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AND MIXED CULTURES OF *Rhodococcus rhodochrous* IGTS8**

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AND MIXED CULTURES OF Rhodococcus rhodochrous IGTS8

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ABSTRACT

Growth assays reveal that Rhodococcus rhodochrous IGTS8 can utilize a wide range of organosulfur compounds as the sole source of sulfur. Compounds that are utilized include thiophenes, sulfides, disulfides, mercaptans, sulfoxides, and sulfones. None of the organosulfur compounds tested can serve as a carbon source. A convenient spectrophotometric assay (Gibbs assay) based on the chromogenic reaction of 2,6-dichloroquinone-4-chloroimide with aromatic hydroxyl groups was developed and used in conjunction with GC/MS analyses to examine the kinetics of carbon-sulfur bond cleavage by axenic and mixed cell cultures of Rhodococcus rhodochrous IGTS8. The desulfurization trait is expressed at uniform levels during the mid-exponential phase, reaches a maximum during idiophase, and then declines in stationary-phase cells. Desulfurization rates for dibenzothiophene (DBT) range from 8 to 15 μM of DBT/ 10^{12} cells/hour. Mixtures of genetically marked Rhodococcus rhodochrous IGTS8 and an organism incapable of cleaving carbon-sulfur bonds in relevant test compounds, Enterobacter cloacae, were prepared in ratios that varied over six orders of magnitude. Growth studies revealed that Enterobacter cloacae was able to gain access to sulfur liberated from organosulfur compounds by IGTS8; however, cell-to-cell contact was required. These data also indicate that the desulfurization activity of IGTS8 cells in mixed cultures may be as much as 200-fold higher than in axenic cultures.

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INTRODUCTION

Sulfur dioxide released into the atmosphere remains one of the most prominent and intractable issues of environmental concern. The combustion of crude oil and coal produces sulfur dioxide, which is a major contributor to the generation of acid rain. The removal of sulfur from coal and crude oil, prior to combustion, will lower sulfur dioxide emissions into the atmosphere. Several technologies for the removal of sulfur from coal and crude oil are currently being employed, but they remain expensive and, for the most part, they address only post-combustion sulfur removal. Microbial desulfurization may provide an improved technology for the pre-combustion removal of organic sulfur from fossil fuels. Many reports have shown that several organisms possess the ability to remove the inorganic sulfur from coal [1,3,4,15], but the organic sulfur content continues to be a tenacious problem. Coal and crude oil contain many of the same organic sulfur structures, which include thiophenes, disulfides, thiols, sulfides, sulfones, mercaptans, and sulfoxides. The compound dibenzothiophene has received the majority of attention in recent biodesulfurization studies because it is an accepted model compound representative of thiophenic structures found in coal and petroleum. Several researchers have isolated organisms that possess the ability to use DBT as a carbon and a sulfur source for growth [2,11-13]. Most of these organisms use the carbon-destructive pathway proposed by Kodama [10], in which degradation is initiated by the cleavage of carbon-carbon bonds. Some organisms have been shown to completely mineralize DBT [16]. The specific cleavage of carbon-sulfur bonds, rather than degradation/mineralization of organosulfur compounds, is preferred for a biodesulfurization process so that sulfur is removed but the carbon and calorific value remain intact. Rhodococcus rhodochrous IGTS8 has proven capable of the selective cleavage of carbon-sulfur bonds in DBT, yielding 2-hydroxybiphenyl (2-HBP) as the sole detectable metabolite [6,8]. The removal of sulfur from coal and petroleum by IGTS8 has also been demonstrated [9]. Recently, an additional organism, Corynebacterium sp. [14], has been reported to be capable of selectively cleaving carbon-sulfur bonds. This culture uses DBT as a sole sulfur source and produces 2-HBP, as well as several hydroxynitrobiphenyls (from subsequent nitration). Currently, the novel biochemical reaction resulting in the selective cleavage of carbon-sulfur bonds is almost wholly uncharacterized. In this paper, the range of organosulfur compounds metabolized by Rhodococcus rhodochrous IGTS8 and the kinetics of DBT desulfurization by axenic and mixed cell cultures of Rhodococcus rhodochrous IGTS8 are reported.

MATERIALS AND METHODS

Chemicals

All organic sulfur compounds were of the highest quality available and were obtained from Aldrich Chemical, Sigma Chemical, ICN Biomedicals, K and K Labs, or Eastman Kodak Chemical. Chemical compounds were filter-sterilized using an Acrodisc-Cr 0.2- μ m-pore-size membrane filter (Gelman) or were sterilized by autoclaving. Concentrations given indicate weight per volume, except for liquid compounds, which indicate volume per volume.

