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NAPHTHALENE DEGRADATION THROUGH THE GENTISATE PATHWAY BY *BURKHOLDERIA CEPACIA* STRAIN JS150

A Thesis

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Degree

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ABSTRACT

This research was conducted to identify the metabolic pathway used by *Burkholderia cepacia* strain JS150 for catabolism of naphthalene and salicylate. The relationship between the naphthalene pathway and pathways for the catabolism of other aromatic compounds by JS150 was also investigated. DNA-DNA hybridization under high stringency conditions revealed significant homology between JS150 DNA and the *nahA* gene of naphthalene catabolic plasmid NAH7. JS150 DNA did not, however, hybridize to *nahG* or *nahR* probes. Gentisate was detected in culture medium by HPLC and GC-MS when the growth substrate was either naphthalene or salicylate. Gentisate dioxygenase, but not catechol 1,2- or 2,3-dioxygenase, was induced by growth on salicylate. Complete degradation of gentisate by extracts of induced cells was shown to be dependent on the presence of reduced glutathione. The above results indicate that JS150 degrades naphthalene and salicylate through a reduced glutathione-dependent gentisate pathway. No other role for the gentisate pathway in JS150 was identified. *m*-hydroxybenzoate, which is degraded through the gentisate pathway by some bacteria, was found to be degraded through protocatechuate *ortho* cleavage in JS150. Anthranilic acid, another potential gentisate pathway substrate, was also shown not to be degraded through the gentisate pathway in JS150. The actual catabolic pathway for anthranilic acid was not identified.

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1. INTRODUCTION

Bacteria that are capable of degrading aromatic pollutants have been known to science for many years. The metabolic pathways responsible for this degradation have, in many cases, been extensively characterized at the biochemical and genetic levels. For recent reviews, see Harayama *et al* (1992), Mason and Cammack (1992), Mohn and Tiedje (1992), Van der Meer *et al* (1992).

Characterization of the pathways and genes involved in the degradation of aromatic compounds by bacteria has so far focused on the degradation of single carbon sources by pure cultures. Pollutants usually occur as mixtures of compounds that should, ideally, be degraded simultaneously. When two or more substrates are degraded simultaneously, interactions between the pathways may occur, such as cross-pathway inhibition, induction, or substrate routing. The design of bioremediation processes and of recombinant bacterial strains for use in bioremediation would greatly benefit from further research into the simultaneous degradation of aromatic compounds by bacteria (Gibson and Sayler, 1992).

Thoroughly characterized bacteria with the ability to simultaneously degrade multiple aromatic substrates will be useful tools for the characterizations of pathway interactions, especially the development of kinetic models. The study of such strains will also allow researchers to identify the most efficient

combinations of pathways for degradation of specific mixtures under specific conditions. The strain used in the research reported in this thesis, *Burkholderia cepacia* JS150, should be especially useful, due to its exceptional metabolic versatility (Gibson and Sayler, 1992).

The pathway by which strain JS150 degrades naphthalene, a bicyclic fused aromatic ring, (figure 1) was the focus of this investigation. Naphthalene, 2-methylnaphthalene, 2-chloronaphthalene, and several higher molecular weight polycyclic aromatic hydrocarbons (PAHs) have been placed on the EPA and ATSDR priority pollutant lists (Sittig, 1981, National Research Council, 1991). PAHs, including naphthalene, are major soil pollutants at manufactured gas plant sites (Srivastava *et al*, 1990). Creosote, a wood preservative that often contaminates soil during storage or application, is composed of 85% PAHs. Thirteen percent of the PAH fraction of creosote is naphthalene and 29% is methylated and dimethylated naphthalenes (Mueller, *et al*, 1989). Chlorinated and alkylated naphthalenes are found in sludge from kraft mills (Kolstinen and Nevalalnen, 1992).

In the cases where genetic information exists, it has been shown that a separate set of genes is involved in the degradation of each ring (Yen and Serdar, 1988). There is also evidence that at least one compound with more than two fused aromatic rings, the three-ring compound phenanthrene, can be degraded by multiple passes through the same pathway that degrades naphthalene to the single-ring compound salicylate (Yang, *et al*, 1994, Sanseverino, *et al*, 1993). The study

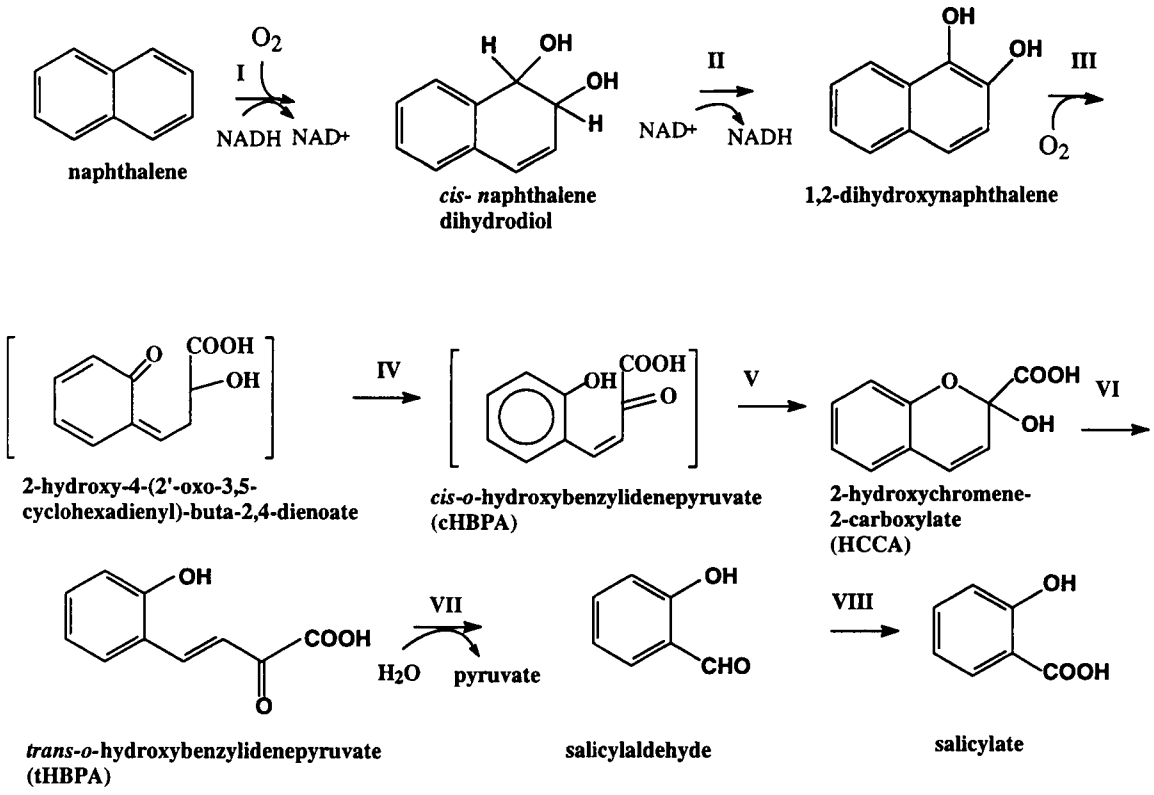

Figure 1. The naphthalene upper pathway of plasmid NAH7. Compounds in brackets are hypothetical. Enzymes: I) naphthalene dioxygenase; II) *cis*-naphthalene dihydrodiol dehydrogenase; III) 1,2-dihydroxynaphthalene dioxygenase; IV) spontaneous (Eaton and Chapman), possibly catalyzed by the isomerase encoded by *nahD* (as designated by Yen and Gunsalus (1982); V) same as IV; VI) 2-hydroxychromene-2-carboxylase isomerase; VII) *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase; VIII) salicylaldehyde dehydrogenase. Gene designations: I) *nahAa-d*; II) *nahB*; III) *nahC*; IV) possibly *nahD /doxH*, as designated by Yen and Gunsalus (1982) and Denome *et al* (1993), respectively; V) same as IV; VI) *nahD /doxJ*, as designated by Eaton and Chapman (1992) and Denome *et al* (1993), respectively; VII) *nahE*; VIII) *nahF*.
Adapted from: Eaton, R. W., and P. J. Chapman. 1992. J. Bacteriol. **174:** 7542-7554.

of the degradation of naphthalene may therefore yield information relevant to the degradation of the far more recalcitrant PAHs.

The goal of the research described in this thesis was to contribute to the characterization of the naphthalene catabolic pathway of strain JS150. Specifically, the goals were:

1) to determine which, if any, of the known naphthalene pathways is used by strain JS150.
2) to determine which, if any, of the genes of the canonical NAH7 naphthalene catabolic plasmid hybridize with JS150 DNA.
3) to determine which, if any, aromatic substrates of strain JS150 converge with the naphthalene pathway prior to the second ring cleavage step.

The initial hypothesis was that JS150 would degrade naphthalene via the pathway encoded by the NAH7 plasmid, since this is by far the most commonly reported pathway. The genes encoding this pathway are highly conserved and are usually found on catabolic plasmids in naphthalene-degrading bacteria (Yen and Serdar, 1988). It was therefore expected that JS150 DNA would hybridize to any NAH7 catabolic gene with which it was probed. The naphthalene catabolic pathway encoded by NAH7 cleaves both rings through hydroxylation followed by *meta* cleavage (Davies and Evans, 1964). JS150 degrades at least three single-ring aromatic substrates *via meta* cleavage: phenol, benzene and toluene

(Haigler, *et al*, 1992). By analogy with the NAH7 pathway, it was expected that these pathways would share common intermediates, but that the degradation of the naphthalene metabolite salicylate would be encoded by separate genes and that these genes would be specifically induced by salicylate (Barnsley, 1975).

2. LITERATURE REVIEW

Naphthalene catabolic plasmids

The most extensively studied naphthalene pathways are those found in the NAH7 plasmid *Pseudomonas putida* PpG7 (Dunn and Gunsalus, 1973) and in plasmids NAH3, pWW60, and pDTG1 of *P. putida* NCIB 9816-2, NCIB 9816-3, and NCIB 9816-4, respectively (Connors and Barnsley, 1982, Cane and Williams, 1982). The three versions of NCIB 9816 are variants of NCIB 9816-1, which arose during maintenance in separate laboratories (Yen and Serdar,1988). The plasmid pWW60-1 (Cane and Williams, 1982) is the designation given to pWW60 following its transfer from its original host to *Pseudomonas putida* PaW701. Many of the naphthalene catabolic genes of NAH7 and related plasmids have been mapped (Eaton and Chapman, 1992, Yen and Gunsalus, 1982, 1985, Cane and Williams, 1986, Schell, 1983) and/or sequenced (Schell and Sukordhaman, 1989, Harayama *et al*, 1987, Ghosal *et al*, 1987, Denome *et al*, 1993, Simon *et al*, 1993, Harayama and Rekik, 1989, Takizawa, *et al*, 1994). Gene order and sequence data have shown that the NAH7 catabolic pathway is closely related to the NCIB 9816 pathway (Cane and Williams, 1986). Simon *et al* (1993) report 93% nucleotide sequence identity between *nahAaAb* of NAH7 and that of pDTG1.

The pathway encoded by NAH7 is shown in figure 1. The pathway is traditionally divided into an "upper" and "lower pathway, as indicated in the figure.

The upper pathway attacks one of the two rings of naphthalene, releasing three carbons in the form of pyruvate. The single-ring product of the upper pathway, salicylate, is degraded to 4-hydroxy-2-oxovalerate and CO_2 by the lower pathway. 4-hydroxy-2-oxovalerate is cleaved to the TCA cycle intermediates pyruvate and acetaldehyde by chromosomally encoded enzymes.

The catabolic genes of the NAH7 plasmid are organized into two operons, *nah* and *sal,* which encode the enzymes of the upper and lower pathways, respectively. A positive regulatory protein, NahR, is the product of the *nahR* gene. *nahR* is transcribed divergently from *sal* by a promoter that partially overlaps p*sal.* When bound to its effector, salicylate, NahR stimulates induced-level transcription of both naphthalene catabolic operons.

The upper pathway

The first step of the upper pathway is oxidation to *cis* -naphthalene dihydrodiol (Jerina, *et al*, 1971). The reaction is catalyzed by naphthalene dioxygenase, which has been extensively studied. Naphthalene dioxygenase from NCIB 9816-4, pDTG1 plasmid, was first purified by Ensley *et al* (1982). Naphthalene dioxygenase was found to be a three-component dioxygenase consisting of NADH-ferredoxin$_{NAP}$ reductase , ferredoxin$_{NAP}$, and ISP$_{NAP}$. The reductase and ferredoxin components for an electron transport chain that transfers electrons from NADH to the two-subunit iron-sulfur protein (ISP) (Haigler and Gibson, 1990a, 1990b). Reduced ISP uses molecular oxygen to

oxidize naphthalene to *cis* -naphthalene dihydrodiol (Ensley and Gibson, 1983). All three components of naphthalene dioxygenase have been individually purified and characterized. (Haigler and Gibson, 1990a, 1990b, Suen and Gibson, 1993, Ensley and Gibson, 1983). The gene cluster encoding naphthalene dioxygenase, *nahAaAbAcAd,* has been cloned and sequenced from several strains (Denome *et al*, 1993, Simon *et al*, 1993, Takizawa *et al*, 1994).

The second intermediate on the upper pathway is 1,2-dihydroxynaphthalene. 1,2-dihydroxynaphthalene was first demonstrated to be a naphthalene intermediate by the sequential induction experiments of Fernley and Evans (1958). The enzyme responsible for the NAD-dependent oxidation of *cis*-naphthalene dihydrodiol to 1,2-dihydroxynaphthalene, *cis*-naphthalene dihydrodiol dehydrogenase, has been purified from *Pseudomonas putida* strain NP and characterized (Patel and Gibson, 1974).

The ring-cleavage enzyme 1,2-dihydroxynaphthalene dioxygenase was purified from NCIB 9816 and characterized by Patel and Barnsley (1980). The cleavage reaction utilizes one mol O_2 per mol of substrate cleaved. *nah*C from the NAH7 plasmid, which encodes 1,2-dihydroxynaphthalene dioxygenase, has been sequenced by Harayama and Rekik (1989).

