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Metabolism of Chlorinated Guaiacols by a Guaiacol-Degrading *Acinetobacter junii* Strain

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The metabolism of chlorinated guaiacols by a pure bacterial strain identified by its ability to use guaiacol as the sole carbon and energy source was studied. This strain, identified as *Acinetobacter junii* 5ga, was unable to grow on several chlorinated guaiacols and catechols. However, strain 5ga grown on guaiacol degraded 4- and 5-chloroguaiacol and 4,5-dichloroguaiacol. Under the same conditions, these cells did not degrade 6-chloroguaiacol, 4,6-dichloroguaiacol, 4,5,6-trichloroguaiacol, or tetrachloroguaiacol, suggesting that the substitution at the 6 position in the ring prevents metabolism of the compound. Degradation of 4-chloroguaiacol was dependent on the initial ratio between the chlorinated compound and viable cells. Transient formation of chlorocatechols resulting from incubation of cells with 4-chloroguaiacol or 4,5-dichloroguaiacol was suggested by UV spectroscopy. Gas chromatography analyses of samples from cultures of strain 5ga grown on guaiacol and incubated with 4- and 4,5-dichloroguaiacol confirmed the presence of 4-chlorocatechol and 4,5-dichlorocatechol, respectively. The formation of the latter was corroborated by gas chromatography-mass spectrometry. Thus, this strain is able to initiate metabolism of specific chlorinated guaiacols by O-demethylation. The starting chlorinated guaiacols and their O-demethylated metabolites inhibited the growth of *A. junii* 5ga on guaiacol.

Bleaching of kraft pulp with chlorine produces large amounts of lignin-derived material termed chlorolignins (16). In the low-molecular-weight fraction of this material are chlorinated phenols, guaiacols, and catechols. As a group, they account for about 47% of the low-molecular-weight chlorinated material produced per ton of kraft softwood pulp (8). Chlorophenolics may be responsible for the acute toxicity observed in the biota exposed to pulp mill effluents (18).

There have been very few studies on the microbial degradation of chloroguaiacols (for a recent review, see reference 9). Work from Neilson's group in Sweden has revealed that some chlorinated guaiacols are biotransformed by bacteria from sediments exposed to effluents from bleach plant wastes (4). O-methylation of these chlorinated phenols by sediments is the main transformation observed (22). O-methylation of chlorinated guaiacols after *p*-dechlorination/hydroxylation has been also reported for *Rhodococcus chlorophenolicus* PCP-1 (10), a pentachlorophenol degrader. Studies performed with this strain and strains PCP-2, CG-1, and CP-2 showed that degradation of several chloroguaiacols may also take place (11, 12).

The scarcity of studies on this subject could be related to the lack of stable, pure bacterial strains that are able to grow on chlorinated guaiacols. In the present work, we report the metabolism of chlorinated guaiacols by a strain that is able to use guaiacol, the parent compound, as the sole carbon and energy source.

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MATERIALS AND METHODS

Bacterial isolation and identification. A bacterial strain able to grow on guaiacol was isolated as follows. Culture tubes (5 ml) containing 1 ml of a chloride-free saline minimal medium, pH 7.2, supplemented with a solution of trace elements (17) and 1.0 mM guaiacol, were prepared. Culture tubes were inoculated separately with three soil and four water samples and incubated at 30°C with agitation. Transfers to fresh medium were performed when cultures exhibited both turbidity and substrate consumption. The isolated strain was transferred periodically to fresh guaiacol-containing liquid medium or, to assess purity, to Luria-Bertani (LB) plates. LB medium was also used to determine viable cells by plating appropriate dilutions (10^{-4} to 10^{-7}) of strain 5ga-containing samples. Standard tests for bacterial identification were performed as described in *Bergey's Manual of Systematic Bacteriology* (15). Additional tests for the assignment of the strain species were also carried out (5, 24).