Bacteria and Growth Conditions

Media (BSM) and growth conditions for Rhodococcus rhodochrous IGTS8 (ATCC No. 53968) have been previously described [6,8,9]. The majority of mixed cell culture experiments were mixtures of Enterobacter cloacae and Rhodococcus rhodochrous IGTS8; however, mixtures of IGTS8 and Enterobacter agglomerans, Klebsiella pneumonia, or Pseudomonas aeruginosa were used in some experiments. The sulfur bioavailability assay [6,8,9] was used to monitor the ability of IGTS8 to use various organosulfur compounds as sole sources of sulfur. Growth tests using BSM lacking glycerol were performed to determine if IGTS8 could use organosulfur compounds as carbon sources [7]. General inhibition of the growth of IGTS8 in BSM glycerol medium was assessed using 20mM sulfate and 20mM of the test compound. The ability to specifically inhibit the desulfurization activity of IGTS8 was assessed using 20mM DBT and 20mM of the test compound in growth experiments employing BSM glycerol media. The involvement of the desulfurization trait for metabolism of a test compound was investigated by growing IGTS8 either with the test compound as a sole sulfur source or in the presence of the organosulfur test compound and sulfate, obtaining a washed-cell suspension, and quantifying the conversion of DBT to 2-HBP during a 1-hour incubation using the standardized conditions of the Gibbs assay. Growth was monitored using a Klett-Summerson colorimeter equipped with a green filter such that 100 Klett units corresponds to approximately 5×10^8 cells/ml. Cell density was also monitored spectrophotometrically at 600 nm. Colony-forming units were determined on nutrient agar (Difco).

Analytical Methods

Desulfurization activity was monitored using the Gibbs reagent (2,6-dichloroquinone-4-chloroimide, Sigma) and gas chromatography/mass spectrometry (GC/MS). Gibbs reagent reacts with aromatic hydroxyl groups such as 2-HBP at a pH of 8.0 to form a blue-colored complex that can be monitored spectrophotometrically at 610 nm. (To obtain the maximum accuracy using the Gibbs assay it was found that the pH and time of incubation/color development must be precisely controlled.) The Gibbs assay was used in two ways to monitor the desulfurization activity of bacterial cultures. The cumulative production of desulfurized product was determined

by assaying aliquots removed at various time points from cultures, growing at the expense of an organosulfur compound such as DBT, that yield a hydroxylated aromatic compound as a desulfurization product. Alternatively, the desulfurization activity of cultures grown using any substrate, organosulfur or otherwise, was monitored by obtaining washed-cell suspensions in BSM, adjusting to a constant cell density of 1 absorbance unit (measured at 600 nm), adding 20mM DBT, incubating at 30°C for 60 minutes, and then using the Gibbs assay to determine the quantity of desulfurized product. The Gibbs assay was conducted as follows: 5 ml of microbial culture was placed into a 15-ml test tube, the pH was adjusted to 8.0 with Na₂CO₃, then 50 µl of Gibbs reagent (10 mg/ml ethanol solution) was added. The solution was allowed to incubate for 30 minutes to produce full color development. The reaction was centrifuged to remove cells, and the absorbance of the supernatant was determined at 610 nm (Beckman DU-65) and then converted to ppm based upon a 2-HBP generated standard curve. Samples for gas chromatography analysis were extracted by liquid-liquid extraction using dichloromethane and analyzed using a Perkin-Elmer 8320 with a AS-100B autosampler. The column used was a 30-meter, 0.25-mm-ID, 0.25-µm film, DB-5 type. The concentration range is 5 to 2000 µg/ml using the internal standard method of quantification.

RESULTS

IGTS8 Growth on Organosulfur Compounds

A diverse collection of organosulfur compounds including thiophenes, sulfides, disulfides, sulfoxides, sulfones, and mercaptans were examined to determine if they can serve as sole sulfur sources for the growth of IGTS8. Additionally, the ability of these compounds to serve as carbon sources, their ability to inhibit growth generally, their effect on the ability of IGTS8 to utilize DBT, and their ability to serve as inducers of the desulfurization trait were investigated. The results of these tests are shown in Table 1. Initially, IGTS8 appeared to be unable to use many of the compounds in Table 1 as sulfur sources; however, an adaptation/adjustment period prior to desulfurization/growth testing yielded the results shown. Adaptation consisted of subculturing IGTS8 cells from sulfur bioavailability assays that exhibited good growth into fresh BSM glycerol medium containing the organosulfur compounds of interest. None of the organosulfur compounds listed in Table 1 were capable of serving as carbon as well as sulfur sources. GC/MS was used to examine the reaction products of several of the organosulfur compounds listed in Table 1 that served as sulfur sources, but no products resulting from carbon-carbon bond cleavage were observed. Each item of data in Table 1 represents the results of at least three independent growth tests.