There has been much dispute over the identity of the cleavage product of 1,2-dihydroxynaphthalene and the pathway by which it is degraded to salicylaldehyde and pyruvate. Davies and Evans (1964) proposed that the initial cleavage product is 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate,

an unstable compound which would spontaneously isomerize to form *cis*-2-hydroxybenzylidenepyruvate(cHBPA), which is then degraded to salicylaldehyde and pyruvate. They identified both cHBPA and another compound, 2-hydroxychromene-2-carboxylate (HCCA) in their 1,2-dihydroxynaphthalene transformation experiment, but due to their inability to demonstrate degradation of HCCA by cell extracts, regarded it as an artifact of their experimental procedure. Barnsley (1976) proposed that HCCA is the ring cleavage product and that it is converted enzymatically to cHBPA, which is degraded to salicylaldehyde and pyruvate. HCCA was considered to be a precursor of cHBPA because HCCA was detected earlier than cHBPA. Furthermore, an inducible enzyme capable of converting HCCA to cHBPA was partially purified from cell extract. Barnsley (1976) did note, however, that the HCCA isolated from his transformation experiment was not optically active, even though HCCA is a chiral molecule. This observation does not support enzymatic production of HCCA.

Eaton and Chapman (1992), using a *nahABC* clone, have been able to conduct transformation experiments with naphthalene, rather than the unstable 1,2-dihydroxynaphthalene used in the previous experiments. The first ring cleavage product detected was HCCA, which isomerized to a mixture of HCCA and *trans*-2-hydroxylbenzalpyruvate (tHBPA). The identifications of tHBPA and HCCA by UV absorbance spectroscopy were confirmed by proton and ^{13}C NMR. The authors propose that Davies and Evans (1965) and Barnsley (1976)

misidentified tHBPA as cHBPA and that the actual upper pathway is that shown in figure 1. According to the Eaton and Chapman proposed pathway, the actual ring cleavage product is the unstable 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate, in agreement with Davies and Evans. This hypothetical intermediate is proposed to spontaneously isomerize to cHBPA, then to HCCA. HCCA is thus generated non-enzymatically, explaining the finding that HCCA isolated from 1,2-dihydroxynaphthalene transformations is racemic (Barnsley,1976).

Although isomerization of HCCA to tHBPA occurs spontaneously, Eaton and Chapman (1992) showed that the product of the *nahD* gene, HCCA isomerase, greatly increases the rate of the isomerization. They also demonstrated that tHBPA is cleaved to pyruvate and salicylaldehyde by a single enzyme, the product of the *nahE* gene.

Davies and Evans (1964) showed that salicylaldehyde is oxidized to salicylate by an NAD^+-dependent dehydrogenase. Eaton and Chapman (1992) also demonstrated salicylaldehyde dehydrogenase activity in crude cell extract from an *E. coli* strain carrying cloned NAH7 upper pathway genes. The salicylaldehyde dehydrogenase gene has been designated *nahF* (Schell, 1983).

Diversity of the upper pathway

All the naphthalene-degrading strains that have been studied at the genetic level appear to be related to the NAH7 pathway. In two cases,

nucleotide sequences of upper pathway genes reveals a high degree of similarity to *nahA* from NAH7 and from pDTG1. The naphthalene dioxygenase genes from *Pseudomonas putida* OUS82 (*pahAa-Ad*), ranged from 94-97% nucleotide sequence identity to *nahAa-Ad* from NAH7 and 90-93% nucleotide sequence identity to the corresponding genes from pDTG1 (Takizawa *et al*, 1994). The predicted amino acid sequences of DoxABD (ferredoxin$_{NAP}$, ISPα_{NAP}, ISPβ_{NAP}) from *Pseudomonas* sp. C18 was were 100% identical to the predicted sequences of the corresponding genes from pDTG1 (Denome *et al*, 1993). The predicted amino acid sequence of DoxG was 97% identical to that of NahC from NAH7 (Denome *et al*, 1993). Sequences of other NAH7 and pDTG1 genes are not available for comparison to Dox sequences, but the Dox pathway gene order appears to be the same as that determined for NAH7 by Eaton and Chapman (1992), *nahABFCED*. A possible difference between *dox* and *nah* is the presence in the *dox* operon of a gene, designated *doxH*, which has no known NAH7 equivalent. Although the function of *doxH* has not been determined, *doxH* and *doxJ* (*nahD*) partially complement each other. Both *doxH* and *doxJ* deletion mutants are able to produce salicylate from naphthalene, but *doxH doxJ* double mutants do not produce salicylate (Denome *et al*, 1993). Based on its position, phenotype, and restriction pattern, Denome *et al* (1993) identified *doxJ* as equivalent to the *nahD* described by Eaton and Chapman (1992) as encoding HCCA isomerase. The phenotype of *doxH* and *doxH doxJ* double mutants suggests that *doxH* also encodes an enzyme involved in part of the proposed

two-step conversion of 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate to HCCA. Based on the failure of double mutants to accumulate cHBPA, Denome *et al* (1993) suggest that the product of *doxH* catalyses the first step, conversion of 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate to cHBPA, and that cHBPA is spontaneously converted to HCCA, but there is no direct evidence for this. It is likely that *doxH* corresponds to the NAH7 gene which Yen and Gunsalus (1982) designated *nahD* and which they mapped to a position between *nahC* and *nahE* equivalent to that occupied by *doxH*. In contrast to the results of Eaton and Chapman, Yen and Gunsalus were able to demonstrate expression of an isomerase activity between *nahC* and *nahE*.

Naphthalene catabolic plasmids that hybridize to *nahA* probes are numerous. Some *nahA*-hybridizing plasmids include pUTK21 (King *et al*, 1990), pKA1, pKA2, and pKA3 (Sanseverino *et al*, 1993). Chromosomal DNA from JS150 also hybridizes to *nahA* (see results section). A high percentage of naphthalene-degrading colonies cultured from PAH-contaminated soil was found to hybridize to *nahA*. Naphthalene-degrading strains that fail to hybridize *nahA* have been isolated, but none has ever been cloned or sequenced. Although the naphthalene catabolic genes from these *nahA*-hybridization negative strains are clearly significantly different from the NAH7 family genes, their relationship to the NAH7 genes cannot be evaluated until sequence data for non-NAH7-hybridizing pathways are available.

There is evidence for a biochemically novel naphthalene upper pathway in a Gram positive strain, *Rhodococcus* sp. B4 (Grund *et al*, 1992). Although these workers failed to detect oxidation of naphthalene by cell-free extracts of B4, they were able to detect 1,2-dihydroxynaphthalene-dependent oxygen-consumption and salicylaldehyde dehydrogenase activity. In contrast to 1,2-dihydroxynaphthalene dioxygenase from NAH7, 1,2-dihydroxynaphthalene-dependent oxygen consumption by B4 extracts required NADH. This suggests that a different ring cleavage mechanism might be used by B4.

The upper pathway of B4 also has unusual regulatory characteristics. It is not induced by salicylate, but it is induced by either naphthalene or by an upper pathway intermediate. Salicylaldehyde dehydrogenase and 1,2-dihydroxynaphthalene oxidation activities are both detected in extracts of B4 cells grown on naphthalene, but not in extracts of B4 cells grown on salicylate. Salicylate has been shown to be the only inducer of both upper and lower pathways in NAH7 (Barnsley, 1975).

The lower pathway

The lower pathway degrades salicylate into TCA cycle intermediates. Four biochemically distinct salicylate pathways are known (see figure 2). The first step of the lower pathway is oxidation of salicylate either to catechol or to gentisate. Catechol is cleaved by either the *meta* ring cleavage pathway, as in NAH7 (Dunn and Gunsalus, 1973) and most of its relatives, or the *ortho* ring

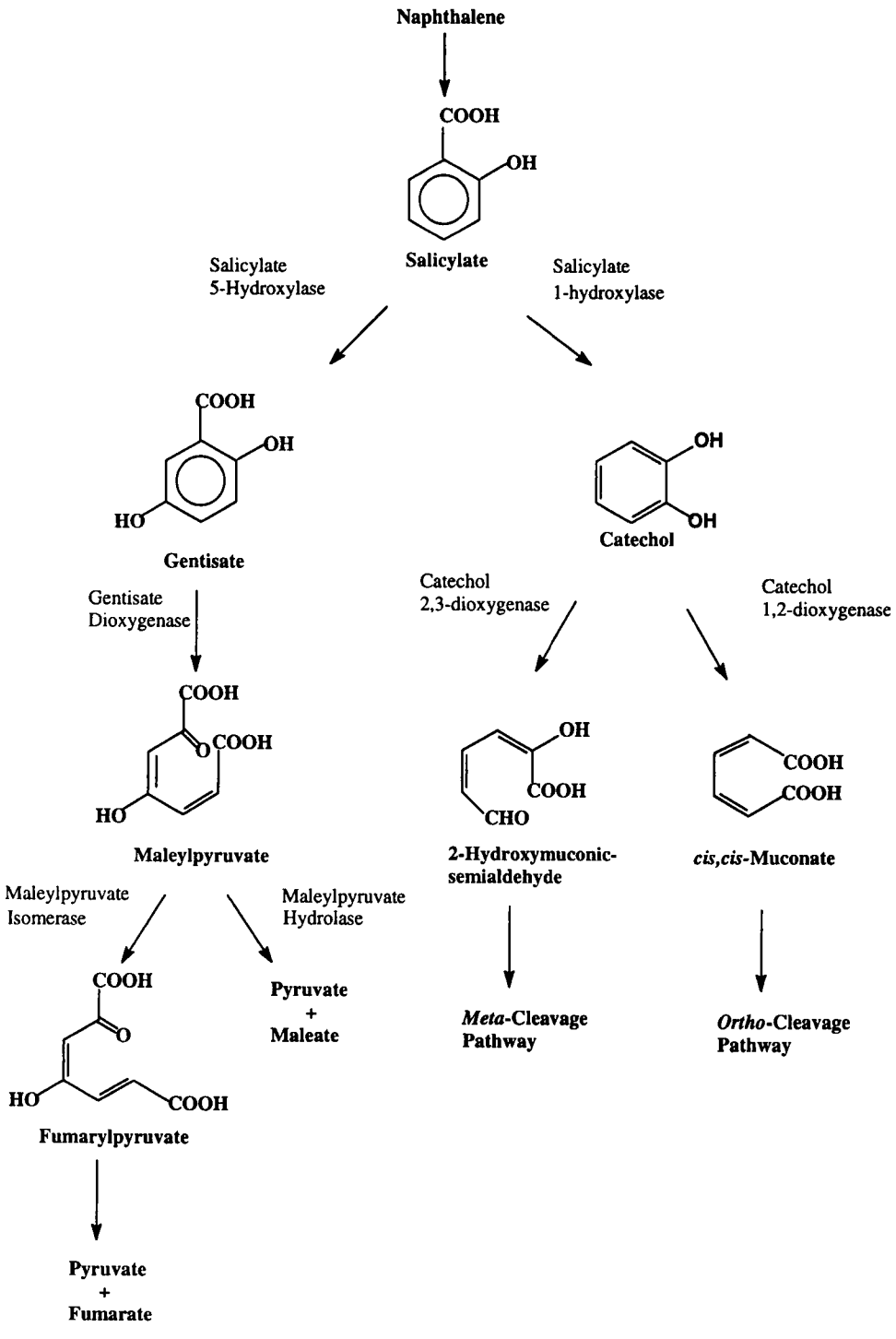

Figure 2. Diversity of naphthalene metabolism in Gram negative bacteria.

cleavage pathway, as in the NCIB 9816- family plasmids pWW60-1 and pDTG1 (Cane and Williams, 1982). The ring cleavage product of gentisate is maleylpyruvate (Lack, 1959). Maleylpyruvate is either hydrolyzed directly to pyruvate and maleate (Hopper *et al*, 1971) or first isomerized to fumarylpyruvate, then hydrolyzed to pyruvate and fumerate (Lack, 1959). Throughout the remainder of this document, these two branches of the gentisate pathway will referred to as the hydrolase branch and isomerase branch, respectively.

Catechol *meta* cleavage

On the NAH7 plasmid, the lower pathway, like the upper pathway, is encoded by a single operon, regulated by the product of an independently transcribed gene, *nahR.* Yen and Serdar (1988) have proposed that the NAH7 lower pathway is composed of two independently evolved genetic modules, *nahG* - *nahR*, and the genes of the catechol *meta* cleavage pathway. This hypothesis is supported by the high degree of similarity between the *meta* pathway genes of the NAH7 lower pathway operon and the benzoate-degrading lower pathway operon of the toluene catabolic plasmid pWWO. Ghosal *et al* (1987) found 81% nucleotide and 85% amino acid sequence identity between the catechol 2,3-dioxygenase genes *nahH* and *xylE*. Furthermore, there is a large gap (831 bp) between *nahG* and *nahH* which includes a 327bp ORF of no known function (You *et al*, 1991). It is possible that this gap is a remnant of a disrupted operon which once included either *nahG* or the *meta* pathway. *nahR* is

likely to have evolved in association with *nahG,* rather than the upper pathway, because *nahR* and *nahG* have overlapping, divergent promoters.

Catechol *ortho* cleavage

The only well-characterized example of a naphthalene lower pathway proceeding through catechol *ortho* cleavage is that of the plasmid pWW60-1 (Cane and Williams, 1982), originally isolated from NCIB 9816-3. pWW60-1 confers on its host the ability to convert naphthalene and salicylate to catechol. The genes of the catechol *ortho* cleavage pathway are carried by the NCIB 9816 chromosome. It has been shown that pWW60-1, like NAH7, actually carries the genes for the *meta* cleavage pathway, but expression of these genes is blocked by a small insertion. A mutant strain from which the insertion was spontaneously deleted grows on naphthalene and salicylate through the meta pathway (Cane and Williams, 1982). The *ortho* pathway is not induced in the mutant because catechol is not allowed to accumulate. pDTG1, from NCIB 9816-4, also uses catechol *ortho* cleavage in its lower pathway. Because pDTG1 and pWW60-1 are both derivatives of the naphthalene plasmid of the original NCIB 9816 isolate, pDTG1 is presumed to have the same *meta* pathway insertional mutation as pWW60-1.

