt/vol) glucose as the carbon source.

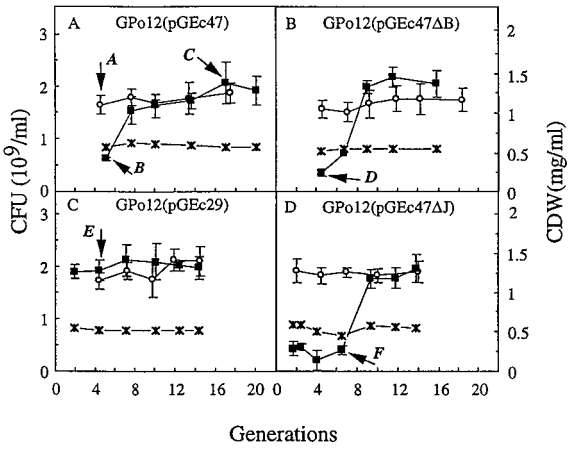


FIG. 3. Effects of *alk* induction on CFU of GPo12 recombinants carrying different *alk* genes and grown for 15 to 20 generations. The cells were grown continuously on minimal medium containing 1.1% (wt/vol) trisodium citrate dihydrate at a dilution rate of about 0.1 $h^{-1}$ in the presence (squares) or absence (circles) of DCPK. DCPK (0.05%) was added at the zero time point. The cell densities (CDW) (×) of the induced and uninduced cultures were similar. Arrows, time points at which cells were sampled for microscopy. The italic capital letters correspond to the panels of Fig. 4.

the different recombinants was constant, as expected. In the absence of DCPK, the number of CFU also remained constant (Fig. 3), and only rod-shaped cells were seen (Fig. 4A). Similar results were obtained when GPo12 recombinants containing vector pLAFRI, pGEc74, or pGEc29 were grown in the presence of DCPK (Fig. 3C and 4E and data not shown). However, for GPo12(pGEc47), GPo12(pGEc47Δ*B*), and GPo12 (pGEc47Δ*J*), growth in the presence of DCPK resulted in a three- to fivefold reduction of CFU during the first two to seven generations (Fig. 3A, B, and D), due to the formation of filaments (Fig. 4B, D, and F). After the seventh generation, normal rod-shaped cells reappeared and the number of CFU increased; these events coincided with the appearance of $Alk^-$ colonies and a reduction of AlkB levels as shown in Fig. 5. After about 15 generations, the cells were once again indistinguishable from uninduced cells (Fig. 4C and data not shown), finally reaching levels equal to those of the corresponding uninduced cultures. These results suggest that induction and expression of the *alk* genes inhibit division of the host.

**Characterization of $Alk^-$ strains generated during continuous growth in the presence of DCPK.** The alkane oxidation capacity of GPo12(pGEc47), GPo12(pGEc47Δ*J*), and GPo1 during growth in continuous cultures was monitored by check-