Experiments were also performed using prolonged (weeks to months) incubations of IGTS8 as well as populations of IGTS8 mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine or ultra-

Table 1. METABOLISM OF ORGANOSULFUR COMPOUNDS BY *R. rhodochrous* IGTS8

COMPOUNDS	UTILIZATION AS CARBON SOURCE	UTILIZATION AS SULFUR SOURCE	GROWTH IN THE PRESENCE OF TEST COMPOUND PLUS SULFATE	GROWTH IN THE PRESENCE OF TEST COMPOUND PLUS DBT	DESULFURIZATION ACTIVITY
1 DBT	-	+	+	+	+
2 DBT SULFOXIDE	-	+	+	+	ND
3 DBT SULFONE	-	+	+	+	ND
4 THIANTHRENE	-	+	ND	ND	ND
5 THIANAPHTHENE	-	-	-	-	ND
6 THIOXANTHREN-9-ONE	-	+	ND	-	ND
7 TRITHIANE	-	+	+	+	+
8 METHIONINE	-	+	ND	-	-
9 CYSTINE	-	+	ND	-	ND
10 BIOTIN	-	-	+	+	ND
11 THIAMINE	-	+	ND	ND	+
12 1,4,7-TRITHIACYCLONANE	-	+	ND	ND	ND
13 1,5-DITHIACYCLOOCTAN-3-OL	-	+	+	+	ND
14 2-THIOPHENE-CARBOXYLIC ACID	-	-	-	-	ND
15 1,3-DITHIANE	-	-	ND	ND	+
16 1,4-DITHIANE	-	+	ND	-	ND
17 1,3-PROPANETHIOL	-	-	ND	-	ND
18 TRANS 1,2-DITHIANE 4,5-DIOL	-	+	ND	ND	ND
19 2-TRIMETHYLSULYL-1,3-DITHIANE	-	+	ND	ND	ND
20 L-CYSTEINE HYDROCHLORIDE HYDRATE	-	+	ND	ND	-
21 BENZYL DISULFIDE	-	+	ND	ND	ND
22 PHENYL DISULFIDE	-	-	-	-	ND
23 PHENYL SULFOXIDE	-	-	-	-	ND
24 2-NITROPHENYL DISULFIDE	-	+	ND	ND	ND
25 4-AMINOPHENYL DISULFIDE	-	-	ND	ND	ND
26 4-NITROPHENYL DISULFIDE	-	+	ND	ND	ND
27 3-NITROPHENYL DISULFIDE	-	-	ND	ND	ND
28 TOLYL DISULFIDE	-	-	+	-	ND
29 THIONIN	-	-	+	-	ND
30 SULFANILAMIDE	-	-	+	-	ND
31 PHENOL RED	-	+	ND	ND	ND
32 METHYLENE BLUE	-	+	ND	ND	ND
33 THIAZOLE YELLOW	-	+	ND	ND	ND
34 THYMOL BLUE	-	-	-	-	ND
35 THIAZOLE	-	+	ND	ND	+
DIMETHYLSULFOXIDE	-	+	ND	ND	+

violet irradiation, using liquid and solid media containing various organosulfur compounds as sole carbon sources. No derivative of IGTS8 was obtained that is capable of using organosulfur compounds as carbon sources.