Gentisate cleavage

The gentisate pathway has been shown to be part of the naphthalene catabolic pathways of a few strains. These include *Pseudomonas* species strains DBT2 (Monticello *et al*, 1985), A3 (Brilon *et al*, 1981), and C22 (Brilon *et al*, 1981), *Pseudomonas fluorescens* BS291 (Yen and Serdar, 1988), and *Rhodococcus* species strain B4 (Grund *et al*, 1992). The gentisate pathway is also known to be involved in the degradation of substituted naphthalenes. *Pseudomonas* sp. DS-1 (Ohe and Watanabe, 1988) and *Moraxella* sp. ASL4 (Wittich, *et al*, 1988) degrade 1,6- and 2,6-naphthalenedisulfonic acids through gentisate.

None of the above strain has been characterized at the molecular level. However, some comparisons between their naphthalene pathways and NAH7's can be made. Only two naphthalene-gentisate pathways, those of DBT2 (Monticello *et al*, 1985) and BS291 (Skyrabin, *et al*, 1980), have been associated with a plasmid. Regulation of naphthalene-gentisate pathways does not always conform to the NAH7 model. Neither 5-sulfosalicylate nor gentisate induces oxidation of 2,6-naphthalene disulfonic acid by ASL4 (Wittich, *et al*, 1988). Salicylate is unable to induce oxidation of naphthalene by strain B4 (Grund, *et al*, 1992). Salicylate can induce the upper pathways of JS150, A3 and C22, however (Brilon, *et al*, 1981). It is not known whether there is any cross-hybridization between NAH7 and the genes of any of the naphthalene-gentisate pathways discussed above.

Role in aromatic metabolism of monocyclic aromatic ring cleavage pathways involved in the degradation of naphthalene and salicylate

Catechol *meta* cleavage pathway

Catechol *meta* cleavage, in which cleavage is between carbons 2 and 3, is involved in the degradation of a wide variety of aromatic substrates. Because the *ortho* pathway cleavage mechanism is incompatible with degradation of 3- and 4-methyl catechols (Dagley, 1986), the *meta* pathway is commonly used to degrade methylated substrates, such as toluene, cresols, and xylenes (Dagley, 1986, Bayly and Barbour, 1978, Kukor and Olsen, 1991, Worsey and Williams, 1975, Gibson *et al*, 1968). Catechol *meta* cleavage is also the most common degradative pathway for monoaromatic rings produced by the cleavage of bicyclic substrates, such as naphthalene and biphenyl (Dagley, 1986, Bayly and Barbour, 1978, Yen and Serdar, 1988, Barnsly, 1976, Lloyd-Jones *et al*, 1994, Sanseverino *et al*, 1993, Taira *et al*, 1992). The *meta* pathway is also frequently employed for the catabolism of apparently *ortho*-cleavage compatible substrates such as phenol and benzene (Feist and Hegeman, 1969, Kukor and Olsen, 1991, Murray and Williams, 1974).

Curiously, the catechol *meta* pathway is rarely used to degrade exogenously provided benzoate, even though the *meta* pathway is used to degrade benzoate that is produced by metabolism of toluene and biphenyl. In fact, "benzoate curing" has been documented in strains carrying the TOL plasmid

pWWO and, recently, in the biphenyl catabolic plasmid pWW100 (Lloyd-Jones *et al*, 1994). These plasmids are unstable and undergo deletions of the catabolic genes or are lost completely during cultivation on benzoate, but are stable during cultivation on most other carbon sources. Apparently, cells that degrade benzoate via the *ortho* pathway have a substantial advantage over those that use the *meta* pathway. There must be an equally substantial, but unexplained, advantage to avoiding the induction of the *ortho* pathway during degradation of toluene or biphenyl. Otherwise, the *meta* pathways of pWWO and pWW100 would have been eliminated by natural selection. Because the *ortho* pathway is induced by *cis, cis,*-muconate (Ornston, 1966a) (see below), its induction can be prevented by a *meta* cleavage pathway that is sufficiently active to prevent accumulation of catechol (Cane and Williams, 1982).

Halogenated benzenes and benzoates are generally degraded through the *ortho* pathway rather than through the *meta* pathway. It has been shown by Bartels *et al* (1984) that cleavage of 3-halocatechols by catechol 2,3-dioxygenase results in the formation of a reactive aryl halide which irreversibly inactivates the enzyme that catalyzes its formation. This results in the accumulation of toxic halocatechols. Consequently, simultaneous exposure to toluene and either chlorobenzene or 3-chlorobenzoate can be lethal to toluene-degrading bacteria.

Degradation of multiple substrates through the catechol *meta* cleavage pathway can involve either a coordinate or a sequential induction scheme. A

common element of is that neither catechol nor any metabolite of catechol is able to induce the genes of the catechol *meta* cleavage pathway. The inducer is usually a primary substrate.

In a coordinately induced system, structurally similar substrates are degraded through the same pathway using the same broad-specificity enzymes. Those substrates which can serve as sole carbon sources are presumably all able to interact with the same broad-specificity regulatory protein. This scheme is exemplified by the *tod* operon of *Pseudomonas putida* F1 (Gibson *et al*, 1968). The *tod* operon encodes degradation of toluene and benzene through the *meta* cleavage or 3-methylcatechol and catechol, respectively (Gibson *et al*, 1968, Rodgers and Gibson, 1977, Gibson *et al*, 1990). Benzene and toluene are both able to induce the *tod* operon (Finette and Gibson, 1988). The toluene dioxygenase that catalyzes the first step in the *tod* pathway can also oxidize phenol to catechol. F1 cannot use phenol as a sole carbon source, however, due to the inability of phenol to serve as an inducer (Gibson *et al*, 1968).

In a sequentially induced system, a series of two or more operons in which the product(s) of the pathway encoded by one operon are the inducers and substrates of another. Sequential induction allows the organism to use several substrates as carbon sources, but does not require a single enzyme or regulatory protein to recognize every substrate.

Such a scheme was recently demonstrated in *P. pickettii* PKO1. Strain PKO1 can utilize benzene, toluene, *m*-cresol, and phenol as sole carbon and

energy sources. The pathway through which these substrates are degraded is composed of three operons of structural genes. A toluene 3-monooxygenase encoded by *tbuABC* is induced by benzene, toluene, ethylbenzene, and *m*-cresol (Olsen *et al*, 1994). The products of the reaction catalyzed by TbuABC, phenol and *m*-cresol, induce phenol hydroxylase and the *meta* cleavage operon (Kukor and Olsen, 1990, Kukor and Olsen 1991). Thus, the *meta* operon is used for growth on four substrates, even though its regulatory protein, TbuS, recognizes only two of them.

Sequential induction is also seen in the pathway encoded by the TOL plasmid. The products of the upper pathway operon, *xylCAB*, oxidize toluene and *m*-xylene to benzoate and *m*-toluate. The enzymes encoded by the lower pathway operon, *xylDLEGF*, degrade benzoate and *m*-toluate through the *meta* cleavage pathway (Worsey and Williams, 1975). It has been shown that *m*-xylene and *m*-methylbenzyl alcohol (an upper pathway intermediate), by interacting with the regulatory protein *xylR* , induce *xylCAB* (Inouye *et al* 1983) and *xylS* (Inouye *et al*, 1987a, Ramos *et al*, 1987). When expressed at the induced level, *xylS* is capable of inducing *xylDLEGF* (Inouye *et al* 1987b, Inouye *et al*, 1983, Mermod *et al*, 1987). When expressed at the uninduced level, *xylS* must interact with its substrate, *m*-toluate, in order to significantly induce *xylDLEGF* (Inouye *et al*, 1981). Thus, the upper pathway substrates can induce all the enzymes required for their degradation. Lower pathway substrates also induce the genes required for their degradation without inducing unnecessary

upper pathway genes. Although *xylDLEGF* can be induced by all the substrates of the TOL system, the XylS protein does not have to recognize all of them.

The NAH7 system, although coordinately induced has some of the advantages of a coordinate induction system. Both the upper pathway and lower pathway operons are induced by salicylate, the product of the upper pathway, rather than by naphthalene. This allows both naphthalene and salicylate to serve as sole carbon sources, while requiring NahR to recognize only salicylate, not naphthalene. The disadvantage of this form of coordinate induction is that during growth on salicylate results in the superfluous induction of the *nah* operon.

The catechol *ortho* cleavage pathway

The type I *ortho* pathway is mainly used to degrade substrates which have neither methyl nor halo substitutions. This chromosomally encoded pathway is widespread among Gram positive and Gram negative bacteria and is possessed by most *Pseudomonas* strains. It is used for the degradation of benzoate (Feist and Hegeman, 1969, Kemp and Hegeman, 1968, Ornston 1966b), anthranilic acid (Kemp and Hegeman, 1968), tryptophan (Palleroni and Stanier, 1964), and, in some cases, phenol (Ehrt *et al*, 1994, Fewson, 1967).

Unlike the *meta* pathway, the catechol *ortho* pathway is induced by a ring cleavage product. *cis, cis*-muconate has been shown to be the inducer of *catA* (type I catechol 1,2-dioxygenase), and *catBC* (type I *cis, cis*-muconate

lactonizing enzyme and muconolactone isomerase) in both *P. aeruginosa* (Kemp and Hegeman, 1968) and *P. putida* (Ornston, 1966b) and of the *catBCEFD* operon in *Alcaligenes calcoaceticus* (Neidle *et al*, 1989). In *P. putida*, the functions of *catE*, *catF*, and *catD*, are carried out by genes of the protocatechuate pathway, which are induced by ß-ketoadipate (Ornston, 1966a).

Phenol, benzoate, anthranilate, and tryptophan are often degraded through the catechol *ortho* cleavage pathway. In all cases, the genes encoding degradation of the substrate to catechol are regulated independently of the genes of the catechol *ortho* cleavage pathway. Enzymes catalyzing the degradation of tryptophan to anthranilate are induced by L-kynurenine, a pathway intermediate (Palleroni and Stanier, 1964). Dioxygenases that convert phenol (Feist and Hegeman, 1969, Ehrt *et al*, 1994), benzoate (Feist and Hegeman, 1969, Kemp and Hegeman, 1968, Ornston 1966b), and anthranilate (Palleroni and Stanier, 1964) to catechol are induced by their own substrates. Catechol, regardless of its origin, is then degraded through the *ortho* pathway as described above.

An evolutionarily related pathway, the type II or modified *ortho* pathway, has an expanded substrate range (Schmidt and Knackmuss, 1980a and 1980b). Ring cleavage is accomplished by a broad-specificity dioxygenase, catechol 1,2-dioxygenase type II, which, unlike the type I catechol 1,2-dioxygenase, is more active against 3- and 4-chlorocatechols than against unsubstituted catechol (Reineke and Knackmuss, 1980). Chloromuconate cycloisomerase was likewise

shown to have a broadened substrate specificity relative to muconate cycloisomerase (Schmidt and Knackmuss, 1980). Degradation of 3-chlorobenzoate by strain B13 proceeds through 3-chlorocatechol and 2-chloro-*cis,cis* -muconate, the chlorinated analogues of type I pathway intermediates. The remaining steps in the pathway differ, however. In the type I *ortho* pathway, muconolactone is converted to ß-ketoadipate enol-lactone, then cleaved to ß-ketoadipate by enol-lactone hydrolase. In the modified *ortho* pathway, a hypothetical chlorinated muconolactone is proposed to decay spontaneously, with concomitant chloride loss, to a *trans*-dienelactone. Cleavage of the dienelactone requires an enzyme, dienelactone hydrolase, which is not active against ß-ketoadipate enol-lactone (Schmidt and Knackmuss, 1980). Dienelactone hydrolase of pJP4 also fails to cleave the *trans*-2-chlorodienelactone generated by degradation of 3,5-dichlorocatechol, although it can cleave *cis*-2-chlorodienelactone. Consequently, degradation of 3,5-dichlorocatechol requires a fourth enzyme, *trans*-chlorodienelactone isomerase (Don *et al*, 1985). The dienelactone cleavage product, maleylacetate, is believed to be converted to ß-ketoadipate by a reductase that is not linked to the rest of the pathway (Don *et al*, 1985). Neither muconolactone isomerase nor enol-lactone hydrolase participates in the modified *ortho* pathway.

Substrates degraded through the modified *ortho* pathway include chlorobenzoates (Fetzner *et al*, 1992, Romanov and Hausinger, 1994, Dorn *et al*, 1974, Chatterjee *et al*, 1981), chlorophenols (Mal'tseva, *et al*, 1991),

chlorobenzenes (Reineke and Knackmuss, 1984, van der Meer *et al*, 1991c, Spain and Nishino, 1987), chlorosalicylates (Schindowski *et al*, 1991), 2,4-dichlorophenoxyacetic acid (Pieper *et al*, 1988), and 4-chlorotoluene (Haigler and Spain, 1989). The pathways utilized for the degradation of these substrates are similar to that used by B13, although variations in substrate range and predominate dienelactone isomer have been reported (Schlömann, *et al*, 1990).

The genes for three different modified *ortho* pathways have been cloned and sequenced (Perkins *et al*, 1990, Frantz and Chakrabarty, 1987, van der Meer *et al*, 1991a). In each case, the genes have been found to be plasmid-encoded and organized into a single operon. One or more separate operons encodes the degradation of a primary substrate to a chlorocatechol. Sequence analysis has shown that the modified *ortho* cleavage pathways from different plasmids are closely related to each other and have significant homology with the genes of the type I *ortho* cleavage pathway. Based on this relationship, which extends to the regulatory genes (van der Meer *et al*, 1991b, Coco *et al*, 1993), it has been assumed that chloro-*cis, cis*-muconates are the inducers of the modified *ortho* cleavage pathways, but this has never been demonstrated.

Gentisate cleavage pathways

The gentisate dioxygenases which have been studied can accommodate methyl and halo substituents at the 3 and 4 positions to varying degrees (Harpel and Lipscomb, 1990a, Crawford *et al*, 1975). However, they have a nearly

absolute requirement for the carboxyl group at position 1 and the hydroxyls at positions 2 and 5. Harpel and Lipscomb (1990a) tested the ability of gentisate dioxygenases from *C. testosteroni* and *C. acidovorans* for activity against gentisate analogs in which one of the three substituents was removed or replaced. Gentisate dioxygenase from *C. testosteroni* and *C. acidovorans* cleaved 5-aminosalicylate with a V_{max} that was 7% and 1%, respectively, of the its V_{max} for gentisate. Neither enzyme had any appreciable activity against any of the other analogs. The gentisate dioxygenase of strain OA3 (Crawford *et al*, 1975) was inactive against 6-methylgentisate, although it degraded 3- and 4-substituted methyl and halo-gentisates.

