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Regulation of Phenol Metabolism in *Ralstonia eutropha* JMP134

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Ralstonia eutrupha JMP134 is a well-known soil bacterium which can metabolite diverse aromatic compounds and xenobiotics, such as phenol, 2,4-dichlorophenoxy acetic acid (2, 4-D), and trichloroethylene (TCE), etc. Phenol is degraded through chromosomally encoded phenol degradation pathway. Phenol is first metabolized into catechol by a multicomponent phenol hydroxylase, which is further metabolized to TCA cycle intermediates via a meta-cleavage pathway. The nucleotide sequences of the genes for the phenol hydroxylase have previously been determined, and found to composed of eight genes *phlKLMNOPRX* in an operon structure The *phlR*, whose gene product is a NtrC-like transcriptional activator, was found to be located at the internal region of the structural genes, which is not the case in most bacteria where the regulatory genes lie near the structural genes. In addition to this regulatory gene, we found other regulatory genes, the *phlA* and *phlR2*, downstream of the phlX. These genes were found to be overlapped and hence likely to be co-transcribed. The protein similarity analysis has revealed that the PhlA belongs to the GntR family, which are known to be negative regulators, whereas the PhlR2 shares high homology with the NtrC-type family of transcriptional activators like the PhlR. Disruption of the *phlA* by insertional mutation has led to the constitutive expression of the activity of phenol hydroxylase in JMP134, indicating that PhlA is a negative regulator. Possible regulatory mechanisms of phenol metabolism in *R. eutropha* JMP134 has been discussed.

Introduction

Phenols are present in many industrial processes and listed as hazardous pollutants by the Environmental Protection Agency. A number of microorganisms have been found to degrade phenol (Antai *et al.*, 1983; Bartilson *et al.*, 1990; Gurujeyalakshmi and Oriel, 1989; Hughes and Bayly, 1983; Kulor and Olsen, 1990; Pieper *et al.*, 1989; Shield *et al.*, 1991; Shingler *et al.*, 1993; Straube, 1987). Phenol is first metabolized into catechol by various phenol hydroxylases. Catechol is a key intermediate in many aromatic degradation pathways, and is further metabolized by two distinct sets of enzymes. The *ortho*-cleavage pathway (*beta*-ketoadipate pathway) and the *meta*-cleavage pathway (*alpa*-ketoacid pathway) convert catechol to TCA cycle intermediates. Although phenol metabolism has been extensively studied in *Alcaligenes eutrophus* and *Pseudomonas putida* U by using various mutant strains (Bayly *et al.*, 1977; Hughes *et al.*, 1984; Wigmore *et al.* 1977), little is known about the regulatory mechanisms of the chromosomally-encoded phenol pathway at the

molecular level in these strains.

Previously we have shown that the 5.0kb fragment downstream of the phenol hydroxylase genes entirely repressed the phenol hydroxylase activity in *P. aeruginosa* PAO1c, whereas the presence of this fragment increased the enzyme activity in *R. eutropha* JMP134 (Kim and Harker, 1997). From this result we suggested that this trans-acting factor is both an activator and a repressor for phenol hydroxylase. In this study, we found that this fragment was consisted of two genes, which were overlapped and hence likely to be co-transcribed. The nucleotide sequence analysis of this region revealed that one gene (*phlR2*) encoded another putative transcriptional activator which also shares high homology with the members of NtrC family such as XylR and DmpR, and the other one (*phlS*) encoded a GntR-type negative regulator.

It has not been much reported that negative regulators are involved in the metabolism of aromatic compounds. However, Mouz et al. described the BphS on the transposon Tn4371 isolated from R. eutropha A5, which is a GntR-like negative regulator involved in byphenyl degradation (Mouz et al., 1999). Another BphS from *Pseudomonas sp.* KKS102 was also reported by Ohtsubo et al. (2001). Arai et al. (1998) have found a GntR-like regulatory protein, AphS, from an adapted strain of *Comamonas testosteroni* TA441, which is negatively controls phenol catabolism. Recently, Teramoto et al. (2001) have identified PhcS from C. testosteroni R5, which is similar to AphS and also involved in phenol metabolism. The PhcS, belonging to the GntR family of transcriptional regulators, was found to repress gratuitous expression of phenol-metabolizing enzymes in the absence of the genuine substrate.