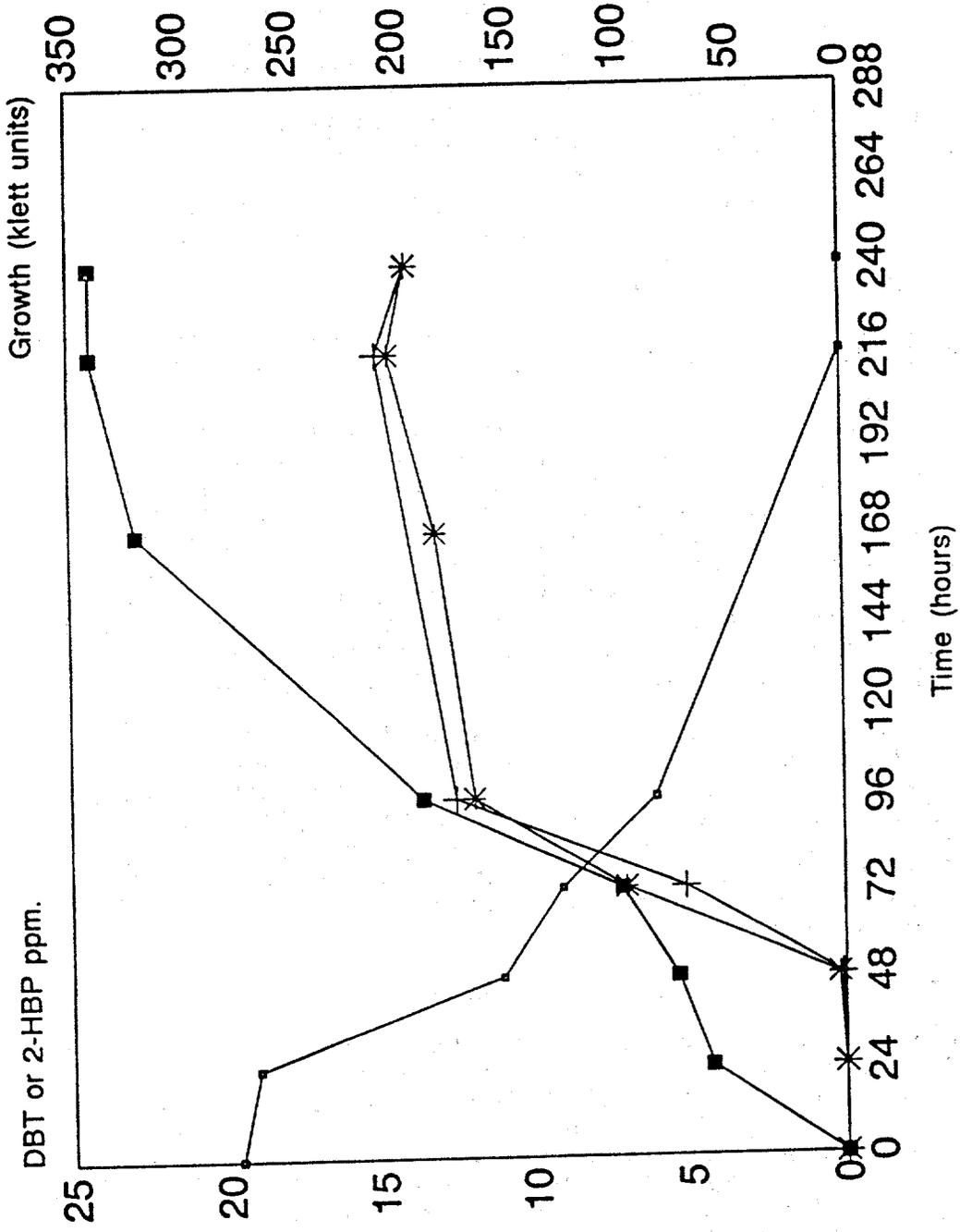
Perhaps the most interesting data in Table 1 are those compounds that appear to be either general or specific inhibitors of growth. Thianaphthalene, phenyl sulfoxide, 2-aminophenyl disulfide, and Thymol blue all appear to be generally inhibitory, because the presence of these compounds inhibits growth under all conditions. The compounds tolyl disulfide, thionin, and sulfanilamide appear to be specific inhibitors of the desulfurization activity of IGTS8, because they will not serve as sole sources of sulfur; they will allow growth of IGTS8 when present with sulfate but will not allow growth when present with DBT. The data in Table 1 also reveal that some compounds that make excellent sulfur sources, such as methionine, do not induce the expression of the desulfurization trait. Growth studies also revealed that the expression of the desulfurization trait of IGTS8 is subject to both repression and induction. IGTS8 will not metabolize DBT in the presence of sulfate. The Gibbs assay was used to detect mutants of IGTS8 that are not repressed by sulfate and will convert DBT to 2-HBP even in the presence of 20mM sulfate. However, these mutant cultures do not express the desulfurization trait when grown with sulfate as the sole source of sulfur. They require the presence of a suitable organosulfur compound to induce the expression of the desulfurization trait.

The concentration of sulfur required for the growth of IGTS8 was investigated using sulfate, sulfite, DBT, dibenzothiophene sulfoxide (DBTSO), and trithiane as sulfur sources at concentrations ranging from 0.005 to 5.0mM. About 0.1mM of a sulfur source is required to achieve the maximum amount of growth of IGTS8 in BSM, and the various sulfur sources mentioned above yielded similar results.

Kinetics of Desulfurization

DBT, DBTSO, and dibenzothiophene sulfone (DBTSO₂) are each converted uniquely to 2-HBP by IGTS8 as determined by GC/MS. No intermediate products of desulfurization were observed nor is 2-HBP degraded further or modified. The expression of the desulfurization trait of IGTS8 in relationship to the bacterial growth cycle was studied using two methodologies. IGTS8 was grown with DBT serving as sole source of sulfur, and the conversion/accumulation of 2-HBP from DBT (or similar substrates) was monitored throughout the growth cycle. Also, IGTS8 was grown using a sulfur source other than DBTSO₂, and cells were harvested at various stages of growth and their ability to convert DBTSO₂ to 2-HBP during brief incubations was quantified.

DBT was added at concentrations of 20 ppm (in excess of the 0.1mM sulfur requirement), and the conversion of 2-HBP to DBT was monitored using both the Gibbs assay and GC/MS. The results shown in Figure 1 illustrate that growth and 2-HBP



○ DBT ppm; ■ Gibbs 2-HBP ppm; * GC/MS 2-HBP ppm; × Growth
 Figure 1. IGTS8 GROWING-CELL DESULFURIZATION OF DBT. AVERAGE STANDARD DEVIATION IS ±0.82 ppm FOR GC/MS OF DBT OR 2-HBP.

production ran parallel; DBT was converted to 2-HBP in near-stoichiometrical amounts. The DBT and 2-HBP ppm data points in Figure 1 are averages of three uniform samples from each time point, with five separate determinations for each sample submitted to GC/MS analysis, totaling fifteen samples for each DBT and 2-HBP GC/MS data point and three samples for each 2-HBP Gibbs assay data point. The average standard deviation for the data in Figure 1 is 0.82 ppm. These data indicate that DBT is metabolized maximally by cells in the early to middle log phase of growth and that stationary-phase cells do not continue to metabolize DBT. A possible complicating factor in these experiments is that 2-HBP, the product of DBT metabolism, is somewhat inhibitory, and its accumulation may bias the results. Moreover, because the cell concentration changes throughout the experiment, changes in the specific activity are not readily apparent.