The ability of strain OA3 to metabolize substituted gentisates is limited by its maleylpyruvate isomerase, as well as by the specificity of the gentisate dioxygenase. The addition of reduced glutathione to partially purified cell extracts resulted in degradation of maleylpyruvate, but the substituted maleylpyruvates resulting from cleavage of 3- or 4- halogentisates were not degraded (Crawford *et al*, 1975).

Although the ability of gentisate dioxygenases to cleave 3- and 4-halogentisates suggests that degradation of haloaromatics through a gentisate dioxygenase pathway may be possible, no such pathway has yet been described. Otherwise, the substrate specificity observed for the gentisate dioxygenases studied by Harpel and Lipscomb is consistent with the observed range of substrates that are degraded through the gentisate pathway. The use

of the gentisate pathway by bacteria appears to be restricted to the degradation of naphthalene derivatives, aromatic acids, cresols, and xylenols, which are readily converted to gentisate or to 3- or 4- methylgentisate.

Only a few examples of the degradation of naphthalene through the gentisate pathway have been published. These are described above. Sulfo- and amino- substituted naphthalenes are also degraded through the gentisate pathway. Strain TA-2 degrades tobias acid (2-naphthylamine 1-sulfonic acid) and 1-naphthalenesulfonic acid to salicylate, which is metabolized through the gentisate pathway (Ohmoto *et al*, 1991). Pseudomonas sp. DS-1(Ohe and Watanabe, 1988) and Moraxella sp. ASL4 (Wittich, *et al*, 1988) degrade 1,6- and 2,6-naphthalenedisulfonic acids to 5-sulfosalicylate, which is converted to gentisate by a 5-sulfosalicylate-specific desulfonating 5-hydroxylase. An isolate from a 1-naphthylamine enrichment degraded 1-naphthylamine to 3-aminosalicylate (Babcock and Stenstrom, 1993). The isolate expressed a high level of gentisate dioxygenase activity after growth on 1-naphthylamine, suggesting that 1-naphthylamine and 3-aminosalicylate are degraded through the gentisate pathway.

The ability to degrade unsubstituted salicylate through gentisate is widespread among bacteria. It has been identified in members of the Gram negative genera *Pseudomonas* (Brilon *et al*, 1981, Haigler *et al*, 1992, Kuhm *et al*, 1991, Ohe and Watanabe, 1988) and *Comamonas* (Wheelis *et al*, 1967, Harpel and Lipscomb, 1990a) and in the Gram positive genera *Streptomyces*

(Grund *et al*, 1990), *Nocardia* (Hagedorn *et al*, 1985), *Bacillus* (Crawford and Olson, 1979), and *Rhodococcus* (Grund *et al*, 1992). In fact, Crawford and Olson (1979) found that within the genus *Bacillus*, degradation of salicylate through gentisate was more common than degradation of salicylate through catechol.

Like salicylate, *m*-hydroxybenzoate is degraded through gentisate in many bacteria. Catabolism of *m*-hydroxybenzoate through gentisate has been found among the Gram negative genera *Pseudomonas* (Hagedorn *et al*, 1985, Hopper and Chapman, 1970, Stolz *et al*, 1992, Sugiyama *et al*, 1958), *Alcaligenes* (Hagedorn *et al*, 1985), *Acinetobacter* (Hagedorn *et al*, 1985), *Salmonella* (Goetz and Harmuth, 1992), *Comamonas* (Wheelis *et al*, 1967, Harpel and Lipscomb, 1990a) and *Klebsiella* (Jones and Cooper, 1990), and in the Gram positive genera *Amycolatopsis* (Grund *et al*, 1990), *Streptomyces* (Grund *et al*, 1990), *Bacillus* (Hagedorn *et al*, 1985), *Nocardia* (Hagedorn *et al*, 1985), *Corynebacterium*, (Hagedorn *et al*, 1985), and *Arthrobacter* (Hagedorn *et al*, 1985). Yano and Arima (1958) showed that bacteria which degrade *m*-hydroxybenzoate through gentisate are readily isolated from soil. In a screening of 133 unidentified isolates, 24 isolates were shown to produce gentisate during growth on *m*-hydroxybenzoate.

A novel benzoate pathway on which *m*-hydroxybenzoate and gentisate are both intermediates was recently reported in *Pseudomonas* sp. KB740 (Altenschmidt, *et al*, 1993), which is also capable of growing on benzoate under

denitrifying conditions. Under aerobic conditions, strain KB740 degrades benzoate via benzoyl-CoA ligase, which is normally associated only with anaerobic degradation of aromatic acids. *m*-hydroxybenzoyl-CoA and gentisate were both formed from benzoate by cell-free extracts of KB740 cells that were grown aerobically on benzoate. Benzoate-grown cells can transform gentisate without a lag, but there is no information on gentisate degradation products. A complete gentisate pathway clearly exists in this strain, since gentisate is used as sole carbon and energy source. *m*-hydroxybenzoate is apparently degraded through the same pathway via a *m*-hydroxybenzoate-CoA ligase. A similar pathway was proposed for the degradation of salicylate by *Rhodococcus* sp. B4 (Grund *et al*, 1992).

Bacteria which grow on cresols or xylenols through *m*-hydroxybenzoate and gentisate intermediates have been reported. Examples are a *Pseudomonas putida* (NCIB 9869) and a *Pseudomonas alcaligenes* (NCIB 9867) isolated by Hopper and Chapman (1970). Both strains grow on *m*-cresol (1-hydroxy-3-methylbenzene) and 3,5-xylenol (1-hydroxy-3,5-dimethylbenzene). The *P. alcaligenes* strain also grows on 2,5-xylenol. The pathway starts with oxidation of the 3-methyl group to a carboxyl group, yielding *m*-hydroxybenzoate or a methyl-substituted *m*-hydroxybenzoate. A second hydroxyl is then added *para* to the first hydroxyl group, yielding either gentisate or 3- or 4- methylgentisate. An interesting feature of NCIB 9867 is the presence of two maleylpyruvate hydrolases, two gentisate dioxygenases, two *m*-hydroxybenzoate 6-

hydroxylases, and an apparently unnecessary fumarylpyruvate hydrolase (Poh and Bayly, 1980). The isofunctional enzymes differ in their substrate ranges and regulation. There is evidence that strain NCIB 9867 has an incomplete plasmid-encoded *m*-hydroxybenzoate pathway and a complete chromosomally encoded cresol /xylenol pathway (Poh and Bayly, 1980, Tham and Poh, 1993).

Harpel and Lipscomb (1990a) report that *Pseudomonas testosteroni* ATCC 49249 may use the gentisate pathway to grow on the *m*-hydroxybenzoate analog *m*-anisate (3-*O*-methylbenzoate). Crude extracts of cells grown on *m*-anisate contain a high level of gentisate dioxygenase. 5-*O*-methylsalicylate, which can be generated by hydroxylation of *m*-anisate, is also a growth substrate and an inducer of gentisate dioxygenase activity. 5-*O*-methylsalicylate is not cleaved either by purified gentisate dioxygenase or by cell-free extracts of *m*-anisate or 5-*O*-methylsalicylate -grown cells. It was therefore suggested by Harpel and Lipscomb that 5-*O*-methylsalicylate is an intermediate between *m*-anisate and gentisate. Although their proposed pathway is plausible, there is not yet adequate evidence to support it. No proposed intermediate nor any of the enzymes involved in converting *m*-anisate to gentisate has been detected in an *m*-anisate-grown culture.

The organization of the gentisate pathway has not yet been determined for any strain, and very little information is available concerning its regulation. Most gentisate- degrading strains that have tested are able to use gentisate as a sole carbon source, indicating that either gentisate or one of its metabolites is

able to induce the gentisate catabolic genes (Altenschmidt, *et al*, 1993, Goetz and Harmuth, 1992, Hopper and Kemp, 1980, Jones and Cooper, 1990, Poh and Bayly, 1980, Stolz *et al*, 1992, Wheelis *et al*, 1967, Wittich *et al* 1988). In *Klebsiella pneumonia* M5a1 (Jones and Cooper, 1990), normal induction was observed in a maleylpyruvate isomerase mutant, indicating that fumarylpyruvate was not the inducer of the gentisate pathway. In the other strains, no experiments have been done to distinguish between induction by gentisate and induction by one of its metabolites.

In some strains, growth on gentisate induces the degradative enzymes of gentisate precursors, as well as inducing gentisate dioxygenase. Growth of *Salmonella typhimurium* TA1530 (Goetz and Harmuth, 1992), *Comamonas acidovorans* strain 14 (Wheelis *et al*, 1967), and *K. pneumoniae* M5a1 (Jones and Cooper, 1990) on gentisate led to the induction of *m*-hydroxybenzoate 6-hydroxylase activity. NCIB 9869 (Hopper and Kemp, 1980) induced *m*-hydroxybenzaldehyde and *m*-hydroxybenzyl alcohol dehydrogenase activities after growth on gentisate.

In other strains, the gentisate cleavage enzymes are induced separately from degradative enzymes of gentisate precursors. Oxidation of sulfonated naphthalenes is not induced by gentisate in strain A3, C22 (Brilon *et al*, 1981), or the *Moraxella* sp. of Wittich *et al* (1988). The ability of these three strains to grow on gentisate is evidence that gentisate or one of its metabolites can induce the enzymes of the gentisate pathway. This differential regulation of sulfo-

naphthalene dioxygenase and gentisate pathway genes indicates that the genes of sulfonated naphthalene degradation in the above three strains are organized into at least two operons. Salicylate is able to induce the sulfonaphthalene dioxygenases of strains A3 and C22, as in the standard naphthalene pathway.

A DNA fragment encoding xylenol methyl hydroxylase, *m*-hydroxybenzoate 6-hydroxylase, and gentisate dioxygenase activities has been cloned from NCIB 9867 (Tham and Poh, 1993). A vector sequence containing a transcription terminator and translation stop codons blocked expression of xylenol methyl hydroxylase and *m*-hydroxybenzoate 6-hydroxylase in the original clone, but gentisate dioxygenase expression was merely reduced. Deletion of the terminator and stop codons resulted in expression of xylenol methyl hydroxylase and *m*-hydroxylbenzoate 6-hydroxylase and elevated expression of gentisate dioxygenase. The differential effect of the terminator and stop codons on expression of the three genes indicates that gentisate dioxygenase is expressed from a different promoter than xylenol methyl hydroxylase and *m*-hydroxylbenzoate 6-hydroxylase.

***Burkholderia cepacia* JS1 and its mutants**

B. cepacia JS1 was isolated by Spain and Nishino (1987) from a *p*-dichlorobenzene enrichment of sewage treatment plant samples collected from Tyndall Air Force Base and nearby Panama City, Florida. It was found to have the ability to grow on or cometabolize a large number of aromatic compounds.

Aromatic compounds that can serve as sole carbon sources include chlorobenzene, *p*-dichlorobenzene, toluene, benzene, phenol, benzoate, *m*- and *p*- hydroxybenzoate, naphthalene, and salicylate (Spain, 1990). Mutants of JS1 have been isolated which can grow on *o*-, *m*-, and *p*- chlorobenzoate and on *p*-chlorotoluene (Haigler and Spain, 1989). Because of its broad substrate range, JS1 should be valuable for the study of bacterial degradation of complex wastes.

JS1 has a mucoid phenotype, which can interfere with some types of experiments, such as bioreactor work, probing, sonication, and cell harvesting. The non-mucoid mutant JS6 was therefore isolated following treatment with ethyl methanesulfonate (Spain and Nishino, 1987). Another non-mucoid mutant, JS150, was isolated from JS1 when it was discovered that JS6 has lost the ability to grow on naphthalene and salicylate (Haigler *et al*, 1992). JS150 was found to retain all known degradative capabilities of JS1, including the ability to grow on naphthalene and salicylate.

JS150 utilizes at least five monoaromatic ring cleavage pathways, type I and type II catechol *ortho*, catechol *meta*, gentisate, and protocatechuate *ortho* cleavage pathways. Early work with JS6 showed that it uses the type II catechol *ortho* pathway to degrade chlorobenzene and *p*-dichlorobenzene (Spain and Nishino, 1987). Toluene is degraded exclusively through a *meta* cleavage pathway which has, not surprisingly, a high activity against 3-methylcatechol (Haigler and Spain, 1989). Spain and Nishino (1987) report that JS6 induces both type I catechol 1,2-dioxygenase and catechol 2,3-dioxygenase during

growth on benzene, with catechol 1,2-dioxygenase being the dominant activity. Haigler *et al* (1992) on the other hand, report that JS150 primarily uses a catechol 2,3-dioxygenase to grow on benzene. The ratio of catechol-specific *meta* cleavage to 3-chlorocatechol specific *meta* cleavage activity is similar in benzene and toluene-induced cell extracts, suggesting that the same catechol 2,3-dioxygenase is involved in both pathways.

The way JS150 handles simultaneous exposure to chlorinated and methylated aromatics is of considerable interest from the standpoint of the degradation of complex wastes. As described above, such exposure results in inactivation of catechol 2,3-dioxygenase by chlorocatechols, delay of induction of the type II *ortho* pathway by misrouting chlorocatechols away from proper cleavage into the pathway inducer, and subsequent accumulation of toxic chloro- and methyl-catechols. JS6 is able to grow on toluene and chlorobenzene simultaneously by degrading both chloro- and methyl-catechols through the type II *ortho* pathway. However, it adapts easily to chlorobenzene-toluene mixtures only if the type II *ortho* pathway was induced prior to exposure to the mixture. The problem is that in the presence of toluene and chlorobenzene the induction of the type II *ortho* pathway is much slower than induction of the enzymes of the toluene pathway. During this lag (three hours in the chemostat experiment of Pettigrew *et al* (1991)), *meta* cleavage is inactivated, *ortho* cleavage is uninduced, and substituted catechols temporarily accumulate to toxic levels. Oddly, JS6 and JS150 have both been shown to express a significant amount of

meta cleavage activity during growth on chlorobenzene. This *meta* cleavage activity, possibly the result of cross-induction of toluene pathway enzymes by chlorobenzene, reduces the efficiency of growth on chlorobenzene. This was demonstrated through studied of a JS6 mutant, JS62, which lacks a functional catechol 2,3-dioxygenase and uses only the type II *ortho* pathway for growth on toluene. JS62 grows to a higher steady-state density and expresses a higher type II catechol 1,2-dioxygenase specific activity in chemostat culture on chlorobenzene than does JS6 (Pettigrew *et al*, 1991).

