

## Sequential Transhydroxylations Converting Hydroxyhydroquinone to Phloroglucinol in the Strictly Anaerobic, Fermentative Bacterium *Pelobacter massiliensis*

ANDREAS BRUNE,<sup>†\*</sup> SYLVIA SCHNELL, AND BERNHARD SCHINK<sup>‡</sup>

*Mikrobiologie I, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, Germany*

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The recently isolated fermenting bacterium *Pelobacter massiliensis* is the only strict anaerobe known to grow on hydroxyhydroquinone (1,2,4-trihydroxybenzene) as the sole source of carbon and energy, converting it to stoichiometric amounts of acetate. In this paper, we report on the enzymatic reactions involved in the conversion of hydroxyhydroquinone and pyrogallol (1,2,3-trihydroxybenzene) to phloroglucinol (1,3,5-trihydroxybenzene). Cell extracts of *P. massiliensis* transhydroxylate pyrogallol to phloroglucinol after addition of 1,2,3,5-tetrahydroxybenzene (1,2,3,5-TTHB) as cosubstrate in a reaction identical to that found earlier with *Pelobacter acidigallici* (A. Brune and B. Schink, *J. Bacteriol.* 172:1070–1076, 1990). Hydroxyhydroquinone conversion to phloroglucinol is initiated in cell extracts without an external addition of cosubstrates. It involves a minimum of three consecutive transhydroxylation reactions characterized by the transient accumulation of two different TTHB isomers. Chemical synthesis of the TTHB intermediates allowed the resolution of the distinct transhydroxylation steps in this sequence. In an initial transhydroxylation, the hydroxyl group in the 1-position of a molecule of hydroxyhydroquinone is transferred to the 5-position of another molecule of hydroxyhydroquinone to give 1,2,4,5-TTHB and resorcinol (1,3-dihydroxybenzene) as products. Following this disproportionation of hydroxyhydroquinone, the 1,2,4,5-isomer is converted to 1,2,3,5-TTHB, an enzymatic activity present only in hydroxyhydroquinone-grown cells. Finally, phloroglucinol is formed from 1,2,3,5-TTHB by transfer of the 2-hydroxyl group to either hydroxyhydroquinone or resorcinol. The resulting coproducts are again cosubstrates in earlier reactions of this sequence. From the spectrum of hydroxybenzenes transhydroxylated by the cell extracts, the minimum structural prerequisites that render a hydroxybenzene a hydroxyl donor or acceptor are deduced.

Trihydroxybenzenes are common intermediates formed in the degradation of plant materials such as glycosides, flavonoids, tannins, and lignin. Several anaerobic bacteria have been shown to mineralize pyrogallol (1,2,3-trihydroxybenzene), phloroglucinol (1,3,5-trihydroxybenzene) and their derivatives (for reviews, see references 7 and 14). The metabolic pathway proposed for trihydroxybenzene degradation in *Eubacterium oxidoreducens* (11) differs significantly from the phloroglucinol pathway recently established in *Pelobacter acidigallici* (5) with respect to the fate of the aliphatic ring cleavage product. In both cases, however, phloroglucinol is the initial substrate of a reductive ring-cleaving reaction sequence and is itself formed from pyrogallol in a so far unique and highly unusual transhydroxylation reaction. Using cell extracts of *P. acidigallici*, we demonstrated that in an intermolecular hydroxyl transfer, the 2-hydroxyl group of the cosubstrate, 1,2,3,5-tetrahydroxybenzene (1,2,3,5-TTHB), is shifted to the 5-position of pyrogallol (3). Dehydroxylation of 1,2,3,5-TTHB yields the product, phloroglucinol, while hydroxylation of pyrogallol regenerates the cosubstrate (Fig. 1).

No transhydroxylation of hydroxyhydroquinone (1,2,4-trihydroxybenzene) to phloroglucinol occurs in *P. acidigal-*

*lici*. Hydroxylation of hydroxyhydroquinone with 1,2,3,5-TTHB does not regenerate the cosubstrate but leads to the formation of a new product, tentatively identified as a different isomer of tetrahydroxybenzene. The reaction comes to a standstill when 1,2,3,5-TTHB is depleted (3). These results are in agreement with the inability of *P. acidigallici* to utilize hydroxyhydroquinone as a substrate.

Only recently, we isolated the strictly anaerobic bacterium *Pelobacter massiliensis* from anaerobic enrichment cultures, growing with hydroxyhydroquinone as a sole source of carbon and energy (16). It ferments hydroxyhydroquinone, pyrogallol, phloroglucinol, and, after decarboxylation, gallic acid to 3 acetate. With respect to its substrate spectrum, excluding hydroxyhydroquinone, and the formation of acetate as the sole product of energy metabolism, it strongly resembles *P. acidigallici* (15). When growing on hydroxyhydroquinone, *P. massiliensis* forms traces of resorcinol and phloroglucinol but finally converts all substrate to acetate. A degradation of hydroxyhydroquinone via phloroglucinol, facilitated by a transhydroxylating activity present only in hydroxyhydroquinone-grown cells, was postulated (16).

In this paper, we demonstrate the presence of the enzyme activities in cell extracts of *P. massiliensis* responsible for the reaction sequence transforming hydroxyhydroquinone to phloroglucinol. A legend to trivial names, structures, and graphical symbols used throughout is provided in Fig. 2.

### MATERIALS AND METHODS

**Medium and growth conditions.** *P. massiliensis* HHQ7 (DSM 6233) was grown in 125-ml infusion bottles in a

\* Corresponding author.

<sup>†</sup> Present address: Department of Microbiology and Public Health, Michigan State University, Giltner Hall, East Lansing, MI 48824-1101.

<sup>‡</sup> Present address and address for reprint requests: Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany.