Chlorinated guaiacol metabolism studies. (i) Use as sole carbon source. Growth on chloroorganic and organic compounds was tested in cultures prepared with the minimal saline medium described above, supplemented with the specific compound as the sole carbon and energy source (0.1 to 2.0 mM). Incubations were performed in 5-ml culture tubes at 28 to 30°C for up to 30 days, with agitation.

(ii) Metabolism by resting cells. The degradation of chlorinated guaiacols by resting cells was assessed in cells harvested in the late log phase of growth on 1 mM guaiacol, washed twice with saline minimal medium, and resuspended in the same solution (approximately 10^8 viable cells per ml) containing a 0.05 to 0.25 mM concentration of each chloroguaiacol. All chlorinated guaiacols and catechols used in this work, including tetrachloroguaiacol (TeCG), 4,5,6-trichloroguaiacol (4,5,6-TCG), 4,5-dichloroguaiacol (4,5-DCG), 4,6-dichloroguaiacol (4,6-DCG), 4-chloroguaiacol (4-CG),

5-chloroguaiacol (5-CG), 6-chloroguaiacol (6-CG), 4,5-dichlorocatechol (4,5-DCC), and 4-chlorocatechol (4-CC), were purchased from Helix Biotech. Corp., Richmond, British Columbia, Canada, and were used as provided by the supplier (purities were 95 to 99%). Incubations were performed in agitated 125-ml Erlenmeyer flasks containing 30 ml of minimal saline medium at 30°C. Samples were taken at different times (0 to 72 h) and prepared for analysis by removal of cells by filtration (0.2-μm pore size; Sartorius, Göttingen, Germany). To test the effect of the starting amount of chlorinated compound and the number of viable guaiacol-grown cells on degradation of 4-CG, experiments were done with 0.2 or 0.4 mM 4-CG and different amounts of viable cells (0.03 to 1.8 mg of viable cells per ml). Results were related to the concentration of 4-CG and the dry weight of viable cells and expressed as millimolar per milligram. A cell suspension containing $3.1 \times 10^8 \pm 0.8 \times 10^8$ viable cells per ml had 0.98 ± 0.19 mg of cells per ml. Controls without organic compounds and without cells were run to monitor cell viability and abiotic transformation, respectively.

(iii) Effect of chlorinated compounds on growth with guaiacol. Growth on guaiacol as the primary substrate in the presence of a chlorinated compound was studied in cultures prepared as described above, supplemented with 1 mM guaiacol and a 0 to 0.5 mM concentration of either chloroguaiacol or catechol, and incubated at 30°C. Three cultures were run for each condition. After 3 days, bacterial growth and chloride release were determined.

Spectrophotometry. Cell turbidity and removal of the aromatic compound were monitored in a Shimadzu UV-160 spectrophotometer. Readings were corrected for absorbance of noninoculated culture samples and/or cultures free of carbon source. Spectrophotometric measurements were obtained with cell-free samples collected at different incubation times. Culture medium without aromatic compound was used as the background reading. When dilutions were necessary, they were made in minimal saline medium.

Chloride determination. Chloride measurements were carried out with an Orion ion-selective electrode, model 96-17B, connected to an Orion meter, model 720A. Each sample was measured three times. Each average (standard deviations were usually lower than 5%) was converted to chloride concentration by interpolation from KCl standards, freshly prepared in deionized water containing 50 mM $NaNO_3$ as the ionic strength adjustor. Pairs of standards chosen to be as near as possible to specific readings were usually run every 6 to 10 sample readings. In all other procedures, the instructions from the supplier were followed. Results were expressed as a percentage of the maximum theoretical chloride production.