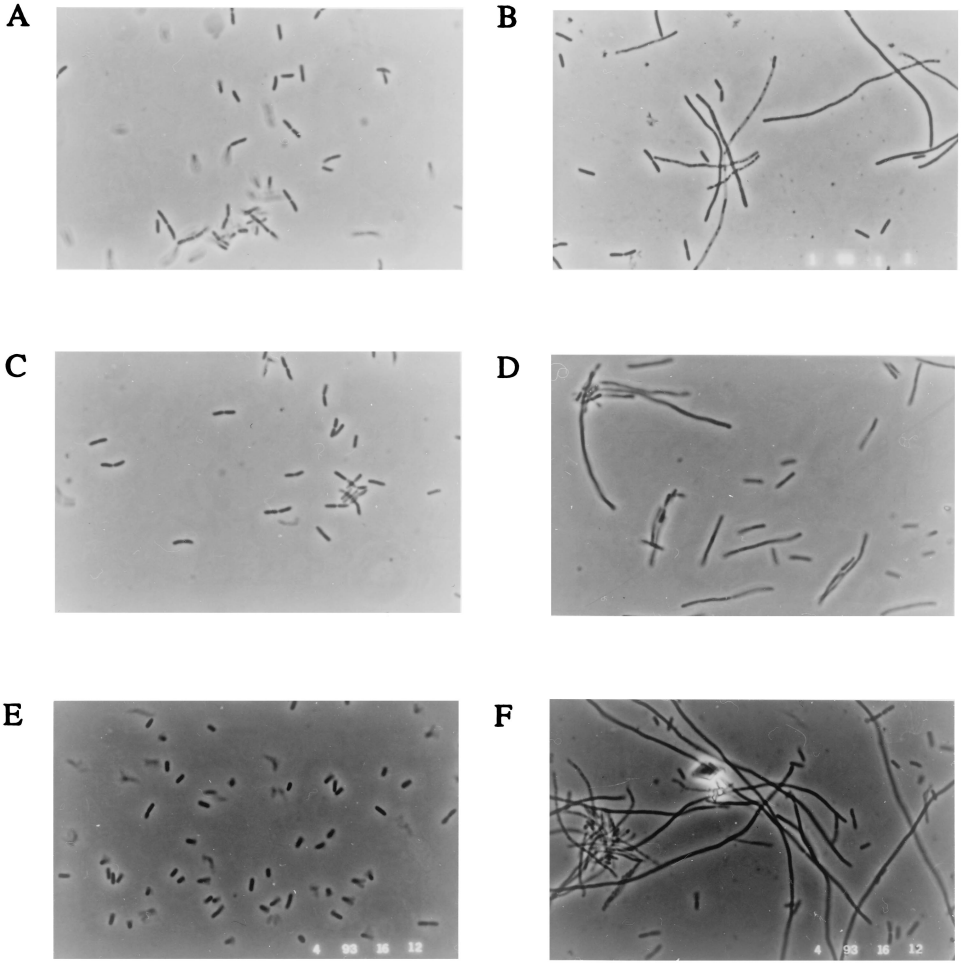


FIG. 4. Morphology of different recombinant cells grown in the presence or absence of DCPK. The cells were sampled at various generations from the cultures in Fig. 3 and examined by phase-contrast microscopy. (A) GPo12(pGEc47) grown in the absence of DCPK for 4.2 generations; (B) GPo12(pGEc47) grown in the presence of DCPK for 4.3 generations; (C) GPo12(pGEc47) grown in the presence of DCPK for 17.5 generations; (D) GPo12(pGEc47Δ*B*) grown in the presence of DCPK for 4.6 generations; (E) GPo12(pGEc29) grown in the presence of DCPK for 4.8 generations; (F) GPo12(pGEc47Δ*J*) grown in the presence of DCPK for 6.5 generations.

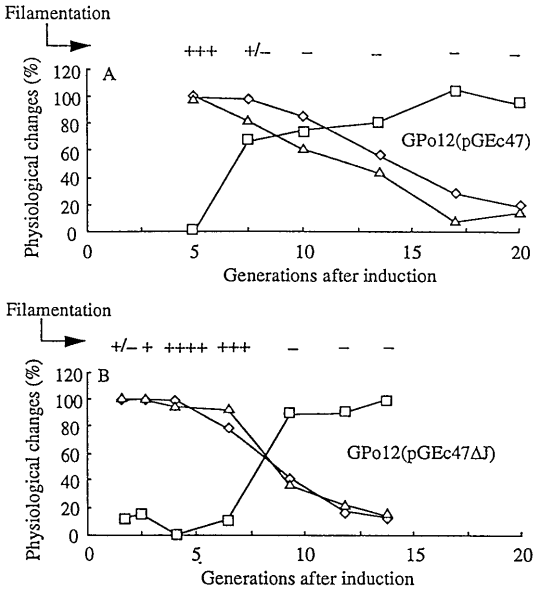


FIG. 5. Summary of physiological and morphological changes in GPo12 (pGEc47) (A) and GPo12(pGEc47Δ*J*) (B) during continuous growth in the presence of DCPK. The amount of AlkB (△) was estimated by densitometry of SDS-polyacrylamide gels. Filamentation was checked by phase-contrast light microscopy at the times indicated (+, filamentation; −, no filamentation). ◇, percent *alk*$^+$; □, CFU.