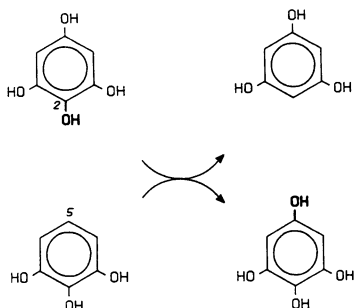


FIG. 1. Transhydroxylation of pyrogallol to phloroglucinol in *P. acidigallici*. In an intermolecular hydroxyl transfer reaction, 1,2,3,5-TTHB serves as cosubstrate and coproduct (3).

bicarbonate-buffered, sulfide-reduced saltwater mineral medium under an  $N_2$ - $CO_2$  atmosphere at 30°C. Hydroxyhydroquinone or pyrogallol (8 mM, fed in two portions of 4 mM to avoid substrate intoxication of cultures) was used as the sole source of carbon and energy. A more detailed description of medium composition and preparation has been previously published (16).

**Cell suspensions and cell extracts.** Cells were harvested by centrifugation in the infusion bottles, washed once, and finally resuspended in 1 ml of  $N_2$ -sparged sodium phosphate buffer (50 mM, pH 7.0) containing the same amounts of NaCl and  $MgCl_2$  as the medium to prevent lysis. Cell suspensions (5 mg [dry weight] per ml) were transferred into smaller  $N_2$ -gassed vials (5 ml) with butyl rubber septa and kept on ice until used. Cells were permeabilized by adding the mild detergent cetyltrimethylammonium bromide to a final concentration of 0.1 mg  $ml^{-1}$  to the assays (125  $\mu g$  of protein  $ml^{-1}$ ). This concentration was found to be sufficient for permeabilization to pyridine nucleotides by measuring dihydrophloroglucinol reductase activity in cell suspensions (3). Cell-free extracts were prepared by French press treatment and centrifugation to remove cell debris, maintaining anaerobic conditions and a temperature of 0 to 4°C throughout the procedure. A more detailed description of these procedures has been published (3).

**Enzyme assays.** Tetrahydroxybenzene-pyrogallol hydroxyltransferase (EC number not yet assigned) and the other transhydroxylating activities reported in Results were as-

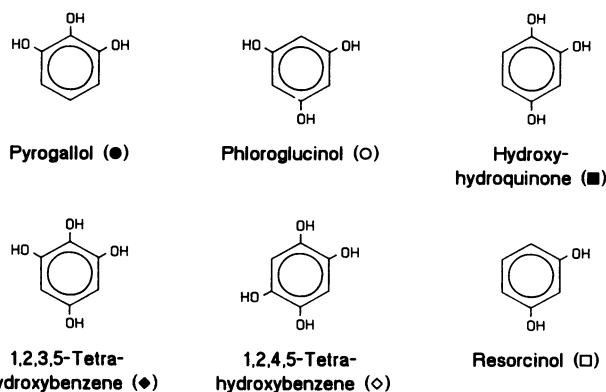


FIG. 2. Structural formulas of the most frequently mentioned hydroxybenzenes. The respective graphic symbols are used in all subsequent figures.

sayed by monitoring disappearance and formation of the respective metabolites by discontinuous high-pressure liquid chromatography (HPLC) analysis of the assay mixture. Assay mixtures (2 ml) contained 100 mM Na-phosphate buffer, pH 7.0; the respective hydroxyl donor and acceptor, usually 1 mM each; and permeabilized cell suspension or cell extract (125  $\mu g$  of protein  $ml^{-1}$ ). Assays were performed under  $N_2$  in 5-ml vials with butyl rubber septa. Additions were made from anoxic stock solutions with gas-tight Unimetrics microliter syringes (Macherey-Nagel, Düren, Germany), to prevent access of air.

Samples (100  $\mu l$ ) were taken at regular intervals, and the reaction was stopped by injection of the samples into 1-ml HPLC vials gassed with  $N_2$  and containing 400  $\mu l$  of 0.1 M  $H_3PO_4$ . Samples were immediately frozen in liquid  $N_2$  and stored at  $-20^\circ C$  until analyzed. General conditions used for analysis of hydroxybenzenes by reversed-phase HPLC have been published previously (3). A mobile phase of 0.1 M ammonium phosphate buffer (pH 2.6) (A) and methanol (B) at a flow rate of 1  $ml\ min^{-1}$  was used; the solvent gradient was 0% B for 0.1 min to 17.5% B in 7 min (linear). Retention times (in minutes) for hydroxybenzenes were as follows: pentahydroxybenzene, 2.02; 1,2,4,5-TTHB, 2.68; 1,2,3,5-TTHB, 2.91; hydroxyhydroquinone, 4.35; pyrogallol, 5.44; phloroglucinol, 5.89; and resorcinol, 8.36. Concentrations of metabolites were determined by comparing peak areas with external and internal standards of known composition. Peak identity was verified by on-line spectral scans and compared with retention times and UV spectra of reference compounds.

All activities were determined at 25°C with both substrate and cosubstrate at 1 mM and are means of several independent assays. Quantitative variations (in the range of 10 to 20%) with different cell preparations were typical, but the reaction patterns never differed qualitatively from the results presented. Protein was quantitated by the microprotein assay described by Bradford (2), with bovine serum albumin as a standard.

**Chemicals.** 1,2,3,5-TTHB was prepared by the method of Baxter and Brown (1) and purified as described earlier (3). 1,2,4,5-TTHB was synthesized from 2,5-dihydroxybenzoquinone by catalytic hydration over Pd on activated charcoal (5%), using the procedure described in reference 1. The reaction was followed by HPLC and yielded 1,2,4,5-TTHB as the sole product. Reoxidation to the quinone occurred as soon as the reaction mixture was exposed to air; hence, filtration of catalyst and evaporation of solvent had to be performed under  $N_2$ .  $^1H$ -nuclear magnetic resonance ( $^1H$ -NMR) analysis of the crystalline product was performed in  $CD_3COD$ . UV spectra were recorded with a Uvikon 860 double-beam spectrophotometer (Kontron, Zürich, Switzerland) in HPLC buffer after collection of the respective peak from the HPLC separation or from gravimetrically prepared standard solutions.