The data shown in Figure 2 were obtained by growing IGTS8 using dimethyl sulfoxide (DMSO) as the sole sulfur source and harvesting cells at various stages of growth, adjusting to the same cell density ($A_{600\text{ nm}} = 1.00$), exposing the culture to DBTSO_2 for 60 minutes, and assaying using the Gibbs reagent. This method of analysis has the advantages of testing cells at identical cell densities and avoiding the accumulation of high concentrations of the potentially inhibitory metabolite 2-HBP. Each data point in Figure 2 is the average of triplicate analyses. The results shown in Figure 2 indicate that the specific desulfurization activity of IGTS8 cells appears to be relatively constant in early to mid log-phase cells, peaks in late log-phase cells, and declines in stationary-phase cells.

Tests using the Gibbs assay, as well as GC/MS analysis, indicate that the rate at which these compounds are metabolized by IGTS8 cells during a 1-hour incubation varies with $\text{DBTSO}_2 > \text{DBT} > \text{DBTSO}$. The corresponding yields of 2-HBP produced are 5.0, 3.0, and 2.75 ppm, respectively. Prolonged incubation of IGTS8 cells for as long as 18 hours yielded the same ranking of these three substrates with respect to their rates of metabolism.

Axenic and Mixed Cell Culture Kinetics

Figure 3 shows quantitative cell-growth data, 2-HBP production data, and viable cell counts of pure IGTS8 cell cultures and mixed cultures of IGTS8 and Enterobacter cloacae. Standard deviation of DBT and 2-HBP ppm data points in Figure 3 was based upon three uniform samples from each time point, with five separate determinations for each sample submitted to GC/MS analysis, totaling fifteen samples for each 2-HBP GC/MS data point and three samples for each 2-HBP Gibbs assay data point. The IGTS8 strain used in these mixed culture experiments was resistant to 500 $\mu\text{g/ml}$ of streptomycin. The mixed cultures were plated onto nutrient agar with and without streptomycin, which readily allowed IGTS8 to be quantified in these mixed cultures. The total cell density of the mixed IGTS8/Enterobacter culture after 360 hours was 5.5×10^8 as compared with 2.2×10^9 cells/ml in the pure IGTS8 cultures. The relative abundance of IGTS8 in these mixed

