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Catabolism of 5-Chlorosalicylate by a *Bacillus* Isolated from the Mississippi River

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A strain of *Bacillus brevis* isolated from a polluted section of the Mississippi River was shown to utilize 5-chloro-2-hydroxybenzoate (5-chlorosalicylate) as a sole source of carbon and energy. Enzymic analyses of cell-free extracts prepared from 5-chlorosalicylate-grown cells demonstrated that the initial step in the pathway involved cleavage of the aromatic ring between C1 and C2 by a specific 5-chlorosalicylate 1,2-dioxygenase. Loss of chloride from the growth substrate occurred after ring fission and was probably enzyme mediated. An intermediate chlorolactone apparently lost chloride by enzymatic hydrolysis with formation of maleylpyruvate. Maleylpyruvate was further degraded by both glutathione-dependent and glutathione-independent mechanisms, with these reactions being identical to the terminal reactions of the gentisate pathway. It was suggested that this novel 5-chlorosalicylate pathway may have evolved by recruitment of enzymes from an ancestral gentisate pathway.

The capability to use non-halogenated benzenoid compounds as sole sources of carbon and energy is common among microorganisms (9); however, halogenated aromatic compounds are often markedly resistant to microbial degradation (11). Despite this general observation, it is occasionally possible to isolate pure microbial strains that are able to use simple mono- or dihalogenated aromatic compounds as sole carbon and energy sources (1, 11, 13). In such cases careful examination of the catabolic pathways being used by particular microbial strains usually shows that halide elimination is nonenzymatic, occurring fortuitously after hydroxylation (1, 11, 15) or oxygenolytic cleavage (11, 21) of the aromatic nucleus. There are, however, a few known instances where catabolic pathways have apparently evolved so that degradative enzymes are quite specific for chlorinated substrates (11, 13).

Another general observation concerning microbial degradation of benzenoid molecules is that prior to ring fission the aromatic nucleus must be substituted with at least two hydroxyl groups (9). Also, these hydroxyls must be oriented either *ortho* or *para* in relationship to one another (9). We are aware of only one possible exception to this general rule, and in that instance (1-hydroxy-2-naphthoate 1,2-dioxygenase) an adjacent benzene ring substitutes for one of the usual two hydroxyls (16).

Here we report isolation of a strain of *Bacillus brevis* that is able to use 2-hydroxy-5-chlorobenzoate (5-chlorosalicylate) as a sole source of carbon and energy. The catabolic sequence used by this bacterium to degrade 5-chlorosalicylate does not follow the general patterns discussed above. The bacterium synthesizes a specific 5-chlorosalicylate 1,2-dioxygenase, a novel enzyme that cleaves the aromatic ring while it is substituted with only one hydroxyl group. Unlike pathways of most previously studied halo-aromatic compound-degrading microorganisms, loss of halide from the growth substrate appears to be enzymatically speeded.

MATERIALS AND METHODS

Isolation, identification, and growth of the microorganism. The bacterium used during this investigation was isolated from the Mississippi River (just downstream from the Twin Cities, Minnesota sewage treatment plant) by selective enrichment on 5-chlorosalicylate as the sole source of carbon and energy. It was tentatively identified as a strain of *B. brevis* by using the keys and procedures of Gordon et al. (14). Stock cultures were maintained on slants of minimal media (see below) containing 0.05% (wt/vol) 5-chlorosalicylic acid as the sole source of carbon and energy. Slants were stored at 4°C and subcultured biweekly. The *Bacillus* was grown in the minimal medium previously described (5), except that 5-chlorosalicylic acid (0.05%) or succinate (0.2%) replaced *p*-hydroxyphenylpropionic acid. A total of 100 ml of medium in a 500-ml flask was inoculated with a loop of cells taken from a stock slant, and the culture was shaken for 24 to 48 h at 30°C before being used to inoculate 1 liter of the same medium in a 2-liter flask. The larger culture was shaken at 30°C on a gyratory platform shaker at

approximately 150 rpm until cells reached late log phase (18 to 24 h). Cells were collected by centrifugation and washed by suspension in 0.1 M MOPS (morpholinopropanesulfonic acid) buffer (pH 7.0). This buffer was also used in all reaction mixtures, unless specified otherwise.

Preparation of cell extracts. Washed cell pastes were suspended in 2 to 3 volumes of buffer, and the resulting cell suspensions were passed through a French pressure cell at 15,000 lb/in^2 (4). Extracted cell suspensions were centrifuged at 26,000 × *g* for 20 min to give cell extracts containing 10 to 25 mg of protein per ml as determined by the method of Lowry et al. (18). All procedures were performed at 0 to 5°C.