2,5-Dihydroxybenzoquinone was prepared by oxidizing hydroquinone in concentrated NaOH with 30%  $H_2O_2$  as described by Jones and Shonle (10).

Hydroxyhydroquinone (99%) was obtained from Aldrich (Steinheim, Germany); all other chemicals were of analytical grade or of the highest commercially available purity and were obtained from Fluka (Neu-Ulm, Germany). Gases (>99.999% pure) were purchased from Messer Griesheim (Ludwigshafen, Germany).

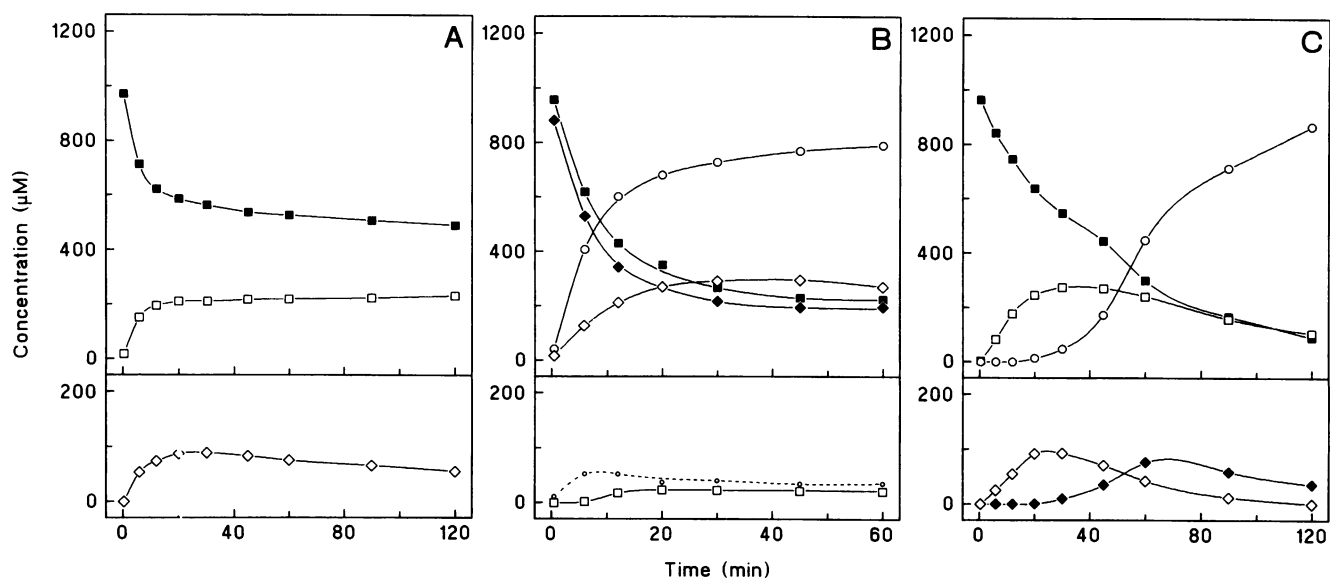


FIG. 3. Transhydroxylation reactions catalyzed by cell extracts of *P. massiliensis*. Cells were grown on pyrogallol (A and B) or hydroxyhydroquinone (C). Assay mixtures ( $125 \mu\text{g}$  of protein  $\text{ml}^{-1}$ ) contained hydroxyhydroquinone alone (■) (A and C) or in the presence of 1,2,3,5-TTHB (◆) (B). The other symbols represent resorcinol (□), phloroglucinol (○), 1,2,4,5-TTHB (◇) and a compound tentatively identified as pentahydroxybenzene (---). For structural formulas, see Fig. 2.

## RESULTS

In a previous publication, we reported that dense cell suspensions of *Pelobacter massiliensis* grown on pyrogallol or hydroxyhydroquinone consume their respective substrate at *in vivo* rates (16). We now present the transhydroxylating activities in the respective cell extracts acting on these and other hydroxylated benzenes. Figure 2 illustrates the structures of the most frequently mentioned hydroxybenzenes together with their trivial names and the graphic symbols used in all subsequent figures to facilitate interpretation of the data.

**Pyrogallol-grown cells.** Upon permeabilization of cells with cetyltrimethylammonium bromide or after French press treatment, no pyrogallol consumption was observed. If incubated with hydroxyhydroquinone (Fig. 3A), cell extracts converted the latter to resorcinol and a second compound which had already been observed in similar studies with *P. acidigallici* (3) and which was then tentatively identified as a tetrahydroxybenzene isomer (TTHB') different from 1,2,3,5-TTHB. In both cases, no phloroglucinol was formed.

Conversion of pyrogallol to phloroglucinol by cell extracts of *P. acidigallici* can be restored by supplementing the assay with 1,2,3,5-TTHB (3). This was confirmed for *P. massiliensis*; the reaction rate depended on the tetrahydroxybenzene concentration (data not shown) with a maximum velocity of  $670 \text{ nmol min}^{-1}$  per mg of protein at  $1 \text{ mM}$  1,2,3,5-TTHB. As with *P. acidigallici*, substoichiometric amounts of 1,2,3,5-TTHB were sufficient for stoichiometric pyrogallol-to-phloroglucinol conversion. The tetrahydroxybenzene concentration remained constant during the assay; a small amount, however, dismutated to phloroglucinol and a compound tentatively identified as pentahydroxybenzene (3).

If 1,2,3,5-TTHB was added to cell extracts incubated with hydroxyhydroquinone, both substrates decreased concomitantly with the formation of phloroglucinol and TTHB' (Fig. 3B). Apparently, TTHB' was formed by hydroxylation of hydroxyhydroquinone, while dehydroxylation of 1,2,3,5-TTHB yielded phloroglucinol. Yet phloroglucinol formation

never exceeded 1,2,3,5-TTHB consumption, indicating that no regeneration of 1,2,3,5-TTHB occurred.