GC-MS. Two procedures were used to prepare samples for gas chromatography-mass spectrometry (GC-MS). Samples from incubations of resting 5ga cells with 4-CG or 4,5-DCG were extracted and acetylated by a procedure described previously (28). Five milliliters of 1 M potassium carbonate was added to 50-ml samples and derivatized by addition of 1 ml of acetic anhydride. After 5 min, they were extracted with 5 ml of hexane, and the phases were separated by centrifugation. The organic phase was concentrated to about 0.7 ml by flushing with nitrogen. An internal standard (0.2 mg of 4,5,6-TCG) was added to each sample, and the samples were derivatized again by addition of 0.1 ml of acetic anhydride and 0.02 ml of pyridine. Samples taken from a culture in log-phase growth on guaiacol at different times after addition of 0.2 mM 4,5-DCG were acidified with 5 ml of 0.1 N H_2SO_4. They were then extracted three times with 50 ml of ethyl acetate, concentrated in a standard rotary evaporator, and resuspended in 5 ml of ethyl acetate. Portions (ca. 0.7 ml) of each extract to which 2,4-dichlorophenol (1 mg) was added as the internal standard were acetylated by addition of 0.1 ml of acetic anhydride and 0.03 ml of pyridine.

GC analysis was performed in a Hewlett-Packard 8990-A system equipped with an automatic sampler and ECD detector. With an open tubular column (30 m by 0.31 mm inner diameter; DB-5 phase of film thickness, 0.25 μm; J&W Scientific) and helium as the carrier gas, the following temperature program was applied: hold at 45°C for 1 min, rise from 45 to 120°C at 15°C/min, and rise from 120 to 230°C at 2°C/min. Under these conditions, the retention times of authentic derivatized standards of 4-CG, 4-CC, 5-CG, 4,5-DCC, 4,5-dichloroveratrole, 4,5-DCG, and the internal standard were 16.1, 20.0, 17.7, 27.6, 19.4, 23.3, and 30.7 min, respectively. The response factors relative to the internal standard for 4-CC, 4-CG, 4,5-DCC, and 4,5-DCG were 0.09, 0.0036, 0.381, and 0.129, respectively. To confirm the identity of the peak with a retention time of 27.6 min, the samples were subjected to GC-MS analysis on a Hewlett-Packard 5985b system (electron impact spectra taken at 70 eV). The chromatographic conditions described above were used.

RESULTS AND DISCUSSION

Isolation and characterization of a guaiacol-degrading bacterial strain. The isolation of a pure bacterial strain able to grow on guaiacol was accomplished through an enrichment batch procedure. After successive transfers of three cultures (two from soil and one from a water sample) to fresh medium, stable growth was attained with culture 5ga, which came from a farm soil sample. Plating on LB medium revealed colonies of a single bacterial strain, which was subjected to further study. The bacterial isolate cells are coccoid (usually in pairs) or short rods; it was gram negative. Negatively stained cells observed by electron microscopy had dimensions of 1.2 to 1.3 μm by 0.8 to 0.9 μm. Standard tests for identification (summarized in Table 1, left) suggested that this strain belongs to the genus *Acinetobacter*. Descriptions of bacterial species of this genus and others from the family *Moraxellaceae* have recently been revised (5, 24). Based on the work of Bouvet and Grimont (5), additional biochemical tests were made (Table 1, right). With the sole exception of the lack of growth on DL-lactate, these results support the identification of strain 5ga as *Acinetobacter junii*.

Strain 5ga grew on 1 mM guaiacol (generation time, 4.5 h), catechol (0.3 to 1 mM), syringol (0.3 mM), and benzoic acid (0.5 to 2.5 mM). Vanillic acid, anisic acid, 3-methoxycatechol, *o*-cresol, 4-methylcatechol, *m*-hydroxybenzoic acid, and *m*- and *p*-nitrophenol failed to support growth. Strain 5ga was unable to grow on the following chlorinated compounds (0.1 to 2.0 mM): pentachlorophenol; 2,4,6- and 2,4,5-trichlorophenol; 4-chloro-3-methylphenol; 2- and 3-chlorobenzoic and 2,3-dichlorobenzoic and 3,5-dichloro-4-hydroxybenzoic acids; and 4-CG, 5-CG, 6-CG, 4,5-DCG, 4,6-DCG, 4,5,6-TCG, TeCG, 4-CC, and 4,5-DCC. Lack of growth on chlorinated guaiacols was also observed when cultures were incubated in the presence of 20 μM catechol, benzoic acid, or guaiacol as the inducer.

