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Characterization of Benzoate Degradation via *ortho*-Cleavage by *Streptomyces setonii*

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Abstract *Streptomyces* are widespread in nature and play a very important role in the biosynthesis as well as biodegradation of natural and unnatural aromatic compounds. Both qualitatively and quantitatively through TLC and UV spectrophotometric assays, it was observed that the thermophilic soil bacteria *S. setonii* (ATCC 39116), which can utilize a benzoate as a sole carbon and energy source in a minimal liquid culture, was not very sensitive to the benzoate concentration and to the culture conditions such as the pH and temperature. The *in vitro* conversion of a catechol to a *cis,cis*-muconic acid by a crude *S. setonii* lysate implies that the aromatic ring cleavage by *S. setonii* is initiated by a thermostable catechol-1,2-dioxygenase, the key enzyme in the *ortho*-cleavage pathway of aromatic compound biodegradation. Unlike non-degrading *S. lividans, S. setonii* was also highly resistant to other similar hazardous aromatic compounds, exhibiting almost no adverse effect on its growth in a complex liquid culture.

Key words: Benzoate, *Streptomyces*, biodegradation, *ortho*-cleavage

Natural and unnatural aromatic compounds, some of which are quite recalcitrant or toxic when released into the natural environment, are both widespread in nature. As environmental problems related to hazardous aromatic compounds (HACs) have become increasingly serious to daily life, various ways to eliminate or reduce these HACs in nature have been pursued, including bioremediation using soil microorganisms [2, 16]. It has been well documented that certain groups of gram-negative soil bacteria, such as the *Pseudomonas* and *Acinetobacter* species, are capable of completely degrading relatively simple HACs including phenol, benzene (B), toluene (T), ethylbenzene (E), or xylene (X) [12, 17, 20, 23]. In addition, the enzymes as well as the genes involved in HAC-biodegradation have also been extensively elucidated at both the biochemical and genetic levels [3, 18]. In general, most simple HACs are aerobically degraded through common intermediates, catechol or protocatechuate [9, 25]. This catechol or protocatechuate are then further degraded either by cleavage between two hydroxyl groups by catechol-1,2-dioxygenase (*ortho*-cleavage) or by cleavage adjacent to the hydroxyl groups by catechol-2,3-dioxygenase (*meta*-cleavage) [3, 8, 9]. Recently, some Gram-positive soil bacteria such as the *Bacillus, Arthrobactor*, and *Rhodococcus* species were isolated from various environments and characterized to contain a similar HAC-degrading pathway as the ones found in gram-negative bacteria [5, 7, 13, 19]. Interestingly, however, the overall characteristics of HAC-degrading enzymes and genes found in gram-positive bacteria seem to be quite different from the ones in the Gram-negative bacteria, implying that these two systems probably originated from different evolutionary ancestors [5, 7].

Streptomyces are gram-positive filamentous soil bacteria which undergo a unique morphological development [4]. For many years, *Streptomyces* have been considered as one of the most important industrial microorganisms, mainly because they produce many valuable secondary metabolites, including antibiotics, anti-cancer drugs, immunosuppresors, enzyme inhibitors, and dyes [10]. In addition, some *Streptomyces* species have also been recognized as being ecologically and environmentally valuable microorganisms due to their superior capabilities of degrading diverse structures of a variety of recalcitrant substances [14, 15, 22, 26]. Considering the fact that most soil *Streptomyces* mycelia can easily penetrate into soil and participate in the biodegradation of various xenobiotics scattered in the natural soil system, they are considered to be an ideal host for *in situ* soil bioremediation [6]. In the case of HAC-mineralizing *Streptomyces*, the thermophilic soil *S. setonii* (ATCC 39116), originally isolated from an Idaho soil by Crawford's group, is the only characterized *Streptomyces* species reported to utilize a benzoate or a phenol as a sole carbon and energy source [1, 21, 24]. Unfortunately, however, very limited information is available on the general degradation characteristics of HAC-degrading *S. setonii*.