ing the ability of randomly picked colonies from LB-tetracycline plates to grow on octane vapor and was found to be lost when the *alk* system was induced under nonselecting conditions, as shown for GPo12(pGEc47) in a previous paper (6). This test cannot be carried out for GPo12(pGEc47Δ*B*), which is not able to grow on octane, but it can be performed with GPo12(pGEc47Δ*J*), since the *alkJ* deletion is complemented by a chromosome-encoded alkanol dehydrogenase (16). Data for GPo12(pGEc47) and GPo12(pGEc47Δ*J*) are summarized in Fig. 5. These GPo12 recombinants, which were initially able to grow on octane, started to lose this ability after continuous growth in the presence of DCPK for four or five [GPo12 (pGEc47Δ*J*)] or six to eight [GPo12(pGEc47)] generations in spite of the presence of tetracycline. The amount of AlkB, which was initially a major band after SDS-PAGE of total cell lysates, also decreased and could finally hardly be detected (Fig. 5). However, when the same cells were grown in the absence of DCPK, the *alk* genes remained fully active, indicating that the instability of the *alk* genes was triggered by induction and expression of the same *alk* genes. Such DCPK-induced instability of the *alk* genes was found not only in the recombinant strains but also for the wild-type strain GPo1.

The loss of the *alk* phenotype could be due to the loss of the entire plasmid or to structural instability of the *alk* genes. To distinguish between these two possibilities, 18 Alk$^-$ mutants of GPo12(pGEc47Δ*J*) were selected randomly to test for the presence of the plasmid. Seventeen isolates were found to contain the plasmid, indicating that *alk* genes were inactivated in GPo12(pGEc47Δ*J*) without loss of the entire plasmid, in agreement with the finding that these recombinants retained their tetracycline resistance. Furthermore, samples were taken at regular intervals from the GPo12(pGEc47Δ*J*) culture used for Fig. 3D, and plasmids were analyzed by agarose gel electrophoresis. *Eco*RV restriction of these plasmids showed the same restriction map for the parent plasmid and for all of the samples taken from 1.6 to 14 generations, although the percentage of *alk*$^+$ cells in these samples decreased rapidly during that time: within 8 generations 50% of the cells in the continuous culture had lost the ability to utilize octane (Fig. 5B).

## DISCUSSION

The stresses which plasmids impose on host strains have been well documented, especially for *E. coli* (3, 14, 21). Generally, the observed negative effects are believed to result from sequestration of cellular resources for plasmid replication, transcription, and translation (2, 3) or from the toxicity of cloned gene products (5, 25). The negative effects of *alk* gene expression on *Pseudomonas* reported here were not due to plasmid replication, since in the absence of induction *Pseudomonas* (*alk*$^+$) recombinants and the host strain grew equally well. Energy depletion due to transcription and translation of the *alk* genes also cannot explain the observed twofold decrease in the growth rate, because the Alk proteins produced by *P. oleovorans* on induction accounted for only a few percent of total protein synthesis.

Figure 5 summarizes the main changes of GPo12(pGEc47) and GPo12(pGEc47Δ*J*) after induction of the *alk* genes in continuous culture. For the first five or six generations, the cells synthesized a maximum amount of AlkB and formed filaments, which resulted in a decreased number of CFU. Towards the end of this period, when cell filamentation had reached a maximum, the cells began to lose their *alk*$^+$ activity. As most of the cells lost their *alk* activity and no longer produced AlkB, the number of CFU increased once again to reach the level found for uninduced cells; the size and shape of these induced cells were indistinguishable from those of the uninduced cells. These changes of physiology and morphology were not observed when the *alk* genes were not induced, when Alk$^-$ recombinants were induced, when recombinants containing only *alkB-L* or only *alkST* were induced, or when the *alk* genes had been inactivated.