To our knowledge, this is the first report of an *Acinetobacter* species capable of growing on guaiacol. Relatively few studies on aromatic metabolism have been performed

TABLE 1. Identification tests for strain 5ga[a]

Test	Result	Test	Result
Gram stain	−	Growth at:	
		37°C	+
Motility	−	41°C	+
H_2S production	−	44°C	−
Pigment production	−	Acid from glucose	−
Oxidase	−	Gelatin hydrolysis	−
Catalase	+	Simmons's citrate	+
Nitrate (nitrite) reduction	−	Utilization of:	
Lactose	−	Malonate	−
Sucrose	−	L-Histidine	+
Mannose	−	L-Arginine	+
Sorbitol	−	Ethanol	+
Xylose	−	L-Leucine	−
Voges-Proskauer	−	L-Ornithine	−
Methyl red	−	L-Tyrosine	−
Urease	−	Phenylacetate	−
Indole	−	L-Phenylalanine	−
Capsule in lactose agar	+	DL-Lactate	−
Capsule in nutrient agar	−	L-Aspartate	−
		Gluconate	−
Antibiotic susceptibility		DL-4-Aminobutyrate	+
Polymyxin B (500 U/ml)	R	*trans*-Aconitate	−
Penicillin (15 U/ml)	R	L-Malate	−

[a] Carbon sources were tested at 0.1 and 0.5%. Histidine was utilized only at 0.5%. R, resistant.

with *Acinetobacter* strains; work with *A. calcoaceticus* is the exception (see reference 20 as an example).

Metabolism of chlorinated guaiacols by *A. junii* 5ga. Although strain 5ga cannot grow on specific chloroguaiacols, its cells may be able to degrade them when a primary carbon source is provided. Preliminary information indicated that this strain degraded some chlorinated guaiacols while it was growing on guaiacol. To test this possibility further, the chloride production of strain 5ga growing on guaiacol in the presence of a 0.05 to 0.25 mM concentration of each chlorinated guaiacol was determined, and the results are shown in Fig. 1. The dechlorination values suggested that 4-CG, 5-CG, and 4,5-DCG were degraded and that TeCG, 6-CG, 4,5,6-TCG, and 4,6-DCG (not shown) were not. The dechlorination rates for 5-CG and 4,5-DCG were dependent on the concentration of chloroguaiacol.

The chloride production results were supported by UV spectrum analysis. None of the compounds containing a chlorine atom in position 6 showed spectral changes after incubation with cells. However, UV spectrophotometric measurements of cultures growing with guaiacol in the presence of 4-CG and 5-CG showed a transient accumulation of UV-absorbing material different from the starting compound, followed by complete removal (data not shown). In these cases, the spectral patterns of accumulated material resembled those of 4-CC and 4,5-DCC.

Lower-than-expected chloride production and removal of UV-absorbing material were observed in some incubations with 4-CG, 5-CG, and 4,5-DCG. A close inspection of these experiments suggested that, in addition to the concentration of chloroguaiacol, the actual number of viable cells in the assay was also important. To learn more about the ability of strain 5ga to metabolize chlorinated guaiacols, we studied the effect of the 4-CG concentration and the number of viable cells grown on guaiacol. At a 4-CG/cell ratio of 0.11, significant removal of 4-CG was observed in spectrophoto-