A possible explanation for the retention of functional *meta* pathway genes by JS6 is the evident superiority of the JS6 *meta* pathway for growth on toluene. The cell density of a chemostat culture of JS6 increases slightly when switched from chlorobenzene to toluene. The density of JS62, however, drops considerably when switched from chlorobenzene to toluene (Pettigrew *et al*, 1991).

Chemostat studies with JS150 (Haigler *et al*, 1992) show that it can grow on mixtures of several aromatic compounds, provided that the culture is first brought to steady state on chlorobenzene. In one chemostat experiment, JS150 degraded a mixture of chlorobenzene, benzene, toluene, 4-chlorotoluene, 1,4-dichlorobenzene, and naphthalene. Less than 4% of each substrate in the influent was recovered in the effluent, indicating that none of the substrates was significantly repressing the degradation of any other. No metabolites were detected in the effluent. The ability of JS150 to simultaneously degrade complex

mixtures of aromatics makes it a promising candidate for bioremediation of complex wastes. The ease with which its substrate range can be expanded through mutation (Haigler *et al*, 1992, Haigler and Spain, 1989), and possibly through the introduction of cloned pathway fragments, increases its potential usefulness. In order to fully understand and exploit this strain, it is important to characterize each of its aromatic degradative pathways.

3. MATERIALS AND METHODS

Preparation of genomic DNA

Genomic DNA was prepared according to the method of Murray and Thompson (1981). A 100 ml was grown overnight in YEPSS broth (0.2 g /l yeast extract, 1.0 g /l peptone, 0.5 g/l sodium salicylate, 2.7 g/l sodium succinate, 0.2 g/l NH_4NO_3, pH 7.0) then harvested by centrifugation at 10,000 RPM. Cells were resuspended in 9.5 ml TE and incubated with 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K for 1 hour at 37°C. 1.8 ml of 5 M NaCl was added and mixed by inversion, followed by 1.5 ml of a solution containing 10% hexadecyltrimethyl ammonium bromide (CTAB) and 0.7 M NaCl. The lysate was then incubated 20 minutes at 65°C and extracted with an equal volume of a 24:24:1 mixture of phenol, chloroform , and isoamyl alcohol. The supernatant was collected and extraction was repeated two more times. The supernatant was then extracted once with 24:1 chloroform/ isoamyl alcohol. DNA was precipitated by addition of 0.6 volumes of isopropanol. Precipitated DNA was spooled on a sealed Pasteur pipette and washed with 70% ethanol. The crude DNA was resuspended in 8ml TE buffer and purified by CsCl ultracentrifugation.

CsCl ultracentrifugation

CsCl ultracentrifugation was performed according to Sambrook, *et al* (1989). Eight ml of crude DNA solution was mixed with 8 g of CsCl and the

volume was adjusted to 10 ml by the addition of TE. The solution was transferred to a 15ml Quick-seal™ ultracentrifuge tube (Beckman) along with 8 mg of ethidium bromide in a volume of 0.8 ml. The tube was topped off with light mineral oil, sealed, and centrifuged for 16 hours at 55,000 rpm in a Beckman model L8-80M ultracentrifuge with a Ti-80 fixed-angle rotor. Genomic DNA (upper band) was recovered from the centrifuge tube with a 3 ml syringe fitted with an 18 ga. needle. Ethidium bromide was removed from the DNA solution by repeated extraction with 2 volumes of water-saturated *sec*-butanol. DNA was precipitated from the aqueous phase by the addition of 2 volumes of sterile deionized water and 6 volumes of absolute ethanol, followed by centrifugation in a JA-20 rotor (Beckman) at 20,000 RPM for 20 minutes at 4°C . Precipitated DNA was washed once with 70% ethanol, dried under vacuum, and resuspended in a minimal volume of TE. DNA solutions were stored at -20°C.

Preparation of slot blots

Total DNA was denatured by boiling 100 µl of the DNA with 400 µl of 0.5M NaOH for 10 minutes. A biodot SF slot blot apparatus (Bio-Rad, Melville, NY) was used to filter the solution through a Biotrans™ nylon membrane (ICN Biochemicals, Cleveland, Ohio) that had been pre-moistened with 0.4 M NaOH. DNA solution was drawn through the filter under a vacuum of 40 cm of H_2O. The DNA solution was followed with two washes of 500 µl of 0.4 M NaOH. The filters

were then baked for 1 hour at 80°C and neutralized by washing in 2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate).

Preparation of colony blots

Colony blots were prepared according to the method recommended by ICN Biomedicals. Biotrans™ nylon membranes were placed over colonies growing on agar plates for 1 minute. Bacteria adhered to filters were lysed by incubating filters colony-side up in 2 ml of denaturing solution on plastic wrap for 5 minutes. Denaturing solution contained 1.5M NaCl and 0.5 N NaOH. Filters were neutralized by incubation for 5 minutes in 2 ml of 3 M sodium acetate, pH 5.5. Filters were blotted on filter paper and allowed to air dry, then baked at 80°C for 1 hour to fix the DNA to the filter. Filters were stored in plastic bags at room temperature before being probed.

Preparation of radiolabeled gene probes

Gene probes were prepared by asymmetric PCR. Templates were produced by amplifying the desired fragment from the source plasmid by standard PCR as described below. This fragment was reamplified twice to minimize the quantity of extraneous DNA. Radiolabeled probe was synthesized under the same conditions as were used for standard PCR except that one primer was eliminated from the reaction mix and the dCTP was substituted with an equimolar amount of α-^{32}P-dCTP.

DNA-DNA hybridization

Hybridizations were done according to the method of Church and Gilbert (1984). Membranes were incubated in prehybridization solution (0.5 M Na_2HPO_4, 1mM EDTA, 7% SDS, pH 7.2) for one hour at 65°C in a shaking water bath. Radiolabeled probe was added to the prehybridization solution and incubation was continued overnight. The membrane was washed three times with high stringency wash buffer (0.59 g of NaCl, 2.42 g of Tris, 0.37 g of EDTA, and 5 g of SDS per liter, pH 7.2). Blots were incubated with X-ray film (Kodak XRP-5 or AR, Eastman Kodak, Rochester, NY) at -70°C with intensifier screens. Film was developed after an overnight, and if necessary, a 3-day exposure.

Polymerase chain reaction (PCR)

PCR was performed with the GeneAmp® PCR kit (Perkin Elmer Cetus, Norwalk, CT) and a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). Each amplification cycle consisted of 1 minute at 94°C (melting), 1 minute at 55°C (annealing), and 2-3 minutes at 72°C (extension). Thirty-eight amplification cycles were used for each program. The amplification cycles were preceded by a 5 minute melting phase at 94°C and followed by a 7 minute final extension at 72°C. When the product of a PCR reaction was used as a template, 1µl was always used. All primers were synthesized by Genosys (The Woodlands, TX). The sequences of the *nah*Ac primers were: CCCTAGCGCGTAACTACCC and GGTCCAGACCTCGGTGGTG. These sequences flank a 1011 bp region which

spans the 5' region of *nah*Ac (ISP-α). The sequences of the primers used for amplification of the *nahG* probe are: GCGCATCGGTATCGTCGGCGGCGG and ACGATTTTTTCCGAATTCTGCGG. The first primer is taken from the sequence of *nahG* published by Schell (1986) and the second primer is taken from the sequence of the insertion element of composite transposon Tn4431 (Schmitt *et al*, 1981). These primers amplify a 300 bp fragment from the *nahG* gene of strain 5RL, which has been inactivated by an insertion of Tn4431 (King, *et al*, 1990). The sequences of the *nahR* primers are ATGGAACTGCGTGACCTG and TCAATTCTCTCTATCCTGCG. These primers amplify a 1125 bp fragment encompassing the entire *nahR* coding region reported by You *et al* (1988). Schell and Sukordhaman (1989) report a significantly shorter coding sequence for *nahR,* which more closely matches the size predicted by the analysis of the NahR protein. Assuming that the sequence of Schell and Sukordhaman is correct, the *nahR* probe extends approximately 285 base pairs downstream from *nahR.*

Cultivation of strains

B. cepacia JS1 and JS150 were routinely cultivated on YEPSS agar containing (per liter) 0.2 g of yeast extract, 2 g of peptone, 0.5 g of sodium salicylate, 2.7 g of sodium succinate, 0.2 g of NH_4NO_3, and 15 g agar. The pH was adjusted to 7.0 with 1 N NaOH. Plates were incubated 2 days at 25°C.

Time-course analysis of salicylate metabolism

A single colony from a YEPSS plate was inoculated into 100 ml of YEPSS broth in a 250 ml flask and incubated overnight at 25°C on a rotary shaker at 150 rpm. The seed culture was harvested by centrifugation, washed twice with PBS (8.0 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4), and resuspended in 100 ml PBS. Two ml quantities of basal salts medium + 40 mM sodium salicylate was dispensed into 25 ml vials (Pierce Chemical Co., Rockford, Ill.). Basal salts medium contained (per liter) 4.0 g of $NaNO_3$, 1.5 g KH_2PO_4, 0.0005 g of $FeCl_3$, 0.2 g $MgSO_4$, 0.01 g $CaCl_2$, and 0.5 g Na_2HPO_4. Each vial was inoculated with 20 µl of washed seed culture suspension and incubated at room temperature with shaking at 120 rpm. Control vials were killed immediately after inoculation by the addition of 0.5 ml 2N H_2SO_4. Two killed controls were sacrificed immediately. Two live vials and 1 killed control were sacrificed after 1, 2, and 5 days of incubation. Metabolic activity in live vials was stopped by addition of 0.5 ml 2N H_2SO_4. Culture supernatants were filtered through 0.2 µm filters to remove cells. Cell-free acidified culture supernatants were analyzed by HPLC as described below.

Identification of metabolites of naphthalene and salicylate

Two hundred fifty ml seed flasks containing 100 ml of basal salts medium + 10 mM sodium succinate were inoculated with isolated colonies from YEPSS plates and incubated at 25°C on a rotary shaker at 150 rpm. Turbid seed

cultures were harvested by centrifugation, washed twice with PBS and resuspended in 100 ml of PBS to be used as inocula.

Metabolite identification experiments were performed by growing cultures in 2 liter flasks containing 1 liter of basal salts medium supplemented with the appropriate carbon source. Sodium salicylate was added at a concentration of 5mM. Naphthalene was added as 1 g of solid crystals. Because the aqueous solubility of naphthalene is only 32 mg /l, undissolved crystals remained in the flask throughout the growth of the culture. This was done because of the anticipated difficulty of detecting readily degradable metabolites of a sparingly soluble substrate. Keeping the naphthalene at near saturation was expected to increase the concentrations of metabolites. Log phase cultures were harvested by centrifugation and supernatants were analyzed for metabolites.

Samples were acidified (pH<2) with concentrated H_2SO_4 and extracted three times with 100 ml ethyl acetate (Menn *et al*, 1993). The ethyl acetate extracts were pooled and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation and the residue was dissolved in methanol. Methanol solutions were transferred to amber glass bottles. The methanol was removed under nitrogen and the residue was stored at -20°C prior to analysis by gas chromatography-mass spectrometry (GC-MS).

Analytical techniques

HPLC was done according to the method of Sanseverino *et al* (1993). Sample volumes of 200 µl were injected onto a Supelcosil LC-18 column (Supelco, Bellefonte, Pa.). The column was eluted for 5 minutes with water (pH 2.5), followed by a linear gradient to 60% acetonitrile-40% water over 12 minutes. The flow rate was 2 ml per minute. Metabolites were detected with a photodiode array detector at a wavelength of 255 nm. Standard curves prepared from authentic samples of salicylic and gentisic acids were used for quantitation.

GC-MS analysis was done according to Menn *et al* (1993) using a Hewlett-Packard model 5995A with a model 18964A GC-MS capillary interface and a model 18965A flame ionization detector. A 0.22 mm by 12 m BP5 capillary column from SGE, inc., Austin, Tex. was used for the gas chromatography. Injection temperature was 50°C. Two minutes after injection, oven temperature was increased at a rate of 10°C /minute to a maximum temperature of 250°C. Mass spectrometry was carried out using electron impact mode at an ionization voltage of 70 eV.

Preparation of crude cell-free extracts

Cultures were grown on basal salts medium + appropriate carbon source as described above. Cultures were harvested in log phase, washed once with 3mM Tris buffer, and resuspended in 10 ml 33mM Tris buffer + 10% glycerol. Suspensions were transferred to scintillation vials for sonic disruption.

Sonic disruption was carried out with a Virsonic 300 ultrasonic cell disrupter model 175893 (VirTis Company, Gardiner, NY). A 1/2" diameter disrupter horn with a microtip was inserted into the cell suspension, which was kept on ice throughout the disruption procedure. Ten pulses of 30 second duration were applied with the probe amplitude set at 30-50% of maximum. Pulses were separated by 1 minute intervals to prevent overheating of the sample. Particulate material was removed by ultracentrifugation for 1 hour at 36,000g, 4°C. Supernatants were stored at -70°C until enzyme assays could be performed.

Enzyme assays

A unit of specific activity is defined as the amount of extract required to produce 1 µmol of product per minute per mg total protein. Specific activity units are calculated from the formula:

$$units = \frac{da}{dt}\left(\frac{V}{(\varepsilon_p - \varepsilon_s)P}\right)$$

where a = absorbance at assay wavelength, t is time in minutes, ε_p = the extinction coefficient of the reaction product in absorbance unit ml /µmol, ε_s = the

extinction coefficient of the reaction substrate, V= reaction volume in ml, and P is the protein concentration of the reaction mixture in mg /ml. Total protein concentration was measured by the BCA protein assay kit (Pierce, Rockford, IL). Spectrophotometric assays were carried out in a Beckman model DU ®-70. Assays were carried out in 1 ml quartz cuvettes with a pathlength of 1 cm. Reaction mixtures prepared without substrate were used as blanks. Reactions were started by adding substrate to blanks and quickly mixing by inversion. Absorbance was recorded by the spectrophotometer at 1-10 second intervals over a period of 1 minute.