In addition to the finding of PhcS as a negative regulator of phenol-hydroxylating enzyme in *C. testosteroni* R5 by Teramoto et al., we also found a GntR-type transcriptional regulator, PhlA, from JMP134, which is similar to PhcS in its regulatory function on phenol metabolism, demonstrating that this type of negative regulation on aromatic degradation pathway is not uncommon in bacteria. In this study, we also discussed possible regulatory mechanisms of phenol metabolism in relation to the function of PhlR, PhlR2 and PhlA in *R. eutropha* JMP134.

Results and Discussion

In this study, we identified two putative transcriptional regulators in downstream region of the PH structural genes in *R. eutropha* JMP134 (Figure 1). ORF1, named the PhlR2, might be an activator and ORF2 the PhlA, is a negative regulator. These two ORFs are found to be overlapped, implying that they are co-transcribed. The PhlR2 showed 80% identity of the amino acid sequences to the PhlR, another putative transcriptional activator whose gene is located inside of the PH structural genes.

PhlR and PhlR2 share greater homology with the members of the NtrC family of positive transcriptional activators such as PoxR of *R.eutropha* E2 (Hino *et al*,1998), PhhR of *P.putida* P35X (Burchhardt *et al.*,1997), DmpR of *P.putida* sp.CF100 (Shingler et al. 1994) and the TubR regulator (Byrne et al. 1996), an so on. Members of this family also require an alternative sigma factor (RpoN) for initiating transcription in response to different environmental signals. The promoter region of the phlK in pYK3021 shows the typical binding

site for RpoN (sigma-54) and two transcriptional acitivator binding sites similar to those for TbuT, XylR, and DmpR etc. The NtrC class of activator proteins have three distinct functional domains namely the central, carboxy terminal and amino terminal domains of which the first two are conserved. As predicted by Singler et al.(1994) for the DmpR regulator, the amino terminal domains of these proteins mediate the specificity of activation in response to effector molecules. The sequence similarity of PhlR and PhlR2 to the above class of positive regulatory proteins indicates that the effector specificity of the regulator may be the limiting factor for the catabolic potential of *R.eutropha* JMP134. We don't know how these two regulators act differently on the expression of phenol degradation pathway at this moment, except that PAO1c with pRK3029, which contains the phlR gene in internal site of the PH genes, expressed the PH activity only in the presence of phenol, implying that PhlR is involved in the activation of the PH genes in response to phenol. The exact regulatory roles of PhlR and PhlR2 in related to substrate specificity and promoter binding ability are under investigation.

In addition to the positive regulators, PhlR and PhlR2, a negative regulator which shares high homology with the members of GntR family was found to exert on the regulation of phenol metabolism in JMP134. GntR-like regulators, though it is rare, were recently found to be involved in the degradation of aromatic compounds. Mouz et al. (1999) reported that a GntR-like negative regulator, BphS, was involved in the control of the biphenyl degradation pathway in *R. eutropha* A5. Moreover, Teramoto et al. (2001) isolated PhcS, which also belongs to the GntR family of transcriptional regulators, related to the expression of multicomponent phenol hydroxylase in Comamonas testosteroni R5. Interestingly, the regulatory mode observed with *C. testosteroni* R5 was the same as that of JMP134, where both PhcR (PhlR in JMP134), a positive transcriptional regulator, and PhcS (PhlA in JMP134), a negative regulator, were involved in the regulation of the PH activity.

The GntR protein is a negative regulator involved in gluconate-inducible expression of the Bacillus subtilis gnt operon which is responsible for gluconate metabolism. The GntR binds to the promoter region of the gnt operon and the binding was specifically inhibited by the substrat, gluconate, or glucono-delta-lactone, one of its metabolites. It is conceivable that similar situation occurs in JMP134 or in R5 where some metabolite acts as an inducer or an effector to inhibit the function of the PhlA or the PhcS. However, PAO1c containing the PhcS didn't express the PH activity even in the presence of phenol. Teramoto et al. (2001) pointed out that *C. testosteroni* R5 would employ an unknown mechanism which is different from that of PAO1c, where the PhcS-mediated repression was overcome in the presence of genuine substrate, other than phenol. The same result was observed with the PhlA in PAO1c, indicating that phenol or catechol is not an inducer. PAO1c possesses the catabolic enzymes for only the ortho-cleavage pathway of catechol, whereas JMP134 carries both the ortho- and the meta-cleavage systems. Moreover, the gene for catechol 2,3-dioxygenase was specifically induced when phenol is present, indicating that the genes for meta-cleavage enzymes are coordinately regulated in phenol metabolism. Hence, it is much likely that one of down metabolites of the meta pathway would be an effector molecule, though we can't rule out the possibility that other gene(s) are involved in this particular regulatory mode of PhlA in JMP134.

