Spontaneous mutations affecting transcriptional regulation by protocatechuate in *Acinetobacter*

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Abstract

Positive selection yields *Acinetobacter* strains with a spontaneous mutation blocking catabolism of protocatechuate. For this study, the growth temperature during selection was lowered to 22°C: growth at 37°C was found to mask the role of the protocatechuate-responsive transcriptional regulator PcaU. The resulting mutants included those with amino acid substitutions useful for understanding PcaU structure and function, a 20-bp deletion whose repeated isolation suggested genetic instability of DNA in the putative PcaU operator, and a large deletion whose phenotype revealed that the supraoperonic cluster of genes for the protocatechuate branch of the β-ketoadipate pathway extends to genes for the utilization of C₆–C₁₀ straight-chain dicarboxylic acids including adipate. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Two closely related transcriptional regulators [1], PobR [2,3] and PcaU [4,5], govern sequential steps in the catabolism of p-hydroxybenzoate in *Acinetobacter* sp. strain ADP1 (Fig. 1), but only PobR is indispensable for growth with this aromatic compound. PcaU may therefore have a regulatory mechanism that is distinct from PobR and that may be a good model for exploring how members of a regulatory protein family evolve new physiological functions.

In *Acinetobacter*, aromatic catabolism is particularly amenable to genetic analysis; positive selection yields mutants blocked in either of the first two steps of p-hydroxybenzoate degradation (Fig. 1). Such selection yielded strains with spontaneous loss-of-function mutations in the structural gene *pobA* [6,7] or the regulatory gene *pobR* [2,8], or in the structural genes *pcaH* and *pcaG* [9,10]. Nearly completely missing, however, were regulatory mutations affecting protocatechuate catabolism: only the *pcaP1* promoter mutation has been described to date [9]. To extend the genetic analysis to PcaU, the selection protocol was modified in this study by lowering the growth temperature to 22°C from 37°C; the latter temperature was found to mask the role of PcaU and is probably not representative of the soil environment in which decaying plant material provides an abundant source of aromatic compounds for consumption by *Acinetobacter*.

2. Materials and methods

2.1. Chemicals, bacterial strains, and culture conditions

Wild-type *Acinetobacter* sp. strain ADP1, originally designated *Acinetobacter calcoaceticus* strain BD413 [11], was routinely grown with 10 mM succinate in a mineral medium at 37°C. For growth tests, 3 mM protocatechuate, 8 mM cis,cis-muconate, or 10 mM C₆–C₁₀ straight-chain dicarboxylic acids (from stock solutions prepared by dis-
solving the solid in 1 M ammonia) were used as sole carbon and energy source. Stock solutions of protocatechuate (pH 7.0) stored frozen were added to agar and only freshly prepared plates with this carbon source were used. Strain ADP500 contains the engineered \(\Delta pcaBDK1\) deletion, which allows selection of mutant derivatives blocked in \(\beta\)-hydroxybenzoate catabolism (Fig. 1), and \(catD101::Km\), which allows correction of \(\Delta pcaBDK1\) by transformation and selection for growth with benzoate [9].

2.2. Selection and characterization of mutant strains

Single colonies of succinate-grown ADP500 [9] were transferred to patches on agar with 10 mM succinate and 3 mM protocatechuate incubated at 22°C. Derivatives of ADP500 in which a spontaneous mutation blocked protocatechuate catabolism were isolated as described previously [10] but with the following exceptions: only the smallest colonies of cells resistant to protocatechuate were chosen (after incubation for 2–6 days); of these strains, those with better growth in the presence of \(\beta\)-hydroxybenzoate at 22°C than at 37°C (20% of those tested, on average) were saved and purified on Luria–Bertani agar; after correction of \(\Delta pcaBDK1\), the spontaneous mutation was localized to regulatory elements of protocatechuate catabolism (and not to the structural \(pcaH\) and \(-G\) genes) by marker rescue using plasmid pZR15 [4]; and template DNA for sequencing was amplified using primers I8PCAU1 (5'-CTATTGAGGTTACACGTAGATCG-3') and I8PCAU4 (5'-GATGAATCAGATCGATATGGCA-3').