The reaction mixture for the gentisate dioxygenase assay contained 20µl cell-free extract, 970 µl 100 mM phosphate buffer, pH 8.0, and 10 µl of 10mM gentisic acid made up in 100 mM phosphate buffer, pH 7.0. Absorbance was measured at 334 nm, the absorbance maximum of maleylpyruvate. The extinction coefficient (ε) of maleylpyruvate at 334 nm under assay conditions is 10.8 ml / µmol. The extinction coefficient of gentisate at 334 nm under assay conditions is 2.77 ml /µmol.

The reaction mixture for the catechol 1,2-dioxygenase assay contains 870 µl of 10mM Na-K phosphate buffer, pH 7.0, 100 µl of 10 mM Na_2EDTA, 20 µl cell extract, and 10 µl of freshly prepared 10 mM catechol. Where a volume of cell extract greater than 20 µl was used, the volume of buffer was reduced to make a total volume of 1 ml. Absorbance was measured at 260 nm, the absorbance maximum of the reaction product, *cis*, *cis*-muconate. The extinction coefficient

for *cis, cis*-muconate is 16.8 absorbance unit ml /µmol (Feist and Hegeman, 1969). Catechol has negligible absorbance at 260 nm.

The reaction mixture for the catechol 2,3-dioxygenase assay contains 970 µl 50 mM Na-K phosphate buffer, pH 7.5, 20 µl cell extract, and 10 µl freshly prepared 10 mM catechol. Absorbance was measured at 375 nm, the absorbance maximum of the reaction product, α-hydroxy-muconic semialdehyde. The extinction coefficient for α-hydroxy-muconic semialdehyde is 29.4 absorbance unit ml /µmol (Feist and Hegeman, 1969). Catechol has negligible absorbance at 375 nm.

The reaction mixture for the protocatechuate 3,4-dioxygenase assay contains 965 µl of 66 mM Na-K phosphate buffer, pH 7.0, 20 µl cell extract, and 15 µl of 10 mM protocatechuic acid solution in assay buffer. Absorbance is measured at 270 nm, the absorbance maximum of the reaction product, ß-carboxy-*cis, cis*-muconate. The extinction coefficients of ß-carboxy-*cis,cis*-muconate and protocatechuate under assay conditions are 6.40 and 2.73 absorbance unit ml / µmol, respectively (Stanier and Ingraham, 1954).

Qualitative assays for *ortho* and *meta* cleavage

Cultures were grown in 50 ml of basal salts medium supplemented with a test aromatic carbon source at a concentration of 5 mM. Cells were harvested in log phase, washed twice in PBS, and resuspended in 4 ml of 0.02M Tris buffer (pH 8). Cell suspensions were divided into 2 ml portions and transferred to glass

tubes. Cells were permeabilized by adding 1 drop of toluene to each tube. Substrate (protocatechuate or catechol) was added to permeabilized cells as 200 µl of a freshly prepared 0.1 M aqueous solution. The presence of *meta* cleavage enzymes able to cleave the substrate results in the development of a yellow color within one minute (Stanier *et al*, 1966). Tubes were then incubated for 1 hour with shaking at 30°C. Protein from lysed cells was precipitated by saturation with ammonium sulfate. The Rothera test for *ortho* cleavage was then performed on the supernatant. The pH was brought up to approximately 10 by addition of 2 drops of 5 N ammonium hydroxide. The formation of a purple product following the addition of one drop of 25% sodium nitroprusside is a positive test for the presence of *cis,cis*-muconate or ß-carboxy-*cis,cis,*-muconate (Stanier *et al*, 1966).

4. RESULTS

DNA-DNA hybridization

JS150 colony blots were probed with a radiolabeled 1 kb fragment amplified from the *nahAc* (naphthalene dioxygenase iron-sulfur protein α subunit) gene of NAH7. Colonies of strain 5R, which carries the naphthalene catabolic plasmid pKA1 (Sanseverino, *et al*, 1993) and JS150 hybridized to this probe, while colonies of the control strain *Pseudomonas putida* PB2440 (Bagdasarian *et al*, 1981) failed to hybridize (figure 3B). This result indicates that JS150 contains a gene with a high degree of nucleotide sequence similarity to *nahA*. It is highly probable that this gene is the naphthalene dioxygenase of JS150.

JS150 colonies hybridized to the probe less strongly than the 5R colonies. This could be due to incomplete lysis of the JS150 colonies, lower *nahA* copy in JS150 number due to a chromosomal rather than plasmid location, or lower cell density in JS150 colonies than in 5R colonies.

JS150 colony blots were probed with a radiolabeled segment of the *nahG* (salicylate 1-hydroxylase) gene amplified from strain 5RL. Strain 5RL carries a NAH7- type naphthalene catabolic plasmid and has been shown to hybridize to NAH7 genes. Strain 5R colonies hybridized strongly to the *nahG* probe under high stringency conditions, but JS150 and PB2440 colonies failed to hybridize to the probe (figure 3A). The JS150 colony blot was reprobed with the *nahA* probe.

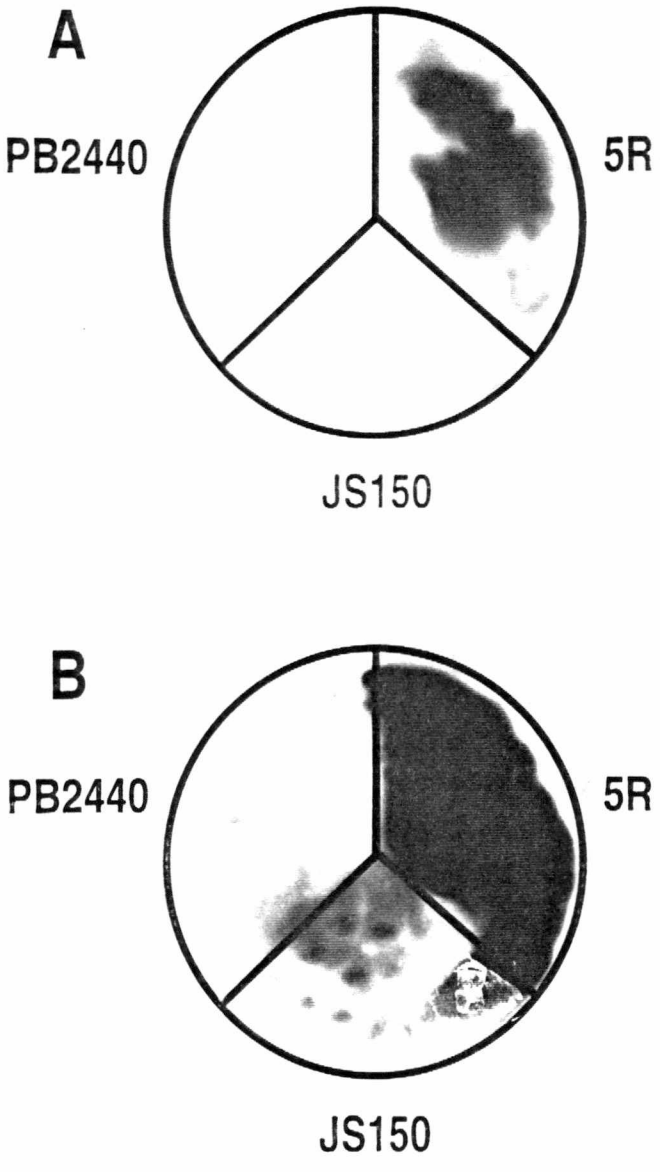

Figure 3. Probing of *B. cepacia* JS150 colonies with *nahG* and *nahA*. **A)** Strains JS150, PB2440, and 5R probed with *nahG*. **B)** The same blots reprobed with *nahA*.

The colony blot hybridized strongly to this probe (figure 3B). Reprobing the *nahG* negative blot with *nahAc* shows that the cells were lysed and that a sufficient amount of hybridizable DNA was present on the blot to permit detection of homologous DNA. The results show that JS150 does not contain a gene of sufficient similarity to *nahG* to hybridize under high stringency conditions. They do not , however, preclude the presence of a gene with some degree of similarity to *nahG* or of a salicylate 1-hydroxylase unrelated to *nahG*.

The NahR-salicylate complex induces transcription from both *pnah* and *psal* the promoters of the NAH7 upper and lower pathway operons, respectively. Since JS150 contains a *nahA* homologue but lacks a close *nahG* homologue it was of interest to determine whether JS150 hybridizes to a *nahR* probe. To rule out the possibility that a *nahR* homologue existed in JS1 but was lost from JS150 as a result of mutagenesis, JS1 genomic DNA was probed with a radiolabeled DNA fragment amplified from the *nahR* gene of NAH7 (figure 4). The slot blot technique was used because JS1 colony blots gave unreliable results. JS1 genomic DNA failed to hybridize to the *nahR* probe, but did hybridize to the *nahAc* probe, suggesting that the naphthalene pathway of JS1 and JS150 may be regulated by a different system than the naphthalene pathway of NAH7. It is also possible that JS150 has a *nahR* homologue which is further diverged from *nahR* than the *nahR* homologues typically found on naphthalene catabolic plasmids, although it seems unlikely that the sequences of *nahA* and *nahR* in JS150 would diverge from their NAH7 counterparts to a significantly different

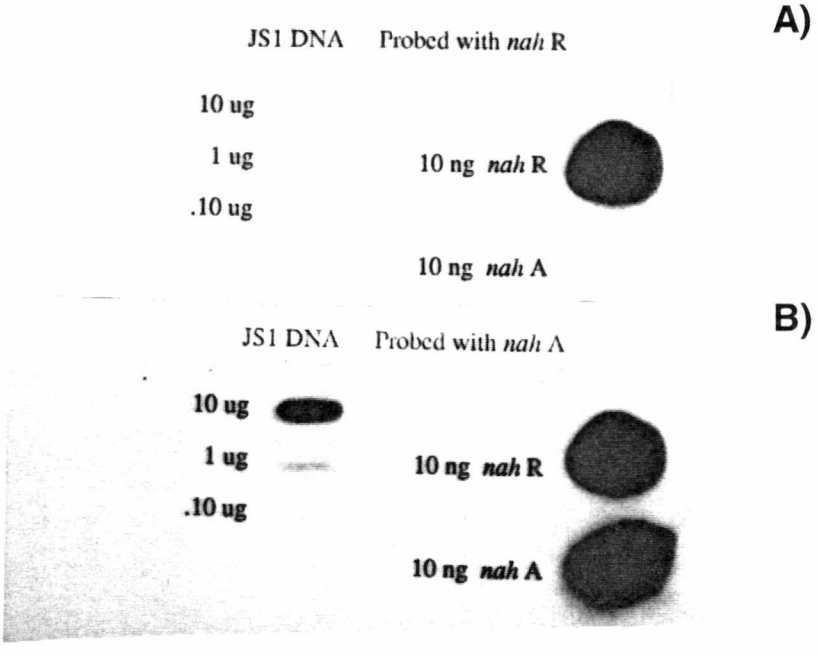

Figure 4. Probing of *B. cepacia* JS1 genomic DNA with *nahR* and *nahA*. **A)** JS1 genomic DNA probed with *nahR*. The control DNAs, PCR-amplified *nahR* and *nahA* fragments, are identical to the *nahR* and *nahA* probes. **B)** The same blot as in A), reprobed with *nahA*.

degree. Haigler *et al* (1992) showed that naphthalene oxidation by JS150 is inducible by growth on naphthalene or salicylate. JS150 must therefore have one or more functional naphthalene regulatory gene(s).

DNA-DNA hybridization results are summarized in table 1.

PCR amplification of JS150 DNA with *nahA* primers

B. cepacia JS150 total genomic DNA was used as the substrate for PCR amplification using *nahAc* primers (figure 5). The amplification was successful, resulting in a fragment of the expected size. This result supports the conclusion from hybridization data of the presence in strain JS150 of a *nahA* homologue.

Identification of metabolites

HPLC

Reverse-phase HPLC was used to follow the degradation of salicylate and appearance of polar metabolites (figure 6, table 2). A major peak with a retention time similar to that of gentisate was detected in samples incubated with viable JS150 cells for 1 day or more (figure 6B, figure 6C), but not in time zero samples (figure 6A) or controls incubated with acid- killed cells (figure 6D). The average retention time for this peak was 7.9 minutes, compared to a retention time of 7.6 minutes for the gentisate standard.

Table 1. Probing JS1 and JS150 genomic DNA with NAH7 genes

DNA-DNA Hybridization

Probe	Strain	
	JS1	JS150
nahA	Positive	Positive
nahG	Negative	Not done
nahR	Negative	Not done

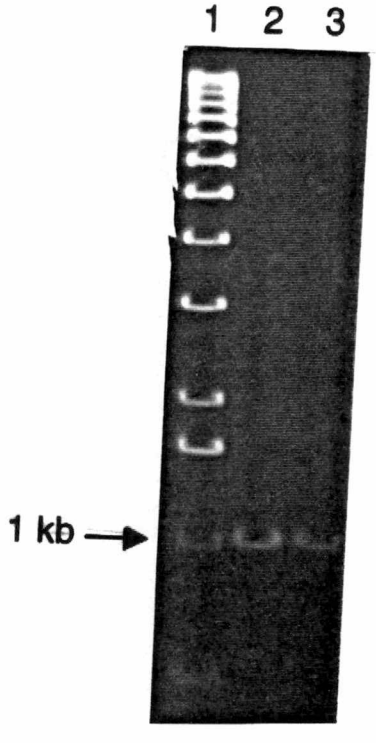

Figure 5. PCR amplification of *nahA* from JS150 DNA. Lane 1, 1 kb size marker; lane 2, amplification of JS150 DNA from *nahAc* primers; lane 3, amplification of NAH7 DNA using *nahAc* primers.