**Identification of 1,2,4,5-TTHB.** TTHB', the putative product of hydroxyhydroquinone hydroxylation (Fig. 3A and B), was positively identified as 1,2,4,5-TTHB by comparison with a chemically synthesized reference compound on the basis of its UV spectrum and the retention time in the HPLC chromatogram. Identity of the reference compound was confirmed by  $^1\text{H-NMR}$  spectroscopy, which yielded resonance signals at 4.98 ppm (s, 4 H, aryl-OH) and 6.41 ppm (s, 2 H, aryl-H) without any significant background. This spectrum is in agreement with chemically pure 1,2,4,5-TTHB, in which all hydroxyl or hydrogen substituents are expected to be chemically equivalent. The UV spectrum of 1,2,4,5-TTHB differs from that of 1,2,3,5-TTHB, with respect to the absorbance maximum (294 nm and 279 nm, respectively) and an approximately twofold increase in the extinction coefficient (Fig. 4).

**Hydroxyhydroquinone-grown cells.** While pyrogallol metabolism by cell extracts or by permeabilized cells was basically identical to that of pyrogallol-grown cells (data not shown), there was a significant difference with respect to hydroxyhydroquinone metabolism. Figure 3C shows that extracts of hydroxyhydroquinone-grown cells transformed hydroxyhydroquinone stoichiometrically to phloroglucinol without any addition of other cosubstrates. The reaction consisted of several phases. Initially, hydroxyhydroquinone was disproportionated to resorcinol and 1,2,4,5-TTHB, as observed with pyrogallol-grown cells (Fig. 3A). However, after 20 min of incubation, 1,2,3,5-TTHB appeared in the reaction mixture, while 1,2,4,5-TTHB decreased. Finally, phloroglucinol was formed.

In the following experiment, the individual steps involved in this reaction sequence were resolved. Cell extracts incubated with 1,2,4,5-TTHB alone transformed this compound almost stoichiometrically to 1,2,3,5-TTHB (Fig. 5A). Subsequently, however, the 1,2,3,5-TTHB concentration decreased again, accompanied by the formation of phloroglu-

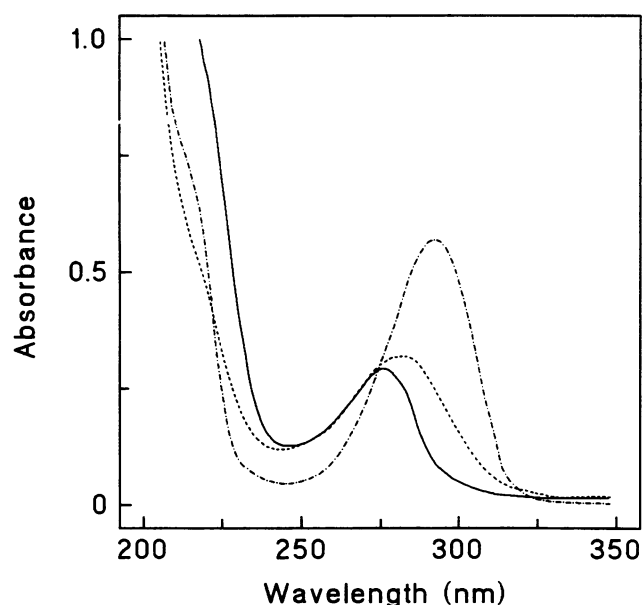


FIG. 4. UV absorption spectra of 1,2,3,5-TTHB (—), 1,2,4,5-TTHB (---), and the compound designated pentahydroxybenzene (· · ·). Concentration of tetrahydroxybenzenes was 125  $\mu$ M in 0.1 M ammonium phosphate buffer, pH 2.6. Pentahydroxybenzene was the respective peak fraction collected during HPLC analysis of reaction K (see Fig. 7).

cinol and another compound, tentatively identified as pentahydroxybenzene. This second reaction occurred also if cell extracts were incubated with 1,2,3,5-TTHB alone and can be interpreted as disproportionation of 1,2,3,5-TTHB to phloroglucinol and pentahydroxybenzene.

Figures 5B and C show results from similar assays in which 1,2,3,5-TTHB was combined with either hydroxyhy-

droquinone or resorcinol, respectively. In both cases, the initial substrates decreased and large amounts of phloroglucinol were formed. Apparently, hydroxyhydroquinone was hydroxylated to 1,2,4,5-TTHB (Fig. 5B) and resorcinol was hydroxylated to hydroxyhydroquinone, which again was further hydroxylated to 1,2,4,5-TTHB (Fig. 5C). In both cases, phloroglucinol was formed by dehydroxylation of 1,2,3,5-TTHB. Since 1,2,4,5-TTHB was effectively transformed to 1,2,3,5-TTHB (Fig. 5A), it did not accumulate stoichiometrically, and led to an additional formation of phloroglucinol, whose final concentration (in contrast to the experiment of Fig. 3B) significantly exceeded the initial 1,2,3,5-TTHB concentration.

Initial specific reaction rates (in nanomoles minute<sup>-1</sup> per milligram of protein) of 1,2,4,5-TTHB conversion to 1,2,3,5-TTHB (400) and phloroglucinol formation from 1,2,3,5-TTHB with hydroxyhydroquinone (340) or resorcinol (230) were significantly above *in vivo* rates. Similar rates were measured with permeabilized cell suspensions.