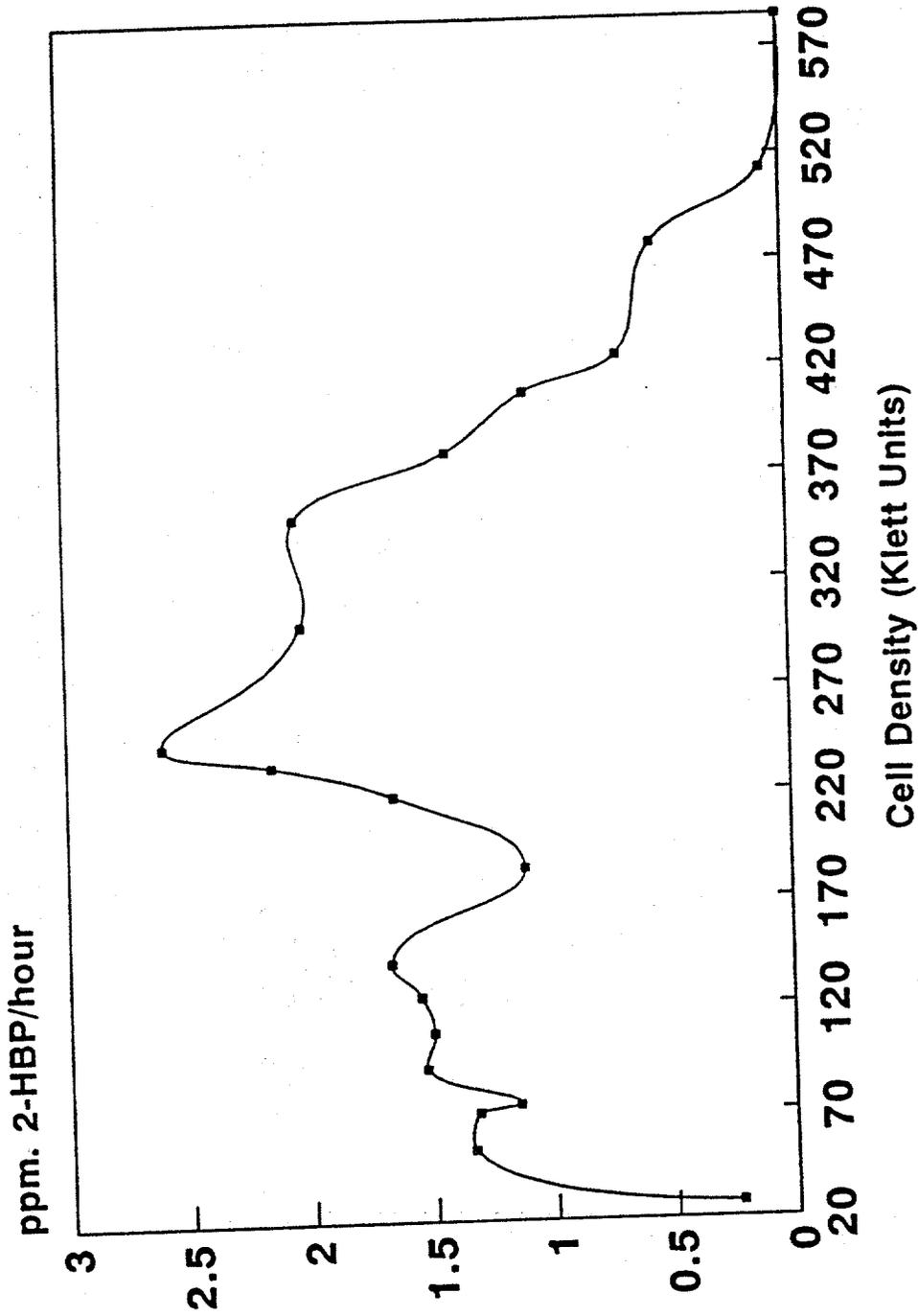


Figure 2. IGTS8 RESTING-CELL DESULFURIZATION OF DBTSO₂. CELL DENSITIES WERE ADJUSTED TO 1.000 ABSORBANCE UNITS AT 600 nm PRIOR TO BEING TESTED USING THE GIBBS ASSAY.