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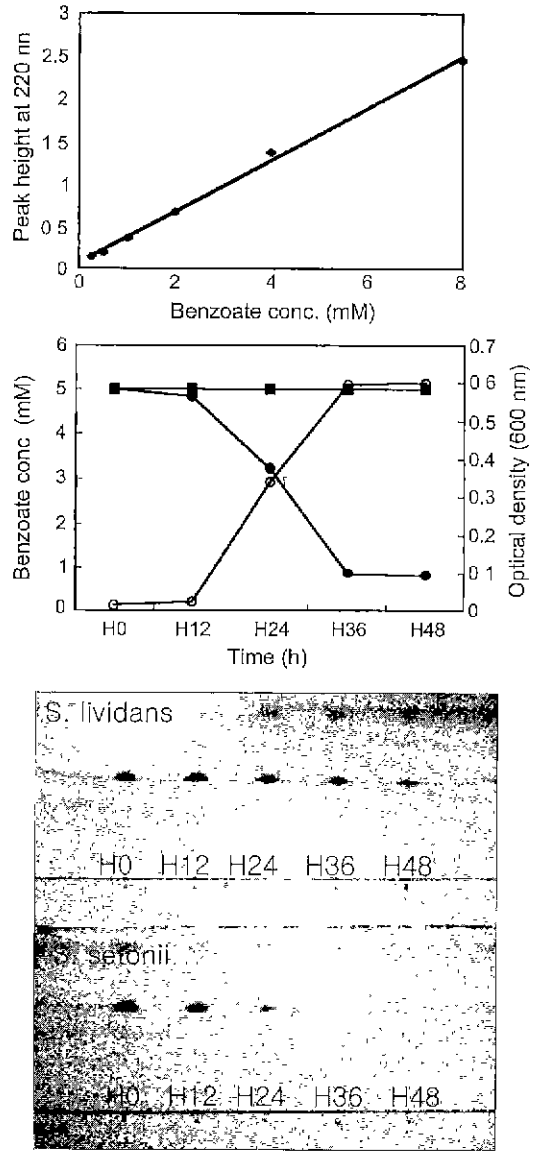

Fig. 1. Benzoate degradation and *Streptomyces* cell growth.
(a) Benzoate standard curve. The benzoate standard curve was obtained by measuring the absorbance at 220 nm with various amounts of benzoate in the minimal media using a UV spectrophotometer (b) UV-spectrophotometric assay of benzoate degradation. Two-ml samples of *S. setonii* or *S. lividans* cultured in 25 ml of 5 mM-containing benzoate minimal media in 250-ml baffeled flasks at 45°C (*S. setonii*) or 30°C (*S. lividans*) were taken every 12 h. One ml of each sample was centrifuged at 15,000 rpm for 10 min, followed by an absorbance measurement of each supernatant at 220 nm to determine the remaining benzoate in the media (closed squares for *S. lividans* and closed circles for *S. setonii*). Another 1 ml of each sample was measured at an absorbance 600 nm to determine the cell growth (the open circles are for *S. setonii* and there was no cell growth for *S. lividans*). (c) TLC assay of benzoate degradation. One-ml samples of *S. setonii* or *S. lividans* cultured in 25 ml of 5 mM-containing benzoate minimal media in 250-ml baffeled flasks at 45°C (*S. setonii*) or 30°C (*S. lividans*) were taken every 12 h. One ml of each sample was centrifuged at 15,000 rpm for 10 min, followed by a 200 µl chloroform extraction of the supernatant for adjusting the pH by adding 40 µl of 2 N HCl. A total of 20 µl of the extracted solution was spotted on a silica-gel TLC coated with a fluorescence indicator (Sigma, USA) and then eluted with a chromatographic solvent of benzene-methanol-acetic acid (45:10:1)

Both qualitatively and quantitatively through TLC and UV spectrophotometric assays, this short note describes that the thermophilic soil *S. setonii* (ATCC 39116), which can utilize a benzoate as a sole carbon and energy source in a minimal liquid culture, is not very sensitive to benzoate concentration and to culture conditions such as pH and temperature. The data also show that a catechol, a common intermediate in HAC-degradation, is cleaved to *cis,cis*-muconic acid by thermostable catechol-1,2-dioxygenase in *S. setonii*, which is the key enzyme in an *ortho*-cleavage pathway. Unlike non-degrading *S. lividans*, *S. setonii* is also highly resistant to other similar HACs exhibiting almost no adverse effect on its growth in a complex liquid culture.

The *S. setonii* strain purchased from ATCC (ATCC 39116) was cultured in 25 ml of benzoate-containing minimal liquid media in a 250-ml baffled flask at 45°C, and the remaining benzoate in the medium was measured using UV spectrophotometery (quantitatively) and TLC (qualitatively). Unlike benzoate non-degrading *S. lividans*, as shown in Fig. 1, *S. setonii* was fully capable of increasing its biomass by consuming the benzoate in the medium. *S. setonii* was able to completely degrade 1 mM benzoate in 12 h and even up to 20 mM in 7 days (Fig. 2), thereby indicating its capability of mineralizing a wide concentration range of benzoate as a sole carbon and energy source in a minimal liquid culture. Moreover, the overall benzoate degradation and growth pattern of the *S. setonii* was not significantly affected by pH of the culture media (Fig. 3). Although there was a slight delay in the benzoate degradation when incubated at a lower than optimum culture temperature (45°C), the *S. setonii* was fully capable of degrading 5 mM benzoate in less than 4 days at both 30°C and 37°C (data not shown). These