**Other transhydroxylation reactions in cell extracts.** The results presented so far prompted us to test whether pyrogallol-grown cells were unable to metabolize the 1,2,4,5-TTHB formed by hydroxyhydroquinone hydroxylation. Figure 6A shows that extracts of pyrogallol-grown cells in fact did not act on 1,2,4,5-TTHB alone, as no other hydroxybenzenes appeared during the assay. This result differs strikingly from that with hydroxyhydroquinone-grown cell extracts, which transformed 1,2,4,5-TTHB almost stoichiometrically to 1,2,3,5-TTHB (Fig. 5A). Yet, if a suitable hydroxyl acceptor such as resorcinol was added (Fig. 6B), 1,2,4,5-TTHB and resorcinol were consumed and hydroxyhydroquinone was formed in a 1:1:2 stoichiometry, indicating that pyrogallol-grown cells are able to catalyze the hydroxylation of resorcinol to hydroxyhydroquinone by dehydroxylation of 1,2,4,5-TTHB to hydroxyhydroquinone. This reaction is the reversal of the hydroxyhydroquinone disproportionation observed in the experiment illustrated in Fig. 3A. If hydroxyhydroquinone-grown cells were used

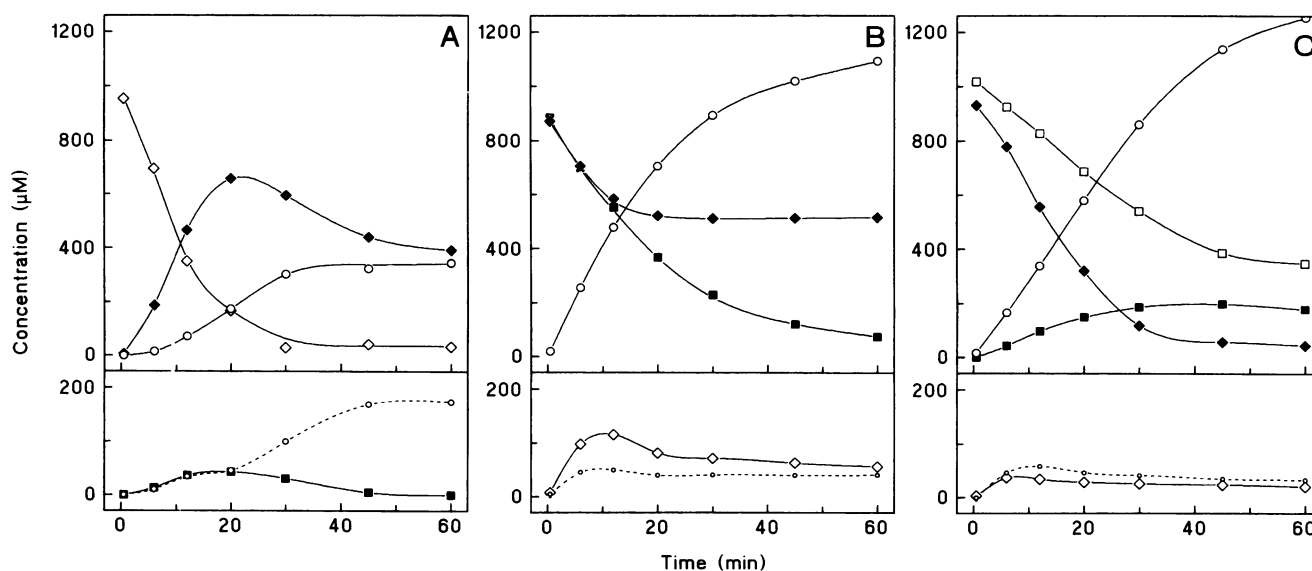


FIG. 5. Transhydroxylation reactions catalyzed by cell extracts of hydroxyhydroquinone-grown *P. massiliensis*. Assay mixtures (125  $\mu$ g of protein ml<sup>-1</sup>) were incubated with 1,2,4,5-TTHB (◇) alone (A), 1,2,3,5-TTHB (◆) plus hydroxyhydroquinone (■) (B), or 1,2,3,5-TTHB (◆) plus resorcinol (□) (C). Other symbols represent phloroglucinol (○) and a compound tentatively identified as pentahydroxybenzene (---). For structural formulas, see Fig. 2.