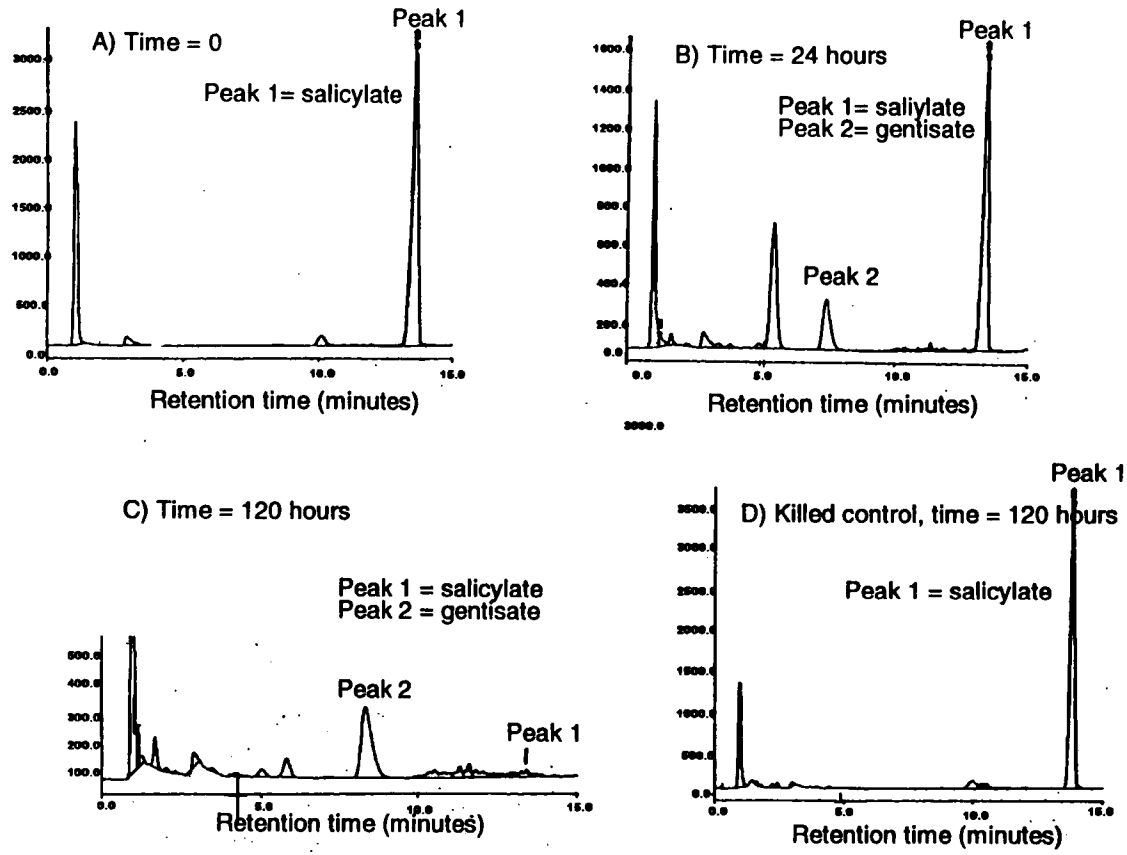

Figure 6. Representative HPLC tracings showing salicylate metabolites of *B. cepacia* JS150. Strain JS150 was grown on salicylate as sole carbon source. Extracts were acidified and separated on a LC-18 column. Metabolites were detected with a photodiode array detector at 255 nm.

Table 2. Salicylate degradation time course

Time (days)	Salicylate concentration (mM)	Salicylate concentration, killed control (mM)	RT of salicylate peaks (min.)	Gentisate concentration (mM)	RT of gentisate peaks (min.)
0	39.0	NA	13.6	no peak	no peak
1	16.3	39.9	13.5	4.2	7.5
2	0.6	37.8	13.5	5.8	8.1
5	0.4	32.3	13.7	4.7	8.3

Acidified supernatants of salicylate-grown cultures of *B. cepacia* JS150 were analyzed by HPLC. Figures for days 0-2 are averages of two replicates. Figures for 5 days are from a single sample.
RT, retention time
Retention time of salicylate standard = 13.7 minutes.
Average retention time of sample peaks identified as salicylate = 13.6 ± 0.2 minutes.
Retention time of gentisate standard = 7.6 minutes.
Average retention time of sample peaks identified as gentisate = 7.9 ± 0.5 minutes.

Gentisate accumulated as salicylate was degraded, its concentration dropped slightly after the salicylate was exhausted, and a significant quantity of gentisate remained undegraded at the end of the experiment. Twelve percent of the salicylate originally present in the cultures was present as gentisate after five days incubation, three days after the salicylate was exhausted. The most likely interpretation of this result is that gentisate cannot be metabolized in the absence of salicylate, which is consistent with the observation that gentisate cannot serve as a growth substrate for JS150. Dependence of gentisate degradation on the presence of salicylate could indicate that the degradative and /or transport genes for salicylate and gentisate are inducible only by salicylate and not by gentisate. However, because only one sample is available for the five day time point, it cannot be concluded with certainty that the concentration of gentisate in the culture medium decreases between days two and five. It is also uncertain that any gentisate degradation that did occur was biologically mediated. The possibility that JS150 is unable to take up or degrade gentisate even in the presence of salicylate can therefore not be ruled out on the basis of this experiment.

GC-MS

GC-MS was used to verify the identification of gentisate as a metabolite of both naphthalene and salicylate in JS150 and its parent strain JS1. JS1 was examined in order to rule out the possibility that the mutagenesis that led to the

isolation of JS150 did not result in major changes in naphthalene metabolism. Compounds with mass spectra matching those of gentisate and salicylate were detected in acidified organic extracts of supernatants of JS1 naphthalene cultures (figure 7B, figure 8B). A compound matching gentisate was also found in JS150 naphthalene cultures (figure 8C), although salicylate could not be detected. Gentisate was also identified in culture supernatant from a JS1 salicylate culture (data not shown). These results demonstrate that gentisate is a metabolite of salicylate during growth of JS150 and its parent strain JS1 on either naphthalene or salicylate.

Enzyme assays

Crude extracts of lysed JS150 cells grown on salicylate and several other carbon sources were tested for gentisate dioxygenase activity through a spectrophotometric assay for the formation of maleylpyruvate, the ring-cleavage product of gentisate. Gentisate dioxygenase activity was detected at an induced level in cells grown on salicylate (table 3). Detection of this activity is evidence that, despite the inability of exogenous gentisate to serve as a growth substrate for JS150, JS150 does have the ability to degrade gentisate when salicylate is present.

Gentisate dioxygenase activity in cells grown on salicylate in combination with glucose or succinate is induced to a significantly higher level than in cells grown on succinate or glucose alone, but to a lower specific activity than in cells

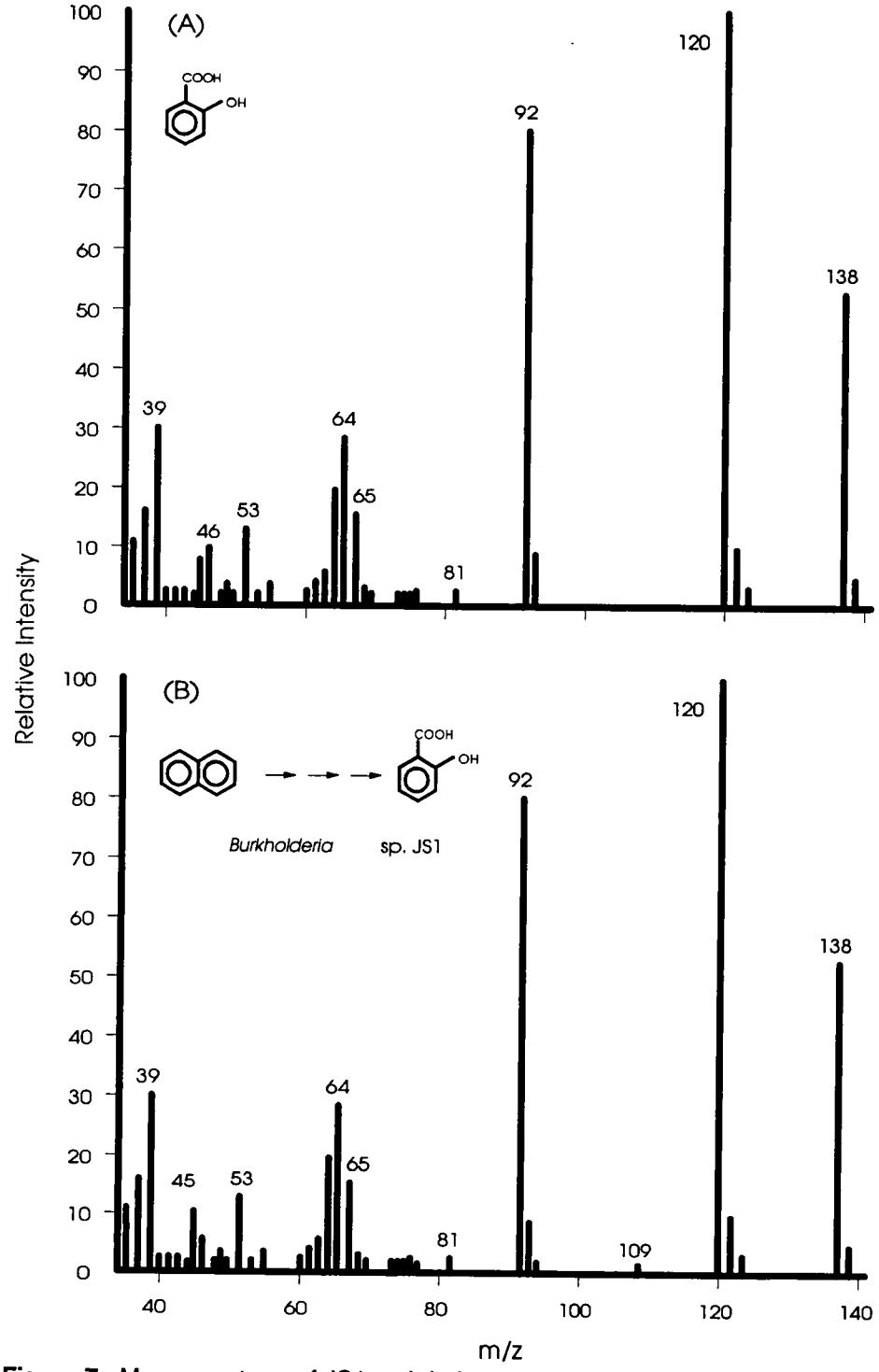

Figure 7. Mass spectrum of JS1naphthalene metabolite (B) compared to that of authentic salicylate (A).

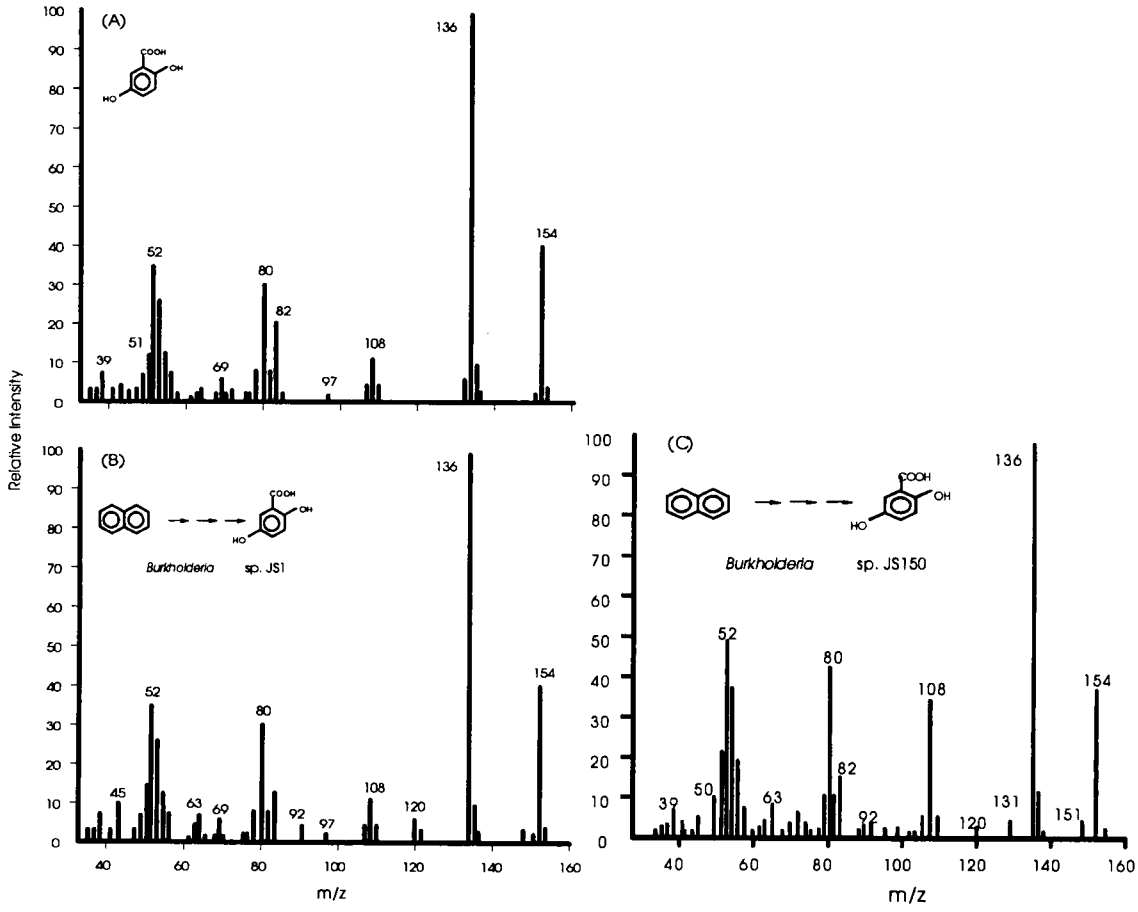

Figure 8. Mass spectrum of naphthalene metabolite from JS1(B) and JS150 (C) compared to authentic gentisate standard (A).

Table 3. Enzyme activities in crude cell extracts of *B. cepacia* JS150 grown on various carbon sources

	Specific activity[a] after growth with:							
Enzyme assayed	Salicylate	Glucose + salicylate	Succinate	Succinate + salicylate	Glucose + gentisate	Glucose	*m*-hydroxy-benzoate	anthranilate
Gentisate dioxygenase	.360 ± .070	.120 ± .030	None detected	.230 ± .010	.030 ± .002	.017 ± .007	.030 ± .002	.022 ± .0075
Catechol 2,3 dioxygenase	None detected	None detected	None detected	Not done	Not done	None detected	Not done	.015 ± .005
Catechol 1,2 dioxygenase	None detected	None detected	None detected	Not done	Not done	None detected	Not done	None detected
PCA 3,4-dioxygenase	Not done	Not done	None detected	Not done	Not done	Not done	.320 ± .040	Not done

[a] µmoles product per ml per minute per mg protein

grown on salicylate alone. This shows that gentisate dioxygenase is incompletely repressed by both glucose and succinate. Glucose appeared to repress gentisate dioxygenase more strongly than succinate. This conclusion cannot be made with certainty because only one experiment each with growth on glucose and glucose plus salicylate was done.