3. Results

3.1. Isolation of spontaneous mutants with a cold-enhanced block in protocatechuate catabolism

An earlier study [10] described a procedure to efficiently isolate strains with a spontaneous heat-sensitive mutation in the genes for protocatechuate dioxygenase [12]. Such strains were identified by their heat-enhanced block in protocatechuate catabolism. In this study, 19 strains with a cold-enhanced block in protocatechuate catabolism were isolated (see Section 2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location in (pcaU)a</th>
<th>(PcaU) mutation</th>
<th>Phenotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP1038</td>
<td>(\Delta(268–307))</td>
<td>Leaky</td>
<td></td>
</tr>
<tr>
<td>ADP1039</td>
<td>2(\times(541–545))</td>
<td>Leaky</td>
<td></td>
</tr>
<tr>
<td>ADP7530</td>
<td>IS(13.3(399–400))</td>
<td>Leaky</td>
<td></td>
</tr>
<tr>
<td>ADP7531</td>
<td>IS(13.3(498–499))</td>
<td>Leaky</td>
<td></td>
</tr>
<tr>
<td>ADP7534</td>
<td>IS(13.3(555–556))</td>
<td>Leaky</td>
<td></td>
</tr>
<tr>
<td>ADP75318, ADP7532c</td>
<td>C32T</td>
<td>Q178stop</td>
<td>Leaky</td>
</tr>
<tr>
<td>ADP7533</td>
<td>C178T</td>
<td>R60stop</td>
<td>Leaky</td>
</tr>
<tr>
<td>ADP7520</td>
<td>C136T</td>
<td>R46C</td>
<td>Cold-sensitive</td>
</tr>
<tr>
<td>ADP1040</td>
<td>G448A</td>
<td>G150R</td>
<td>Heat-sensitive</td>
</tr>
</tbody>
</table>

Nucleotides are numbered starting from the A residue of the second possible ATG, yielding a 822-bp \(pcaU\) gene and a 274-amino acid residue \(PcaU\) protein. The residues deleted (\(\Delta\)) or duplicated (2\(\times\)) are indicated.

Phenotypes were tested by growing mutant strains with \(\beta\)-hydroxybenzoate and with protocatechuate on agar at 37°C and at 22°C.

Two independently isolated mutants have the same \(pcaU\) mutation.
were tested for growth at various temperatures on agar with protocatechuate: based on colony size and time of appearance, one strain had an unambiguous cold-sensitive phenotype with growth more severely impaired at 22°C than at 37°C, nine strains had a weakly cold-sensitive phenotype (particularly noticeable at 16°C), and nine strains had a leaky phenotype with growth only subtly impaired at all temperatures tested. For each of these 19 mutants, excluding one strain described below with a large deletion, DNA sequencing revealed a mutation in one of the known regulatory elements of the pca operon: the pcaU gene [4,5] and the predicted PcaU DNA binding site [1,4]. Apparently, even the leaky mutations prevented the toxic accumulation of carboxymuconate better at the lower growth temperature.

3.2. Mutations in pcaU

All nine of the mutants with a leaky phenotype had a mutation affecting pcaU (Table 1): a 40-bp deletion, a 5-bp tandem duplication, two nonsense mutations (one found in two independently isolated strains), and three different insertions (including insertions in both orientations, each with a 3-bp target site duplication) of the 1.2-kb Acinetobacter insertion sequence IS1236 [8]. In the remaining strain, ADP7529, PCR amplification combined with marker rescue experiments revealed a large deletion removing most of pcaU (leaving no more than the DNA encoding the PcaU N-terminal 15 amino acids) and an unknown amount of downstream DNA but leaving the putative PcaU operon in the pcaU-pcaI intergenic region (Fig. 2). The leaky phenotype is consistent with that of engineered mutations inactivating PcaU [4].

The one mutation conferring a cold-sensitive phenotype produced an amino acid substitution, Arg80 to Cys80, adjacent to the PcaU helix-turn-helix motif [4] (Table 1). In PobR, a cold-sensitive mutation producing a Trp50 to Arg50 substitution near the helix-turn-helix motif generated a protein that bound abnormally tightly to its operator DNA [1]. Another conditional pcaU mutation (also with a stronger phenotype at the restrictive temperature than the nine leaky mutations apparently inactivating PcaU) was identified in this study: in a previously isolated strain [10], a Gly150 to Arg150 substitution conferred heat-sensitive growth with protocatechuate (Table 1).

3.3. Mutations in the pcaU–pcaI intergenic region

All nine of the strains with a weakly cold-sensitive phenotype had a mutation in the putative PcaU binding site in the pcaU–pcaI intergenic region [1,4]: three strains contained ΔpcaO1, a 20-bp deletion removing the 10-bp direct repeat from the putative operator, and six strains had pcaO2, a transition mutation within the inverted repeat (Fig. 2). The isolation of strains with one of only two different spontaneous mutations was reproducible: of three mutants with weakly cold-sensitive growth with protocatechuate that were isolated previously by selection for resistance to p-hydroxybenzoate but not further characterized, DNA sequencing as part of this study revealed that one strain contained ΔpcaO1 and two strains contained pcaO2.