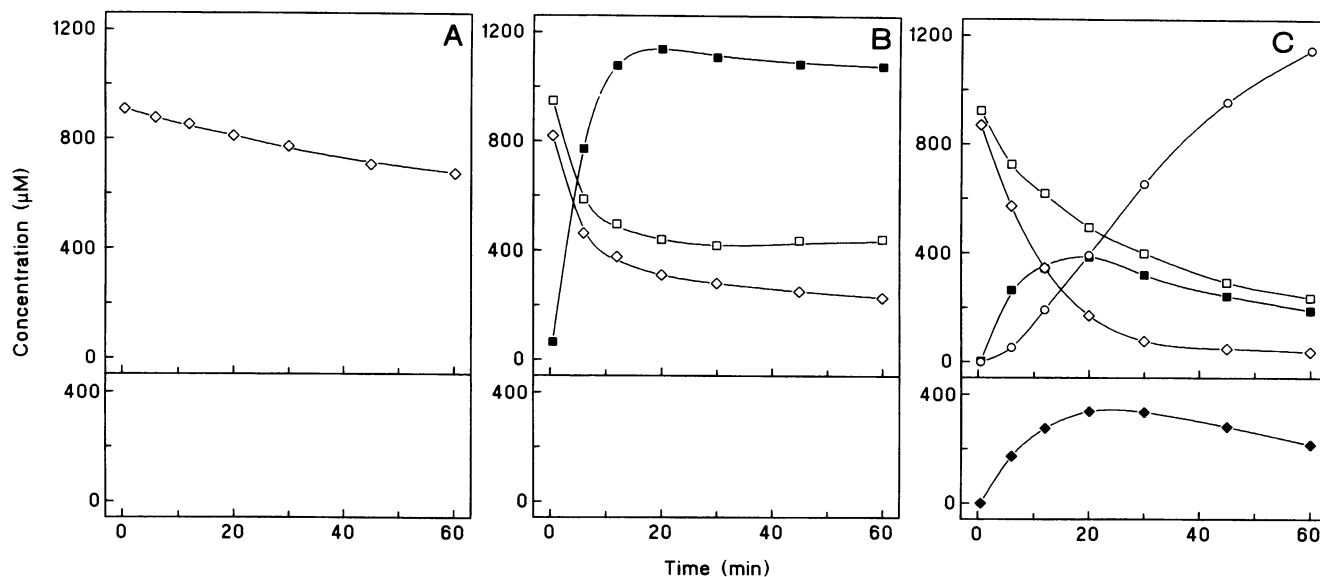


FIG. 6. Transhydroxylation reactions catalyzed by cell extracts of *P. massiliensis*. Cells were grown with pyrogallol (A and B) or hydroxyhydroquinone (C). Assay mixtures ( $125 \mu\text{g}$  of protein  $\text{ml}^{-1}$ ) were incubated with 1,2,4,5-TTHB ( $\diamond$ ) alone (A) and in the presence of resorcinol ( $\square$ ) (B and C). The other symbols represent hydroxyhydroquinone ( $\blacksquare$ ), 1,2,3,5-TTHB ( $\blacklozenge$ ), and phloroglucinol ( $\circ$ ). For structural formulas, see Fig. 2.

(Fig. 6C), phloroglucinol was formed as final product. This was to be expected, since the conversion of 1,2,4,5-TTHB to 1,2,3,5-TTHB (Fig. 5A) creates the same substrate combination as shown in Fig. 5C.

In similar experiments, we tested all possible combinations of di-, tri-, and the two available tetrahydroxybenzenes. For reasons of space and clarity we summarize the observed transhydroxylation reactions in the schematic presentation given in Fig. 7. Three different hydroxybenzenes proved to be effective hydroxyl donors: 1,2,3,5-TTHB (reactions A to C), 1,2,4,5-TTHB (reactions D to F), and hydroxyhydroquinone (reactions G to I). They were dehydroxylated specifically to phloroglucinol, hydroxyhydroquinone, and resorcinol, respectively. The scope of possible hydroxyl acceptors consisted of pyrogallol (reactions A, D, and G), hydroxyhydroquinone (reactions B, E, and H), and resorcinol (reactions C, F, and I), which were specifically hydroxylated to 1,2,3,5-TTHB, 1,2,4,5-TTHB, and hydroxyhydroquinone, respectively, by the hydroxyl donors mentioned above.

However, in the absence of other hydroxyl acceptors or at a very high donor/acceptor ratio, the hydroxyl donors could react with themselves and disproportionated to hydroxylated and dehydroxylated products (reactions H, K, and L). In the case of tetrahydroxybenzenes, hydroxylation yielded the tentatively identified pentahydroxybenzene. It was not obtainable as a reference compound, but the retention time in the HPLC chromatogram, its UV spectrum (Fig. 4), and its occurrence in the respective experiments are consistent with the anticipated structure. The concentrations of pentahydroxybenzene given in Fig. 3 and 5 were calculated with the assumption that in reaction K (Fig. 7), the hydroxylated product, pentahydroxybenzene, accumulates stoichiometrically with the dehydroxylated product, phloroglucinol. This assumption is justified because in this case pentahydroxybenzene was not consumed again by consecutive transhydroxylation reactions (data not shown). The resulting pentahydroxybenzene concentrations are certainly not accurate but are in accordance with a constant carbon and electron balance at

different times during the assays. Pentahydroxybenzene proved to be highly unstable and could not be isolated from the reaction mixture. Only reaction M was not started with equimolar amounts of both substrates, but 1,2,4,5-TTHB-to-1,2,3,5-TTHB conversion nevertheless proceeded at the high rate given above, probably sustained only by the small pentahydroxybenzene pool formed by reaction L.

Reactions A to K were found in permeabilized suspensions and extracts of both hydroxyhydroquinone- and pyrogallol-grown cells. Reactions L and M, i.e., transformation of 1,2,4,5-TTHB to 1,2,3,5-TTHB, were catalyzed only by hydroxyhydroquinone-grown cells.

It should be emphasized that in many experiments the final stoichiometry of hydroxylated and dehydroxylated products did not exactly agree with the initial substrate concentrations. Inevitably, the resulting new combinations of intermediates together with the initially present reactants gave rise to consecutive transhydroxylation reactions. However, time course and final products of such sequential transhydroxylation reactions were always in agreement with the initial reactions observed individually with the respective substrate combinations. Furthermore, the tetrahydroxybenzenes proved to be very labile compounds under assay and sampling conditions, despite extreme precautions in sample handling (see Materials and Methods). This instability caused a slight decrease of tetrahydroxybenzenes during the incubation even if no transhydroxylation reactions were observed, e.g., in cell-free controls or in the experiment whose results are shown in Fig. 6A.

No transhydroxylation reactions were observed with catechol, hydroquinone, or phenol, nor did the presence of these compounds interfere with the reported transhydroxylation reactions, regardless of the hydroxyl donor. No activities were observed in the absence of cell extracts or with heat-denatured controls. It was verified in all cases that the reaction rates were linearly dependent on the protein concentration in the assays.