Enzymatic analyses. Oxygen consumption was measured with an oxygen electrode (Oxygraph; Gilson Medical Electronics, Middleton, Wis.). Dioxygenase assays were performed at 25°C in 1.5 ml of buffer containing 1.0 μmol of substrate and 0.1 to 0.2 mg of cell extract protein. Reactions were initiated by addition of substrate. Oxygenase specific activities were corrected for uptake of oxygen in the absence of substrate. Maleylpyruvate hydrolase and fumarylpyruvate hydrolase activities were measured as previously described (7, 8). The *N*-ethylmaleimide procedure of Crawford and Frick (7) was used to demonstrate reduced glutathione requirement or nonrequirement for maleylpyruvate degradation. All enzyme specific activities are expressed as micromoles of substrate transformed per minute per milligram of protein provided.

Spectrophotometric determinations of pyruvate were performed using lactate dehydrogenase as previously described (3). Stoichiometry of reduced nicotinamide adenine dinucleotide consumption was determined by observing a decrease in absorbance at 340 nm resulting from reduced nicotinamide adenine dinucleotide oxidation (E = 6,200 for this compound).

Partial purifications of 5-chlorosalicylate 1,2-dioxygenase. 5-Chlorosalicylate dioxygenase was purified approximately twofold (specific activity = 0.13 U) by ammonium sulfate fractionation. Ammonium sulfate was added to crude extracts as a saturated neutral solution, and dioxygenase activity precipitated in the 40 to 60% ammonium sulfate saturation range. The dioxygenase was further purified by batch affinity chromatography. 5-Chlorosalicylate was coupled to aminohexyl-Sepharose 4B by the carbodiimide procedure outlined on the information sheet shipped with AH-Sepharose-4B (Sigma Chemical Co., St. Louis, Mo). Equal volumes of the affinity resin and the ammonium sulfate-fractionated enzyme solution (after dialysis for 24 h against 50 mM MOPS, pH 7) were mixed, and the gel with adsorbed dioxygenase was filtered off on a coarse glass funnel. The gel was successively washed with equal volumes of 0.2 M, then 0.5 M, NaCl in 50 mM MOPS (pH 7.2). These fractions were discarded. A final wash with 1.0 M NaCl in 50 mM MOPS (pH 7.2) yielded most of the 5-chlorosalicylate dioxygenase activity (specific activity = 0.578 U).

Before assay, both ammonium sulfate-fractionated dioxygenase and affinity resin-treated dioxygenase required reactivation by incubation with Fe^{2+} ions (8, 10). Purification or even storage of the dioxygenase apparently resulted in loss of catalytically important iron.

Chloride analyses. Chloride ion concentrations in enzyme reaction mixtures and bacterial growth media were determined using an Orion 701-A digital pH/millivolt meter and a chloride-specific electrode (Orion model 94-17A). Chloride concentration was determined using a calibration curve which plotted the log of chloride molarity against millivolts for a series of standard samples. For some experiments a chloride-limited growth medium was required. This was prepared by using distilled/deionized water and substituting ammonium sulfate for ammonium chloride in the usual growth medium.

Growth of *B. brevis* on 5-chlorosalicylate and release of chloride to the growth medium. *B. brevis*, was grown at 30°C in a 10-liter carboy containing 8 liters of chloride-limited basal medium containing 0.5 g of 5-chlorosalicylic acid per liter. A 1-liter culture (same medium) in logarithmic phase was used as an inoculum. Growth was monitored by removing 100-ml samples at 2-h intervals and determining their relative turbidity with a Klett-Summerson colorimeter equipped with a no. 42 filter (400 to 465 nm transmission). Each sample was then freed of cells by centrifugation, and its chloride concentration was determined with a chloride-specific electrode.

Materials. Enzymes and cofactors were purchased from Sigma. Aromatic compounds were purchased from the Aldrich Chemical Co., Milwaukee, Wis. Maleylpyruvate was prepared enzymatically essentially as described by Lack (17), except that the enzyme used (gentisate 1,2-dioxygenase) was prepared from *Moraxella* OA3 (8). Fumarylpyruvate was prepared nonenzymatically from maleylpyruvate by isomerization with H_2SO_4 (17).