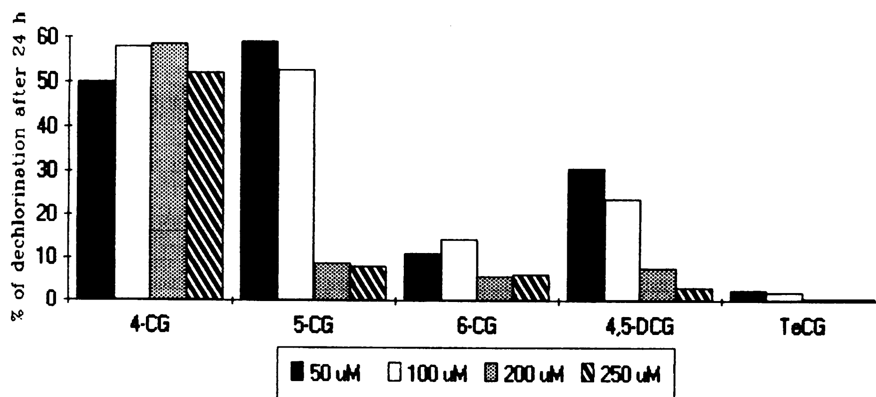

FIG. 1. Chloride release by strain 5ga growing on guaiacol in the presence of several chloroguaiacols at 0.05 to 0.25 mM. Values are from one representative experiment and were corrected for abiotic dechlorination and for the presence of cells.

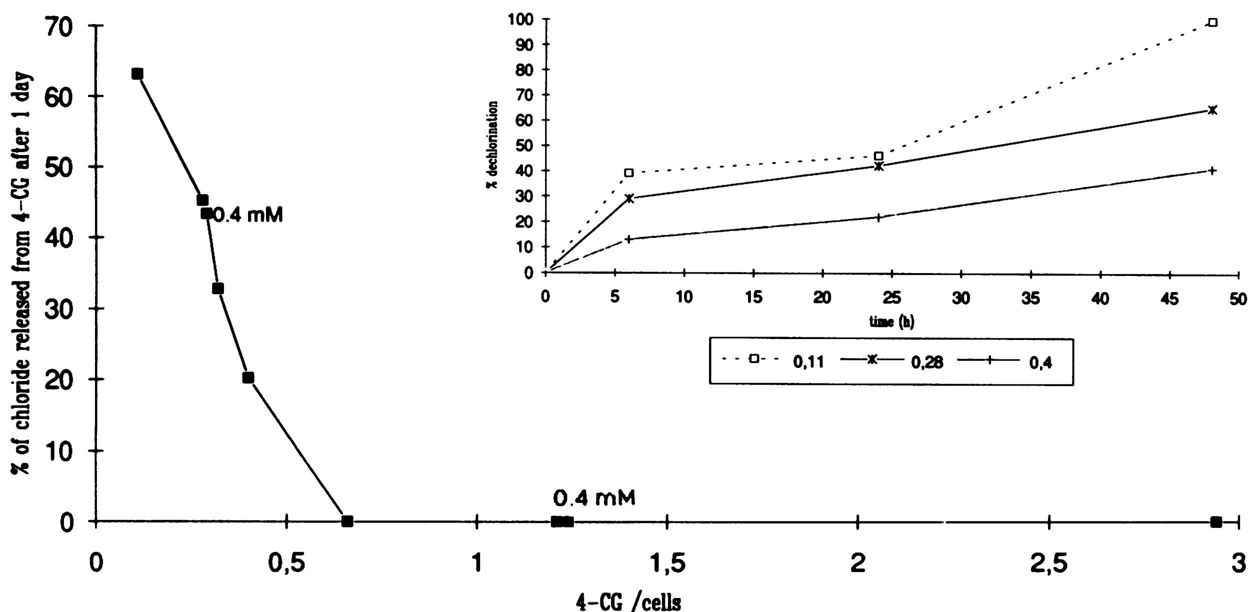

FIG. 2. Chloride released from 4-CG by resting cells of strain 5ga grown on guaiacol at different concentrations of 4-CG (millimolar) per milligram of viable cells. Experiments were made with 0.2 mM 4-CG except when indicated. (Inset) Dechlorination of 4-CG by resting cells of strain 5ga at different concentrations of 4-CG per milligram of viable cells.