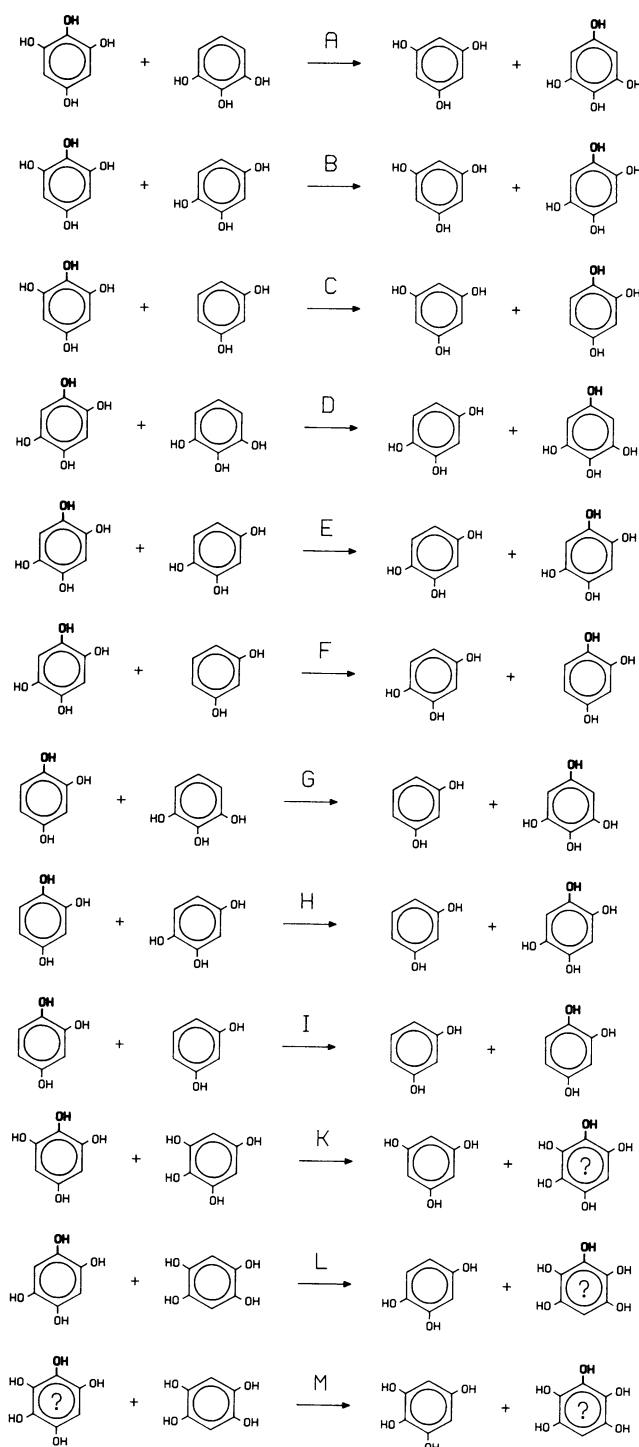


FIG. 7. Transhydroxylation reactions observed in permeabilized cell suspensions and cell extracts of *P. massiliensis* with various substrate combinations. All reactions were observed with pyrogallol- and hydroxyhydroquinone-grown cells except for reactions L and M, which only occurred with hydroxyhydroquinone-grown cells. A participation of pentahydroxybenzene (?) in reaction M is only hypothetical, since no reference compound was available. In reactions E and I, no turnover of metabolites could be measured because of identical substrates and products, but disproportionation of hydroxyhydroquinone (reaction H) markedly decreased. For compound names, see Fig. 2.

## DISCUSSION

This is the first report of an *in vitro* transformation of hydroxyhydroquinone (1,2,4-trihydroxybenzene) to phloroglucinol (1,3,5-trihydroxybenzene). The enzymatic activities responsible for this reaction are shown to consist of a sequence of transhydroxylations which are partly also involved in pyrogallol-to-phloroglucinol transformation and are partly unique to hydroxyhydroquinone-metabolizing cells of *P. massiliensis*.

Transformation of pyrogallol (1,2,3-trihydroxybenzene) to phloroglucinol in *P. massiliensis* shows the same characteristics as already observed with *P. acidigallici* (3). Cell extracts of both organisms catalyze an intermolecular hydroxyl transfer with 1,2,3,5-TTHB as cosubstrate and coproduct of the reaction (Fig. 1). In *P. massiliensis*, this activity was present both in hydroxyhydroquinone- and in pyrogallol-grown cells.

If pyrogallol-grown cells of *P. massiliensis* were incubated with hydroxyhydroquinone in dense suspension, they accumulated large amounts of resorcinol in the supernatant (16). *P. acidigallici* is unable to grow with hydroxyhydroquinone as a substrate, but permeabilized cells in dense suspensions convert this substrate to resorcinol and a second compound tentatively identified as an isomer of tetrahydroxybenzene other than 1,2,3,5-TTHB (3). The same reaction is catalyzed by extracts of pyrogallol-grown *P. massiliensis*; it is now characterized as a disproportionation of hydroxyhydroquinone to resorcinol and 1,2,4,5-TTHB. Both *P. acidigallici* and pyrogallol-grown *P. massiliensis* cells lack the ability to further metabolize these products.

However, if *P. massiliensis* is precultivated on hydroxyhydroquinone, cell extracts exhibit an additional activity rendering them capable of hydroxyhydroquinone-to-phloroglucinol conversion. The complete reaction sequence is summarized in Fig. 8. It consists of transhydroxylating activities also present in pyrogallol-grown cells of *P. massiliensis* as well as in *P. acidigallici* (reactions H, B, and C) and of a novel activity transforming 1,2,4,5-TTHB to 1,2,3,5-TTHB (reaction M) that has not previously been observed.

At first glance, this reaction could be interpreted as an enzymatic isomerization of tetrahydroxybenzenes (refer to Fig. 5A), similar to the initial interpretation of pyrogallol-to-phloroglucinol interconversion (12). However, on the basis of the formation of the tentatively identified pentahydroxybenzene only in assays in which reactions K and L occurred and the possible analogy in the function of pentahydroxybenzene in tetrahydroxybenzene interconversion to the function of 1,2,3,5-TTHB in pyrogallol-to-phloroglucinol conversion (Fig. 1), we suggest that pentahydroxybenzene serves as cosubstrate and coproduct in a similar intermolecular transhydroxylation (Fig. 7, reaction M). The pentahydroxybenzene pool necessary to support this reaction is generated by reaction L.