**AlkB triggers changes of *Pseudomonas* physiology.** AlkB is the most likely *alk* product to play a major role in disturbing the physiology of the host. We have noted a correlation of similar effects with *alkB* expression in *E. coli* as well. Thus, excessive expression of *alkB* altered the physiology and morphology of *E. coli* W3110 *alk* recombinants, resulting in modification of the membrane lipid fatty acid composition and in the formation of intracellular membrane vesicles (22). Observations on a variety of *E. coli alk* recombinants have clearly shown that, whenever *alkB* is expressed, there is a reduction in growth rate (13, 18, 22).

However, in contrast to what we found in *E. coli*, AlkB is not the sole *alk*-encoded membrane protein which has these effects in *Pseudomonas*. When a 528-bp *alkB* deletion was tested, a significant growth reduction and associated effects were still observed. A candidate for producing such effects is AlkJ. In contrast to *E. coli* W3110, for which AlkJ was barely detected by two-dimensional gel electrophoresis after induction (unpublished observation), the AlkJ formed in *P. oleovorans* cells accounts for at least 10% of total membrane proteins (27).

**Genetic instability of the *alk* genes and stable bioconversion strains.** The loss of *alk* activity during induction and expression of the *alk* genes was caused by structural instability of the plasmid since 17 of 18 tested Alk$^-$ mutants of GPo12 (pGEc47Δ*J*) still retained the plasmid. Furthermore, since the *Eco*RV restriction pattern of the plasmids from cultures dominated by Alk$^-$ mutants was the same as that of the parent plasmid, it is unlikely that the loss of the *alk* activity in GPo12(pGEc47Δ*J*) resulted from large deletions or insertions in the *alk* genes.

The loss of the *alk* activity occurred not only in the recombinant strain but also in the wild-type strain GPo1 following induction with DCPK. Thus, *alk* genes are inactivated when they are not vital to growth and impose detrimental effects on host cells. We have observed similar effects when the pGEc47 plasmid was introduced into *E. coli* W3110 to create recombinants for the conversion of alkanes to alkanoic acids; continuous growth and induction of the *alk* genes led to the rapid elimination of the *alk* phenotype from W3110(pGEc47). Since the plasmid-encoded tetracycline resistance was retained, all or some of the *alk* genes were inactivated without concurrent elimination of the entire plasmid (14). Such effects were not seen when plasmid pGEc47 was introduced into *E. coli* HB101. Here, the *alk* genes were retained and remained active even without antibiotic selection (14), probably because the expression of *alkB* is four- to fivefold lower in HB101 than in W3110 (22a). A similar example has been reported for *Pseudomonas putida* harboring the TOL plasmid. When the strain was grown on benzoate, a nonselective carbon source which is the product of toluene oxidation, a modified plasmid lacking the TOL catabolic genes for toluene oxidation was generated (9).

The simplest explanation for the above observations, namely that the affected genes are mutated randomly, seems unlikely. Even if the low-copy-number pLAFRI-based pGEc47 plasmids were present as single copies and random $Alk^-$ mutants were to grow at twofold-higher growth rates compared with the parent recombinants, mutation frequencies of $10^{-4}$ and $10^{-2}$ per cell per generation would be required to explain the loss of the $alk^+$ phenotype in Fig. 5 for GPo12(pGEc47) and GPo12 (pGEc47Δ*J*), respectively. For higher plasmid copy numbers, it is even more difficult to explain loss of the Alk phenotype by random mutations. In either case, the required mutation frequencies are very high, suggesting that there must be specific mechanisms which eliminate or diminish *alk* gene expression if the corresponding *alk* gene product is detrimental to the host, without necessarily eliminating the plasmid or even the gene in question.

This phenomenon has clear implications for whole-cell bioconversions which typically depend on enzyme systems that are vital for good productivity but may be unnecessary or even deleterious to the host. If induction of such systems can lead to gene inactivation by the host independently of antibiotic selection, the resulting biocatalysts will not be suited to long-term continuous bioconversions. It will be interesting to learn how bacteria sense and inactivate undesired gene expression and to determine how such mechanisms might be circumvented for the production of stable biocatalysts.

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