RESULTS

The bacterium used in this investigation was enriched from water taken from a polluted section of the Mississippi River (just downstream from the outflow of the Twin Cities, Minnesota sewage treatment plant). An 80-ml sample of river water was diluted with 20 ml of a five-times-concentrated solution of mineral salts growth medium (5), and 0.05 g of neutralized 5-chlorosalicylic acid was added as a source of carbon. The enrichment was shaken at 30°C until microbial growth was evident (about 5 days). A bacterium was then purified from the enrichment by repeated streaking onto basal medium containing 0.05% 5-chlorosalicylic acid as the sole carbon source. A pure culture was obtained after a final purification on soil extract agar (14). The chlorosalicylate-degrading bacterium was an aerobic, endospore-forming rod and was tentatively identified as a strain of *B. brevis*. The bacillus was able to use the following aromatic compounds as sole sources of carbon and energy for growth: 5-chlorosalicylate, 5-bromosalicylate, 5-fluorosalicylate, and 4-hydroxy-

benzoate. It failed to grow on benzoate, 3-hydroxybenzoate, or salicylate (2-hydroxybenzoate).

Cell-free extracts prepared from 5-chlorosalicylate-grown cells were examined for oxygenase activities. No significant oxygenase activities were detected against protocatechuate (3,4-dihydroxybenzoate), catechol (*o*-hydroxyphenol), or gentisate (2,5-dihydroxybenzoate). An oxygenase activity against 5-chlorosalicylate was present in cell extracts (specific activity, 0.07 U). Each mole of 5-chlorosalicylate provided under our assay conditions elicited consumption of approximately 1 mol of oxygen. Pyridine nucleotide cofactors were not required in the assay solution and did not increase rates of oxygen consumption. These data indicate that the enzymic activity against chlorosalicylate is that of a ring-fission dioxygenase.

Crude extracts prepared from 5-chlorosalicylate-grown cells converted 5-chlorosalicylate to pyruvate and chloride. Each mole of 5-chlorosalicylate provided resulted in the formation of approximately 1 mol of pyruvate and 1 mol of chloride ion. Growth of *B. brevis* in chloride-limited medium containing 5-chlorosalicylate as the sole carbon source resulted in release of chloride ions, with the rate of chloride release corresponding closely to the microbial growth rate. Cellular doubling time for the bacillus growing on 5-chlorosalicylate was 3.3 h, whereas the time required to double the concentration of chloride in the growth medium was 3.1 h. The growth curve (culture turbidity) and chloride release curve were parallel throughout.

The chlorosalicylate-dioxygenase activity was partially purified by a combination of ammonium sulfate fractionation and affinity chromatography. Dioxygenase that had been purified twofold by ammonium sulfate fractionation (40 to 60% saturation range) attacked 5-chlorosalicylate, rapidly forming a nonaromatic product with spectral characteristics identical to maleylpyruvate (17; γ_{max} [pH 7] $\simeq$ 325 nm, shifted to $\simeq$332 nm at pH 11 to 12, and disappeared at pH 1 to 2; Fig. 1). Dioxygenase further purified by affinity chromatography oxidized 5-chlorosalicylate, yielding a compound with a γ_{max} (pH 7) of 292 nm (Fig. 2). This compound disappeared slowly over a period of several hours (see following). These observations indicate that crude enzyme preparations contain an enzyme that converts the immediate ring-fission product (γ_{max} = 292 nm) to maleylpyruvate, since affinity chromatography-purified oxygenase preparations do not accumulate maleylpyruvate except after prolonged incubation. Addition of NaOH (pH 11 to 12) immediately converted the compound of γ_{max} (pH 7) = 292 nm to a compound again identical to maleylpyruvate (Fig. 3). The identity of this compound as maleylpyruvate was confirmed in another experiment by isomerizing it to fumar-

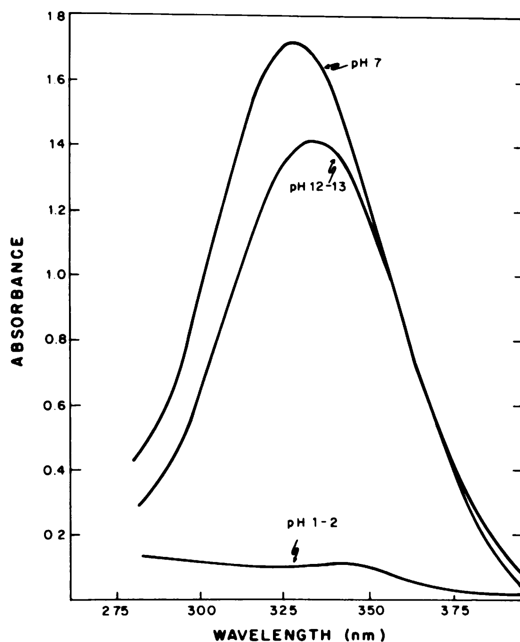

FIG. 1. *Ultraviolet/visible absorption spectrum of maleylpyruvate formed by enzymic oxidation of 5-chlorosalicylate.*