A comparison of the observed reaction patterns given in this scheme allows the formulation of apparent minimal structural prerequisites necessary for a hydroxybenzene to act as hydroxyl donor or acceptor (Fig. 9). With both hydroxyhydroquinone- and pyrogallol-grown cells, a hydroxyl group can only be donated if it is activated by the combined effect of two other hydroxyl substituents in *ortho*- and *para*-positions (Fig. 9A). Further hydroxylations at the 5- and/or 6-position do not change this specificity. There is more variety in the substitution pattern of possible hydroxyl acceptors. With pyrogallol- and hydroxyhydroquinone-grown cells, in addition to the compounds represented by

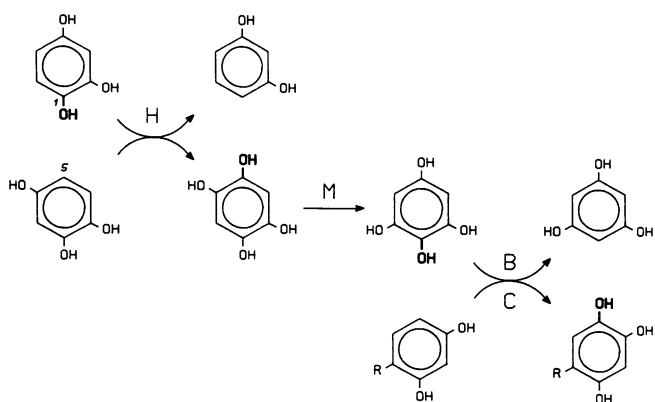


FIG. 8. Proposed sequence of transhydroxylation reactions responsible for net conversion of hydroxyhydroquinone to phloroglucinol in *P. massiliensis* (R = H, OH). All individual steps were demonstrated in extracts of hydroxyhydroquinone-grown cells (Fig. 3C and 4). Letters denote the reactions as summarized in Fig. 7, except for reaction M, in which pentahydroxybenzene was omitted on both sides of the equation.

structure B (Fig. 9B), pyrogallol (Fig. 9C) is a potent acceptor. All these compounds have in common a hydroxyl group in *para*-position to the hydroxylation/dehydroxylation position. 1,2,4,5-TTHB, however, lacks a hydroxyl group positioned *para* to its only possible hydroxylation site (Fig. 9D). Consequently, it is not hydroxylated by pyrogallol-grown cell extracts. Hydroxyhydroquinone-grown cells, though, possess a unique activity enabling them to perform a hydroxylation of 1,2,4,5-TTHB with either 1,2,4,5-TTHB itself or pentahydroxybenzene as a hydroxyl donor, leading either to pentahydroxybenzene formation (reaction L) or to a net transhydroxylation of 1,2,4,5-TTHB to 1,2,3,5-TTHB (reaction M), respectively.

Pyrogallol cannot act as hydroxyl group donor and consequently is not disproportionated to a di- and a tetrahydroxybenzene. This explains why 1,2,3,5-TTHB must be added to the assay to restore pyrogallol-to-phloroglucinol conversion. A net hydroxylation of pyrogallol to 1,2,3,5-TTHB occurring in growing cells has been postulated (3). Chemically, the

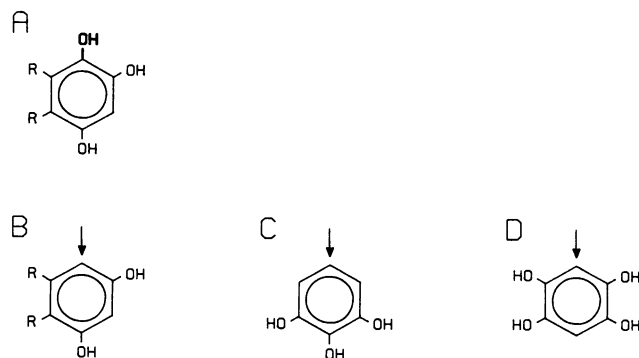


FIG. 9. Apparent minimal structural prerequisites which allow a hydroxybenzene to act as a hydroxyl donor (A) or acceptor (B to D) in the transhydroxylase reactions catalyzed by *P. massiliensis* and *P. acidigallici*. The hydroxyl group which can be donated is shown in boldface, and the accepting ring position is indicated by an arrow (R = H, OH). Structure D can function as hydroxyl acceptor only in hydroxyhydroquinone-grown *P. massiliensis*.

oxidation of a hydroxybenzene (e.g., hydroquinone) to the respective quinone, which in turn undergoes a 1,4-addition of water and rearranges to the next higher hydroxybenzene (8), becomes increasingly easier with the number of hydroxyl substituents at the aromatic nucleus, due to the +M effect of the hydroxyl groups. The corresponding standard redox potential ( $E_0'$ ) of the quinol/quinone half cell (data from references 6 and 9) is shifted to more negative values (+280, +174, and -60 mV for *para*-quinone formation from hydroquinone, hydroxyhydroquinone, and 1,2,4,5-TTHB, respectively). *ortho*-quinones are in general less easily formed, but again an additional hydroxyl group is favorable ( $E_0'$  is +372 mV for 1,2-dihydroxybenzene and +293 mV for pyrogallol). Yet for strictly anaerobic, fermentative bacteria, it is difficult to envisage which oxidant they could employ in such a reaction and how they accomplish reoxidation of this electron acceptor. A reverse electron transport, as already postulated to be necessary for the operation of the complete citric acid cycle demonstrated in *P. acidigallici* (4), would serve this purpose. *Desulfuromonas acetoxidans*, which is closely related to *P. acidigallici* on the basis of 16S rRNA sequences (17), operates a catabolic citric acid cycle including a reversed electron flow from the quinone level to pyridine nucleotides (13).

The actual mechanism underlying the intermolecular hydroxyl transfer among tetrahydroxybenzenes is still open to speculation. The involvement of a diphenyl ether intermediate has been suggested (13a), which could explain the intermolecular nature of hydroxyl transfer and redox reaction. We are currently attempting to purify the transhydroxylase of *P. acidigallici* to study the mechanism of pyrogallol-to-phloroglucinol transhydroxylation in more detail.

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