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# Specificity of a *Flavobacterium* in the metabolism of substituted chlorobenzoates

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## SUMMARY

A strain of *Flavobacterium breve* capable of utilizing 3,5-dichlorosalicylate as a sole source of carbon and energy was identified. Degradation of 3,5-dichlorosalicylate was specific as this strain did not metabolize dicamba (3,6-dichloro-2-methoxybenzoic acid), 3,5-dicamba (3,5-dichloro -2-methoxybenzoic acid), or 3,6-dichlorosalicylate. The organism was able to remove completely 3,5-dichlorosalicylate in the presence of three times as much 3,6-dichlorosalicylate being degraded. The organism was able to utilize 3,5-dichlorosalicylate at concentrations up to 1000 μg/ml. A mixture of 3,5 and 3,6-dichlorosalicylate isomers purified by biological destruction of the unwanted isomer (3,5-dichlorosalicylate) would be useful for producing isomerically pure dicamba, an important herbicide.

## INTRODUCTION

Bacteria capable of the oxidative metabolism of chlorinated aromatic compounds have been isolated, identified and characterized. These bacterial strains are capable of growing on chlorinated compounds such as 5-chlorosalicylate, 2,4-dichlorobenzoate, and 3,4-dichlorotoluene as sole sources of carbon [4,8]. Crawford et al. [4] reported the isolation of a microorganism which catabolizes 5-chlorosalicylate, a compound very similar in structure to 3,5-dichlorosalicylate. The utilization of 5-chlorosalicylate by a strain of *Flavobacterium breve* proceeds by ortho-cleavage or oxygenation at the 1,2 position of the molecule. The dioxygenase enzyme involved in this process is unique in that it cleaves the chlorinated aromatic molecule having only one hydroxyl group on the phenyl ring. Further metabolism appeared to yield common cellular intermediates such as pyruvate, fumarate, and maleic acid.

The specificity of degradative organisms towards

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the utilization of chloroaromatic compounds is often high or even absolute. This specificity is an attribute of the organism's monooxygenase or dioxygenase enzyme system which is responsible for initial oxidation of the substrate [7]. Dorn and Knackmuss found oxygenase enzymes specific for the chlorinated substrate [5]. The specificity of an enzyme for a particular chlorinated substrate could be useful in a process where precise conversions are needed on a single molecular type occurring in a mixture of chemicals.

Schultz [6] reported a number of examples of the use of biocatalysts in organic synthesis. For a bacterial strain to be useful as a biocatalyst in isomeric purification it must be absolute in its specificity towards oxidation of the unwanted isomer and not inhibited by the isomer that is being purified. There is a need to produce isomerically pure 3,6-dichlorosalicylate since it is an intermediate in the production of 3,6-dichloro-2-methoxybenzoic acid, the herbicide dicamba. Dicamba is used as a pre-emergent and post-emergent herbicide for control of annual and perennial broadleaf weeds and several grassy weeds. Technical grade dicamba consists of 8 to 10% (w/w) of the 3,5-isomer, 3,5-dichloro-2-methoxybenzoic acid, which has no herbicidal activity. There is currently no feasible method to separate the isomers or their precursors, 3,5- and 3,6-dichlorosalicylate. This paper demonstrates the potential use of *Flavobacterium breve* for the selective biological removal of the 3,5-isomer.

## MATERIALS AND METHODS

*Organisms and medium*

The bacterial strain was a strain of *Flavobacterium breve* isolated by Crawford et al. [4] which was able to utilize 5-chlorosalicylate as a sole carbon source. Crawford et al. referred to the strain as *Bacillus brevis* 5ClSAL1, however *B. brevis* has been reclassified as *F. breve* [1].

Cultures were maintained on slants of reduced chlorine medium containing 500 $\mu$g/ml of 5-chlorosalicylate. The reduced chlorine medium contained (g/l): $K_2HPO_4 \cdot 3H_2O$, 1.826; $K_2PO_4$, 0.87; $(NH_4)_2HPO_4$, 0.66; $MgSO_4$, 0.097; $MnSO_4 \cdot H_2O$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.005; $CaSO_4$, 0.001; and pH was adjusted to 7.0 with 1.0 N NaOH. The chloride concentration in the medium was below the detection limit (1 ppm) as determined by the method of Bergmann and Sanik [2].

*Chemicals*

5-chlorosalicylate and 3,5-dichlorosalicylate were purchased from Kodak, Rochester, NY and Aldrich Chemical Co., Milwaukee, WI. Dicamba, 3,5-dicamba and 3,6-dichlorosalicylate were provided by the Analytical Standards group of Sandoz Crop Protection Corporation, Des Plaines, IL. All compounds had a purity of 98% or greater.