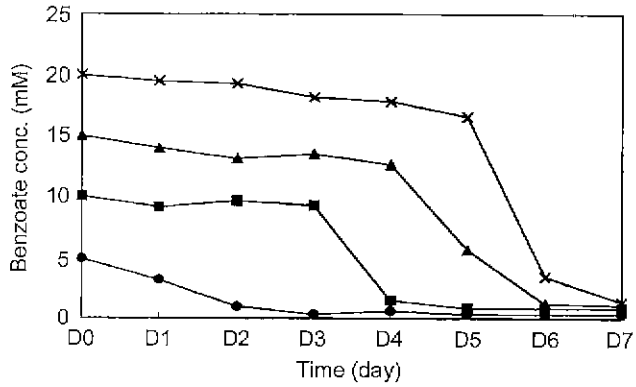

Fig. 2. Benzoate degradation at different concentrations by *S. setonii*.
One-ml samples of *S. setonii* cultured in 25 ml of minimal media containing various amounts (5, 10, 15, and 20 mM) of benzoate in 250-ml baffeled flasks at 45°C were taken every 12 h. One ml of each sample was centrifuged at 15,000 rpm for 10 min, followed by an absorbance measurement of each supernatant at 220 nm to determine the remaining benzoate in the media. A proper dilution was made with a minimal medium to keep the absorbance at 220 nm within the standard curve range

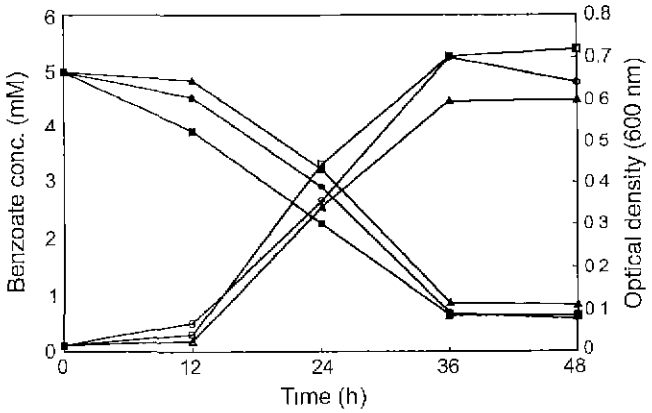

Fig. 3. Benzoate degradation at different pHs by *S. setonii*.
Two-ml samples of *S setonii* in 5 mM benzoate-containing minimal media in 250-ml baffeled flasks at 45°C were taken every 12 h. One ml of each sample was centrifuged at 15,000 rpm for 10 min followed by an absorbance measurement of each supernatant at 220 nm to determine the remaining benzoate in the media (closed squares for pH 5.5, closed triangles for pH 7.5, and closed circles for pH 9.5) Another 1 ml of each sample was measured at an absorbance of 600 nm to determine the cell growth (open squares for pH 5.5, open triangles for pH 7 5, and open circles for pH 9.5)