metric measurements made at 6 h; it was apparently complete after 48 h of incubation (not shown). At a 4-CG/cell ratio of 0.28, a transient accumulation of UV-absorbing material different from that with 4-CG (probably 4-CC) was detected first, but most material was removed before 48 h. Finally, at 4-CG/cell ratios of 0.66 and higher, no significant spectral changes could be observed, suggesting that a minimum threshold 4-CG/cell ratio was necessary for degradation of 4-CG by these resting cells.

The data from UV analysis are supported by the chloride release measurements shown in Fig. 2. The ability of these cells to dechlorinate 4-CG decreased sharply at 4-CG/cell ratios of between 0.11 and 0.66. At higher 4-CG/cell ratios, no dechlorination could be detected even if the incubation was carried out for 72 h. That this 4-CG/cell ratio governs the degradation was further suggested by experiments in which the concentration of 4-CG was doubled (4-CG/cell ratios of 0.32 and 1.23). The inset in Fig. 2 depicts dechlorination rates from a separate experiment and indicates that the initial rates were proportional to the 4-CG/cell ratios used in the incubation. Preliminary experiments performed with 4,5-DCG suggested a similar pattern but with a lower threshold 4-CG/cell ratio necessary for degradation (data not shown). A similar trend was observed in incubations of 4-CG with guaiacol-grown cells of a gram-positive strain that we have recently isolated (unpublished data). Unfortunately, the chemical instability of chlorocatechols prevented us from performing the corresponding analysis.

This part of the work provides evidence that *A. junii* 5ga cells actively growing on guaiacol or resting cells previously grown on it are able to degrade specific chloroguaiacols. This is the first report of such catabolic ability for this bacterial genus. Some *Acinetobacter* strains have been reported to be able to degrade other haloaromatic compounds. *Acinetobacter* strains have been involved in 4-chlorobiphenyl mineralization (26) and transformation of polychlorinated biphenyls (14). More germane is the ability of *Acinetobacter* sp. strain 4-CB1 to cometabolize 3,4-dichlorobenzoate (1). *A. junii* 5ga was isolated from a soil not previously exposed to chlorinated guaiacols. We have recently reported that biotransformation of several chlorinated phenols takes place in this particular soil (6). Although it is limited, the ability of this strain (and possibly others) to degrade chlorinated guaiacols may be important for the biodegradation performed by natural soil microflora.

Detection by GC-MS of chloroguaiacol-derived metabolites produced by *A. junii* 5ga. From the observations reported above, we suspected the production of corresponding chlorocatechols during degradation of chloroguaiacols. To confirm this and also to detect other possible metabolic products, we carried out a GC analysis of the initial products of the incubation of resting cells of strain 5ga with 4-CG or 4,5-DCG. The results indicated that, after 3 h, 4-CG and 4,5-DCG (retention times of 16.1 and 23.3 min, respectively) gave rise to the corresponding chlorocatechols (retention times of 20.0 and 27.6 min, respectively). The percentages of remaining chloroguaiacol and chlorocatechol formed after 3 h of incubation were 30 and 7.1% for 4-CG and 59.4 and 39.6% for 4,5-DCG, respectively. A separate experiment performed with strain 5ga growing on guaiacol showed that only 7% of the initially added 4,5-DCG remained at 22 h. The presence of one main peak with a retention time of 27.6 min at 11 and 22 h also suggested the formation of 4,5-DCC. A GC-MS analysis of this sample showed that the *m/z* (relative intensity) for that peak was 264,M+(3), 262,M+(4), 222,M−42+(10), 220,M−42+(15), 180,M−42−42+(65), 178,M−42−42+(100), 113(4), 43(88); this pattern corresponds to a dichlorodihydroxybenzene diacetate.