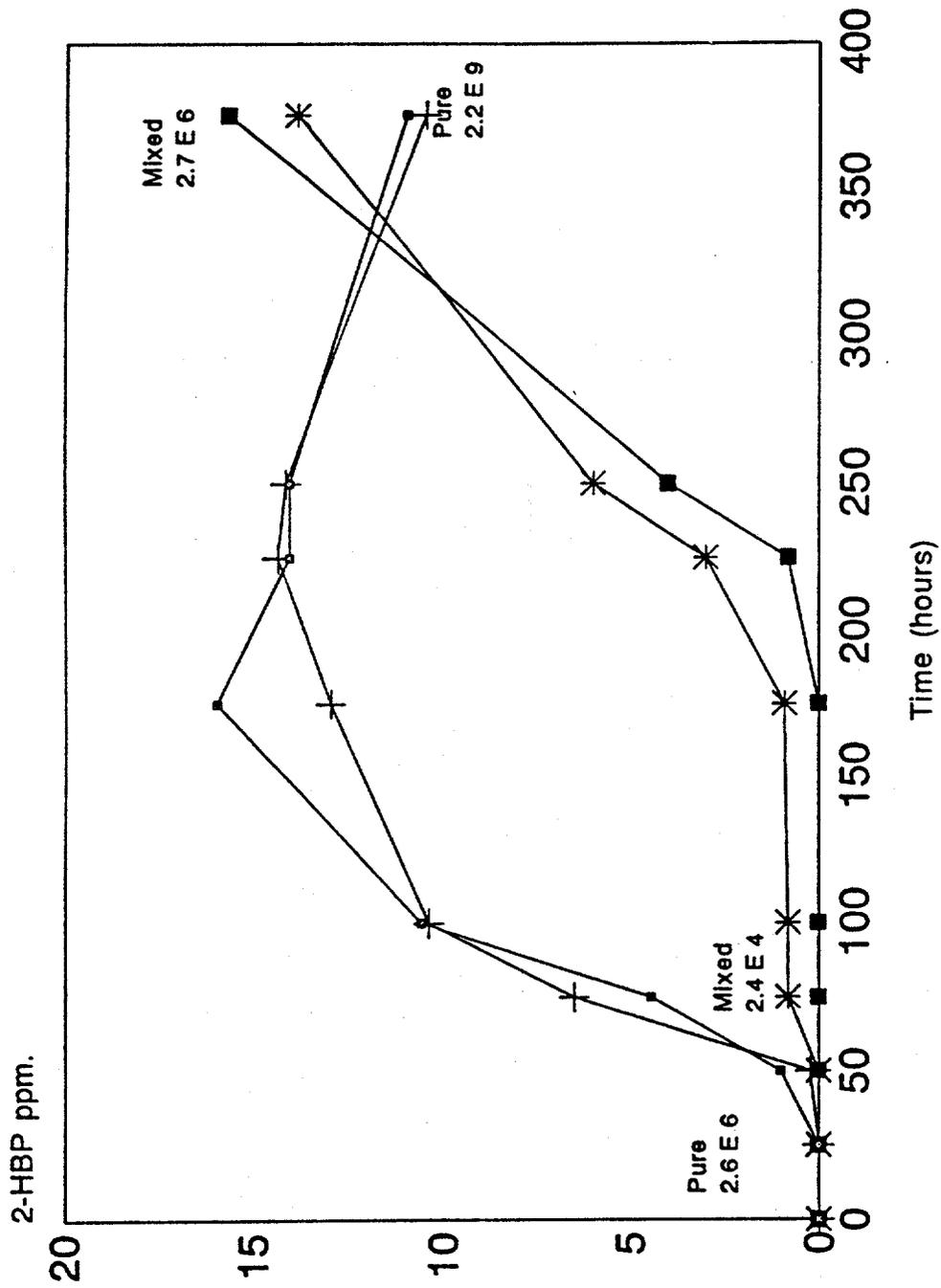


Figure 3. AXENIC AND MIXED-CELL CULTURES OF IGTS8

cultures after 360 hours was 2.7×10^6 cells/ml. Even though these mixed cultures contained 200-fold fewer cells capable of metabolizing DBT, they eventually were capable of producing quantities of 2-HBP nearly identical to those of pure IGTS8 cultures [Enterobacter cloacae in pure culture is unable to grow under sulfur bioavailability assay conditions or convert DBT to detectable quantities of 2-HBP (data not shown).] Figure 3 summarizes these observations for IGTS8 by plotting the 2-HBP production curves as determined by both the Gibbs and GC/MS assays for both a pure and the mixed culture of IGTS8. Also indicated on Figure 3 is the fact that the cell density of IGTS8 in the pure culture was 2.6×10^6 cells/ml after 40 hours and 2.2×10^9 cells/ml after 360 hours; whereas the concentration of IGTS8 in the mixed culture was 2.4×10^4 cells/ml after 40 hours and 2.7×10^6 cells/ml after 360 hours. Similar experiments employing mixtures of IGTS8 with Enterobacter agglomerans, Klebsiella pneumonia, and Pseudomonas aeruginosa yield similar results (data not shown).

To investigate this further, mixtures of IGTS8 and Enterobacter cloacae cultures were prepared in various ratios. These mixed cultures were then used to inoculate test tubes containing BSM glycerol media with DBT serving as the sole source of sulfur. The tubes were incubated at 30°C and growth was monitored over a 15-day period. Viable cell counts and Gibbs assay values were also determined after 15 days of growth. The streptomycin-resistant strain of IGTS8 was used so that populations of Enterobacter and IGTS8 could be monitored. Eight initial cell ratios ranging from pure IGTS8 to 10^6 Enterobacter/IGTS8 cell were investigated using triplicate samples. The results are shown in Table 2. All of the cultures eventually grew to about the same density, although the growth rate was influenced by the ratio of Enterobacter to IGTS8. This effect seemed more pronounced when the initial ratio of Enterobacter to IGTS8 was greater than or equal to 100. In cultures with initial ratios less than 100, the final ratio increased; whereas when the initial ratio was greater than 100, the final ratio decreased. In some cases the final ratio decreased about 1000-fold. Decreased final ratios indicate that IGTS8 grew proportionately more than the Enterobacter culture, but this resulted in an overall decrease in the rate of growth.

Several experiments were performed with IGTS8 and Enterobacter cloacae cultures separated by a dialysis membrane. The bacterial growth medium used was BSM supplemented with 20-ppm DBT. A dialysis membrane separated the cultures and allowed complete transfer of metabolites and nutrients between cells, but the organisms were unable to pass through the membrane. IGTS8 grew well while the Enterobacter culture failed to grow. These studies showed that cell contact is required for the growth of Enterobacter in co-culture with IGTS8 when DBT is used as the sole sulfur source.

Table 2. IGTS8 and Enterobacter cloacae CULTURES PREPARED IN VARIOUS RATIOS

Initial ratio of Enterobacter to IGTS8	Days of Incubation										Final Ratio of Enterobacter to IGTS8	Gibbs Assay Value in ppm.
	1	2	3	6	7	8	9	10	13	15		
IGTS8	33	47	50	46	260	415	520	525	560	590	IGTS8	11.39
1:1	23	23	23	23	85	290	430	470	470	470	1.3:1	9.59
10:1	14	14	14	27	150	320	380	440	460	470	14.5:1	9.66
100:1	12	12	12	12	16	27	180	330	480	500	40.3:1	8.83
1000:1	13	13	13	13	17	24	122	300	460	500	96.8:1	7.82
10000:1	12	12	12	12	12	22	82	190	480	500	260:1	6.65
100000:1	14	14	14	14	16	25	50	170	360	475	498:1	6.33
1000000:1	13	13	13	13	17	22	33	88	425	470	1000:1	5.17

DISCUSSION

Rhodococcus rhodochrous IGTS8 possesses the ability to desulfurize/utilize an extremely broad array of organosulfur compounds as sole sulfur sources. Some very interesting points of IGTS8's desulfurization of DBT can be concluded: carbon infrastructure is maintained during desulfurization, compounds that can serve as sulfur sources do not necessarily induce desulfurization activity, and some organosulfur compounds inhibit the growth and/or desulfurization ability of IGTS8.