3.4. Linkage of pcaU to genes required for growth with C6–C10 dicarboxylic acids

To identify other functions which may have been lost as a consequence of the DNA deletion in ADP7529, this strain was tested for growth with various substrates biochemically related to compounds in the β-ketoadipate pathway. These tests revealed that ADP7529 grew on succinate but unlike all the other regulatory mutants in this study, it did not grow with the higher straight-chain dicarboxylic acids (C6–C10) adipic, pimelic, suberic, azelaic, and sebacic acids (the C6 dicarboxylic acid adipate is shown). The extent and exact location relative to pcaU of the dicarboxylic acid genes is unknown. DNA gel mobility shift assays have shown that PcaU binds in the pcaU–pcaI intergenic region to DNA containing an inverted and direct repeat nearly identical in sequence and arrangement to those in the 35-bp PobR binding site in the pobA–pobR intergenic region. The extent of the ΔpcaO1 deletion and the location of the pcaO2 point mutation (a C-to-T transition changing the half site of the inverted repeat to 5′-TTTGGTTTGA-3′) is shown relative to these direct and inverted DNA repeats. The ΔpcaO1 deletion was found in strains ADP7519, ADP7521, ADP7527, and ADP2511; the pcaO2 mutation was found in ADP7522–7526, ADP7528, and ADP7535–7536.

![Fig. 2. Organization of pca genes. The gene for the transcriptional activator PcaU is divergently transcribed from pca genes for conversion of protocatechuate via β-ketoadipate to citric acid cycle intermediates. The spontaneous deletion in ADP7529 removes most or all of pcaU together with downstream DNA and abolishes growth with C6–C10 dicarboxylic acids: adipic, pimelic, suberic, azelaic, and sebacic acids (the C6 dicarboxylic acid adipate is shown).](https://academic.oup.com/femsle/article-abstract/201/1/15/698065/29-6-01)
strains blocked in protocatechuic catabolism were again selected, this time at 37°C. Of the ADP500 derivatives in which an independent spontaneous mutation allowed growth on succinate at 37°C in the presence of protocatechuic acid, 19% (36/192) had lost the ability to grow with adipate.

4. Discussion

In this study, spontaneous mutations were identified that affect regulation of protocatechuic catabolism; these mutations either change the transcriptional regulator PcaU or lie within the putative PcaU operator (Figs. 1 and 2). All 19 of the mutants isolated in this study produced a cold-enhanced block in protocatechuic catabolism. This result is consistent with an early study [15] using Acinetobacter (formerly Moraxella) which noted that growth temperatures of 30°C or above contributed to apparent constitutive expression of enzymes whose synthesis is regulated by protocatechuic acid. Given that Acinetobacter sp. strain ADP1 is a soil isolate [11,16], it is perhaps not surprising that the effects of PcaU on pca operon expression can be masked by growth at 37°C and are most evident at lower temperatures probably more representative of the soil environment.

The phenotypes of the regulatory mutants described here suggest a model in which PcaU activates transcription by changing the conformation of the operator DNA, and in the absence of PcaU, this conformational change occurs, due to inherent DNA flexibility, to a degree sufficient for growth with protocatechuic acid: PcaU null mutants have a leaky phenotype. In putative PcaU operator mutants (with a weakly cold-sensitive phenotype), PcaU binding may interfere with the DNA conformational change. Similarly, in the unambiguously cold-sensitive mutant, PcaU may bind the operator DNA too tightly, as shown for a cold-sensitive PobR mutant [1]. For both of these classes of cold-sensitive mutant, aberrant PcaU binding may repress transcription of the adjacent structural genes, and higher growth temperatures may restore the protein and DNA flexibility required for efficient transcriptional activation. In the single heat-sensitive mutant, a PcaU domain may be disrupted at higher temperatures, keeping the protein in a conformation in which it represses transcription. Although these explanations remain speculative, they can serve to guide future experiments and are consistent with recent data suggesting that wild-type PcaU can function as both an activator and repressor of pca structural gene expression [5].

Further characterization of the mutations described here should yield insight into the molecular biology of the β-ketoacid pathway [17–19] at the level of both protein and DNA. PcaU mutations causing exceptions to the leaky phenotype should be particularly useful to an understanding of how structure influences function in the protein. The genetic instability leading to the 20-bp ΔpcaO1 deletion can be attributed to replication error caused by slipped DNA strands, therefore suggesting a tradeoff between the benefit of regulation modulated by direct 10-bp sequence repetitions and the detriment of their potential loss by deletion. Lastly, the genes between pcaU and mucK were shown here to be required for utilization of C6–C10 dicarboxylic acids including adipate, opening a new line of investigation into the basis for the clustering of genes with related function that appears to be a conserved feature in the evolution of the β-ketoacid pathway [18,19].

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References