Together, these results strongly suggest that O-demethylation is the initial reaction during metabolism of 4-CG and 4,5-DCG, and probably 5-CG also, by strain 5ga. Bacterial O-demethylation of chlorinated guaiacols has been described for anaerobic systems (19) and for one gram-positive strain (3). O-methylation did not occur, because we did not detect

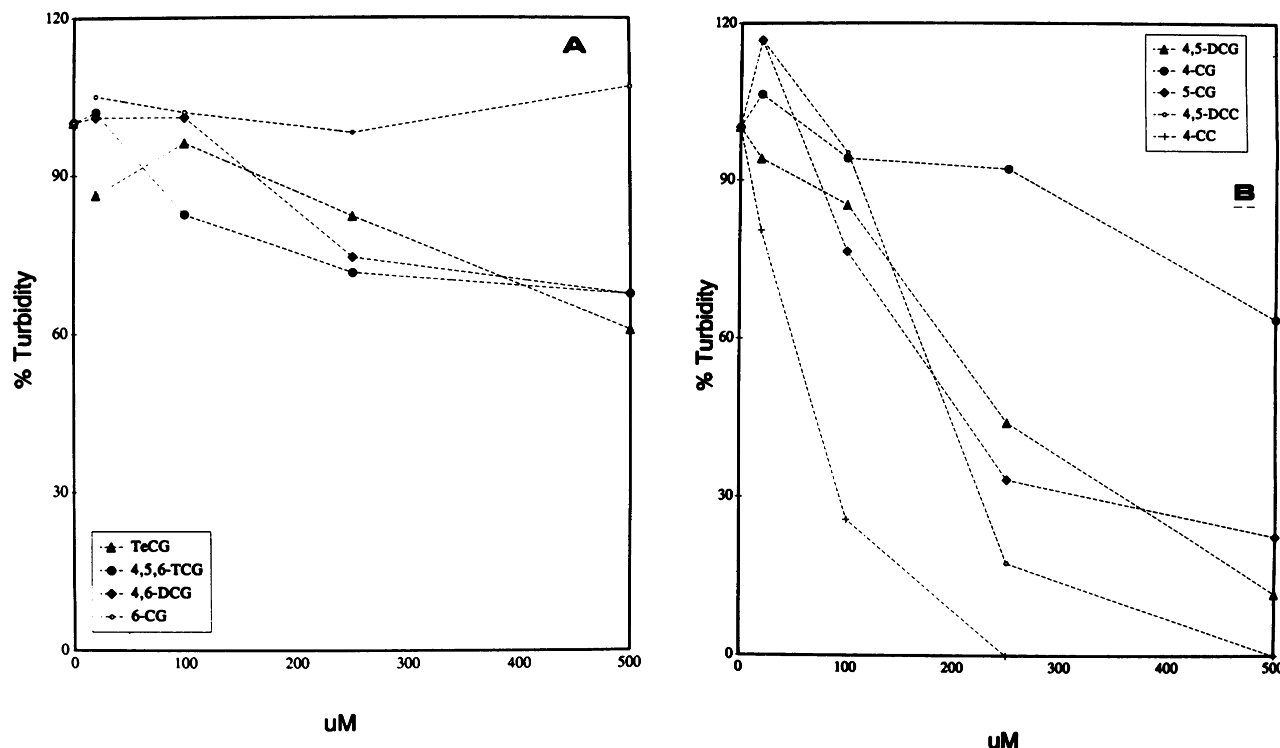

FIG. 3. Effect of different levels of (A) TeCG, 4,5,6-TCG, 4,6-DCG, and 6-CG and (B) 4,5-DCG, 4-CG, 5-CG, 4,5-DCC, and 4-CC on the growth of strain 5ga on guaiacol. Turbidity values are the averages for three samples after 2.5 days. Standard deviations were less than 5%.