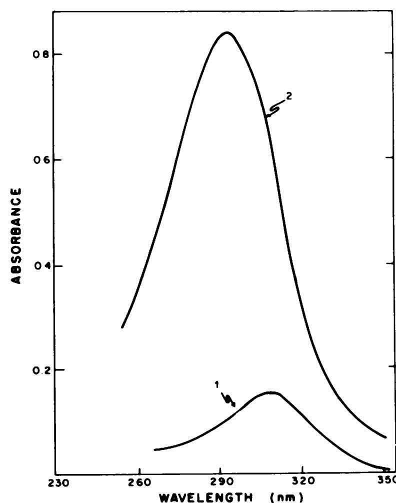

FIG. 2. *Ultraviolet/visible absorption spectrum of the immediate ring-fission product after ring fission of 5-chlorosalicylate. The reaction was performed in 1 ml of MOPS buffer (pH 7). (1) Spectrum of 5-chlorosalicylate (0.05 μmol); (2) spectrum of ring-fission product formed on addition of affinity-purified chlorosalicylate dioxygenase.*

ylpyruvate with H_2SO_4 (Fig. 3). The ring-fission product (γ_{max} = 292 nm) was generated at pH 7 using affinity-purified dioxygenase. Addition of NaOH (pH 11 to 12) shifted the γ_{max} to 330 nm. Readjustment of the pH to 1 to 2 by addition of H_2SO_4 resulted in a disappearance of the absorption spectrum. At this point the acidic solution was boiled for 2 min. Heating resulted in reappearance of the ultraviolet-visible spectrum (γ_{max} $\simeq$ 330 nm). A final adjustment of the pH to 11 to 12 shifted the spectrum to γ_{max} $\simeq$ 350 nm. These are the observations one would expect for the following reactions: 5-chlorosalicylate —O_2→ ring-fission product —NaOH→ maleylpyruvate (17; γ_{max} = 330 nm) —H_2SO_4→ maleylpyruvate (17; ultraviolet-visible light transparent) —100°C→ fumarylpyruvate (17; γ_{max} [pH 0 to 1] $\simeq$ 330 nm) —NaOH→ fumarylpyruvate (17; γ_{max} [pH 11 to 12] $\simeq$ 350 nm). The conversion of the immediate ring-fission product (γ_{max} = 292 nm) to maleylpyruvate apparently occurred non-enzymatically even at pH 7. At neutrality this conversion proceeded slowly, requiring an incubation period of several hours at 25°C.

The disappearance of the ring-fission product (generated from chlorosalicylate by affinity-purified dioxygenase) was speeded dramatically by addition of ammonium sulfate-fractionated crude extract. The increase in rate of disappearance was proportional to the amount of crude ammonium sulfate-fractionated extract added. Again, maleylpyruvate was the new product observed.

When crude extracts of 5-chlorosalicylate-grown cells were examined for the presence of maleylpyruvate hydrolase and fumarylpyruvate hydrolase, both enzymes were found to be present. Also, when formation and disappearance of maleylpyruvate formed from 5-chlorosalicylate was observed spectrophotometrically (330 nm), disappearance of maleylpyruvate was shown to be speeded on addition of reduced glutathione (GSH; Fig. 4). Examination of 5-chlorosalicylate degradation by the *N*-ethylmaleimide procedure (7) indicated that both GSH-dependent and GSH-independent catabolic activities against maleylpyruvate were present in crude extracts.