$^{14}$C-Dicamba acid (U-phenyl $^{14}$C, lot 801106, 98% radiochemical purity, 11.5 mCi/mmol) and $^{14}$C-3,6-dichlorosalicylate (U-phenyl $^{14}$C, lot 840110, 99% radiochemical purity, 14.3 mCi/mmol) were obtained from Pathfinder Laboratories Inc., St. Louis, MO. To increase solubility, stock solutions of all chemicals used as carbon sources were prepared by titration with NaOH to pH 7.0. Each chemical stock solution was filter sterilized through a 0.2-$\mu$m Teflon filter before being added to sterile media. All other chemicals were reagent grade or better, and all other solvents were glass distilled quality.

*Analytical methods*

High-pressure liquid chromatographic analysis of media containing 3,5- and 3,6-dichlorosalicylate was conducted on a Waters Associates Model 440 HPLC. HPLC on a reverse phase carbon-18 column with ion suppression technique using 1% acetic acid in methanol/water (1:1) as mobile phase separated the isomers. Ultraviolet detection at 280 nm was used to quantitate 3,5- and 3,6-dichlorosalicylate.

Liquid scintillation counting was done in a Searle Mark III liquid scintillation counter using an external standard quench correction program. Appropriate backgrounds were subtracted from all samples.

The amount of chloride released from substrate was determined by the method of Bergmann and Sanik [2].

*Growth kinetics on 3,5-dichlorosalicylate*

Cells of *Flavobacterium breve* strain 5ClSAL1 were grown in 100 ml of medium which contained 500 μg/ml 5-chlorosalicylate or 100 μg/ml 3,5-dichlorosalicylate. Cells were harvested by centrifugation, washed and resuspended in 10 ml of sterile medium. Each inoculum was then added separately to one liter of reduced chlorine medium containing 100 μg/ml 3,5-dichlorosalicylate. Cultures were incubated at 30°C on a rotary shaker at 100 rpm. The cultures were sampled immediately, and after 1, 2, 3, 6, 7, 10 and 14 days incubation. Each sample (a 10-ml aliquot) was subjected to chloride release analysis [22]. Data were graphed using the SAS smoothing program (SAS Institute, Cary, NC).

*Biological specificity*

An inoculum of *F. breve* 5ClSAL1 grown on 100 μg/ml 3,5-dichlorosalicylate was prepared as indicated previously. Amber serum bottles (125 ml) were prepared with 50 ml (final volume) of sterile reduced chlorine medium containing 100 μg/ml of filter-sterilized dicamba, 3,5-dicamba, 3,5-dichlorosalicylate or 3,6-dichlorosalicylate. One μCi of $^{14}$C-dicamba per serum bottle was used for dicamba treatments. Duplicate bottles for each chemical were inoculated with 1.0 ml of inoculum. Sterile medium in serum bottles was used as a control. All serum bottles were incubated at 30°C on a rotary shaker at 100 rpm. Cultures were sampled at time 0 and at 4 weeks.

At each sampling time samples were diluted and plated on nutrient agar to determine the number of viable cells present. Chloride release was determined [22]. Aliquots of medium (0.1 ml) from the dicamba culture were radioassayed in duplicate as described above. After 4 weeks incubation all cultures were acidified with 5.0 ml of 20% (v/v) $H_2SO_4$. The dicamba culture was purged with $CO_2$-free air. Purged air was passed through a solution of 0.5 M KOH which served as a $CO_2$ trap. Aliquots of KOH (0.5 ml) were radioassayed in duplicate to determine the extent of mineralization of the substrate.

*Specificity for 3,5-dichlorosalicylate*

*F. breve* 5ClSAL1 was grown for 5 days in 100 ml of medium which contained 100 μg/ml 3,5-dichlorosalicylate. The entire culture was used to inoculate 900 ml of medium which contained 275 μg/ml of 3,5-dichlorosalicylate and 814 μg/ml of 3,6-dichlorosalicylate along with 85.8 μCi of $^{14}$C-3,6-dichlorosalicylate, as determined by HPLC analysis.

The experiment was carried out in a one-liter glass chemostat vessel fitted with a stainless steel headplate (Virtis, Gardner, NY) and equipped with a continuous pH recorder (Queue, Parkersburg, WA). The vessel was incubated at 30°C, continuously aerated, and stirred at 400 rpm.

Immediately after incubation and after 2, 5, 8 and 13 days of incubation aliquots of medium were removed for enumeration of viable cells by plating on nutrient agar. Total radiocarbon in the medium was determined in duplicate by radioassay of 0.1 ml of medium.