Most of the compounds found in Table 1 can be used as sulfur sources, but none can be used as carbon sources. The desulfurization of compounds such as 1,4,7-trithiacyclononane, 1,5-dithiacyclooctan-3-ol, 1,3-dithiane, 1,4-dithiane, 1,3-propanethiol, and trans 1,2-dithiane,4,5-diol would be expected to yield products similar if not identical to ethanol or glycerol. Because IGTS8 can use glycerol and ethanol as carbon sources, it is unexpected that no organosulfur compound has been found to simultaneously serve as a carbon as well as a sulfur source. The carbon infrastructure apparently remains intact not only in desulfurization experiments with organosulfur compounds but also in experiments with petroleum and coal, because previous work showed that caloric value was preserved in biodesulfurized petroleum and coal samples [5].

Another point of interest is the organosulfur compounds that appear to be either general or specific inhibitors. Thianaphthalene, phenyl sulfoxide, 2-aminophenyl disulfide, and Thymol blue all appear to be generally inhibitory, because the presence of these compounds inhibits growth under all conditions. It is not clear if the inhibition is caused by the original compound or some metabolite. The compounds tolyl disulfide, thionin, and sulfanilamide appear to be specific inhibitors of the desulfurization activity of IGTS8 because they will not serve as sole sources of sulfur; they will allow growth of IGTS8 when present with sulfate but will not allow growth when present with DBT. There is no obvious structure common to these three compounds or distinguishable from the other compounds tested that can be cited to explain the nature of the apparent specific inhibition of desulfurization activity. Specific inhibitor compounds can be quite useful in the investigation of biochemical reactions, and the metabolism of these compounds by IGTS8 will be examined further in future studies.

The minimum sulfur requirement for healthy growth of IGTS8 in BSM was found to be about 0.1mM. Similar results were observed for sulfate and a variety of organosulfur compounds. These data suggest that IGTS8 does not metabolize DBT in excess of its requirements for growth. IGTS8 growth studies on various organosulfur compounds in BSM also yield varied doubling times and growth rates. The fastest observed doubling time was 12 hours.

The metabolic pathway for the conversion of DBT to 2-HBP has been speculated to proceed by successive oxidative steps:

DBT/DBTSO/DBTSO₂/DBTSO₃/2-HBP and sulfite/sulfate [14,16]. The data obtained in this report were insufficient to either unequivocally confirm or deny the existence of this pathway. No intermediates in the conversion of DBT, DBTSO, or DBTSO₂ to 2-HBP were observed in this study, although DBT, DBTSO, and DBTSO₂ are each converted uniquely to 2-HBP by IGTS8 and 2-HBP was produced by pre-grown cells at a rate favoring DBTSO₂/DBT/DBTSO. The sulfur liberated from DBT (or other organosulfur compounds) was exclusively found associated with/incorporated into the bacterial cells. Neither sulfate, sulfite, nor any other soluble form of sulfur was observed as a reaction product. Likewise, an analysis of headspace gases failed to detect hydrogen sulfide or any other sulfur-containing gas as a reaction product (data not shown). Interestingly, however, sulfur liberated from DBT (or other organosulfur compounds) by IGTS8 is in a form that is available to other microorganisms.

We examined a variety of bacterial species in mixed cultures with IGTS8, including Enterobacter cloacae, Enterobacter agglomerans, Pseudomonas aeruginosa, and Klebsiella pneumonia. All of the mixed culture experiments yielded similar results in that the quantity of DBT metabolized by mixed cultures was equivalent to that of pure cultures of IGTS8, yet the relative abundance of IGTS8 cells in mixed cultures was several orders of magnitude lower than in pure IGTS8 cultures. The only bacterium present in these mixed cultures that demonstrated any ability to desulfurize DBT is Rhodococcus rhodochrous IGTS8. This implies that DBT is desulfurized by IGTS8 in such a way that the liberated sulfur is available to other fast-growing bacteria in the mixed culture that are themselves unable to metabolize DBT. Sulfur liberated from DBT by IGTS8 can be used by other microorganisms, but the fact that cell contact is required (as demonstrated by examining cultures separated by a dialysis membrane) is further confirmation that a soluble sulfur form, such as sulfate, is not released into the medium by IGTS8 except from DBT. These data suggest a close association between IGTS8 and E. cloacae forms such that E. cloacae is capable of interrupting the uptake of sulfur by IGTS8 cells in the mixed culture, "stealing" sulfur liberated from DBT from the external surface of IGTS8 cells. The result is that IGTS8 cells in mixed cultures are forced to metabolize more organosulfur compounds than in pure culture. That is to say that the per cell activity of IGTS8 cells appears to be as much as 200-fold higher in mixed cultures than in pure cultures. The physiological explanation for this stimulation in activity is currently unknown and will be the subject of further studies. These data with mixed cultures also suggest that DBT is not taken up into the cytoplasm of IGTS8 to be metabolized intracellularly. Rather, the desulfurization activity of IGTS8 cells appears to be associated with the exterior surface of the cell. Moreover, preliminary experiments (data not shown) indicate that desulfurization activity is found in the cell wall/membrane fraction of lysed IGTS8 cells.

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