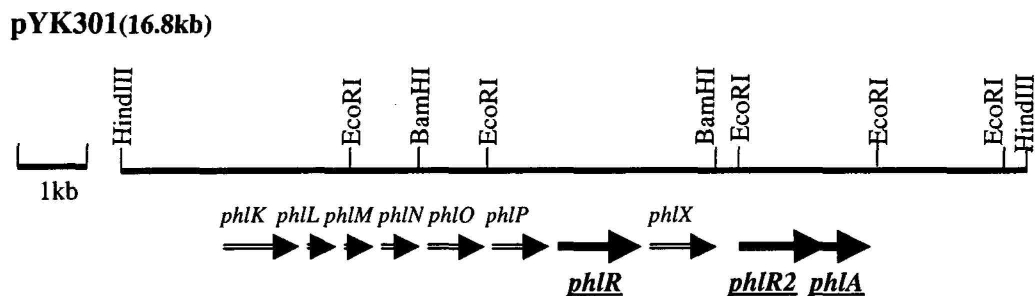

Fig. 1. Localization of phenol hydroxylase genes and their regulatory genes. *phlR* and *phlR2*; putative transcriptional regulatory genes, *phlA*; negative regulatory gene

References

1. Antai, S. P., and Crawford, D. L. (1983) Can. J. Microbial. **29**, 142-143.
2. Arai, H., Saiko A., Tohru O., Michihisa M., Toshiaki K. (1998) Microbiol. **144,** 2895-2903.
3. Arai, H., Saiko A., Tohru O., Toshiaki K. (1999) Mol Microbiol. **33(6),** 1132-1140.
4. Bartilson, M., Nordlund, I., and Shingler, V. (1990) Mol. Gen. Genet. **220**, 294-300.
5. Bayly, R. C., Wigmore, G. J., and McKenzie, D. I. (1977) J. Gen. Microbiol. **100**, 71-79.
6. Byrne, A.M. and Olsen, R.H. (1996) J. Bacteriol., **178**, 6327-6337,
7. Gurujeyalakshmi, G., and Oriel, P. (1989) Appl. Environ. Microbiol. **55**, 500-502.
8. Hino, S.; Watanabe, K and Takahashi, N.: Microbiology, (1998) **144,** 1765-1772,
9. Hughes, E. J. L., Skurray, R., and Bayly, R. C. (1984) J. Bacteriol. **158**, 79-83.
10. Kukor, J., and Olsen, R. H. (1990) J. Bacteriol. **172**, 4624-4630.
11. Kim, Y., and A. R. Harker (1997) Mol. Cell Vol. **7**, 620-629
12. Mouz S., Merlin C., Springael D., Toussaint A. (1999) Mol Gen Genet. **262,** 790-799.
13. Nakada, Y., Ying J., Takayuki N., Yoshifumi I., Chung-dar L. (2001) J. Bacteriol. **183,** 6517-6524.
14. Ohtsubo, Y., Mina D., Kazuhide K., Masamichi T., Akinori O., Yuji N. (2001) J. Bacteriol. **276,** 36146 36154.
15. Pieper, D. H., Engesser, K. H., and Knackmuss, H. J. (1989) Arch. Microbiol. **151**, 356-371.
16. Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J., and Pritchard, P. H. (1991) Appl Environ. Microbiol. **7**, 1935-1941.
17. Shingler, V., Bartilson, M., and Moore, T. (1993) J. Bacateriol. **175**, 1596-1604.
18. Shingler, V. and Moore,T. (1994) J. Bacteriol., **176,** 1555-1560,
19. Straube, G. (1987) J. Basic Microbiol. **4**, 229-232.
20. Teramoto, M., Shigeaki, H., Kazuya, W. (2001) J. Bacteriol. **183,** 4227-4234.
21. Wigmore, G. J., Biberardino, D., and Bayly, R. C. (1977) J. Gen. Microbiol. **100**, 81-87.