10 ml of medium which was removed at each sampling time was acidified to pH $<1$ with 2.0 ml of concentrated $H_2SO_4$. Acidified medium (5.0 ml) was extracted twice with 10.0 ml of diethyl ether. The remaining aqueous fraction was subjected to chloride release analysis. The ether was concentrated to dryness and the residue was taken up in 5.0 ml of methanol. HPLC was used to quantitate 3,5- and 3,6-dichlorosalicylate.

*Temperature optimization*

*F. breve* strain 5ClSAL1 was inoculated into duplicate flasks containing 1000 μg/ml 3,5-dichlorosalicylate. Flasks were incubated on a shaker at 30, 40, or 50°C. One uninoculated control was maintained at each temperature. Cultures were sampled immediately, and after 2, 4, 7, 10 and 14 days inoculation. The number of total viable cells and the amount of chloride released were determined at each sampling time.

*Tolerance to 3,5-dichlorosalicylate*

Duplicate cultures with a cell density of approximately $1.6 \times 10^6$ cells/ml were exposed to 0, 100,

1000, 5000 or 10 000 μg/ml of 3,5-dichlorosalicylate. After 30 min, plate counts were conducted on all samples to determine the contact toxicity of 3,5-dichlorosalicylate. Aliquots were subjected to chloride release analysis after 3 weeks of incubation to determine the percent removal of 3,5-dichlorosalicylate at each concentration.

## RESULTS

*Growth kinetics*

Cultures inoculated with *F. breve* 5ClSAL1 grown on 5-chlorosalicylate or on 3,5-dichlorosalicylate grew on 3,5-dichlorosalicylate at similar rates (Fig. 1). After 14 days of incubation, 100% of the 3,5-dichlorosalicylate was removed. After a 4 to 5-day lag period metabolism of 3,5-dichlorosalicylate was rapid. Between the seventh and tenth day chloride was released at a rate of 8.6 μg per ml of culture per day. This corresponds to 25.1 μg of 3,5-dichlorosalicylate per ml of culture being metabolized per day.

*Biological specificity*

To demonstrate the specificity of *Flavobacterium breve* strain 5ClSAL1 for 3,5-dichlorosalicylate, cells were incubated with 100 μg/ml of 3,5-dichlorosalicylate, 3,6-dichlorosalicylate, dicamba, or 3,5-dicamba. Results of cell counts, chloride release from substrate, and substrate depletion indicate that strain 5ClSAL1 is incapable of degrading 3,6-dichlorosalicylate, dicamba or 3,5-dicamba. No decrease in soluble radiocarbon in the medium or release of $^{14}CO_2$ from radiolabeled dicamba was evident. An increase in the viable count and an increase in the amount of chloride released into the medium was seen only in the cultures containing 3,5-dichlorosalicylate.

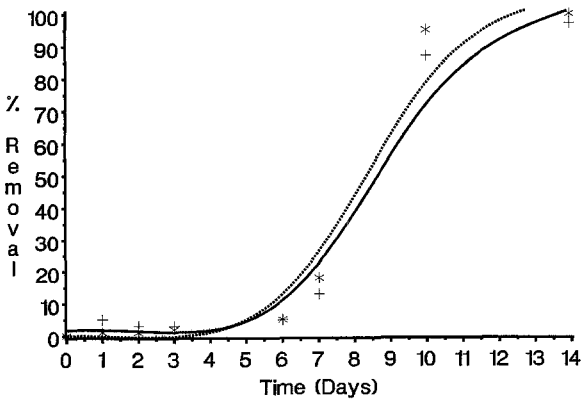


Fig. 1. Removal of 3,5-dichlorosalicylate by *Flavobacterium breve* as determined by chloride release. Cells for inoculum grown on 3,5-dichlorosalicylate (+) or 5-chlorosalicylate (*).

*Isomeric specificity*

Total radiocarbon, HPLC analysis, and chloride release all indicate that substrate specificity of *Flavobacterium breve* was absolute for 3,5-dichlorosalicylate even when 3,6-dichlorosalicylate was present at much higher concentrations (Table 1). The 3,5-dichlorosalicylate (substrate) concentration in the medium decreased dramatically during the course of the experiment while the 3,6-dichlorosalicylate concentration remained constant. 3,6-Dichlorosalicylate did not inhibit metabolism of 3,5-dichlorosalicylate. The rate of metabolism was higher in this experiment than in the growth kinetics experiment where only 3,5-dichlorosalicylate was present, 50.4 μg versus 25.1 μg of 3,5-dichlorosalicylate per ml of culture being metabolized per day.

*Tolerance to 3,5-dichlorosalicylate*

Tolerance of *F. breve* strain 5ClSAL1 to high concentrations of 3,5-dichlorosalicylate in liquid culture is demonstrated in Table 2. There was some contact toxicity of 3,5-dichlorosalicylate at high concentrations. Cell numbers were reduced approximately 2/3 after 30 min exposure to 5000 μg/ml of 3,5-dichlorosalicylate. After three weeks, removal of 3,5-dichlorosalicylate was seen only at the 100 and 1000 μg/ml levels.