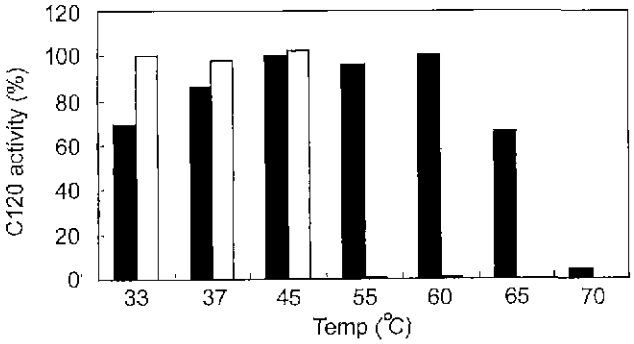

Fig. 4. Catechol-1.2-dioxygenase assays with the lysates from *S. setonii* and *Acinetobacter* sp. CNU8.
S setonii (at 45°C) and *Acinetobacter* sp. CNU8 (at 30°C) were cultured in 5 mM benzoate-containing minimal media for 36 h, followed by centrifugation at 10,000 rpm for 5 min The pellets were washed with 7 ml of a resuspending buffer (50 mM Tris-HCl pH 7 5, 1 mM $MnSO_4$), and then resuspended in the same buffer to a 1/100 culture volume. The resuspended pellets were sonicated with 9-second pulses at a 35% amplitude; 6 times for *S. setonii* and 3 times for *Acinetobacter* sp CNU8, followed by centrifugation at 10 000 rpm for 30 min The protein concentrations in the supernatants of *S. setonii* and *Acinetobacter* sp CNU8 were 0.13 μg/μl and 0.34 μg/μl, respectively. The *in vitro* catechol conversion assay using a UV spectrophotometer has been described elsewhere [7, 8] The assay mixture consisted of the cell lysates (100 μl for *S. setonii* or 25 μl of *Acinetobacter* sp. CNU8), a reaction buffer (30 mM Tris-HCl, pH 8.0, 1 2 mM EDTA, pH 8.0), and 0.3 mM of catechol in a total reaction volume of 1 ml. The reaction was initiated by adding the catechol into the reaction mixture, followed by a scanning of the absorbance between 200 nm and 450 nm. The C12O activity was measured according to the absorbance increase at 260 nm for 1 min (closed bars for *S. setonii* and open bars for *Acinetobacter* sp *CNU8). C12O* activities at 45°C for *S. setonii* and at 33°C for *Acinetobacter* sp. CNU8 were considered as 100%, respectively.

results indicate that the benzoate degradation by *S. setonii* is not very sensitive to benzoate concentration and to culture conditions such as pH and temperature, which strongly supports a possibility of using HAC-mineralizing *S. setonii* as an ideal inoculum for the *in situ* bioremediation of HAC-contaminated soil or wastewater. Recently, a lab-scale *S. setonii*-based bioaugmentation experiment also showed accelerated benzoate degradation in a natural soil system (Kim *et al.*, unpublished data).

In order to further characterize the benzoate-degradation pathway in *S. setonii*, an aromatic ring cleavage step was investigated using a crude *S. setonii* lysate *in vitro*. The *S. setonii* was cultured in 200 ml of 5 mM benzoate-containing minimal medium at 45°C for 36 h, followed by sonication of the harvested cells in a 1/100 volume of TE buffer [11]. The ensuing *in vitro* catechol conversion assay has been previously described elsewhere [7, 8]. The incubation of a catechol in the presence of the crude *S. setonii* lysate resulted in the formation of a *cis,cis*-muconic acid (*ortho*-cleavage intermediate) but not a muconic semialdehyde (*meta*-cleavage intermediate), as evidenced by an increase of absorbance only at 260 nm. The result revealed that the benzoate had been degraded by an *ortho*-cleavage enzyme, the catechol-1.2-dioxygenase in *S. setonii*. Interestingly, unlike the mesophilic benzoate-degrading *Acinetobacter* sp. CNU8 [12], the conversion of the catechol to a *cis,cis*-muconic acid by the crude *S. setonii* lysate was observed within a relatively broad range (from 33°C up to 65°C) of assay temperatures (Fig. 4), suggesting that *S. setonii* contains a thermostable catechol-1.2-dioxygenase.

In order to compare the degree of cell growth inhibition by HACs such as benzoate, phenol, or 4-chlorophenol in different *Streptomyces* species, both *S. setonii* or *S. lividans* were separately cultured in 25 ml of YEME liquid medium [11] containing different amounts of benzoate, phenol, or 4-chlorophenol, and the cell growth was then

Table 1. Degree of *Streptomyces* cell growth inhibition by phenol, benzoate, or 4-chlorophenol.

Strain	Inhibitor conc. (mM)														
	Phenol					Benzoate					4-Chlorophenol				
	0	1	3	5	7	0	1	3	5	7	0	1	3	5	7
S setonii	100	95.9	89.1	839.	84.8	100	98.2	94.6	83.1	80.3	100	98.9	94 3	4.3	0
S. lividans	100	91.6	72.4	29.6	19.9	100	88.6	68.4	63.0	63.0	100	56.9	0.9	0.6	0

One-ml samples of *S. setonii* or *S lividans* cultured in 25 ml of YEME [11] containing different amounts of phenol, benzoate, or 4-chlorophenol in 250-ml baffeled flasks at 45°C (*S setonii*) or at 30°C (*S lividans*) were taken every 12 h for measuring the absorbance at 600 nm to determine the cell growth. The maximun OD at 600 nm for each culture set was then compared with that containing no inhibitor (100%).

measured using an optical density at 600 nm. As shown in Table 1, the *S. setonii* growth was not significantly affected by the presence of phenol or benzoate in the liquid culture. In the case of *S. lividans*, however, a significant reduction of cell growth was observed in the presence of phenol or benzoate, proportional to the amount of HACs in the culture media (Table 1). Although both *S. setonii* and *S. lividans* did not mineralize 4-chlorophenol, *S. setonii* showed much higher resistance to this compound than *S. lividans* (Table 1). These observations suggest that, unlike *S. lividans* which is quite sensitive to the three different HACs tested, *S. setonii* is more resistant to not only benzoate and phenol, but also to a structurally-related non-degradable HAC such as 4-chlorophenol.

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