The 5-chlorosalicylate dioxygenase of *B. brevis* was also active against other substituted salicylates. The following relative rates of activity (in parentheses) were found when dioxygenase activity (using a twofold-purified enzyme preparation) was determined against several 5-chlorosalicylate analogs: 5-chlorosalicylate (1.00), 5-fluorosalicylate (0.51), 5-bromosalicylate (0.35), 5-iodosalicylate (0.01), 5-methoxysalicylate (0.59), gentisate (0.01), salicylate (0.01). Thus, a halogen (other than iodine) or a meth-

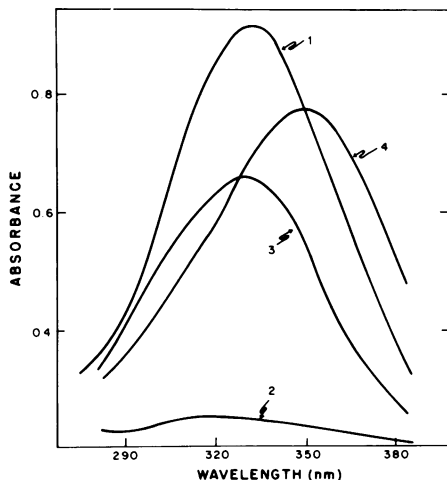

FIG. 3. *Nonenzymatic conversion of the immediate ring-fission product of 5-chlorosalicylate to maleylpyruvate and then fumarylpyruvate. The initial reaction was performed in 1 ml of MOPS buffer (pH 7). Affinity-purified 5-chlorosalicylate dioxygenase was used to generate 0.05 µmol of immediate ring-fission product (γ_{max} = 292 nm, Fig. 2). (1) Spectrum of compound formed on adjustment of the pH from 7 to 11-12; (2) spectrum after adjustment from pH 11-12 to pH 0-1; (3) spectrum observed after boiling the acidic solution for 1 min; (4) spectrum observed after a final adjustment of pH to 11-12.*

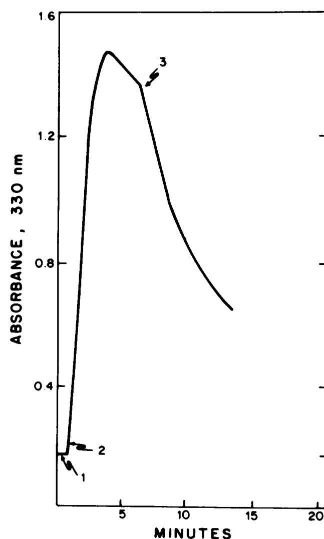

FIG. 4. *Stimulation of maleylpyruvate degradation by GSH. The reaction was observed in 1 ml of MOPS buffer (pH 7). (1) Addition of 0.1 µmol of 5-chlorosalicylate; (2) addition of approximately 0.3 mg of protein (35 to 60% ammonium sulfate cut prepared from 5-chlorosalicylate-grown B. brevis); (3) addition of 100 µM GSH.*

oxyl group is required at the 5 position for a substituted salicylate to be a substrate for the dioxygenase. An important observation is that a hydroxyl group (as in gentisate) will not substitute for the chlorine at position 5.

DISCUSSION

The data presented above are consistent with the catabolic sequence shown in Fig. 5. Our identification of maleylpyruvate as a catabolic product of 5-chlorosalicylate firmly established that ring fission occurred between C1 and C2 of the aromatic ring. Identification of maleylpyruvate along with oxygen consumption stoichiometry (1 O_2 per 5-chlorosalicylate) confirmed that the ring-fission activity was that of a "dioxygenase," that is, both atoms of the O_2 molecule were incorporated into the ring-fission product in a reaction directly analogous to that carried out by gentisate 1,2-dioxygenase (17). However, gentisate is not a substrate for the dioxygenase. In fact, a chlorine atom at ring position 5 is required for maximal activity of the enzyme. Thus, the dioxygenase is not simply a gentisate dioxygenase that also attacks chlorosalicylate. Rather, this novel enzyme is a specific 5-chlorosalicylate 1,2-dioxygenase.

Experiments using affinity-purified dioxygenase established that maleylpyruvate was not the immediate product formed by fission of the 5-chlorosalicylate nucleus. The most probable immediate ring-fission product (γ_{max} [pH 7] ≃ 292 nm) is compound II of Fig. 5. We were unable to prepare pure compound II for testing as an enzyme substrate; however, indirect evidence points to its intermediacy in the catabolic pathway. It is likely that compounds of the maleylpyruvate configuration exist in solution as an equilibrium between the free acid and a 5-membered-ring lactone (12). Such lactones are known to maximally absorb ultraviolet light in the range of 230 to 300 nm (10, 19), as did our observed immediate ring-fission product. Also, an equilibrium between compounds II and III would release chloride ion with ultimate conversion of compound II to maleylpyruvate (compound IV). Alkaline conditions would be expected to greatly increase the rate of conversion of the chlorolactone to maleylpyruvate. We were able to firmly establish that the immediate ring-fission product from 5-chlorosalicylate was slowly converted non-enzymatically to maleylpyruvate and chloride, and that alkalinization of a solution of the ring-fission product resulted in very rapid formation of maleylpyruvate. Thus, our postulation of a chlorolactone intermediate between 5-chlorosalicylate and maleylpyruvate appears reasonable.