4,5-dichloroveratrole. It is worth mentioning that several strains, including one tentatively assigned to the genus *Acinetobacter*, are able to O-methylate chlorophenols (3). We did not observe *p*-dechlorination/hydroxylation products from 4-CG, 5-CG, or 4,5-DCG, as has been reported for metabolism of TeCG by *Rhodococcus* strains (11, 12), nor did we observe dehalogenation products prior to ring cleavage, i.e., monochloroguaiacol or monocatechol. Our data do not allow us to rule out the formation of 1,2-chlorinated benzo-1,2-quinones by incubated cells or by abiotic conditions. In fact, rapid destruction of such 1,2-quinones leading to formation of the corresponding catechols has been reported (23). However, the results of GC-MS analysis do allow us to conclude that dehalogenation of chlorinated guaiacols carried out by *A. junii* 5ga cells takes place after O-demethylation, probably through what has been called fortuitous metabolism (21).

Inhibition of growth of *A. junii* 5ga on guaiacol by chlorinated compounds. After the study of chlorinated guaiacol metabolism by strain 5ga, it was apparent that several results could be explained by inhibitory effects of chlorocatechols. In incubations with resting cells, a rapid decrease in cell viability was observed. Cell viabilities ranging from 40 to 90% of the initial viable-cell counts compared with cells incubated in the absence of an organic compound were determined after 24 h. The loss of viability was greater under those conditions when chlorinated guaiacol metabolism was taking place. In this context, further indirect evidence of such inhibitory effects came from experiments in which strain 5ga growing continuously on guaiacol was able to extensively degrade (dechlorinate) 4,5-DCG at concentrations of up to 100 μM. A rapid decrease in culture viability along with the sudden formation of brown-pink material, probably corresponding to autooxidation products of 4,5-DCC, were observed when the amount of 4,5-DCG added was higher than 100 to 120 μM.

For these reasons, it was important to measure the effect of chlorinated guaiacols and catechols on strain 5ga growing on guaiacol. The results (Fig. 3) showed that inhibition of growth was dependent on each specific chlorinated compound and its concentration. More importantly, compounds which are not attacked by this strain were also weak growth inhibitors. In contrast, compounds which are degraded by this strain have greater inhibition potential, especially chlorocatechols. When strain 5ga was grown on an LB rich medium in the presence of these chlorinated compounds, a comparable tendency was observed, although the extent of inhibition was lower than with guaiacol.

The inhibitory effect of 4-CC is a possible explanation for the behavior of resting cells in incubations with 4-CG. The final concentration of chlorocatechols results from the initial input of reduced cofactors and the channeling of intermediates through catabolic reactions; this allows reduction of the oxidized cofactors. Kinetic data reported for degradation of 4-chlorophenol by *Pseudomonas putida* support the view that there is an optimum ratio for cells and a chlorinated compound (25).

There are a few examples of the inhibition of aromatic metabolism in *Acinetobacter* strains by chlorinated intermediates. *A. calcoaceticus* is able to utilize monofluorobenzoate, but 3-fluorobenzoate causes cell death through accumulation of 3-fluorocatechol (7, 27). On the other hand, during biotransformation of 3,4-dichlorobenzoate by *Acinetobacter* sp. strain 4-CB1, 4-carboxy-1,2-benzoquinone is produced (1). That quinone inhibited the degradation of 4-chlorobenzoate, which was the primary substrate (2). The

inhibition of metabolism by chlorocatechols has been reported for the degradation of methylbenzoates by *Pseudomonas cepacia* MB2. In that case, the inhibitory effect was observed during cometabolism of chlorobenzoates (13).

It seems quite possible that the inability of these chlorinated compounds to support the growth of strain 5ga and others may be explained by the initial formation of O-demethylation products (chlorocatechols).

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