Extracts from salicylate- grown JS150 cells were assayed with spectrophotometric assays for catechol 1,2- and 2,3-dioxygenases to determine whether salicylate might be degraded through both gentisate and catechol. Neither of the above enzymes was induced during growth of JS150 on salicylate. Therefore, catechol cannot be a salicylate metabolite in JS150. The only salicylate catabolic pathways known to occur in bacteria under aerobic conditions proceed through either gentisate or catechol. It is therefore probable that JS150 degrades salicylate exclusively through the gentisate pathway.

Crude cell extracts of JS150 cultures grown on *m*-hydroxybenzoate were tested spectrophotometrically for gentisate dioxygenase and protocatechuate 3,4-dioxygenase. Protocatechuate 3,4-dioxygenase activity was present at a high level, but gentisate dioxygenase was only present at a basal level. *Ortho* cleavage of protocatechuate was also detected by the Rothera test in permeabilized JS150 cultures grown on either *m*-hydroxybenzoate or *p*-hydroxybenzoate. Permeabilized cells grown on either substrate failed to produce a visible yellow product from protocatechuate, indicating that significant *meta* cleavage of protocatechuate does not occur during growth on *m*- or *p*-

hydroxybenzoate. These results indicate that *m*-hydroxybenzoate, like *p*-hydroxybenzoate, is degraded through the protocatechuate *ortho* cleavage pathway, rather than through the gentisate pathway.

The structure of anthranilate is similar to that of salicylate. Anthranilate (*o*-aminobenzoate) has been reported to act as a gratuitous inducer of naphthalene catabolic genes that are induced by salicylate (Shamsuzzaman and Barnsley, 1974). Anthranilate-grown JS150 cultures were tested for induction of gentisate dioxygenase to determine if anthranilate could act as a gratuitous inducer or substrate of gentisate dioxygenase. Growth on anthranilate did not induce gentisate dioxygenase activity. Spectrophotometric assay of cell extracts also failed to detect induction of catechol 1,2- or 2,3-dioxygenase activities. This is unusual, since anthranilate metabolism in aerobic bacteria is almost always reported to proceed through the catechol *ortho* cleavage pathway. Lack of significant catechol *ortho* and *meta* cleavage activity was confirmed by permeabilized cell assays. Permeabilized anthranilate- grown cells failed to convert catechol to a yellow product or to a Rothera- positive product, indicating the absence of 2-hydroxymuconic semialdehyde and *cis, cis*-muconate, respectively. The above results show that anthranilate is degraded through an atypical, possibly novel, pathway in JS150.

Glutathione-dependence of the JS150 gentisate pathway

Maleylpyruvate isomerases are commonly dependent on reduced glutathione (GSH) (Hagedorn *et al*, 1985), but no known maleylpyruvate hydrolase has this requirement (Crawford and Frick, 1977). It was therefore of interest to test the gentisate pathway of JS150 for glutathione dependence. Crude cell extract from a salicylate- grown JS150 culture was incubated with gentisate and the reaction was followed spectrophotometrically (figure 9). The initial increase in absorbance at 334 nm results from the cleavage of gentisate to form maleylpyruvate. Failure of the OD_{334} to decrease indicates that either maleylpyruvate or fumarylpyruvate accumulates without being degraded. When reduced glutathione is added to the reaction, absorbance begins to decrease immediately. The glutathione-dependence demonstrated by this experiment indicates that JS150 probably uses the maleylpyruvate isomerase branch of the gentisate pathway (Crawford and Frick, 1977).

Substrate range of the JS150 gentisate pathway

The ability of JS150 to grow on several potential gentisate pathway substrates was tested (table 4). Of those that could serve as growth substrates for JS150, only naphthalene and salicylate were degraded through the gentisate pathway. This was somewhat surprising in view of the large number of aromatic substrates that serve as growth substrates for JS150 (Haigler, *et al*, 1992). Use of the gentisate pathway rather than the more common catechol *meta* cleavage

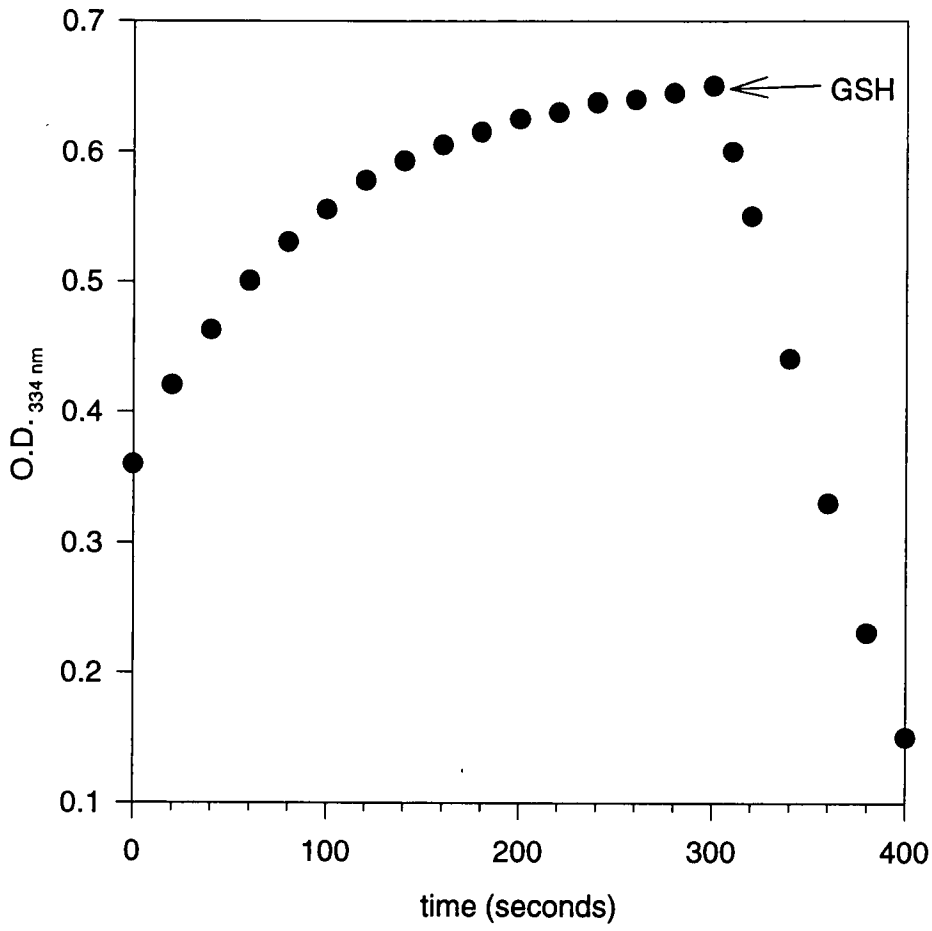

Figure 9. Glutathione-dependence of gentisate degradation in JS150. At time 0, the reaction was started by adding gentisate (final concentration 0.1 mM) to extract of salicylate-grown JS150 cells (40 µg/ml final protein concentration) in 0.1 M Na-K phosphate buffer, pH 7.0. Final volume was 1 ml. At the arrow, reduced glutathione was added to a final concentration of 0.1 mM in a volume of 10 µl.

Table 4. Substrate range of JS150 gentisate pathway

	Growth	Ring-cleavage pathway	Reference
Naphthalene	+	MPI [a]	This work, Haigler et al
Salicylate	+	MPI	This work, Haigler et al
Gentisate	-	MPI	This work
m-hydroxybenzoate	+	PCA 3,4[b]	This work
anthranilate	+	?[c]	This work, Haigler et al
4-chlorosalicylate	-	ND[d]	This work
3-methylsalicylate	-	ND	This work
4-methylsalicylate	-	ND	This work
Tryptophan	+	?	This work

[a] Gentisate pathway, maleylpyruvate isomerase branch.
[b] Protocatechuate *ortho* cleavage.
[c] Ring cleavage pathway present, but unknown.
[d] Occurance of ring cleavage not determined

pathway does not result in any apparent enhancement of metabolic versatility in JS150. It remains to be determined whether JS150 derives any other benefit from using the gentisate pathway to degrade naphthalene and salicylate.

5. DISCUSSION

Failure of JS150 to hybridize with *nahR* and *nahG* does not necessarily indicate that JS150 lacks homologues of these genes. However, the enzyme assays show that the function encoded by *nahG*, that of salicylate 1-hydroxylase, is not present to a detectable degree during growth on salicylate. Therefore, if a homologue of *nahG* is present in JS150, it does not encode a functional salicylate 1-hydroxylase.

Because naphthalene dioxygenase expression in JS150 is inducible by salicylate (Haigler *et al*, 1992), it cannot be said that JS150 lacks NahR function. Further investigation is needed to determine whether or not the regulatory gene(s) of the JS150 naphthalene pathway has significant similarity to *nahR*.

According to the model proposed by Yen and Serdar (1988), *nahG* and *nahR* constitute a "genetic module" of co-evolved genes which were acquired together by the NAH7 naphthalene catabolic pathway during its evolution. The genes encoding the catechol *meta* cleavage pathway and the upper pathway are also proposed to constitute independent modules. According to this model, *nahG* and its regulator, *nahR*, evolved together, then, after they became associated with the other two modules, the product of *nahR* acquired control over transcription of all the genes in the naphthalene pathway. Control over the *meta* pathway was acquired by fusion of the *meta* pathway operon to the *nahG* operon and loss of the original *meta* pathway promoter. The upper pathway

Demonstration that JS150 genomic DNA hybridizes with *nahA* and not with *nahG* or *nahR*, coupled with the absence of salicylate 1-hydroxylase or salicylate-induced *meta* cleavage activity, supports the genetic module hypothesis. The data reported in this thesis strongly suggest that strain JS150 contains a *nah* operon homologue that is not associated with any of the other elements of the NAH7 naphthalene catabolic pathway. It would be interesting to sequence the promoter of the JS150 *nah* operon homologue and to clone and sequence its regulatory gene. It is expected that the sequences of those elements should have little similarity to the sequences of *nahR* and *pnah*.

The research reported in this thesis shows that JS150 grows on naphthalene and salicylate through the gentisate pathway. There have been no previous reports of a strain using the gentisate pathway but being unable to use exogenous gentisate as a growth substrate. As discussed in the literature review, those strains which have been examined induce genes required for gentisate degradation, and sometimes also genes required for the metabolism of gentisate precursors, in the presence of gentisate. The gentisate pathway may be regulated in an unusual manner in JS150. A gentisate precursor, such as salicylate, could directly induce all the genes involved in salicylate degradation in JS150. Induction of the gentisate degradative genes by salicylate in JS150 is consistent with the restriction of the use of the gentisate pathway to the degradation of naphthalene and salicylate.

The results of the research reported here bring to seven the number of cleavage pathways possessed by strain JS150. These are: 1) the naphthalene upper pathway, 2) the gentisate pathway, 3) the catechol *ortho* cleavage pathway (Haigler *et al,* 1992), 4) at least one catechol *meta* cleavage pathway (Haigler, *et al,* 1992), 5) the modified *ortho* cleavage pathway (Spain and Nishino, 1987), 6) the protocatechuate *ortho* cleavage pathway, and 7) the unidentified pathway through which anthranilate is degraded. This is greater than the number required for the metabolism of the substrates JS150 is known to degrade. The presence of so many ring cleavage pathways in JS150 makes JS150 a good strain in which to investigate the relative efficiencies of few versus many ring cleavage pathways for the simultaneous degradation of multiple aromatic substrates.

In order to engineer strains for the degradation of pollutant mixtures, it is important to develop an understanding of how multiple aromatic compounds are most efficiently degraded. The combinations of pathways found together in strains such as JS150 may indicate which combinations are most efficient under specific conditions. The factors, if any, that influence the pathway through which a substrate is degraded need to be identified. It should be useful to determine the effect of replacing a JS150 pathway with an alternative pathway for the degradation of the same substrate.

For example, there could be an advantage to using the gentisate pathway, as opposed to the more common catechol *meta* pathway, in the presence of a

chlorobenzene catabolic pathway. It is well known that the chlorobenzene metabolite 3-chlorocatechol is cleaved by catechol 2,3-dioxygenases to form a reactive aryl halide, disabling the catechol 2,3-dioxygenase and delaying induction of the chlorocatechol 1,2-dioxygenase, if present (Bartels, *et al*, 1984). This results in chlorobenzene having considerable toxicity to bacteria that are growing on toluene (Pettigrew *et al*, 1991). Once 3-chlorocatechol 1,2-dioxygenase is induced, chlorocatechols and methylcatechols no longer accumulate to toxic levels (Pettigrew *et al*, 1991), but chlorocatechols are continuously shunted into the unproductive pathway leading to aryl halide formation. Despite the harmful effects of the *meta* pathway for chlorobenzene-degraders, bacteria that use this toxic combination of ring cleavage pathways, including JS150, exist. The work of Haigler and Spain (1989) suggests that the superiority of the *meta* pathway to the modified *ortho* pathway for growth on toluene is the likely explanation for the retention of both pathways.

Degradation of salicylate through a catechol *meta* cleavage pathway would be expected to have effects on chlorobenzene degradation analogous to the effects of toluene. Therefore, an alternative salicylate pathway, such as the gentisate pathway or catechol *ortho* pathway, may allow more efficient growth on naphthalene and/or salicylate in the presence of chlorobenzene than would the pathway encoded by the NAH7 plasmid. The *ortho* pathway, however, is unable to metabolize chloro- or methyl- substituted catechols (Dagley, 1986). The modified *ortho* pathway, which metabolizes both methyl and chloro substitutions,

has been used to expand the substrate range of *Pseudomonas* strain B13 and WR401 to include chlorosalicylate and chloronaphthalene (Lehrbach *et al*, 1984, Rubio *et al*, 1986). A broad substrate-range gentisate pathway could also be used for this purpose. JS150 itself is not likely to contain any genes suitable for such a construction, as it did not grow on any of the tested chloro- or methyl-substituted salicylates. A strain that degrades salicylate through a broad substrate range gentisate pathway, comparable to the gentisate pathways of strains NCIB 9867 and NCIB 9869 (Hopper and Chapman, 1970) needs to be isolated.

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