*Temperature optimization*

*F. breve* strain 5ClSAL1 incubated at 30°C increased in cell numbers and removed 100% of the 3,5-dichlorosalicylate after 10 days. No increase in cell number or removal of 3,5-dichlorosalicylate was evident at 40 or 50°C.

Table 1

Specificity of 3,5-dichlorosalicylate (3,5-DCSA) removal in the presence of three-fold greater concentration of 3,6-dichlorosalicylate (3,6-DCSA)

| Incubation time (days) | Viable cells/ml ($\times 10^5$) | $^{14}$C-3,6-DCSA (DPM/ml $\times 10^3$)[4] | 3,6-DCSA (ppm)[b] | 3,5-DCSA (ppm)[b] | % Removal of 3,5-DCSA[b] | Chloride release from substrate (ppm) | % Removal of 3,5-DCSA[d] |
|---|---|---|---|---|---|---|---|
| 0 | 0.4 | 189 | 814 | 275 | 0 | 0 | 0 |
| 2 | 0.1 | 189 | 805 | 277 | 0 | 1.3 | 2 |
| 5 | 1.6 | 186 | 810 | 289 | 0 | 1.3 | 2 |
| 8 | 51.0 | 196 | 802 | 252 | 8 | 7.1 | 10 |
| 13 | 1900.0 | 188 | 812 | ND[c] | 100 | 72.2 | 100 |

[a] All values in this column are virtually identical since they are all within expeirmental error of each other.
[b] As determined by high pressure liquid chromatography.
[c] None detected, limit of detection = 8.6 ppm.
[d] As determined by chloride release.

## DISCUSSION

*F. breve* strain 5ClSAL1 may be a useful biocatalyst for purification of mixtures of 3,5- and 3,6-dichlorosalicylate. Purified mixtures could then be utilized for producing isomerically pure dicamba. The biological specificity towards 3,5-dichlorosalicylate was absolute even when the culture was incubated with mixtures of 3,6- and 3,5-dichlorosalicylate at a ratio of 3:1. 3,6-Dichlorosalicylate did not inhibit the oxidation of 3,5-dichlorosalicylate and the presence of the 3,6-isomer did not decrease the rate of 3,5-dichlorosalicylate removal. The ability of this organism to utilize 3,5-dichlorosalicylate appears to be stable as long as the culture is maintained in the presence of 3,5-dichlorosalicylate or 5-chlorosalicylate.

Table 2

Tolerance of *Flavobacterium breve* to 3,5-dichlorosalicylate

| 3,5-dichlorosalicylate ($\mu$g/ml) | Replicate | % Removal of 3,5-dichlorosalicylate[a] | CFU per ml after 0.5 h ($\times 10^4$) |
|---|---|---|---|
| 0 | 1 | – | 176 |
| | 2 | – | 150 |
| 100 | 1 | 100 | 155 |
| | 2 | 100 | 160 |
| 1 000 | 1 | 54 | 175 |
| | 2 | 49 | 160 |
| 5 000 | 1 | 0 | 51 |
| | 2 | 0 | 52 |
| 10 000 | 1 | 0 | 0 |
| | 2 | 0 | 0 |

[a] After 3 weeks incubation at 30°C.

No metabolites were detected by any of the analytical methods used. The kinetics of removal (Fig. 1) suggest that the degradative process required for 5-chlorosalicylate and 3,5-dichlorosalicylate degradation are the same. Inocula grown on 5-chlorosalicylate or 3,5-dichlorosalicylate had the same growth kinetics when grown on 3,5-dichlorosalicylate. The enzyme or enzymes involved appear to be specific for salicylates with a chlorine at the five position, as no oxidation of dicamba, 3,6-dichlorosalicylate, or 3,5-dicamba was evident.

The process of eliminating 3,5-dichlorosalicylate might be further optimized by controlling growth conditions such as temperature, aeration, and nutrient concentration. Further investigation is needed on the ability of this organism to utilize the 3,5-isomer in medium where the ratio of the 3,6- to the 3,5-isomer is higher. Whole cell or enzyme immobilization might provide a useful processing system since operation at dilution rates greater than the apparent maximum exponential growth rate of the free cells is possible [3].

A reasonable approach to utilizing the degradative abilities of *F. breve* may be to incorporate a microbial treatment system into the existing process used for the synthesis of dicamba. Benefits would include a purified final product as well as reduced treatment and disposal of waste. Further investigation should be undertaken on the cost and feasibility of this approach.

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