Catabolism of maleylpyruvate appears to proceed by the classical reactions of the gentisate pathway (8, 17), with both the GSH-dependent and GSH-independent variations of the pathway being operative (7). Both of these variations are common among other representatives of the genus *Bacillus* (6, 7), where maleylpyruvate seems to be a highly important catabolic intermediate (6).

This is apparently the first observation of a specific dioxygenase that cleaves as the enzyme's primary substrate a single-ring benzenoid molecule having only one hydroxyl on the ring. Que (20) reported that a catechol 2,3-dioxygenase would also cleave the ring of *o*-aminophenol; however, the reaction rate was very slow in comparison with the rate of cleavage of the ring of the enzyme's normal substrate, catechol. Kiyohara and Kakao (16) reported the existence of

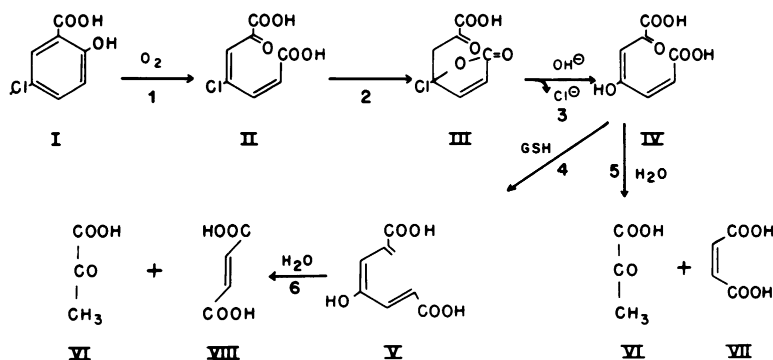

FIG. 5. *Catabolism of 5-chlorosalicylic acid by B. brevis. (I) 5-Chlorosalicylic acid; (II) 7-carboxy-4-chloro-2-keto-hept-3,5-dieneoic acid; (III) 7-carboxy-4-chloro-2-keto-hept-3,5-dieneoic acid 4,7-lactone; (IV) maleylpyruvic acid; (V) fumarylpyruvic acid; (VI) pyruvic acid; (VII) maleic acid; (VIII) fumaric acid. (1) 5-Chlorosalicylic acid 1,2-dioxygenase; (2) nonenzymic (?); (3) "lactone hydrolase" (?); (4) maleylpyruvate isomerase; (5) maleylpyruvate hydrolase; (6) fumarylpyruvate hydrolase.*

a 1-hydroxy-2-naphthoate 1,2-dioxygenase, where a diphenyl ring structure was oxidatively cleaved while bearing only one hydroxyl group. In the instance reported here, the mono-hydroxylated molecule (5-chlorosalicylate) is the primary substrate, whereas the corresponding dihydroxy compound (gentisate) is not attacked at a significant rate.

Another important observation is that loss of the halogen substituent as halide ion from ring carbon 5 appears to be enzymatically mediated. The enzyme involved is probably a lactone hydrolase. Unfortunately, at this time we are unable to examine the substrate specificity of the hydrolase because of the unavailability of suitable substrates for enzyme assays.

It is interesting to speculate on the evolution of this novel pathway for dissimilation of 5-chlorosalicylate. It seems reasonable to suggest that the pathway of Fig. 5 evolved by recruitment of enzymes from an ancestral gentisate pathway. This would require that the specificity of the ancestral gentisate 1,2-dioxygenase (common among *Bacillus* sp.; 4, 6) change so that 5-chlorosalicylate is bound and cleaved in preference to gentisate. Initially, the remainder of the pathway need not change, since loss of chloride and formation of maleylpyruvate occur slowly nonenzymatically. Further evolution could then improve the efficiency of the new pathway, particularly in the recruitment of an enzyme to speed the dehalogenation reaction.

A selective force does indeed exist in nature for an evolution of enzyme specificity as postulated above. 5-Chlorosalicylate is known to be present in environments, such as the Mississippi River, that receive chlorinated sewage effluents (1). 5-Chlorosalicylate is probably a recalcitrant molecule in comparison to its non-halogenated analogs; therefore it should become a selective advantage for a bacterial mutant to attain the ability to use 5-chlorosalicylate as a source of carbon and energy while its competitors are unable to perform the same feat.

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